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Detection of *icaA* Gene Expression in Clinical Biofilm-Producing *Staphylococcus Aureus* Isolates

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Abstract

The pathogenicity resulting from *Staphylococcus aureus* infection has remarkable importance as one of the community-associated bacterial infections, due to the virulent ability of these bacteria to produce biofilms. This study was designed to detect biofilm production in clinical isolates from samples of wounds and urinary tract infections. The expression levels of the *icaA* gene that is responsible of slime layer production in biofilms was compared in isolates with different biofilm producing capabilities. Fifty seven samples that included 32 samples from urine and 25 samples from wounds were collected from Alwasti Hospital, Al-Kindi Teaching Hospital, and Alzahraa Clinic, Baghdad, Iraq. The bacteria was identified according to biochemical tests, API20 strip test, and PCR assay. The results of 16S rRNA PCR detection revealed that nine isolates were identified as *S. aureus*. The biofilm assay showed that 46.15% of the isolates were strong biofilm producers, 46.15% had moderate ability to produce biofilm, and 7.70% were weak producers. Quantitative PCR assay was carried out on three isolates with different biofilm-producing abilities. The results demonstrated that the strong biofilm-producing isolates had significantly higher ($P \leq 0.01$) gene expression level (6.508) compared with the moderate (1.624) and the weak (1.231) isolates.

Keywords: *Staphylococcus aureus* , Biofilm, Gene expression of *icaA*

الكشف عن التعبير الجيني للـ *icaA* في العزلات السريرية لبكتريا المكورات العنقودية الذهبية المنتجة للغشاء الحيوي

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الخلاصة

للامراضيه الناجمة عن عدوى المكورات العنقودية الذهبية اهميه كبيره باعتبارها واحده من الالتهابات البكتيرية المرتبطة بالمجتمع وهذا ربما بسبب قدرتها على إنتاج الاغشيه الحيوية. تم تصميم هذه الدراسة للكشف عن إنتاج الغشاء الحيوي من قبل العزلات الماخوذه من المصابين بالجروح ومرضي عدوى المسالك البولية ومن ثم مقارنه مستويات التعبير لجين ال*icaA* المسؤول عن تكوين الطبقة اللزجة للغشاء الحيوي في العزلات مع قدرات مختلفة لإنتاج الغشاء الحيوي. جمعت 57 عينة التي تتضمن32 عينة من الادرار و25 عينة من الجروح من مستشفى الواسطي ، ومستشفى الكندي التعليمي ، ومستوصف الزهراء ، بغداد ، العراق . تم تشخيص البكتيريا وفقا للاختبارات الكيموحياتية، شريط API 20 و اختبارالPCR. التحري عن جين

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16S rRNA باستخدام تقنية تفاعل سلسلة البوليميراز , اظهرت النتائج ان تسع عزلات كانت تنتمي الى بكتريا المكورات العنقودية الذهبية, كما اظهر فحص الغشاء الحيوي ان 46.15% من العزلات كانت عالية الانتاجية للغشاء الحيوي و 46.15% كانت تمتلك قدره متوسطة لإنتاج البايوفلم و 7.70% من العزلات كانت ضعيفة. تم اجراء اختبار ال PCR الكمي لثلاث عزلات مع مختلف القدرات الانتاجية للغشاء الحيوي و أظهرت النتائج فرقا معنويا كبيرا عند ($P \leq 0.01$) حيث العزلة قوية الانتاج للغشاء الحيوي كان مستوى تعبيرها الجيني أعلى معنويا (6.508) مقارنة مع المتوسطة (1.624) ثم الضعيفة (1.231).

Introduction

Staphylococci are a diverse group of microscopic organisms that cause infections extending from minor skin diseases to life-threatening bacteremia. Despite expansive scale efforts to control their spread, they continue to represent a major cause of both hospital and community-acquired diseases around the world [1]. They are non-motile, non-spore forming, facultative anaerobes that grow by aerobic respiration or by fermentation [2]. Species of this genus can be distinguished by their ability to produce the coagulase enzyme that causes blood clotting [3]. *Staphylococci* are known to elaborate many surface adhesions which contribute in the attachment to several host proteins, such as fibronectin, vitronectin, laminin, and collagen that are lining up the endothelial matrix [4].

