RESEARCH ARTICLE

The Role of Genetic Variation for *ica*A Gene *Staphylococcus aureus* in Producing Biofilm

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ABSTRACT

The current study aimed to evaluate the *in vitro* biofilm production and the presence of the *ica*A gene in *Staphylococcus aureus* isolated from burn samples. 45 burn swabs specimens were collected from the patients at the Burns Unit of Al –Kindy Hospital; S. *aureus* isolates identified by biochemical tests and confirmed the tests by VITEK-2 System. The isolates of *S. aureus* were tested for biofilm production in Congo red agar and for presence of *ica*A gene presence by PCR. Products of polymerase chain reaction (PCR) were sequenced and aligned with the previous recorded sequences online. Out of 45 specimens, 16 (35.5%) were identified as *S. aureus*. A weak correlation between the presence *ica*A gene that detected by PCR and the formation of biofilm on Congo red agar plates. Out of 16 isolates as screened by CRA method, 11 isolates (68.7%) were biofilm producing, and 5 isolates (31.3%) were non-producing biofilm while the results of PCR showed that 13 isolates (81.3%) possesses *icaA* gene, 2/13 isolates were not produce biofilm on CRA plates, but positive for *ica*A gene. The obtained data form this study suggests that the presence of *ica*A gene requires for ability of isolate to form biofilm on Congo red plates but it is affected by genotyping variation of *ica*A gene.

Keywords: Biofilm, CRA, *ica*A gene, PCR, Sequencing, *Staphylococcus aureus.*

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INTRODUCTION

Staphylococcus aureus is one of the most frequent pathogens in hospitals that cause a wide variety of infections. Historically, *Staphylococci* were the commonest organism causing burn wound infection in early part of the century.[1] The ability of this bacterium to produce biofilm as a risk factor contributing to chronic or persistent infections.[2] Biofilm is defined as the organization of microorganisms on different surfaces, it is often linked with burns infection that provides a suitable site for colonization bacteria and may support complex biofilm flora with large numbers of bacteria, including *S. aureus*. The ability of *S. aureus* to form biofilm increased antibiotics resistance and the spread of multi-drug resistance strains within the hospital environment.[3,4]

Biofilm formation is mediated by the products of intercellular adhesion (*ica*) locus, this locus consists of *icaADBC* operon that contains 4 genes encoding the essential proteins for the production of polysaccharide intercellular adhesion (PIA) in *Staphylococcus spp.*[5], and the expression of *ica* genes for biofilm production is very variable in *S. aureus* strains. Although numerous genes are involved in biofilm formation, the *icaA* gene is the specific gene detects in *S. aureus* strains associated with the ability of the production biofilm.[7,8] The product of *icaA* gene is a protein named transmembrane; this protein, with *N*-acetylglucosaminyl transferases enzymatic activity, led to the synthesis poly-*N*-acetyl glucosamine polymer.[9] Thus, the current study aimed to evaluate the correlation between biofilm production and the presence of *icaA* gene associated with *variation for this gene* in the S. *aureus* isolated from burn cases.

METHODS

Isolation and Phenotypic Identification of *S. aureus*

Forty-five specimens were collected by swabbing from burn infections of hospitalized patients, and specimens were obtained from the burn unit of AL-Kindy hospital (Baghdad) for 4 months from September 2019 to January 2020. All isolates were identified as S. *aureus* by standard laboratory[10] and the procedure described in Figure 1.

All swabs were inoculated on the brain, heart infusion broth (BHI), incubated at 37°C for 24 hours, and the suspension was streaked onto Manitol Salt Agar (MSA). The suspected isolates were tested by gram stain and colonial morphology

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Table 1: Primer used to amplify biofilm gene *(ica*A) in this study

Primer type	*Primer sequence (5'-3')*	*Product size (bp)*	*Reference*
*ica*A-F	5'-ACTTGCTGGCGCAGTCAATA-3'	630	design by a researchers
*ica*A-R	5'- GACCATGTTGCGTAACCACC-3'		

