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Original Article

Phenotypic and Molecular Detection of Biofilm Formation in Methicillin-Resistant *Staphylococcus Aureus* Isolated from Different Clinical Sources in Erbil City

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Competing interests: The authors declare no conflict of Interest.

Abstract. ***Background:*** ***Staphylococcus aureus*** **is an important causative pathogen. The production of biofilms is an important factor and makes these bacteria resistant to antimicrobial therapy.**
Objectives: the current study aimed to assess the prevalence of resistance to antibacterial agents and to evaluate the phenotypic and genotypic characterization of biofilm formation among *S. aureus* strains.
Methods: **This study included 50 isolates of Methicillin-resistant *S. aureus* (MRSA) and Methicillin-Susceptible *S. aureus* (MSSA). *S. aureus* was identified by molecular and conventional methods, and antimicrobial resistance was tested with a disc diffusion method. The biofilm formation was performed through the Microtiter plate method. Strains were subjected to PCR to determine the presence of *nuc*, *mecA*, *icaA*, *icaB*, *icaC*, and *icaD* genes.**
Results: **Of the 50 *S. aureus* isolates, 32(64%) and 18(36%) were MRSA and MSSA, respectively. A large number of MRSA and MSSA isolates showed resistance to Penicillin and Azithromycin, and a lower number of MRSA and MSSA isolates showed resistance to Amikacin Gentamicin. None of the isolates was resistant to Vancomycin. The MRSA strains had significantly higher resistance against antibiotics than MSSA strains (P = 0.0154). All isolates (MRSA and MSSA) were able to produce biofilm with levels ranging from strong (31.25 %), (16.6%) to moderate (53.12%), (50%) to weak (15.6%), (33.3%) respectively. The MRSA strains had a significantly higher biofilm formation ability than the MSSA strains (P = 0.0079). The biofilm-encoding genes were detected among isolates with different frequencies. The majority of *S. aureus* isolates, 42 (84%), were positive for the icaA. The prevalence rates of the icaB, icaC and icaD genes were found to be 37 (74%), 40 (80%) and 41 (82%), respectively.**
Conclusions: **The prevalence of biofilm encoding genes associated with multidrug resistance in *S. aureus* strains is high. Therefore, identifying epidemiology, molecular characteristics, and biofilm management of *S. aureus* infection would be helpful.**

Keywords: MRSA, MSSA, Biofilm-related genes, Antibiotic resistance.

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Introduction. The most prevalent agents of the hospital- and community-acquired infections are *Staphylococcus aureus* (*S. aureus*). Furthermore, one of the main human pathogens is methicillin-resistant *Staphylococcus aureus* (MRSA). It is a bacterial pathogen that causes many illnesses, including skin infections, dangerous invasive infections like pneumonia, soft tissue infections, bones, heart valves, and even deadly human septicemia. It also

quickly develops antibiotic resistance.[1,2] In recent years, *S. aureus* infections have become more deadly due to the increasing incidence of antimicrobial resistance in S. aureus due to extensive antibiotic use.[3] The risk of death and the treatment cost were higher in the infections caused by antibiotic-resistant strains than by susceptible strains.[4] Antimicrobial resistance in methicillin-resistant strains of S. aureus (MRSA) is linked with the acquisition of a mobile genetic element named the staphylococcal cassette chromosome mec, which carries the mecA gene, encoding the low-affinity penicillin-binding protein 2a and confers resistance to the β-lactam antibiotics.[5] Although MRSA are resistant to β-lactam antibiotics, many MRSA isolates are multidrug-resistant (MDR) because they exhibit resistance to other antimicrobial agents, such as macrolides, tetracycline, aminoglycosides, chloramphenicol, and fluoroquinolones, which are frequently used in the treatment of infections caused on by these microorganisms.[6]

