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Original Research Article

Random Amplified Polymorphic DNA (RAPD)-PCR analysis of genotypic and phenotypic characteristics of Methicillin-Resistant *Staphylococcus epidermidis* (MRSE) strains involved in biofilm formation

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Abstract

Purpose: *The current study aims to investigate genotypic and phenotypic aspects of methicillin-resistant Staphylococcus epidermidis (MRSE) strains involved in the biofilm formation and the attendance of the icaADB gene and genotypic characterization of this gene by random amplified polymorphic DNA (RAPD) PCR.*
Methods: *60 Staphylococcus epidermidis strains were isolated from clinical specimens, suspected of having the bacteria, from the laboratories of Isfahan. Biofilm formation was measured by the microtiter plate method. The attendance of biofilm formation genes was studied using PCR and all isolates producing biofilm (strong and moderate) were genetically classified by RAPD-PCR.*
Results: *37 isolates (61.7%) were MRSE and all positive biofilm strains. The prevalence of biofilm-related genes in the isolates was SesC (100%), SesI (45.9%), icaA (29.7%), icaB (37.8%), icaC (81.08%), icaD (70.2%), arcA (81.08%), and* opp3AB *(70%). PCR analysis showed that among 30 isolates of strong and medium biofilm production, 70% (21/30) positive for the icaADB gene. The Dendrograms obtained from RAPD-PCR results showed that all nine main clusters were at an 80% similarity level, and there were four isolates of a single type.*
Conclusion: *These findings confirmed the high genotypic diversity of biofilm-producing MRSE strains and the relative diffusion of specific clones among clinical samples.*

Keywords: *Biofilm formation, icaADBC gene, MRSE, RAPD-PCR*

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INTRODUCTION

Staphylococcus epidermidis (S. epidermidis) is an important part of the normal microbial flora of the skin and mucous membranes [1]. Today, this bacterium is responsible for the majority of infections, which are usually without severe clinical signs [2,3]. *S. epidermidis* is a major cause of nosocomial infections in infants and people with fixed medical prostheses. Biofilm formation is one of the important factors in the pathogenicity and antibiotic resistance of microorganisms in the environment [4]. Many *S. epidermidis* strains transport the *icaADBC*

operon that encodes proteins participating in the structure of the exopolysaccharide PIA (Polysaccharide Intercellular Adhesin). PIA is made through the rein of the intercellular adhesion (*ica*) locus. The *ica* locus comprises *icaR* (regulatory) and *ica ADBC* (biosynthetic) genes [5]. In *ica* genes, the *icaA* and *icaD* play a significant role in the maximal expression of biofilm formation [6-8]. Today, cell-wall-correlated surface proteins have been introduced as *S. epidermidis* surface (*Ses*) proteins (e.g., *SesI* and *SesC*). When patients are infected by *S. epidermidis* isolate, they show *SesI* and *SesC* which are responsible for bacterial biofilm adherence.

SesI consists of 201 amino acids and *SesC* includes 676 amino acids indicating the LPETG motif as a sortase substrate [7-9]. The *SesI* gene is expressed in invasive strains [7, 8]. *In vitro*, the amount of biofilm produced by each *S. epidermidis* isolate varies substantially. It is significantly affected by glucose as well as growth and environmental conditions [10, 11]. In *S. epidermidis*, arginine catabolic motility (ACME) is a new genomic island that enhances the colonization and biofilm capacity of *S. epidermidis* species in the skin and mucus, as well as encoding specific virulence factors. The organism's survival and pathogenesis are significant factors. ACME has a gene cluster of *opp3* and *arc* that are homological together and intervene in the transmission of bacterial diseases [12]. *S. epidermidis* can produce biofilm and develop drug resistance and tolerance to antimicrobial drugs by acquiring mobile genetic elements [12]. As a result, early detection and management of biofilm-forming *Staphylococci* are one of the required stages in the prevention and control of device-associated nosocomial infections [11]. Molecular markers are a valuable tool for the description and assessment of genetic diversity within species it has been shown that different markers and different classes of they show diversity. In this regard, the RAPD molecular marker is one of the most common molecular markers used in various fields. RAPD is a PCR-based marker in this regard RAPD-PCR is a cost-effective, fast, and reliable technology for pathogenic strain discrimination in this era of molecular taxonomy [13, 14, 15]. Understanding the types and origins of *S. epidermidis* isolates, particularly MRSE strains, is a definitive step for epidemiological superintendence characterization, and determination of circulating isolates and finally is useful for control of infections due to the above bacterium [15-17]. Therefore, the purpose of our study was to investigate genotypic and phenotypic aspects of MRSE strains involved in biofilm formation and identify the genetic variation of these strains by RAPD-PCR.