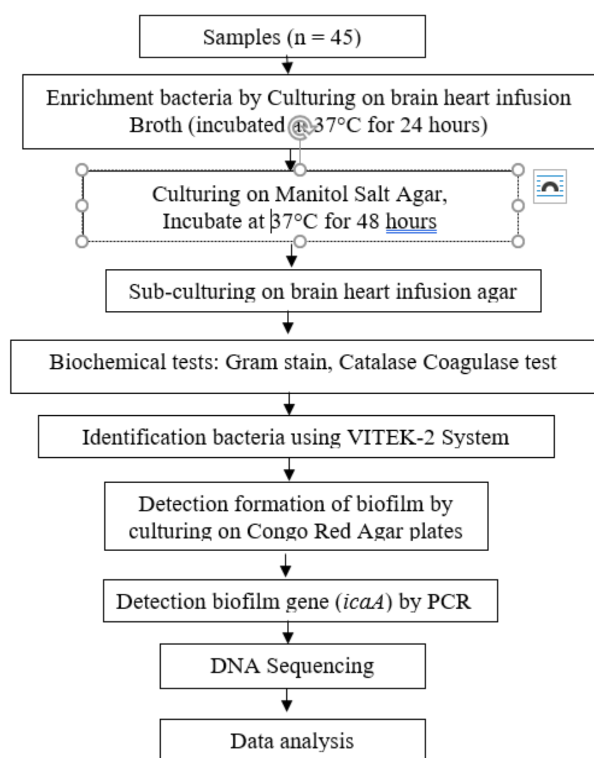

Figure 1: The procedure of the study

of *Staphylococcus* on (MSA). Isolates were identified as *S. aureus* by biochemical tests like catalase and coagulase, then confirmed the results by VITEK-2 System (BioMerieux-France).

Production of biofilm on Congo Red Agar (CRA) Plates

The qualitative test for biofilm production was performed by culturing *S. aureus* isolates on Congo Red Agar plates (CRA).[11] The colonies were streaked on CRA plates and were incubated aerobically for 24 hours at 37°C. Red colonies were recorded as non-biofilm-producing isolates, while black was recognized as biofilm-producing isolates.

The study's reference strain of S. aureus (ATCC 6538) was supplied from the Ministry of Science and Technology / Microbiology Division and equipped with diagnostic kits included in the study as biofilm-positive control.

Primer design and genotyping characterizations of *S. aureus*

Extraction genomic DNA

Genomic DNA was extracted from all isolates of *S. aureus* by using genomic DNA extraction kit (Promega, USA) supplemented with lysozyme enzyme (30 µg/mL). *Concentrations and purity of DNA (ng/µl) were* measured *by Nanodrop (*ThermoFisher/ USA).

Detection of Biofilm gene (icaA)

Isolates of *S. aureus* were examined for the presence of biofilm gene (*ica*A), PCR is used to amplify (*ica*A) gene using *Sequence-Specific Primer (SSP-PCR). For designing primers, and free online programs were used as below:*

http:// primer3plus.com/cgi-bin/dev/primer3plus.cgi and http:// eu. idtdna. com/ calc/ analyzer *In addition, the primer blasting was made.* https:// blast. ncbi. nlm. nih. gov/ Blast. cgi

Primers of (*ica*A) gene were provided by DNA Alpha/ Canada, *and the PCR program was designed according to its specific primer characters.*

Optimal conditions are identified in the methodology. Several experiments have proved the purpose of reaching these conditions. The 25 µL reaction mixture of PCR is containing 12 µL master mix (1 X), 4 µl DNA template (200 ng/µL), 1 µL forward and 1 µL reverse primers (10 pm/µL) and 7 µL deionized water. The PCR program was carried out with the following reaction: an initial denaturation at 95°C for 5 minutes. for one cycle followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 56°C for 40 seconds, and extension at 72°C for 1 minutes, and final extension for one cycle at 72°C for 5 minutes.

The amplified DNA products were separated by electrophoresis on 1.5% agarose gel at 70 volts for 90 min, using the 2000-bp DNA ladder and stained with 0.3 µg/mL ethidium bromide (Biotium, USA) and examined DNA bands under ultraviolet light using gel documentation system.