In addition to the bacteria's resistance to antibiotics, its ability to develop biofilm, a dynamic structurally complex multilayered cellular matrix, is another significant complicated factor for a better understanding of the molecular pathogenesis of *S. aureus*. As a result, new preventative and therapeutic approaches may be developed. Biofilm production, which is required for the survival and persistence of MRSA in its hosts, is considered to be a significant virulence factor, as well as one of many, including extracellular toxins and surface features that is effective in the induction and maintenance of infection in the host.[7] Initial attachment, biofilm maturation, and dispersal are the three main processes that can be classified as stages in the evolution of biofilms, according to several definitions. An individual planktonic cell will associate reversibly with a surface during initial attachment, and if it does not disassociate, it will bond irrevocably to the surface. Attachment is facilitated through surface proteins, referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs).[8] During infection, these proteins play major roles in attachment to host factors such as fibrinogen, fibronectin, and collagen. Biofilm maturation occurs through cell division and the production of the extracellular polymeric matrix. The composition of the biofilm matrix varies between strains but generally can contain host factors, polysaccharides, proteins, and extracellular DNA (eDNA).[9,10] Following biofilm accumulation, cells within the biofilm can reactivate to a planktonic state through dispersal.[11]

Production of biofilms is crucial during infection because it protects the bacteria against various human defense mechanisms and shields them from antimicrobial agents.[12] The ability to form biofilm is a trait associated with bacterial virulence and many chronic bacterial infections.[13] Several genes are involved in the production and maintenance of biofilms by staphylococci, of which the most widely studied are the icaA and icaD (intercellular adhesion A and B) genes responsible for the production of polysaccharide intercellular adhesion (PIA) that includes N-acetyl glucosamine as a primary component of the exopolysaccharide matrix surrounding the bacterial cells within the biofilm.[14] The aim of this study was to determine antimicrobial resistance profiles and detection of biofilm formation of MRSA and MSSA isolates from different clinical sources from patients in hospitals of Erbil city-Iraq.

Materials and Methods

Bacterial Strains. A total of 50 *S. aureus* strains from a clinical source, such as swabs from urine (25), a wound (10), and a catheter (10) throat (5), were used in this work. The strains were obtained from hospitals in Erbil City, Erbil, Iraq. The isolates were identified using traditional microbiological techniques. Besides, the VITEK 2 compact system was utilized to reidentify them (BioMerieux, France). Finally, the isolates' identities were verified using polymerase chain reaction (PCR) (Alpha PCRmax, UK) by detecting nuc genes. **Table 1** contains the gene sequences and PCR setup.

Antibiotic Susceptibility Testing. The disc diffusion technique was used to test all staphylococcal isolates for methicillin resistance. PCR verified methicillin resistance to detect the *mecA* gene.[16] The isolates' susceptibility was evaluated using the disc diffusion technique[20] using the following antibiotic discs (Bioanalyse, Turkey): Amikacin AK 30 µg, Azithromycin AZM 15 µg, Clindamycin CD 2 µg, Ciprofloxacin CIP 5 µg, Erythromycin E 15 µg, Gentamicin G 10 µg, Levofloxacin LEV 5 µg, Norfloxacin NOR 10 µg, Penicillin P 10 U, Tetracycline TE 30 µg, Trimethoprim+Sulfamethoxazole SXT 1.25+23.75 µg, and Vancomycin VA 30 µg. *S. aureus* ATCC 25923 was used as the control strain.

Biofilm Formation Assay. Biofilm generation was quantified using a Microtiter plate (MTP) approach described by Yousefi M. et al.[21] In brief, bacterial isolates were cultured in trypticase soy broth (TSB) (Merck, Germany) with 0.5 percent glucose and incubated at 37ºC overnight. Cultures with 0.5 percent glucose with 1:40 in fresh TSB were diluted (Sigma, USA). Two hundred µL of the diluted solution was put into Microtiter plate wells and incubated for 48 hours at 37ºC. Only 200 µL of TSB-0.5% glucose was present in the negative control wells, and there was no bacterial suspension. Wells were carefully cleaned three times

Table 1. Sequences of oligonucleotide primers used for PCR amplification of biofilm-associated genes with nuc and mecA genes used in this study.

Gene name	Primers detail			References
	Primer Sequence (5´ – 3´) (Oligonucleotide)	size (bp)	PCR Conditions	
nuc	GCG ATT GAT GGT GAT ACG GTT AGC CAA GCC TTG ACG AAC TAA AGC	279	95°C–30 s; 53°C–45 s; 72°C–40 s; 40 cycles	15
mecA	ATG TCT GCA GTA CCG GAG CTT T AAA AT CGA TGG TAA AGG TTG GC	533	94°C–30 s; 55°C–45 s; 72°C–1 min; 40 cycles	16
icaA	ACA CTT GCT GGC GCA GTC AA TCT GGA ACC AAC ATC CAA CA	188	94°C–30 s; 56°C–60 s; 72°C–45 s; 30 cycles	17
icaB	CCC AAC GCT AAA ATC GC ATT GGA GTT CGG AGT GAC TGC	1080	95°C–30 s; 58°C–30 s; 72°C–45 s; 40 cycles	18
icaC	CTT GGG TAT TTG CAC GCA TT GCA ATA TCA TGC CGA CAC CT	209	95°C–30 s; 55°C–40 s; 72°C–45 s; 40 cycles	19
icaD	ATG GTC AAG CCC AGA CAG AG CGT GTT TTC AAC ATT TAA TGC AA	198	94°C–30 s; 55°C–40 s; 72°C–45 s; 30 cycles	17

