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Molecular Detection of Virulence Genes, Biofilm Formation, and Antibiotic Resistance in Pathogenic *Staphylococcus aureus* from Taif Hospitals

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Abstract

The bacterium *Staphylococcus* can cause various health problems, particularly in hospitalized patients. Therefore, the current study aimed to isolate methicillin-resistant *Staphylococcus aureus* (MRSA) strains, test their capability to form a biofilm, and detect genes related to virulence and biofilm formation. Bacterial isolates were collected from the King Faisal Specialist Hospital and Children's hospital in Taif Governorate, Saudi Arabia, and identified using primers for *mecA* and *nuc1.* They were tested for resistance against twelve widely distributed antibiotics and biofilm formation capability. The MRSA isolates were tested for *fnbA, fnbB,* and *SCCmec.* Among 100 isolates, 24 were identified as *Staphylococcus aureus,* and most of them were MRSA. Most isolates were resistant to cefrizine and cefepime (96%). The isolates showed higher resistance to amoxicillin and ampicillin (92%), followed by aztreonam (83%). Two isolates, S15 and S17, were high-grade positive for biofilm formation, 62.5% were medium-grade, and 20.8% were low-grade positive. Two of the isolates, S11 and S16, tested negative for biofilm formation. Furthermore, mecAI. ncu1 was found in all of the isolates, except S11. Most isolates had *SCCmecIII* and *SCCmecV.* All isolates were habituated to *fnbB,* while *fnbA* was not found in S3 and S11. These results indicated that PCR techniques offer rapid, simple, and accurate determination of the genetic profile and biofilm production capability of MRSA, and can be used in clinical diagnosis as well as to monitor the spread of antibiotic-resistant *S. aureus* strains.

Keywords: *Staphylococcus aureus*, Biofilm Formation, Virulence Genes, Antibiotic Resistance

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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most pathogenic microbes that cause diseases in humans and animals. This gram-positive bacterium is found on human skin and nasal mucous membranes as a part of normal bacterial flora.[1-3] Indiscriminate use of antibiotics has resulted in an increase in antibiotic-resistant bacteria, owing to which patients fail to respond to antibiotic treatment. The effectiveness of antibiotics used for treating humans and animals is threatened by resistant species and strains of microorganisms.[4-6] *S. aureus* is a prevalent and endemic pathogen found in hospitals, and biofilm-forming and MRSA strains have become a serious clinical problem.[7-9]

S. aureus is cluster-shaped and has the ability to form a three-dimensional biofilm that surrounds the cluster of cells, allowing them to resist unfavorable conditions. MRSA can develop antibiotic resistance owing to its biofilm formation capability and hence poses a great threat to hospitalized patients.[2,9] The biofilm is an organized structure and a major virulence factor that is important in protecting *Staphylococcus* cells from exposure to antibiotics.[10] Biofilm-forming bacteria infect biofilms in most human infections, with *Staphylococcus aureus* being the most harmful biofilm-producing species.[7,8] MRSA biofilms can spread to the hippocampus, and are not affected by antibiotics as the biofilm acts as a protective shield that increases resistance to antibiotics and other immune factors.[9,11]

MRSA causes chronic infections owing to its ability to resist various antibiotics by forming a biofilm on artificial heart valves, catheters, and medically implanted prostheses.[12,13] The spread of MRSA along with other staphylococcal diseases has led to a significant increase in the use of antibiotics at an estimated annual cost of $450 million, with increased disease rates associated with biofilm-mediated infection.[14] Therefore, an understanding of the evolution ofstaphylococcal biofilms at the molecular level is necessary to generate new treatment strategies for biofilm-associated infections and reduce the burden caused by these pathogens. Therefore, this study aimed to isolate antibiotic-resistant staphylococci and study their genetic details in 50 relation to the capability of biofilm formation.

MATERIALS AND METHODS

Collection of *Staphylococcus* isolates

The study protocol was approved by Taif University Medical Ethics Review Board (Project No. 1-437-5371) in accordance with the guidelines for human protection. Samples were collected from patients at King Faisal Hospital and Children's hospital in Taif City, Saudi Arabia, from October 2020 to November 2021, with documented patient consent. Approximately 100 bacterial isolates were collected and identified using a fully automated VITEK-2 COMPACT microbiology system (Bio Mtrieux, Inc., Durham, NC, USA).