DNA Sequencing

All the PCR products were purified by QIAquick PCR purification kit (QIAGEN, USA) according to the manufacturer's guidelines and were sent to Macrogen company / Korea for DNA sequencing, DNA sequence analysis was performed by using Geneious software 11.1.5 and other software (for data sent by the Macrogen/Korea sequence service). Sequences were analyzed using the NCBI BLAST program to detect SNPs and any other change within genomic areas studied.

RESULTS

From a total of 45 burn specimens, 16 (35.5%) isolates of *S. aureus* were identified by bacteriological and biochemical tests. First, *S. aureus* isolates appeared yellow colonies on mannitol salt agar; gram stain (+), catalase, and coagulase (+), then the isolates were confirmed as *S. aureus* by VITEK-2 compact device.

In this study, the CRA test is a simple qualitative phenotypic method was used to detect biofilm production. Out of 16 *S. aureus* isolates,11 (68.7 %) isolates were produced black colonies with dry crystalline density on CRA, while 5 (31.3%) isolates did not produce biofilm and showed red colonies on CRA (Figure 2).

Detection *ica*A gene by PCR and the correlation with biofilm formation

PCR is used to identify the gene responsible for the formation of biofilm among 16 isolates of *S. aureus. The* 13 isolates (81.3%) have the *icaA* gene (molecular weight is 630 bp) while only 3 isolates (18.7%) do not have it as shown in Figure 3, among 13 *ica*A positive isolates, 11 isolates were previously positive for biofilm production on Congo Red Agar while 2 isolates that were not produced biofilm on this media as shown in Table 2.

Sequencing *icaA* gene of *S. aureus*

The 13 *ica*A positive isolates detected by PCR, 11/13 isolates were positive for biofilm production on congo red agar, while 2 isolates (isolates number 2 and 7) were not produced biofilm. Therefore, the biofilm formation test results should be confirmed by genotypic characterization methods to detect (*ica*A).

To evaluate the role of *ica*A gene sequence, all 13 isolates were sent for sequencing. The sequences of the nitrogen bases were determined after reading the DNA sequences of the forward and reverse for strands, recollecting the two strands, deleting the anomalies in either of them, analyzing them, and matching them to NCBI online at (www.ncbi.nlm.nih.gov). At the same time, Geneious Software has shown that reference sequences are very similar to *icaA* gene sequences 99 % (Table 3). Comparing the observed DNA sequences of these samples with their stored reference sequences (Gen Bank: CP002388.1.1).

According to Table 3, the lower value of expecting equal to zero indicates that the studied sequence identical with reference sequence, besides the higher value of bites (817), indicates a great degree of similarity.

Analyzing the present results of the sequencing of *icaA* gene the data in Table 4 and Figure 4 showed that 1 (4%) Insertion mutation, others were substitution type, 14 (56%) of them transition and 10 (40%) of them transversion mutations, while isolates number 8,10,11,12,13 showed no variation comparing to reference data at NCBI.

Figure 2: Biofilm production on Congo Red Agar of the test isolates.

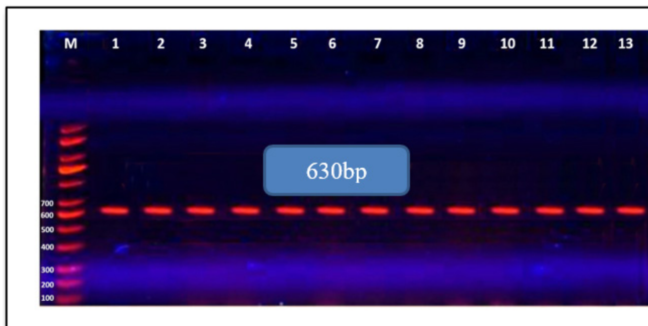

Figure 3: Gel electrophoresis of PCR products from DNA of *S. aureus* using primer *icaA* (630bp). The electrophoresis was performed at 70 volt/cm for 90 min. M: DNA Ladder (2000bp), lanes 1-13: positive results of *ica*A gene.

Table 2: Association between biofilm formation as detected by CRA plates and the presence of biofilm *ica*A gene as detected by PCR in *S. aureus* isolates.