METHODS

The present study was approved by the Ethics Committee of Islamic Azad University - Shahrekord Branch, Iran (Code No. IR.IAU.SHK.REC.1401.022). A microbiologist from each medical diagnostic laboratory prescreened patients to determine their inclusion or exclusion in the study. Inclusion criteria included all samples that were related to people over 18 years, who had not taken any antibiotics for three weeks prior to the visit and had no history of hospitalization Exclusion criteria was failure to provide informed consent.

Bacterial isolates and identification

In this cross-sectional study that was conducted from May to August 2021, a total of 100 clinical samples suspected of *S. epidermidis* were collected from the medical diagnostic Nobel laboratory of Isfahan. The clinical samples included urine 35 samples, blood 25 samples, wounds 20 samples, catheters 10 samples, abscesses 5 samples, sputum 3 samples, and eyes 2 samples. Clinical samples were incubated at 37°C for 24 hours in blood agar (Merck, Germany). *S. epidermidis* strains were specifically identified using the following microbiological methods: observing colony morphology, performing Gram staining, assessing growth on mannitol salt agar, conducting tube coagulase tests, and evaluating susceptibility to the novobiocin assay. Once these methods confirmed the isolates, they were stored at -70°C for further analysis [18].

Bacterial DNA was extracted utilizing a DNA extraction kit (CinnaGen, Iran) and then detection of the *mecA* gene were performed as earlier reported by Havaei et al. (2015) [19].

The mixture for PCR amplification included the following components: PCR master mix contained 2.5 µl 10x Buffer, 0.5 µM each primer, 0.4 µl dNTP, 0.6 µl MgCl2, 5 µg template DNA and 15.2 µl ddH2O, 0.5 µl of each primer (10 pmol/ µl). Amplification conditions were initial denaturation at 94°C for 5 min; followed by 35 cycles each of denaturation at 94°C for 15s; annealing at 55°C for 15s; extension at 72°C for 5 min extension at 72°C for 5 min and final extension at 72°C for 5 min. The primer sequence used in this reaction is shown in Table 1 [7, 19-21].

Table 1: Target genes and oligonucleotide primers used for the detection of virulence factors in the *S. epidermidis* strains isolated from various types of clinical samples

Target gene	Primer sequence	PCR product (bp)	Reference
mec	F: TGGCTATCGTGTCACAATCG R: CTGGAACTTGTTGAGCAGAG	310	[19]
SesC	F: GTTGATAACCGTCAACAAGG R: CATGTTGATCTTTTGAATCCC	388	[7]
SesI	F :GCTGATTATGTAAATGACTCAAAT R: AGCTTTTGTTGTTTGAGCTTC	408	[20]
ica A	F: ACACTTGCTGGCGCAGTCAA R: TCTGGAACCAACATCCAACA	188	[21]
ica B	F: AGAATCGTGAAGTATAGAAAATT R: TCTAATCTTTTTCATGGAATCCGT	900	[21]
Ica C	F: ATGGGACGGATTCCATGAAAAAGA R: TAATAAGCATTAATGTTCAATT	1100	[21]
ica D	F: ATGGTCAAGCCCAGACAGAGR: AGTATTTTCAATGTTTAAAGCAA	198	[21]
arcA	F: CTAACACTGAACCCCAATG R: GAGCCAGAAGTACGCGAG	1946	[20]
opp3AB	F: GCAAATCTGTAAATGGTCTGTTC R: GAAGATTGGCAGCACAAAGTG	1183	[20]
icaADB	F: TTATCAATG CCGCAGTTGTC R: GTTTAACGCGAGTGCGCTAT	546	[20]