Antibiotics susceptibility

The antibiotic susceptibility of *S. aureus* was determined according to the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 2018)[15] by disc diffusion method using Mueller-Hinton (MH) agar. The inocula were streaked onto MH agar plates using a sterile swab, and discs were incubated. The antibiotics tested were cefotaxime (30 µg), amoxicillin (25 µg), cefepime (30 µg), ampicillin (10 µg), cefixime (10 µg), cefatrizine (10 µg), gentamicin (10 µg), chloramphenicol (30 µg), lincomycin (15 µg), aztreonam (30 µg), oxacillin (5 µg), and norfloxacine (5 µg).

Biofilm forming capability

Biofilm formation by *S. aureus* isolates was determined using crystal violet assay on microtiter plates as described previously[16]. The optical density of each well was measured at 570 nm (OD570) using an automated Multiskan reader (Bio-Rad, Germany). Biofilm formation was interpreted as highly positive (OD570 ≥ 1), moderate-grade positive (0.4 ≤ OD570 < 0.9), low-72 grade positive (0.1 ≤ OD570 < 0.4), or negative (OD570 < 0.1). Each isolate was tested three times.

DNA isolation

DNA was extracted from the isolates

Table 1. Primer sequences and amplicon sizes of tested genes fibronectin-binding protein *mecA* and *SCCmec*

Primers	Sequence	Size (bp)	Annealing temp.
mecAI-F	(F) TCC AGA TTA CAA CTT CAC CAG G	162	56
	(R) CAA TTC ATA TCT TGT AAC G		
ncu1	(F) TCC AGA TTA CAA CTT CAC CAG G	301	52
SCCmec-III	(R) CAA TTC ATA TCT TGT AAC G	243	58
	(F) CATTTGTGAAACACAGTACG		
	(R) GTTATTGAGACTCCTAAAGC		
SCCmec-V	(F) GAACATTGTTACTTAAATGAGCG	325	58
	(R) TGAAAGTTGTACCCTTGACACC		
fnbA	(F) CAT AAA TTG GGA GCA GCA TCA	127	54
	(R) ATC AGC AGC TGA ATT CCC ATT		
fnbB	(F) GTA ACA GCT AAT GGT CGA ATT GAT ACT		
	(R) CAA GTT CGA TAG GAG TAC TAT GTT C	524	54

using a DNeasy Bacterial Mini Kit (QIAGEN, USA) according to the manufacturer's instructions.

Detection of the *mecA*, SCC*mec*, and fibronectin-binding protein genes

PCR was performed using Go Taq® Green Master Mix (Promega, USA), according to the manufacturer's instructions. The primers used and conditions for each gene are listed in Table 1.[2,17] Amplicons were observed after electrophoresis on 1.5% agarose gel using a 100 bp DNA ladder (Fermentas, Lithuania, USA).

Data analyses

Pearson's simple linear correlation coefficient (r) and their significance (P) were assessed using SPSS 20.

RESULTS

Isolation of antibiotic-resistant bacteria

Approximately 100 clinical samples including urine and stool swabs were collected and analyzed for antibiotic-resistant bacteria. Of the 100 cultures tested, 24 bacterial isolates resistant to multiple antibiotics were identified as *Staphylococcus* and tested for biofilm formation, antibiotic resistance, and detection of virulence and biofilm genes. These isolates were assigned codes S1–S24.

Antibiotic susceptibility testing

In total, 12 antibiotics were tested, and the isolates showed high variability of resistance.

Table 2. The number of isolates and antibiotic resistance profile of *Staphylococcus aureus* isolates

Isolates	Antibiotic Profile
S -1	*Sxt, Amx, Cef, Amp, Cx, Caz, Lin,*
S -2	*Amx, Cef, Amp, Cx, Caz, Azt*
S -3	*Amx, Cef, Amp, Cx, Caz, Azt, Oxa*
S -4	*Sxt, Amx, Cef, Amp, Caz, Azt, Oxa*
S -5	*Amx, Cef, Amp, Cx, Caz, Azt*
S -6	*Sxt, Amx, Cef, Amp, Caz, Azt, Oxa, Nor*
S -7	*Sxt, Amx, Cef, Amp, Caz, Azt, Oxa, Nor*
S -8	*Cef, Amp, Cx, Caz, Lin, Azt, Oxa*
S -9	*Amx, Cef, Amp, Cx, Caz, Azt*
S -10	*Sxt, Cef, Amp, Cx, Caz, Azt*
S -11	*Amx, Cef, Caz, Azt*
S -12	*Amx, Cef, Amp, Cx, Caz*
S -13	*Sxt, Amx, Cef, Amp, Caz, Azt, Oxa*
S -14	*Sxt, Amx, Cef, Amp, Caz, Azt, Oxa*
S -15	*Sxt, Amx, Cef, Amp, Cx, Caz, Azt, Gen, Oxa, Nor*
S -16	*Amx, Cef, Amp, Caz, Azt*
S -17	*Sxt, Amx, Cef, Amp, Cx, Caz, Azt, Gen, Oxa, Nor*
S -18	*Amx, Cef, Cx, Azt*
S -19	*Sxt, Amx, Cef, Amp, Caz, Azt, Oxa, Nor*
S -20	*Sxt, Amx, Cef, Amp, Caz, Azt, Oxa, Nor*
S -21	*Amx, Cef, Amp, Cx, Caz, Azt, Oxa*
S -22	*Amx, Cef, Amp, Cx, Caz*
S -23	*Sxt, Amx, Cef, Amp, Caz, Azt, Oxa*
S -24	*Sxt, Amx, Cef, Amp, Caz, Azt, Oxa*