	Biofilm formation on Congo Red Agar plates	*Biofilm gene (icaA) by PCR*
Result	*No.(%) of isolates*	*No.(%) of isolates*
Positive	11(68.7%)	13(81.3%)
Negative	5(31.3%)	3(18.7%)

DISCUSSION

S. aureus is one of the main causes of nosocomial infections, the nosocomial infections of burn wound are considered the major health problems in the world. In the present study, 35.5% of *S. aureus* isolates were found in samples obtained from burn

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Staphylococcus aureus subsp. aureus 55/2053, complete genome
Sequence ID: CP002388.1  Length: 2756919  Number of Matches: 1

Range 1: 2706717 to 2707544 GenBank  Graphics          ▼ Next Match  ▲ Previous Match

Score            Expect  Identities      Gaps          Strand
1509 bits(817)   0.0     826/830(99%)    2/830(0%)     Plus/Plus

Query  1        ATGATTATGTAATGTGCTTGGATGCAGATACTATCGTTGATCAAGATGCACCATATTATA  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2706717  ATGATTATGTAATGTGCTTGGATGCAGATACTATCGTTGATCAAGATGCACCATATTATA  2706776

Query  61       TGATTGAGAATTTCAAACATGATCCAAAACTTGGTGCAGTTACAGGTAATCCTAGAATTC  120
                ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2706777  TGATTGAAAATTTCAAACATGATCCAAAACTTGGTGCAGTTACAGGTAATCCTAGAATTC  2706836

Query  121      GAAATAAGAGTTCTATTTTAGGTAAAATTCAAACGATAGAATATGCAAGTTTAATTGGCT  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2706837  GAAATAAGAGTTCTATTTTAGGTAAAATTCAAACGATAGAATATGCAAGTTTAATTGGCT  2706896

Query  181      GTATTAAGCGAAGTCAGACACTTGCTGGCGCAGTCAATACTATTTCGGGTGTCTTCACTC  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2706897  GTATTAAGCGAAGTCAGACACTTGCTGGCGCAGTCAATACTATTTCGGGTGTCTTCACTC  2706956

Query  241      TATTTaaaaaaaGTGCAGTTGTCGACGTTGGCTACTGGGATACTGATATGATTACCGAAG  300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2706957  TATTTAAAAAAAGTGCAGTTGTCGACGTTGGCTACTGGGATACTGATATGATTACCGAAG  2707016

Query  301      ATATTGCAGTTTCTTGGAAATTGCATTTACGTGGATATCGTATTAAGTATGAACCGCTTG  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2707017  ATATTGCAGTTTCTTGGAAATTGCATTTACGTGGATATCGTATTAAGTATGAACCGCTTG  2707076

Query  361      CCATGTGTTGGATGTTGGTTCCAGAAACATTGGGAGGTCTTTGGAAGCAACGCGTGAGAT  420
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2707077  CCATGTGTTGGATGTTGGTTCCAGAAACATTGGGAGGTCTTTGGAAGCAACGCGTGAGAT  2707136

Query  421      GGGCTCAAGGGGGACACGAAGTATTACTACGAGACTTATTATTAGCACAATGAAAACGAA  480
                ||||||||||||||||||||||||||||||||||||| || |||||||||||||||||||
Sbjct  2707137  GGGCTCAAGGGGGACACGAAGTATTACTACGAGACTT-TT-TTAGCACAATGAAAACGAA  2707194

Query  481      AAGGTTTCCTTTATATATTTTGATGTTTGAGCAAATCATCTCGATTTTATGGGTATATAT  540
                ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  2707195  AAGGTTTCCTTTATATATTTTGATGTTTGAGCAAATCATCTCAATTTTATGGGTATATAT  2707254

Query  541      AGTGCTTCTATATTTAGGCTATTTGTTCATAACAGCAAACTTCTTAGACTATACATTTAT  600
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2707255  AGTGCTTCTATATTTAGGCTATTTGTTCATAACAGCAAACTTCTTAGACTATACATTTAT  2707314