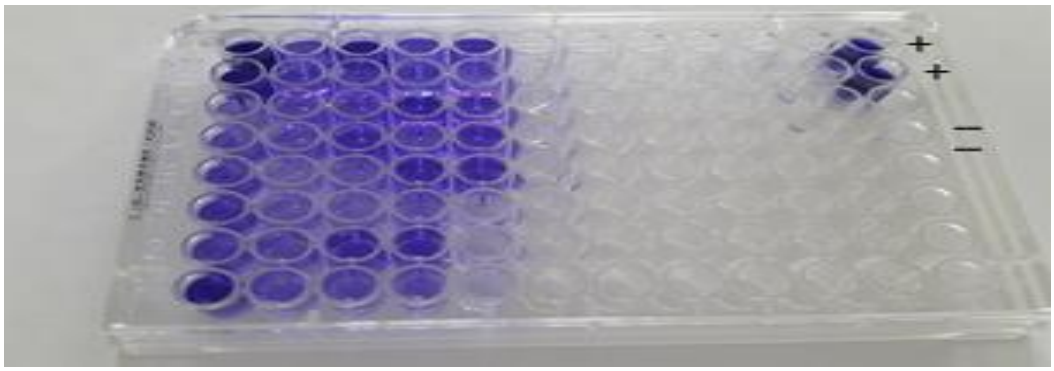

Figure 1: Biofilm formation among methicillin-resistant *S. epidermidis*

Microtiter plate test for detection of biofilm

Biofilm formation assays were carried out following a formerly described procedure [22] with little modifications. All isolates were incubated overnight in tryptic soy broth (TSB, Oxoid) plus 0.25% glucose (Merck, Germany) at 37 °C. A 1:10 dilution of bacterial culture in the TSB medium was used. Then, 200 mL of each dilution was distributed to each broth media in flat-bottom 96-well polystyrene plates and incubated for 24 hours at 37 °C without shaking. After an overnight, the content of each well was removed, and wells were washed with 150 µl of sterile saline to remove all non-adherent bacteria. The bacteria were fixed with 100% ethanol for 15 min. Then, the wells were air-dried for 20 min after which 200 µl crystal violet (1% w/v) solution was added for about 5 min. After the staining step, the wells were washed four times with distilled water to eliminate the additional stain. The bacterial cells were solubilized with 200 µL of 33% (v/v) glacial acetic acid and incubated at 37 °C for 15 min. Finally, the optical density (OD) of each well was read at 570 nm using an ELISA reader (Stat Fax-2100, Awareness Technology, Inc, USA). Each test was performed in triplicate. *S. epidermidis* isolates (*ATCC 35984*) and (*ATCC 12228*) were utilized as positive and negative controls, respectively. The adherence ability of the tested strains was categorized into four according to OD values Negative: ODs < 0.1; Positive: ODs: 0.1-1.199; moderate positive: ODs: 0.2-0.299 and ODs ≤0.3 (Figure 1) [22].

Antibiotic Susceptibility Testing

The antibiotic susceptibility testing was done according to the Kirby-Bauer method [23]. The following antibiotics (Padtan Teb, Iran) were utilized in this test: Oxacillin (1 µg), methicillin (5 µg), tobramycin (10 µg), nitrofurantoin (300 µg), chloramphenicol (30 µg), mupirocin (5 µg), tetracycline (30 µg), lincomycin (1 µg), clindamycin (2 µg), rifampicin (5 µg), gentamycin (10 µg). In line with CLSI (2019) guidelines, the size of inhibition zones by antibiotic disks for resistance and susceptibility were determined [24].

Detection of genes involved in biofilm formation

Genomic DNA was extracted using by DNA extraction kit (CinnaGen, Iran) according to the manufacturer's instructions. The primer sequence used for detection of biofilm-producing genes *icaA*, *icaB, icaC*, *icaD*, *SesC*, *SesI*, *icaADBC*, *arcA*, and *opp3AB* is shown in Table 1. For detection of biofilm-producing genes *icaA*, *icaB*, *icaC*, *icaD* the polymerase chain reaction was performed with a mixture of 9.5 µl DW, 1 µl primer, 1.5 µl MgCl2, 3 µl 10x buffer, 2.5 µl dNTPs, 2µl Taq polymerase, and 5µl DNA template. The amplification program consisted of initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94°C for 60 sec, annealing at 55 °C for 30 sec (*icaC) and then at* 55 °C for 60 sec (*icaA),* 55 °C for 30 sec (*icaD),* 52 °C for 30 sec (*icaB),* and extension at 72 °C for 60 sec and final step of 72 °C for 10 min [21] and the test method of Salgueiro et al. was used to amplify the *SesC* and *SesI*, *opp3AB*, and *arcA* genes [20]. The amplification conditions for these genes, initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 minute, primer annealing at 58 °C and extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 min [20].