Whereas, Sxt = Trimethoprim / sulfamethoxazole (1.25/23.75µg), Amx = Amoxicillin (25µg), Cef = Cefepime (30 µg), Amp = Ampicillin (10µg), Cx = Cefixime (10µg), Caz = Cefatrizine (10µg), Gen =Gentamicin (10µg), Chl = Chloramphenicol (30 µg), Lin = Lincomycin (15 µg), Azt = Aztreonam (30µg), Oxa = Oxacillin (5 µg) and Nor = Norfloxacine (5 µg)

Most isolates showed highest resistance to cefrizine and cefepime (96%). The isolates also showed high resistance to amoxicillin and ampicillin (92%), followed by aztreonam (83%). All the isolates were sensitive to chloramphenicol. The isolates showed low resistance to lincomycin and gentamicin (8%) (Figure 1 and Table 2). The most sensitive isolates were S11, S16, and S18, which were resistant to amoxicillin, cefepime, cefrizine, and aztreonam. Isolates S15 and S17 showed the highest resistance to most of the antibiotics tested. The other isolates showed moderate resistance.

Determination of slime production

Phenotypic slime production was assessed by culturing isolates on CRA plates. Among the isolates, S1, S2, S13, S14, S15, and S17 were slime-producers, developing almost black colonies. The remaining isolates were considered as non-producers because they produced white colonies on CRA plates (Figure 2 and Table 3).

Quantitative biofilm formation

All 24 *S. aureus* isolates were screened for adherence to polystyrene microplates. Most isolates were able to form biofilms, and S15 and S17 were considered high-grade positive (OD570 values of 1.004 ± 0.007 and 1.011 ± 0.017, respectively). Isolates S1, S3, S4, S6, S7, S8, S13, S14, S19, S20, S21, S22, S23, and S24 were considered moderate-grade positive, with OD570 ranging from 0.670 ± 0.056 to 0.939 ± 0.025. Moreover, the isolates S2, S9, S10, S12, and S18 were considered low-grade positive, with OD570 ranging from 0.129 ± 0.003 to 0.390 ± 0.072. while S11 and S16 were negative for biofilm formation (Table 3).

PCR analysis for *mecAI, ncu1*, and SCC*mec* genes

The PCR amplification products of *mecA* and SCC*mec* in *S. aureus* isolates are shown in Figure 3 and Table 4. All isolates carried *mecA I* with a size of approximately 162 bp. The *ncu1* amplicon, approximately 301 bp in size, was found

Table 3. Biofilm formation, grade of biofilm and production of the slime of *Staphylococcus aureus* isolates

Isolates	Biofilm	Grade of biofilm	Production of slime
S -1	0.670± 0.056	Mediate-grade positive	Negative
S -2	0.258±0.003	Low-grade positive	Negative
S -3	0.768±0.037	Mediate-grade positive	Negative
S -4	0.800±0.138	Mediate-grade positive	Negative
S -5	0.728±0.011	Mediate-grade positive	Negative
S -6	0.939±0.025	Mediate-grade positive	Positive
S -7	0.928±0.109	Mediate-grade positive	Positive
S -8	0.762±0.132	Mediate-grade positive	Positive
S -9	0.390±0.072	Low-grade positive	Negative
S -10	0.136±0.036	Low-grade positive	Negative
S -11	0.093±0.002	Negative	Negative
S -12	0.129±0.003	Low-grade positive	Negative
S -13	0.815±0.164	Mediate-grade positive	Positive
S -14	0.899±0.037	Mediate-grade positive	Positive
S -15	1.004±0.007	High-grade positive	Positive
S -16	0.070±0.010	Negative	Negative
S -17	1.011±0.017	High-grade positive	Positive
S -18	0.197±0.071	Low-grade positive	Negative
S -19	0.916±0.043	Mediate-grade positive	Negative
S -20	0.919±0.028	Mediate-grade positive	Negative
S -21	0.837±0.007	Mediate-grade positive	Negative
S -22	0.737±0.117	Mediate-grade positive	Negative
S -23	0.819±0.059	Mediate-grade positive	Negative
S -24	0.827±0.041	Mediate-grade positive	Negative