Table 2. Classification of biofilm formation abilities by Microtiter plate method.

Cut-off value calculation	Mean of OD570 values results	Biofilm formation abilities
OD ≤ ODc	OD > 0.557	Strong
ODc< - ≤ 2×ODc	0.278< OD ≤ 0.557	Moderate
2× ODc< - ≤ 4×ODc	0.139< OD ≤ 0.278	Weak
OD >4×ODc	OD ≤ 0.139	Non

with phosphate buffer saline (PBS) (pH 7.2), fixed for 20 minutes with methanol, dried at room temperature, and stained for 10 minutes with crystal violet 0.1 percent. Then 1 mL of 95% ethanol was added to each well to dissolve the dye bound to the adherent cells. Finally, using an ELISA reader (BioTek ELx800, USA), optical density (OD) at 570 nm (A570) was determined for each well. The average OD of negative control + 3 standard deviation (SD) of negative control was calculated for the optical density cut–off (ODc). Based on the absorbance of crystal violet stain linked to the adhered cells, biofilms formed by various strains have been analyzed and categorized (**Table 2**).

DNA Isolation. DNA extraction from a genome following the manufacturer's instructions, genomic DNA was isolated from pure cultures using the Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan); the extract was then eluted with a 100 µL elution buffer. Extracts were kept at -20°C until PCR was performed. A DNA template was used for PCR using 2 µL of the total extracted material from each test sample.

Primers and PCR Conditions. The primers designed by BioGene (South Korea) specifically for the *nuc*, *mecA*, *icaA, icaB, icaC,* and *icaD* are given in **Table 2**. The monoplex PCR was performed in a 25-*µ*L volume for each gene. All PCR reactions were conducted by employing 2 µl DNA template (density of 10 ng/µl), a Master Mix comprising of 3 mM MgCl2, 0.2% Tween® 20, 20 mM Tris-HCl pH 8.5, (NH4)2S04, 0.4 mM of each dNTP, 0.4 µM of each primer, and 0.2 units/µl Ampliqon Taq DNA polymerase. The PCR amplification conditions were established as shown in **Table 1**.

Statistical Analysis. The T-test was used to examine group differences at the 0.05 level of significance. Utilizing the statistical analysis program GraphPad Prism 7.

Results

Isolation and characterization of S. aureus. 50 *S. aureus* isolates from patients at hospitals in Erbil City, Iraq, were used in this study and were identified using standard microbiological techniques. The VITEK II Compact System was used, and it reidentified all strains of *S. aureus* to support the identification of *S. aureus* isolates made by a standard approach. All isolates of *S. aureus* were further tested for the presence of the *nuc* genes to verify their identities; the presence of *nuc* genes in all of the strains proved that they were all *S. aureus* (**Figure 1**). Disk diffusion assay was used to test *S. aureus* isolates for methicillin resistance and separate them into MRSA (32 strains) and MSSA (18 strains). The *mecA* gene was detected by PCR to verify Methicillin resistance (**Figure 2**).

Antibiotic susceptibility of S. aureus isolates.

Antibiotic resistance pattern of the MRSA isolates. Of the total 50 *S. aureus* isolates, 32 (62%) were Methicillin-resistant *S. aureus* (MRSA). A large number of MRSA isolates showed resistance to Penicillin (100%), Azithromycin (56.25%), Clindamycin (40.6), and Tetracycline (31.25). A lesser number of MRSA isolates showed resistance to Amikacin (6.25%), Gentamicin (9.4%), Levofloxacin (12.5%),