Polymerase Chain Reaction tracing of the icaADB cluster

PCR tracing of the *icaADB* cluster was performed among all the isolates that were strong and moderate biofilm-producing using the sequence of a primer shown in Table 1 for amplification of the gene. The conditions for PCR amplification were: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 minute, primer annealing at 58 °C and extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 min. Amplification products were analyzed using 1.5% agarose gel with KBC power load dye (CinnaGen Co. Iran) [20].

RAPD-PCR profile

All isolates producing strong and moderate biofilms were genetically classified by RAPD-PCR according to the method introduced by Williams [25]. In brief, PCR amplification was carried out in a 25 µl total volume containing 1U Taq DNA polymerase, 2.5 µl of 10x PCR buffer, and 2 µl the primer OLP6 (5` GAGGGAAGAG 3`) and 50ng of DNA template. PCR amplifications were as follows: a cycle of pre-denaturation at 94 °C for 5 min, followed by 40 cycles of 1 min at 93°C, 1.30 min at 37°C, and 1 min at 72°C. A final extension step of 72°C for 8 min is included in all amplifications. Negative control of the same reaction mixture with water instead of chromosomal DNA was included in each run. Amplified PCR fragments were separated using electrophoresis in a 1.5% agarose gel, stained with KBC power load dye (CinnaGen Co. Iran), and scanned by the Gel Documentation Systems. RAPD profiles were analyzed based on the number and position of the major bands according to the Dice formula and similarity coefficients for each pair of lanes and a dendrogram were generated based on the unweighted pair group method with averages (UPGMA) using GelJ software, as described previously [26]. Isolates with a similarity coefficient equal to or above 80% were clustered as the same genotypes.

RESULTS

From 100 clinical samples, 60 *S. epidermidis* isolates were isolated out of which 37 isolates were MRSE (Figure 1) all of which were positive for biofilm formation. Among the *S. epidermidis* strains, strong biofilm formation was observed in 72.9% (27/37), while 8.1% (3/37) displayed moderate biofilm formation, and 18.9% (7/37) exhibited weak biofilm formation. Table 2 shows the percentage of biofilm production of isolates in different clinical samples.

As shown in Table 3, the isolates showed high resistance to oxacillin (91.8%), tobramycin (64.8%), and methicillin (62.1%), but less resistant to mupirocin (27.02%), and nitrofurantoin (10.8%). The prevalence of biofilm constitutive genes was 100% (37/37) *SesC*, 45.9% (17/37*) SesI*, 29.7% (11/37*) icaA*, 37.8% (14/37) *icaB*, 81.08% (30/37) *icaC*, and *icaD* 70.2% (26/37), 81.08% (30/37) *arcA*, and 70% (21/37) *opp3AB* (Figure 2).

In this research, there were a total of 30 (30/37) isolates producing strong and moderate biofilms. The analysis of PCR results showed that among strong and moderate biofilm-producing isolates,

Table 2: Distribution of 37 biofilm forming of MRSE strains according to different clinical samples

Bacteria/ Samples	Biofilm formation					
	High		Moderate		Weak	
	N0	%	NO	%	NO	%
Urine	16	43.2%	2	5.4%	3	8.1%
Blood	7	18.9%	1	2.7%	2	5.4
wound	3	8.1%	0	0	1	2.7
catather	1	2.7	0	0	1	2.7
total	27	72.9%	3	8.1%	7	18.9%

Table 3: Antibiotic-resistance pattern of 37 biofilm-producing MRSE isolates

Antibiotic	No. of strains	Resistance (%)
oxacillin	34	91.8
Erythromycin	28	75.67
Tetracycline	26	70.27
tobramycin	24	64.8
methicillin	23	62.1
Lincomycin	18	48.64
Clindamycin	17	45.94
chloramphenicol	19	51.35
rifampin	20	54
mupirocin	10	27.02
nitrofurantoin	4	10.8
Vancomycin	0	0