Table 4. The number of *Staphylococcus aureus* isolates that habitat the Biofilm and *SCCmec* genes

Isolates	Detection genes					
	mecA1	*ncu1*	*fnbA*	*fnbB*	*SCCmec* III	*SCCmec* V
S -1	+	+	+	+	+	+
S -2	+	+	+	+	+	+
S -3	+	+	.	+	+	+
S -4	+	+	+	+	+	+
S -5	+	+	+	+	+	+
S -6	+	+	+	+	+	+
S -7	+	+	+	+	+	+
S -8	+	+	+	+	+	+
S -9	+	+	+	+	+	+
S -10	+	+	+	+	+	+
S -11	+	.	.	+	+	.
S -12	+	+	+	+	+	+
S -13	+	+	+	+	+	+
S -14	+	+	+	+	+	+
S -15	+	+	+	+	+	+
S -16	+	+	+	+	.	.
S -17	+	+	+	+	+	.
S -18	+	+	+	+	.	.
S -19	+	+	+	+	.	.
S -20	+	+	+	+	.	.
S -21	+	+	+	+	.	.
S -22	+	+	+	.	.	.
S -23	+	+	+	+	.	.
S -24	+	+	+	+	.	.

in most isolates except S11. This suggested that all isolates were MRSA, except S11, which is a weak isolate incapable of biofilm formation. Moreover, PCR amplicons of *SCCmec* were produced in all isolates, as shown in Figure 3. Most isolates had *SCCmec III* with a size of approximately 243 bp; however, isolates S16, S18, S19, S20, S21, S22, S23, and S24 did not contain *SCCmec III*. The *SCCmec V* amplicon, with a size of approximately 325 bp, was found in most tested isolates except in S11, S16, and from S18 to S24.

Detection of *fnbA* and *fnbB*

The presence of adhesive genes was confirmed by 127 bp and 524 bp bands of *fnbA* and *fnbB*, respectively. Almost all isolates were found to possess genes for both the homologous fibronectin-binding proteins fnbA and *fnbB,* except S3 and S11, which contained only the *fnbA* (Table 4 and Figure 2).

DISCUSSION

Biofilm formation is characteristic of several bacterial species and is related to their virulence. Chronic bacterial infections are closely related to biofilm formation.[18,19] The current study showed that two isolates, S15 and S17,