S. aureus belongs to the coagulase-positive staphylococcal species [1]. It is an important human pathogen associated with wide spectrum of infections including numerous skin diseases along with chronic-wound infections. It also colonizes mucosal surfaces [5]. Infection results when a breach in the mucosal barrier or skin allows bacterial cells access to the underlying tissues or to the bloodstream [5, 6] *S. aureus* causes numerous infections, ranging from acute skin abscesses to life-threatening bacteremia and endocarditis [7].

The biofilm-associated polysaccharide of *S. aureus* is referred to as the polysaccharide intercellular adhesion or (PIA) which has been well characterized. Therefore, biofilm formation is an essential step in the pathogenesis of Staphylococci and depends on the expression of the icaADBC operon involved in the synthesis of this polysaccharide intercellular adhesion [8]. It is composed of polymeric N-acetylglucosamine (PNAG) that is synthesized by the products of four genes in the icaADBC operon [9]. In some strains, genetic disruption of the icaADBC genes results in the loss of biofilm formation [8]. The *icaA* gene is known to encode nacetyl-glucosamine tranferase transmembrane synthesizing PNAG polymers [10].

The aim of this study is detecting the biofilm forming capacity of clinical isolates. Then, we compare the gene expression level of *icaA* gene among isolates with different biofilm producing capacities (weak, moderate and strong).

Materials and Methods

1. Isolation and Identification of *Staphylococcus aureus*

1.1. Isolation: Clinical samples of 32 urine and 25 wound swabs were isolated from wounds and urinary tract infection (UTI) patients by sterile transport medium (BIOZEK medical) swabs.. The samples were then cultured on Mannitol salt agar which is a selective medium for *Staphylococcus* spp. After that, the samples were incubated for 24 hours at 37 C° under aerobic conditions. The isolates that were grown on mannitol salt agar were sub-cultured on nutrient agar slants and kept at 4°C until use.

1.2. Identification

1) Growth medium

A- Mannitol salt agar (MSA) is considered as a selective medium due to the presence of high salt concentration (7.5%); hence it only allows the growth of staphylococci and suppresses the growth of other bacteria. In addition, this medium contains mannitol, which has served as a differential agent. *S. aureus* can ferment this sugar into acidic by-products. The reduction of pH is indicated by the production of phenol red, resulting in a yellow halo around the colonies [11].

B- Blood agar: *S. aureus* isolates were cultured on blood agar for activation and testing their ability to produce hemolysin enzyme that causes blood hemolysis and serves as an important virulence factor [12].

2) Microscopic and morphological features

The bacterial isolates were subjected to Gram stain to examine their response and, subsequently, the slides were examined under the oil emersion lense of light microscope. The isolates appeared as Gram-positive cocci arranged in grape-like clusters [13].

3) Biochemical tests

Staphylococcus auras isolates were identified by using catalase and coagulase tests [11], in addition to API 20 test strip.

2. Biofilm assay

The ability of *S. aureus* to form biofilms on abiotic surfaces was quantified. An aliquot (200 µl) of an overnight brain heart infusion broth (BHI) bacterial culture (equivalent to McFarland standard no. 0.5) was dispensed in wells of sterile 96-well polystyrene micro titter plates. Thereafter, all microplates were covered and incubated aerobically at 37°C for 24 hour. Each isolate was assayed in triplicate. Bacteria-free BHI wells were considered as control. To visualize biofilms, the contents of each well were aspirated and the wells were washed thrice with 200 µl distilled water. Thereafter, 200 µl of methanol was added and the microplates were left to air drying for 15 min. Next, 200 µl of 0.1% crystal violet solution was added for 5 min at room temperature. The washing step was repeated as stated earlier. Thereafter, the plates were incubated at 37°C for nearly 30 min to achieve complete dryness. Subsequently, 200 µl of absolute ethanol was added for approximately 10 min. Finally, the optical density of each well was measured at 630 nm via microplate reader [14, 15].