70% (21/30) of isolates were positive for the *icaADB* gene (Figure 2). RAPD-PCR was used to study all 30 isolates producing strong and moderate biofilms *S. epidermidis*. The method that we used was able to classify all 30 isolates in this investigation. DNA profiles generated using primer OLP6 showed 1-13 bands ranging in size from 150 to 2000bp. The dendrogram indicated 9 major clusters on a similarity level of 80% and 4 isolates were single type (Figure 3). From 30 *S. epidermidis* strains, 7 strains originated from cluster A that shared ≥80% similarity. Cluster A had the most isolates, with seven, followed by clusters B, C, and D, which each contained three. All of the isolates in cluster

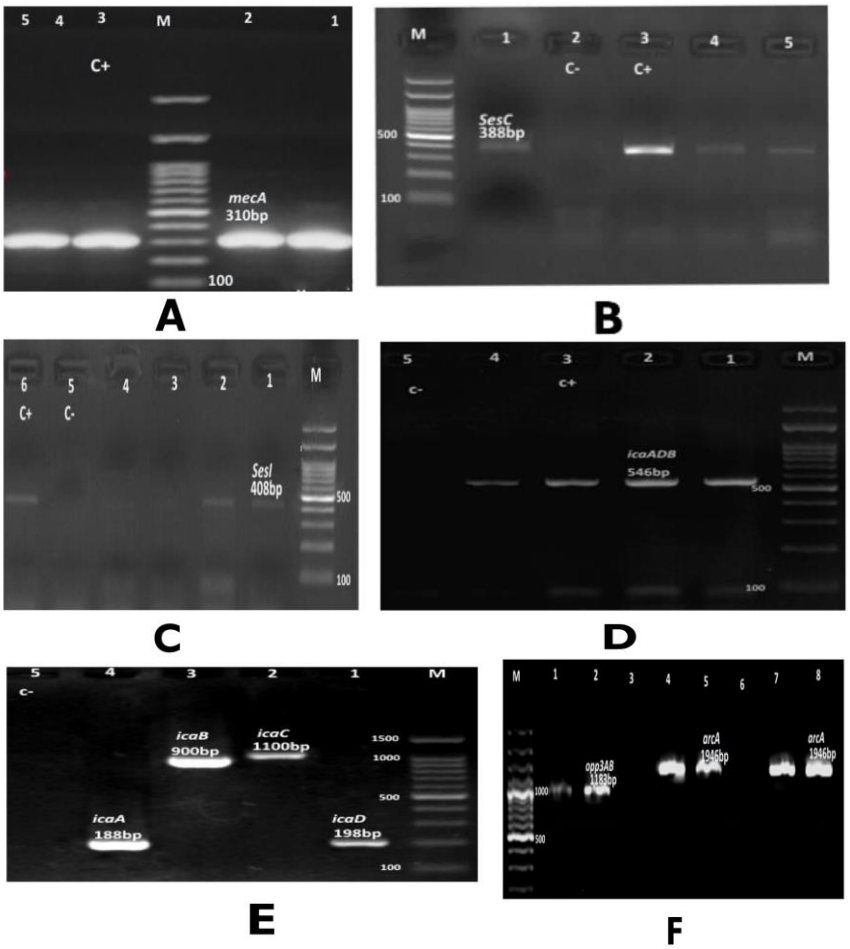

Figure 2: Electrophoresis gel images of *mecA* (A), *SesC* (B), *SesI* (C), *icaADB* (D) genes, multiplex PCR amplification of *icaADBC* (E) as well as *arcA* and *opp3AB* genes (F)