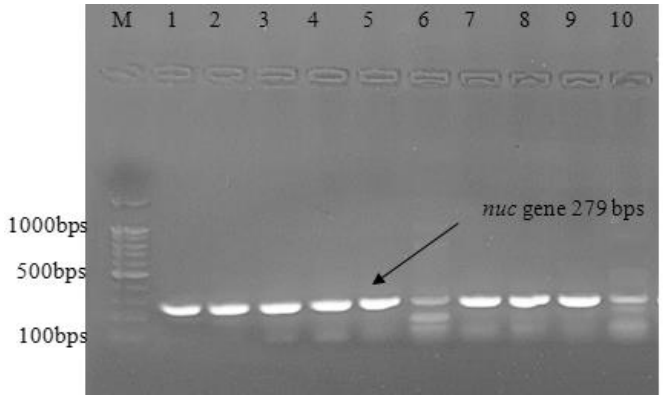

Figure 1 Agarose gel electrophoresis of PCR amplification products for the *nuc* gene of *S. aureus*. **M**: The DNA marker (100 bp ladder), lanes **1, 2, 3, 4, 5, 6, 7, 8, 9,** and **10** positive amplification of **279 bp** for *nuc* gene.

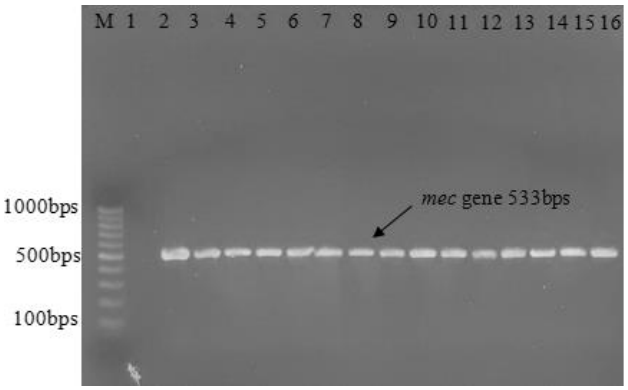

Figure 2 Agarose gel electrophoresis of PCR amplification products for the *nuc* gene of *S. aureus*. **M**: The DNA marker (100 bp ladder), lane **1 negative control**: lanes **2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,** and **16** positive amplification of **533 bp** for *mec* gene.

Ciprofloxacin (15.6%), trimethoprim with sulfamethoxazole (21.9%), Norfloxacin (25%), and Erythromycin (28.1%). Vancomycin resistance was not seen in any MRSA samples (**Table 3**).

The MRSA isolates were screened for resistance to 9 different antimicrobial drug groups, and resistance to at least 3 groups revealed that the isolates were MDRs (multidrug-resistant). Out of 32 MRSA isolates, 19 (59.3%) were MDR (resistant to three or more antibiotics), 3 (9.3%) were resistant to only two antibiotics, and 10 (31.2%) were resistant to only one antibiotic. The most prevalent resistance pattern among MDR MRSA isolates, in addition to resistance to -lactam antibiotics, was resistance to azithromycin, clindamycin, and tetracycline.

Antibiotic resistance pattern of the MSSA isolates. Eighteen isolates (36%) of the 50 *Staphylococcus* isolates were Methicillin-Susceptible *S. aureus* (MSSA). The rate of resistance of the 18 MSSA isolates to the antibiotics shows various sensitivity patterns (**Table 4**). The greatest observed resistance rate was against Penicillin (100%), Azithromycin (55.6%), and Clindamycin (44.4). Also, the lowest resistant percentage of MSSA isolates recorded was against Amikacin and Gentamicin (5.6), then Levofloxacin and Ciprofloxacin (11.1%), trimethoprim with sulfamethoxazole and Erythromycin (16.6%), Norfloxacin and Tetracycline (22.2) respectively. None of the MSSA isolates was resistant to Vancomycin (**Table 2**). The multidrug resistance pattern of the MSSA demonstrated that eleven (61.1%) of the MSSA were multidrug-resistant, two (11.1%) were resistant to only two antibiotics, and five (27.7%) were resistant to only one antibiotic (**Table 3**).

A statistical test comparing the antibiotic resistance pattern between MRSA and MSSA isolates showed a significant difference between MRSA and MSSA isolates, and MRSA strains had significantly higher resistance against antibiotics than MSSA strains ($P = 0.0154$).