Query  601      GACATATAGTTTTTCAATATTTCTACTATCATCATTTACTATGACTTTTATAAACGTTAT  660
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2707315  GACATATAGTTTTTCAATATTTCTACTATCATCATTTACTATGACTTTTATAAACGTTAT  2707374

Query  661      TCAATTTACAGTCGCACTCTTTATTGATAGTCGCTACGAGAAAAAGAATATGGCTGGACT  720
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2707375  TCAATTTACAGTCGCACTCTTTATTGATAGTCGCTACGAGAAAAAGAATATGGCTGGACT  2707434

Query  721      CATATTTGTAAGTTGGTATCCGACAGTATACTGGATTATTAACGCAGCAGTAGTTCTTGT  780
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2707435  CATATTTGTAAGTTGGTATCCGACAGTATACTGGATTATTAACGCAGCAGTAGTTCTTGT  2707494

Query  781      CGCATTTCCAAAAGCATTAAAACGTAAGAAAGGTGGTTACGCAACATGGT  830
                ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2707495  CGCATTTCCAAAAGCATTAAAACGTAAGAAAGGTGGTTACGCAACATGGT  2707544
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Figure 4: A representative sequence alignment of *icaA* gene segment 630 bp of primer amplification results with NCBI.

Table 3: Sequencing ID, Score, and identities for *icaA* gene from Staphylococcus aureus sub sp. Aureus 55/2053, complete genome**.**

Accession	*Identities*	*Score*	*Expect*	*Graps*
ID:CP002388.1	826/830 (99%)	1509bits (817)	0.0	2/830/(0%)

Table 4: *icaA*: Changes in the nitrogen bases of sequences of isolates of *Staphylococcus aureus* Gene Bank: CP002388.1.

No. of sample	*Wild type*	*Mutnt type*	*Location*	*Change in amino acid*	*Type of mutation*	*Effect*	*Type of substitution*
2	TT-	TTT	2706781-2706782	Insertion T	Insertion	Insertion	Frameshift
1,2,3,4, 5,6,7	GAA	GAG	2706784	Glu< Glu	Substitution	Silent	Transition
4	AGG	TGG	2706820	Trp< Arg	Substitution	Missense	Transversion
4	CAG	CAA	2706929	Gln< Gln	Substitution	Silent	Transition
9	TCA	TTA	2706952	Leu < Ser	Substitution	Missense	Transition
7	GAG	GAC	2707169	Asp < Glu	Substitution	Missense	Transversion
1, 2, 4	TTT	TAT	2707172	Tyr < Phe	Substitution	Missense	Transversion
7	TAG	TAT	2707179	Tyr < *	Substitution	Missense	Transversion
7	AAT	CAT	2707183	His < Thr	Substitution	Missense	Transversion
7	AAG	AAT	2707197	Asn < Lys	Substitution	Missense	Transversion
7	TTT	ATT	2707204	Ile < Phe	Substitution	Missense	Transversion
1,6,7	AAT	GAT	2707237	Asp < Asn	Substitution	Missense	Transition
2	AGC	AAC	2707480	Asn < Ser	Substitution	Missense	Transition
2	AGA	ACA	2707522	Thr < Arg	Substitution	Missense	Transversion
2	AAG	GAG	2707524	Glu < Lys	Substitution	Missense	Transition
8,10,1112,13	-	-	-	-	-	-	-

patients, this result was in agreement with the finding reported by Alwash and Saleh,[12] Hatem[13] in Iraq, and Omidi *et al*.[8]

In Iran, which described the occurrence of *S. aureus* in burns was 33.3, 35, and 33.6% respectively, but didn't agree with other studies performed in Iraq by Alwan *et al*.,[14] Hussien *et al*.,[15] and Hezam *et al*.[16] It reported a low frequency of *S. aureus* and also in Pakistan[1] and Saudi Arabia.[17] In contrast, a high prevalence of *S. aureus* (69.5%) was obtained from burn unit in Ethiopia.[18]

The prevalence of *S. aureus* strains varies in different geographic regions; the high percentage of *S. aureus* isolated from burns patients is due to many reasons. The reasons include the burn provide a suitable site for bacterial growth and is more persistent richer sources of infection, long-term hospitalization, and overcrowding in burns units is an important cause of cross-infection.