Table 1-Biofilm degree of bacterial isolates

Mean of OD	Biofilm degree
OD ≤ ODc	Non –adherent
ODc < OD ≤ 2*ODc	Weakly adherent
2*ODc < OD ≤ 4*ODc	Moderately adherent
4*ODc < OD	Strongly adherent

OD= optical density, ODc= cut off value [15].

3. Detection of *S. aureus* isolates by molecular techniques

3.1- DNA extraction: DNA was extracted by using Presto™ Mini gDNA Bacteria Kit. Based on the procedure itemized by the manufacturing company, DNA was extracted from overnight cultures of the carefully selected staphylococcal isolates.

3.2- PCR assay (monoplex PCR for the detection of 16SrRNA)

The 16S rRNA gene primers specific for *S. aureus* were used to amplify the extracted genomic DNA using the PCR technique, as shown in Table-2.

Table 2-Primers used for the amplification of 16sRNA in *S. aurues:*

Primer name	Primer sequence 5′→3′	Target gene	Amplicon size pb	Reference
Sa442-F	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG	16sRNA	108	**[16]**
Sa442-R	CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	16sRNA	108	**[16]**

PCR mixture was set up in a total volume of 20 µl, which included 5 µl of template DNA, 1 µl of sterile nuclease-free water, 2µl of each primer (forward and reverse), and 10 µl of master mix. Then, the mixture was vortexed gently. The amplification conditions were as follows: 3 minutes at 95°C for initial denaturation, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 57°C for 30 seconds, and strand extension at 72°C for 3 min. The PCR products were analyzed by gel electrophoresis on a 1.5 w/v agarose gel in 1x TBE buffer for 50 minutes (80 volt) and visualized by staining with red safe stain.

4. Real time PCR (RT-PCR)

4.1- RNA Extraction from *S. aureus* isolates

The RNA from *S. aureus* cells was isolated by using genezol triRNA pure kit (Gene aid/Thailand). *S. aureus* isolates were cultured on microtiterplate, as mentioned before, for the purpose of having biofilm cells. Methanol was removed from the plates by washing with distilled water to remove all cells not adhering to the wells. Subsequently, biofilm cells were re-suspended in cold sterile normal saline by flushing the wells with this saline by using a pipette until no visible biofilm was left on the glass surface. Then, bacterial cells were transferred to a 1.5 ml microcentrifuge tubes and centrifuged for two minutes at 14000 g, followed by complete discard of the supernatant [17]. RNA isolation from this lysed preparation was performed by following the instructions of the manufacturer (GENZOL TriRNA Pure Kit).

4.2- Complementary DNA (cDNA) synthesis

The cDNAs were used for the quantification of mRNA levels of biofilm encoding genes by utilizing the qRT-PCR, according to RT master mix (Hisenscript TM RH RT premix kit); 15 µl of nuclease-free water was transferred to a specific tube of the kit, then 5 µl of total RNA was added. The mixture was mixed by vortexing, followed by brief centrifugation. The cycling protocol included 1 hour at 50°C for reverse transcription followed by 10 minutes at 85°C for RTase inactivation. The samples with synthesized cDNA were stored at -20°C until use.

4.3- Table 3-Primers used for real time PCR.

Primer name	Primer sequence 5′→3′	Target gene
icaA F	GAGGTAAAGCCAACGCACTC	*ica A*
icaA R	CCTGTAACCGCACCAAGTTT	*ica A*
gmk F	AGCACCTCCAAGTTTAGAACAC	*gmk*
gmk R	ACGCGCTTCGTTAATACGAC	*gmk*

4.4- Quantitative PCR protocol

The reaction mixture was prepared as shown in (Table-4).