Biofilm Formation Assay. The MTP was used to assess *S. aureus* strains for their ability to produce biofilms. In this study, after crystal violet staining, OD570 mean microplate readings ranged from 0.186 to 0.613. The negative control mean was 0.054. Therefore, an ODc570 of biofilm production was defined as 0.139. The strains were classified into four categories: non–biofilm producer (−), OD570≤0.139; weak biofilm producer (+), 0.139<OD570≤0.278; moderate biofilm producer (++), 0.278<OD570≤0.557; strong biofilm producer (+++), 0.557≤OD570. *S. aureus* strains that were included in this work were divided into MRSA (32 strains) and MSSA (18 strains), and the information reveals that all *S. aureus* isolates tested positive for biofilms.

The majority of the 32 MRSA strains examined (n=17) (53.12%) produced moderate biofilms. The strong biofilms were produced by (n=10) 31.25 % of the MRSA strains, while (n=5) 15.6 % of strains were weak biofilm producers. A large group of the 18 MSSA strains examined (n=9) (50%) also formed moderate biofilms, while (n=3) 16.6% and (n=6) 33.3% of MSSA strains were strong and weak producers, respectively (**Table 4**).

Comparison of biofilm biomass (absorbance at 570 nm) Using a statistical analysis, it was determined that MRSA isolates were significantly more capable of forming biofilms than MSSA isolates ($P = 0.0079$) (**Figure 3**).

Detection of biofilm formation genes. The existence of the *ica* operon in the tested strains was verified by amplification of specific segments for *icaA* (188 bp), *icaB* (1080 bp), *icaC* (209 bp), and *icaD* (198 bp) **Figure 4** and the relationship between the formation of biofilm and the four genes associated with biofilm was evaluated. These genes' distribution in *S. aureus* isolates is shown in **Table 5**. All four genes were found among isolates with different occurrences. As observed, a large percentage of S. aureus isolates, 42 (84%), were found to

Table 3. Susceptibility patterns of S. aureus isolate toward antimicrobials.

Antimicrobial agent	MRSA (*n* = 32)		MSSA (*n* = 18)	
	Resistance *n*. (%)	Sensitivity *n*. (%)	Resistance n. (%)	Sensitivity n. (%)
Amikacin (AK)	2 (6.25)	30 (93.75)	1 (5.6)	17 (94.4)
Azithromycin (AZM)	18 (56.25)	14 (43.75)	10 (55.6)	8 (44.4)
Clindamycin (CD)	13 (40.6)	19 (59.4)	8 (44.4)	10 (55.6)
Ciprofloxacin (CIP)	5 (15.6)	27 (84.4)	2 (11.1)	16 (88.9)
Erythromycin (E)	9 (28.1)	23 (71.9)	3 (16.6)	15 (83.4)
Gentamicin (G)	3 (9.4)	29 (90.6)	1 (5.6)	17 (94.4)
Levofloxacin (LEV)	4 (12.5)	28 (87.5)	2 (11.1)	16 (88.9)
Norfloxacin (NOR)	8 (25)	24 (75)	4 (22.2)	14 (77.8)
Penicillin (P)	32 (100)	0 (0)	18 (100)	0 (0)
Tetracycline (TE)	10 (31.25)	22 (68.75)	4 (22.2)	14 (77.8)
Trimethoprim+Sulfamethoxazole (SXT)	7 (21.9)	25 (78.1)	3 (16.6)	15 (83.4)
Vancomycin (VA)	0 (0)	32 (100)	0 (0)	18 (100)

Table 4. Screening of *S. aureus* isolates from biofilm production by MTP method.

Biofilm formation status	Isolates	
	MRSA	MSSA
Strong	10 (31.25%)	3 (16.6%)
Moderate	17 (53.12%)	9 (50%)
Weak	5 (15.6%)	6 (33.3%)

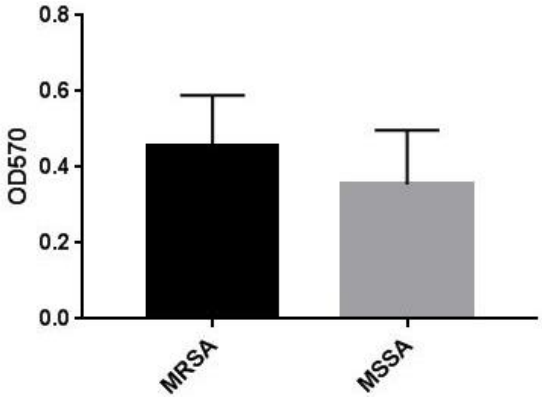

Figure 3. Comparison of biofilm-forming ability by MRSA and MSSA strains.

be *icaA* positive. On the other hand, the prevalence rates of the *icaB*, *icaC*, and *icaD* genes were unswervingly found to be 37 (74%), 40 (80%) and 41 (82%), respectively.