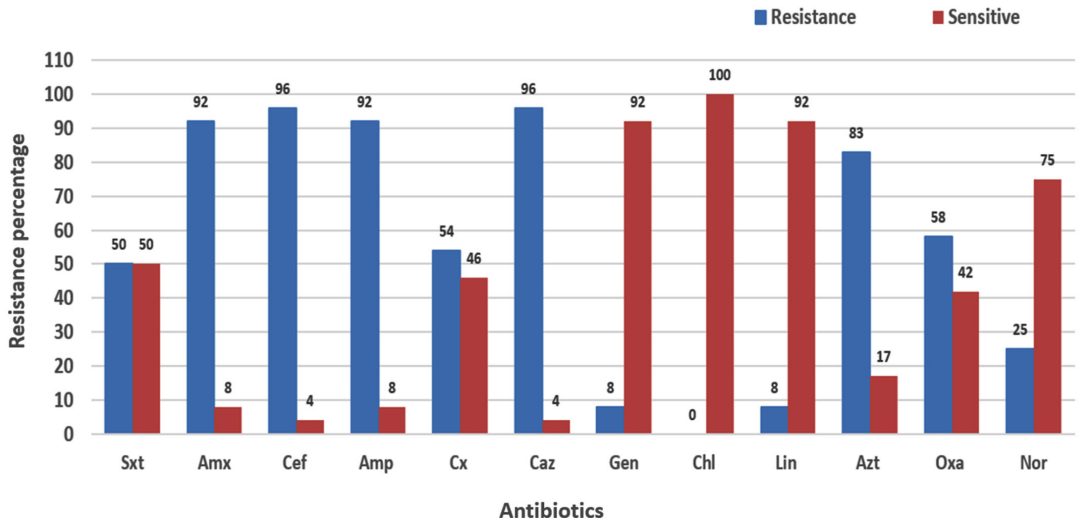

Figure 1. Antibiotic resistance pattern among S. aureus isolates, Whereas, Sxt = Trimethoprim / sulfamethoxazole, Amx = Amoxicillin, Cef = Cefepime, Amp = Ampicillin, Cx = Cefixime, Caz = Cefatrizine, Gen = Gentamicin, Chl = Chloramphenicol, Lin = Lincomycin, Azt = Aztreonam, Oxa = Oxacillin and Nor = Norfloxacine

had high capability to form biofilms. Moreover, 55% of the isolates were medium-grade positive for biofilm formation. The biofilm-forming strains cause chronic infection associated with polymeric implants.[20-22] MRSA is characterized by its adhesiveness, an important trait for infecting humans. The characterization of genes related to biofilm formation may improve the understanding of the phenotypic and genetic characteristics of infection caused by biofilms.[7-9] Several genes responsible for biofilm formation have been characterized, example, genes encoding adhesion molecules in *S. aureus*.[23,24] In this study, a polystyrene microtiter plate was used to detect biofilm formation. Molecular methods to detect the presence of biofilm-forming genes require the development of biofilm and polysaccharide adhesion between bacterial cells, which is brought about by genes encoding intracellular adhesion enzymes.[8,9] The expression of genes related to biofilm formation is regulated by multiple genes, such as *fnbA* and *fnbB*, which may interact with each other and regulate biofilm formation. *fnbA* and *fnbB* contribute to the invasion and adhesion of this bacterial species, and therefore, may be related to its ability to form biofilms.[25] The prevalence of *Staphylococcus* carrying these genes have been previously observed,[26,27] and the differences in availability of the genes might be due to varied primer sequences or the location of these genes in the bacterial chromosome. In the current study, 96% of the *Staphylococcus* isolates carried *fnbB* and 92% had f*nbA*, which is in accordance with previous studies.[25] The presence of *fnbB* may be related to the ability to form biofilms. The antibiotic resistance gene, *mecA*, has recently become prevalent; however, it is not indigenous to *S. aureus* and has been acquired recently from unknown sources.[17] The *mecA* gene product is a penicillin-binding protein (PBP), specifically BP2a. *S. aureus* produces four PBPs,[10] which are cytoplasmic membrane-fixing enzymes involved in cell wall formation.[4,28,29] Eleven species containing SCC*mec* have been assigned to the *Staphylococcus* species.[4,30] However, the prevalence of the fourth type is universal, while the rest of the species differ according to the location of bacterial isolates.[30-32] All the isolates carried *mecA* and 96% of them had *ncu1*. Moreover, 79% of the isolates carried SCC*mec*III, and 71% had SCC*mecV*. Several subtypes of SCC*mec*, including *IIA* to *E*, *IVa* to *IVg*, and *VT* have been reported.[2] Two possible explanations for the ability of *Staphylococcus* species to colonize synthetic materials, such as catheters or hospital plastics, are the production of polysaccharide slime by *Staphylococcus* isolates and the presence of adhesives on biomaterial surfaces to host matrix proteins that are absorbed *in vivo*.[6,9]