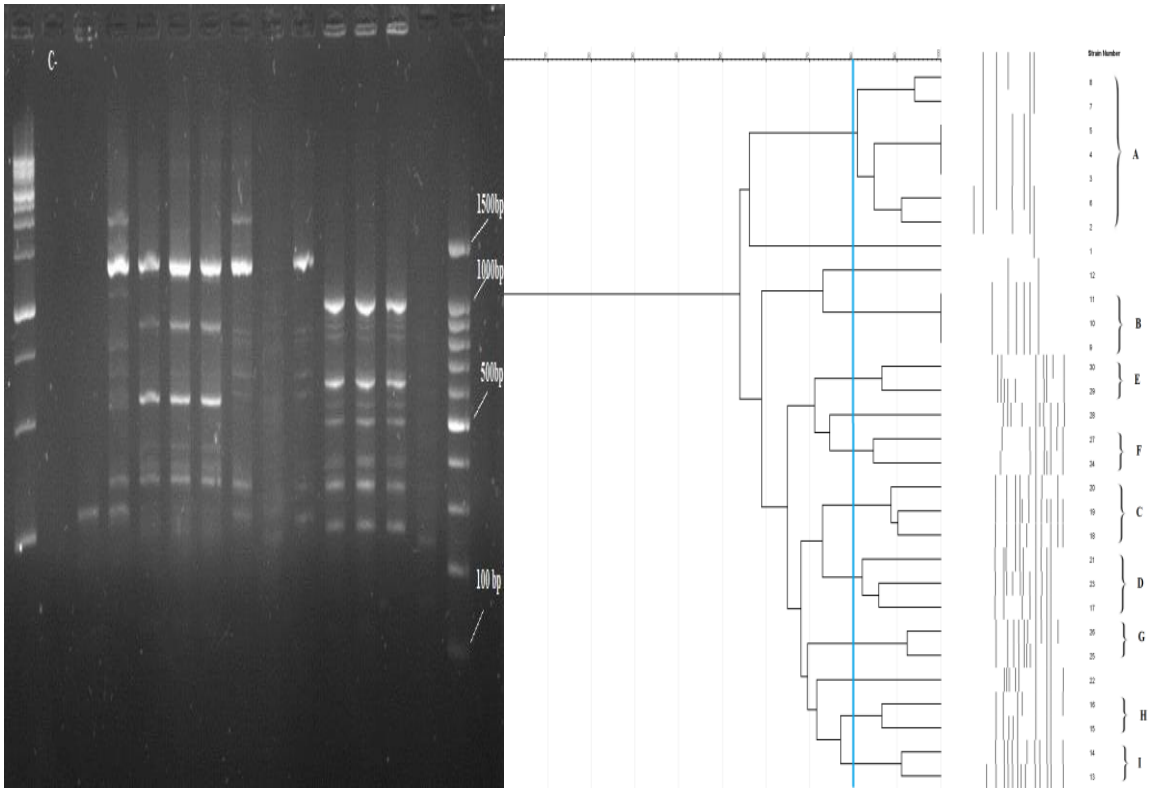

Figure 3: Cluster analysis of MRSE isolates based on Random Amplified Polymorphic DNA typing

Table 4: Distribution of the *icaADB* gene among 30 biofilm-forming (strong and moderate) strains of MRSE according to RAPD_PCR method

No. of isolates	Biofilm formation	*icaADB*	RAPD type
1	Strong	+	Single
2	Strong	+	A
3	Strong	+	A
4	Strong	+	A
5	Strong	+	A
6	Strong	+	A
7	Strong	+	A
8	Strong	+	A
9	Strong	-	B
10	Strong	+	B
11	Strong	+	B
12	Strong	-	Single
13	Strong	+	I
14	Strong	+	I
15	Strong	+	H
16	Strong	+	H
17	Strong	_	D
18	Strong	+	C
19	Strong	+	C
20	Strong	+	C
21	Moderate	-	D
22	Strong	+	Single
23	Moderate	-	D
24	Strong	-	F
25	Moderate	-	F
26	Strong	+	G
27	Strong	-	G
28	Strong	-	Single
29	Strong	+	E
30	Strong	+	E

A were strong biofilm-producing and positive for the *icaADB* gene. Overall, RAPD-PCR showed heterogeneity among the *S. epidermidis* isolates. Table 4 describes the distribution of the *icaADB* gene among 30 biofilm-forming (strong and moderate) strains of MRSE according to the RAPD-PCR method.

DISCUSSION

This study indicates a high prevalence of antibiotic-resistant in the biofilms of MRSE strains. The isolates showed less resistance to mupirocin and nitrofurantoin. Percentage of biofilm production in urine isolates was higher than in other clinical samples.

The high prevalence of biofilm formation of *S. epidermidis* isolates has been reported previously [30-32] Similar to our research, in the study conducted by Borooni et al (2019), t all isolates of *Staphylococcus epidermidis* had the ability to produce biofilm and the prevalence of biofilm-producing genes was *icaA* (32.6%), *icaB* (25.4%), *icaC* (72.3%), *icaD* (64.8%) [30]. Pinheiro et al. (2014) observed *icaADBC* operon in 38.3% of strains; with 58.5 % producing a biofilm [33]. Mekni et al. (2017) reported *icaADBC* genes in 80.7% of the biofilm producer isolates [34]. Moreover, Salgueiro et al. (2017) [20] reported that the *icaADB* genes were observed in 81.2% of the biofilm creator isolates

[20]. The outcomes of the two studies align with our study. Mottaghiyan et al. (2019) found that 71.2% of the isolates were capable of producing biofilms and the prevalence of *icaABD* was 52.2% [35].