Twenty-two 44% of isolates that produce biofilm were revealed to have all four genes. There are great relationships between strong biofilm formation in isolates and the presence of all four biofilm encoding genes. Also, a significant relationship was found between the presence of *icaA* (p= 0.0036), *icaB* (p= 0.0001), *icaC* (p= 0.001), and *icaD* (p= 0.0019) gene and phenotypic biofilm formation in *S. aureus* isolates. The occurrence of icaABCD genes was highly significantly

Table 5. Screening the presence of biofilm encoding genes of *S. aureus* isolates.

No. of isolates	Presence of biofilm encoding genes				
	icaA	*icaB*	*icaC*	*icaD*	*ica ABCD*
50	42	37	40	41	22
100	84%	74%	80%	82%	44%

associated with the phenotypic biofilm formation in *S. aureus* isolates (P = <0.0001).

Discussion. *S. aureus* is an opportunistic pathogen that has long been recognized as a frequent source of human infections. Despite being a natural component of human flora, *S. aureus* has the potential to cause a wide variety of illnesses, from relatively minor skin infections to catastrophic outcomes. Many of these illnesses can quickly become life-threatening if they are not properly controlled and treated.[22] The ability to form biofilm using some phenotypic and genotypic indicators is related to the spread of antibiotic resistance, especially resistance to methicillin in infected samples. It is one of the main concerns related to the ability of *Staphylococcus aureus* to threaten health. Considering the formation of multilayered units in the creation of biofilm structures, the expansion of biofilm samples can be considered a key and indicator step in the increase of infection and the expansion of antibiotic resistance. The objective of this study was to investigate the phenotypic and molecular diagnosis of biofilm formation in methicillin-resistant *Staphylococcus aureus* isolated from different clinical sources.

In this study, antibiotic susceptibility testing and mecA gene identification revealed that 32 strains were MRSA and 18 were MSSA. Kumurya et al. have reported similar validation procedures.[23] A large number of MRSA strains are sensitive to penicillin, azithromycin, clindamycin, tetracycline, sulfamethoxazole, gentamicin,