Table 4- Components of one-step RT-PCR used in *ica A* gene expression

Component	Volume
qPCR Master Mix	10 µl
Forward primer (10 µM ⁄µl)	2 µl
Reverse primer (10 µM ⁄µl)	2 µl
Nuclease-free water	3 µl
Complementary DNA	3 µl

The program used for real time PCR quantification of gmk *icaA* is shown in Table-5.

Table 5-Quantitative RT-PCR protocol

Step	Temperature °C	Duration	Cycles
Reverse transcription	95°C	15 min	1
Enzyme inactivation	95°C	15 sec	40
Denaturation	60°C	30 sec	40
Annealing	72°C	30 sec	40

Results and Discussion

1- Study population

In this study, 57 patients were involved in the sample collected from Alwasti hospital, Al-Kindi Teaching Hospital and Alzahraa Clinic, Baghdad, Iraq, during the period from October 2018 to January 2019. The samples were divided into two groups, wound and UTI patients. These groups were divided into males and females. The age of UTI patients ranged between 16-74 years with a mean of 49.84, as shown in Table-1. This result partially agrees with a study in Iraq that investigated UTI patients with an age ranged between 15-50 years [18,19]. While the age of wound patients ranged between 18-66 years with a mean of 42.04. This result partially agrees with the study of Almeida, were patients’ age was about 18-63 year [20].

Table 6-Mean and range of age of subjects UTI and wound cases.

Source	**No**	**Mean**	**Range of age**
UTI(urinary tract infection)	32	49.84	16.00-74.00
Wound infection	25	42.04	18.00-66.00
P-value	---	0.0392 *	---
* (P<0.05).			

In this study, the percentage of males in the wound infection patients was 56% while that of females was 44%. These percentages agree with those reported in the study of Almeida, who showed a percentage of 50.40% for males and 49.60% for females [20].

In the case of UTI patients, the male percentage was 28.13% and the female percentage was 71.87%. This result agrees with a study conducted in Iraq and involved the detection of biofilm formation by methicillin-resistant *S. aureus* within hospital and community-acquired urinary tract infections, where male percentage was 46% while that of females was 54% [21].

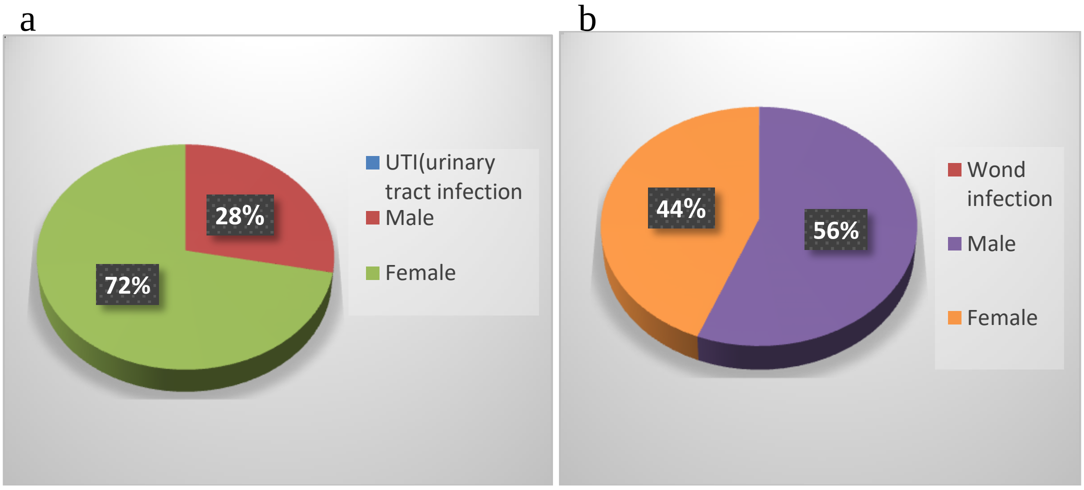


Figure 1-Distribution of sample study according to sex a) UTI samples, b) wound infection samples.