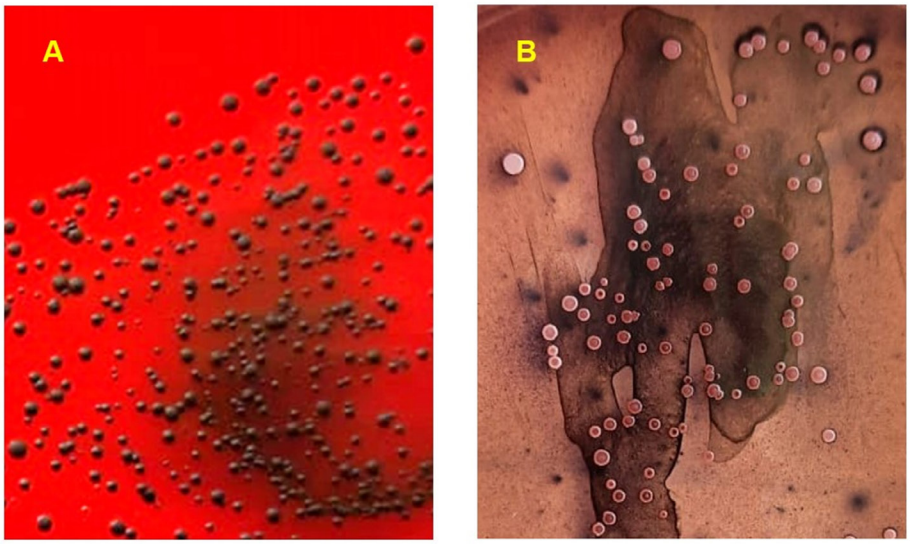

Figure 2. Colorimetric scale for colony analysis of slime production by *Staphylococcus aureus* 288 S15 and S11 using Congo Red agar assay. A: slime-producing strain (almost black); B: non- 289 producing strain (white)

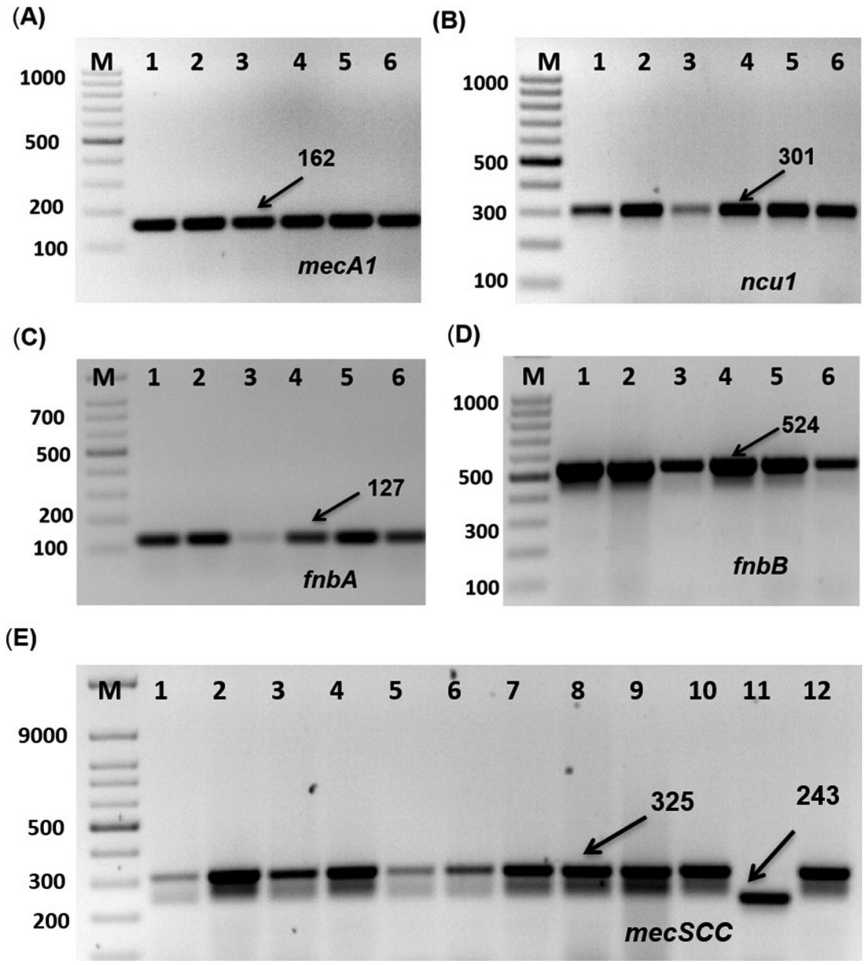

Figure 3. Amplification of Biofilm formation and MRSA genes of *Staphylococcus aureus* isolates by single PCR. (a) Amplification of *mecA1* gene (162 bp). (b) Amplification of *cnu1* gene (301 bp). (c) Amplification of *fnbA* gene (127 bp). (d) Amplification of *fnbB* gene (524 bp). (e) Amplification of *mecSccIII* and *mecSccV* genes (243 and 325bp). M: 100-bp DNA ladder

CONCLUSION

PCR is an easy, fast, and cheap way to characterize pathogenic *S. aureus* isolates capable of forming biofilms and carrying related genes such as fnbA and fnbB. In addition, MRSA isolates are resistant to many antibiotics and can cause health issues. The genes *mecA* and SCC*mec* are virulence markers in *Staphylococcus* isolates.

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FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by Taif University Medical Ethics Review Board (Project No. 1-437-5371).

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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