An important group of *S. epidermidis* surface (*Ses*) proteins in the biofilm are known for their potency to attach to host cells, immunogenicity, and association with invasive strains. Anti-*SesC* antibodies are utilized to prevent the establishment of biofilms by isolates of *S. epidermidis,* because the *SesC* sequence in *S. epidermidis* is highly protected [7,8]. Khodaparast et al. 2016 reported that the *SesC* gene is found in all *S. epidermidis* isolates and can be used as a genetic marker to distinguish *S. epidermidis* from other isolates [7]. Fard et al. (2015) found that out of 40 CoNS, every 20 isolates identified as *S. epidermidis* were *SesC* positive. [8]. *SesC* is an important virulence factor associated with adhesion and Qi X et al. (2018) demonstrated that the prevalence of *SesI* in *S. epidermidis* invasive isolates was significantly higher compared with noninvasive isolates [27]. Also, Soderquist et al. (2009) indicated that there is no *SesI* in the normal *S. epidermidis* flora of healthy subjects, but, it was detected in nearly 50% of invasive isolates correlated with invasive infections [36]. *ACME-arcA* is a genomic isle and a safe sequence in *Staphylococcus* that roles in retention [12, 35]. Diep et al. (2016) showed that 60% of *S. epidermidis* isolates were positive for *arcA* [37]. In study conducted by Xu et al. (2018) the outbreak of *arcA* was reported in 67.7% of MRSE isolates, and 74.1 of *S. epidermidis* isolates, and 83.3% of Methicillin-sensitive *S. epidermidis* (MSSE) strains. [29]. It was determined in the report done by Shamansouri et al. (2016) the *arcA* gene may promote strain with many drugs resistance and 14 (20.58%) of MRSE strains were positive for *arcA* [12]. The distribution of arc and opp3 clusters in *S. epidermidis* was reported by Du et al. (2013) to be 71.6 percent and 32.4 percent, respectively [36]. Machado et al. (2021) reported all strains positive for ACME cluster *opp3AB* were also positive for *arcA.* The outcome of this survey was close to ours [38]. Consequently, the results of our and many studies suggest that due to the high rate of biofilm-formation of *S. epidermidis* isolates, paying attention and successful management of these isolates is recommended. The molecular typing method was evaluated not only for its potential to reveal possible differences in properties between strains but also for its ability to help in the identification of related colonies of these bacteria among various hosts and sources [39].

Today, PCR-based typing techniques are impressive methods to investigate the strain source, the clonal association between strains, and epidemiology. RAPD-PCR typing is a simple, beneficial, and cost-beneficial method that has been commonly used for the genetic diversity of MRSE isolates at a regional scale [40, 41]. A dendrogram analysis revealed 9 clusters with a similarity of 80%, which shows the high diversity of the studied isolates in our study. Out of the 30 strong and moderate biofilm-producing isolates, 7 strains originated from cluster A and all of them carried the i*caADB* genes indicating their significant similarity and the potential of a relatively common origin of spreading in our setting. In addition, clusters B, C, and D, each with three isolates, are the common source of strain expansion in our setting. In a study by Zare et al. (2019). RAPD-PCR of *S. aureus* was performed via OLP6primer. It revealed four groups of a similar clone [13]. In addition, Maghsoudloo et al. made a molecular pattern of 60 *S. epidermidis* strains using the RAPD-PCR technique. Consistent with our findings, their results identified 24 species of RAPD indicating high genotypic variation in *S. epidermidis* isolates [42].

CONCLUSION

This study showed the high genotypic diversity of biofilm-producing in MRSE strains. The use of molecular typing methods such as MLST technique in addition to the RAPD-PCR technique is suggested to check the genetic diversity of MRSE strains and check specific clones among them**.**

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. P.B.: investigation, formal analysis and writing—original draft; E.T.: conceptualization, writing—review and editing, funding acquisition, supervision. All authors discussed the results and agreed on the final manuscript.

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