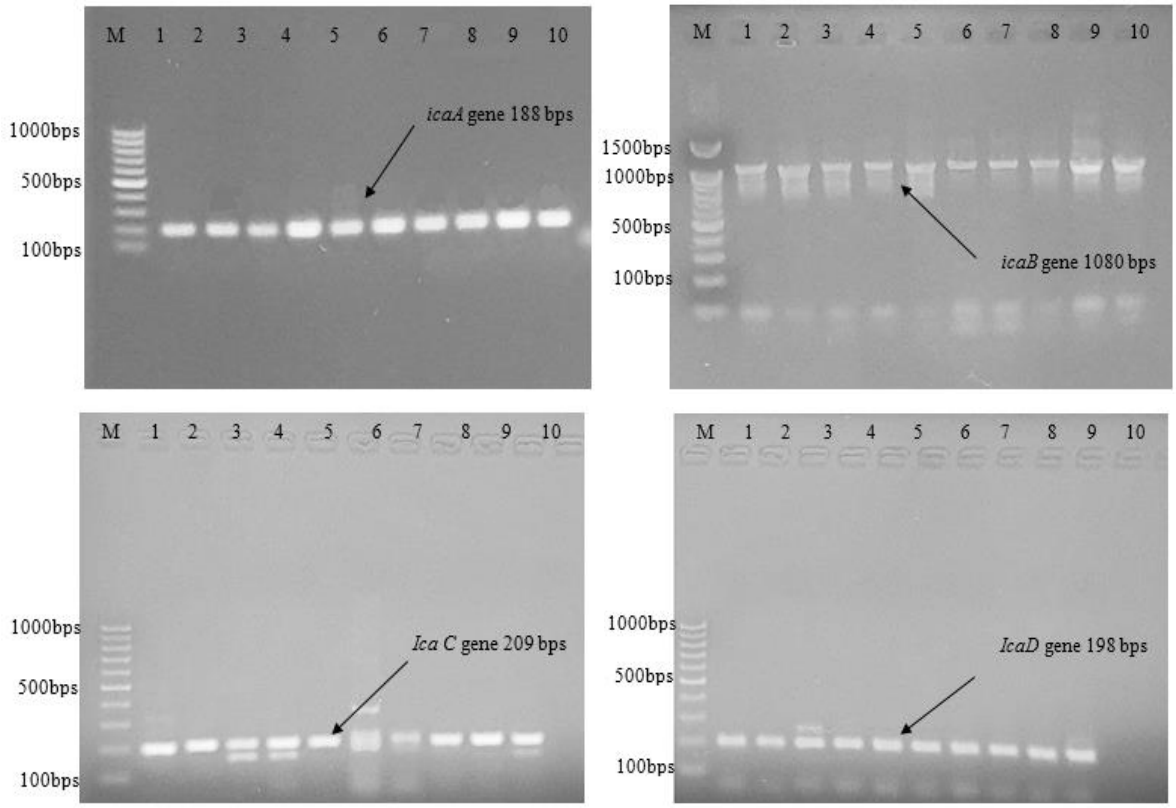

Figure 4. Agarose gel electrophoresis graphic of PCR amplification for biofilm-related genes in *S. aureus* isolates.

levofloxacin, Norfloxacin, ciprofloxacin, Amikacin, as shown by the studies conducted by John Walter et al. and Stefanaki et al. [24,25]

In this study, the rate of resistance to erythromycin was 28.1%, which is not consistent with the study of J Szabó et al., and Romen Singh Naorem et al., which may be due to the different number of strains in the studies.[26,27] The insignificant resistance of MRSA strains to Vancomycin in this study is consistent with studies conducted by Vinay Kumar Moses et al. and Chaudhari CN et al.[22,28] The MSSA isolates were susceptible to most antibiotics tested. However, more resistance was observed to penicillin, azithromycin, clindamycin, and to some extent to clindamycin, tetracycline, tobramycin, Amikacin, gentamicin, ciprofloxacin, Norfloxacin, and levofloxacin. In contrast, in the case of MRSA, multiple drug resistance was common, and only a few antibiotics were active against these isolates. Also, the resistance of MSSA strains to Vancomycin was negligible and zero. These findings are consistent with the findings of Fateh Rahimi et al., Shilpa Arora et al., and H Saderi et al.[29-31] Comparing the two antibiotic resistance patterns of MRSA and MSSA isolates showed that MRSA strains have much higher antibiotic resistance than MSSA strains. This finding is consistent with the findings of Solmaz Dibah et al., and Ricciardi et al.[32,33]

S. aureus has been found to produce biofilms, which are thought to cause chronic or persistent infections because they act as a defense against the immune system and antibiotics.[6] This study used MTP to form the biofilm of *Staphylococcus aureus* strains. A study including 50 clinical isolates of *Staphylococcus aureus* reported that MTP is a more accurate and repeatable method for biofilm detection 34. MRSA strains showed a higher percentage of biofilm formation (moderate and strong) than MSSA strains. Moreover, MSSA strains showed a higher percentage of forming weak biofilms. These findings are consistent with the study findings of Leshem et al. and Omidi et al.[35,36] In addition, the existence of icaABCD operon components is often correlated with biofilm generation, which is consistent with the findings of research by Shivaee et al. and Khasawneh et al.[37,38] In this study, as can be seen, most of the Staphylococcus aureus isolates (from low to high, icaC - icaB - icaD - icaA) were positive for biofilm-related genes. These findings are consistent with the findings of the studies of Mehdi Goudarzi et al. and Ali Haghi et al.[39,40] On the other hand, Azmi et al. pointed out that the icaA gene's low prevalence (16.5%)[41] suggests that alternative mechanisms may be used by these strains in addition to the ica-dependent system, which may not be the only mechanism involved in biofilm development.

Conclusions. The results of the present study have shown that *Staphylococcus aureus* isolates had high resistance to most of the investigated antibiotics. It seems that the biofilm in *Staphylococcus aureus* has a high phenotypic expression, and the high ability of this bacterium to form a biofilm can be particularly important

in the emergence of infections and the creation of multiple antibiotic strains. Considering the importance of *Staphylococcus aureus* bacteria as a hospital pathogen and considering the increasing antibiotic resistance in clinical isolates in order to prevent the formation of biofilm and colonization of bacteria in hospital tools and environment, it is recommended to observe proper sterilization in the therapeutic tools related to the patient, to prevent the transmission of infection and also the formation of biofilm.

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