Biofilm formation is a major virulence factor that protects bacteria against host immunity and antibiotics treatment and increases the ability of bacteria to adhesion and colonization on surfaces.[19] In the current study, biofilm production of *S. aureus* by using CRA plates shown that 68.7% of isolates produced biofilm, this result was in accordance with the result reported by Arslan and Özkardes[20] and Hatem[13] but not in accordance with Al-Hadban *et al*.[21] who found that (38%) of isolates were biofilm producers in Congo Red Agar. In contrast, a higher rate of biofilm formation was reported by Gad *et al*.[22] where 83.3% of *S. aureus* isolates, this difference between various studies might be due to heterogeneity in the origins of strains, source of isolation, environment, and the presence of the biofilm-associated genes and their expression.[23] Mertens and Ghebremedhin[24] reported that biofilm production influenced by environmental signals and induced in response to external stress and inhibitory concentrations of certain antibiotics,

Our findings pointed to an important role of the *icaA* due to the ability to produce biofilm in a high percentage (81.3%) of *S.aureus* isolates collected from patients with burns. The obtained results agree with those of Zmantar *et al*.[25] and Mir *et al*.[26] as they recorded the existence of the *ica*A gene in about 78.3% and 72%, respectively.

Studies by Serray *et al*.[7] and Omidi *et al*.[8] proved that although different genes are involved in biofilm production, the *icaA* gene was the specific gene detected in *S.aureus* isolates that essential for biofilm production. In the current study, when the correlation between the presence of *ica*A gene detected by PCR and biofilm production detected by CRA method, the results obtained from the two methods were not similar. By CRA method, 11 (68.7%) isolates of *S. aureus* were biofilm positive, and 5 (31.3%) isolates were negative biofilm, while by PCR, 13 (81.3%) isolates were carried *icaA (ica*A+*)* and 3(18.7%) were *icaA*-, these results indicate that not all isolates carried *icaA* gene were able to form biofilm *in-vitro*. Hence, the ability of *S.aureus* isolates form biofilm in CRA requires the presence of *icaA* gene, and it is affected by growth conditions. This result agreed with the study by Al-Hadban,[21] who found a week correlation between the presence of *icaA* gene by PCR compared with Congo red agar method. Zhou *et al*.,[27] and Mohamad[28] reported that the co-expression of *icaA* with another gene such as *icaD* could increase biofilm production remarkably. Keikhaie *et al*.[29] also reported that the capability of *S. aureus* to form biofilm might be caused by the differences in the containing of biofilm-related genes which make -up with the physiological situation.

By analyzing the results of the sequencing of *ica*A gene according to table 4, the data showed that 1 (4%) Insertion mutation, others was substitution type, 14 (56%) of the transition and 10 (40%) of them transversion mutations, therefore. The results show that the type and location of

variations found in present sequence may result in different mutations. Moreover, some of these mutations lead to changes in the genetic codes and change in the amino acids, consequently changing translation products. This finding is one of the most important reasons that may increase the resistance of bacteria depending on the new variation in the target gene.[30] Moreover, the high variation of the icaA gene occurs in the extraction DNA from non-producing biofilm, Which belong to isolate DNA No. 2 and 7 that may be related to non-producing biofilm even with the presence of the icaA gene genome. At the same time isolates number 8, 10,11,12,13 were without genetic variation compared to reference sequence at NCBI; however, all last isolates produced biofilm as *ica*A+.

Finally, from the results of PCR and genotyping analysis, an exciting finding could be concluded in which the presence and genetic variation of *ica*A gene related to the capability of *S. aureus* clinical isolates to form biofilm. That means the different structure and diversity may be caused by the differences in biofilm-related genes, which make up with physiological situation.[29] The current interesting results need more research on the other parts of icaA genes in S.aureus strains and other bacteria.

CONCLUSION

The present study concludes that not all present *S. aureus i*solates were capable of forming biofilm; however, the presence of *icaA* gene was not always associated with biofilm formation in vitro, the genetic variation in *icaA* gene sequence may associate with the effectiveness of biofilm-producing *in vitro*.

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