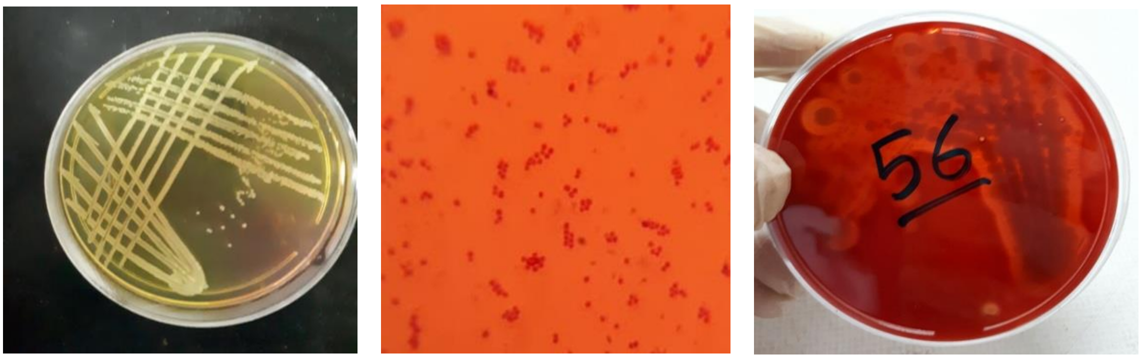
Sample collection

Among the 57 clinical (urine and wound swab) specimens, 37 specimens (66 %) were primarily identified as *S. aureus* according to biochemical tests, and nineteen specimens (34%) were identified as coagulase negative staphylococci.

1- Isolation and identification

The results of culturing on mannitol salt agar showed that 73.68 % of the isolates were able to cause mannitol fermentation and that phenol red changed to the yellow color, as shown in Figure-2(a).

As for culturing on blood agar, the isolates that were able to grow on MSA showed hemolysis around the colonies on blood agar, as shown in Figure-(2-c). The results of microscopic examination demonstrated that the cells appeared as Gram positive cocci, mostly arranged in grape-like clusters, as can be seen in Figure-(2-b).

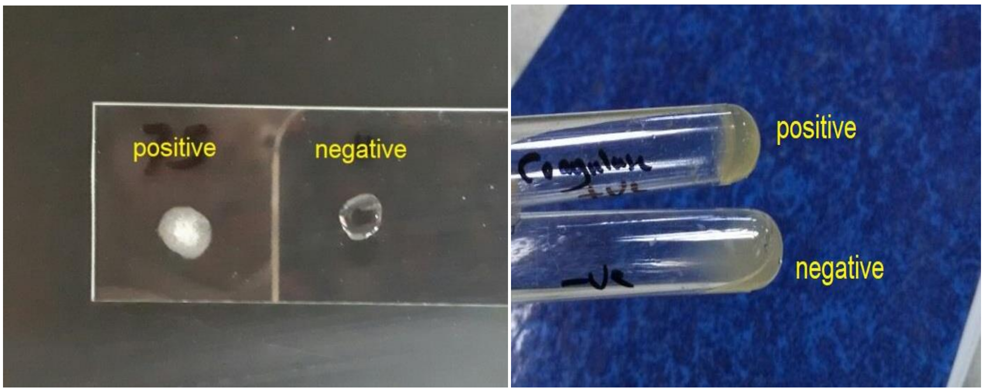


(a) **(b)** **(c)**

Figure 2-(a) *Staphylococcus aureus* on mannitol salt agar.
(b) *Staphylococcus aureus* visualization under microscope.
(c) *Staphylococcus aureus* on blood agar.

Biochemical tests

Coagulase test: (28) % of the isolates were coagulase positive, as shown in fig. (3-b).
Catalase test: (22) % of the isolates were catalase positive, as shown in fig. (3-a).



(a) (b)

Figure 3-Biochemical tests a) catalase test b) coagulase test.

Identification by API 20Staph system

Bacterial isolates were identified by using API20 identification strip system, as shown in Figure-4.

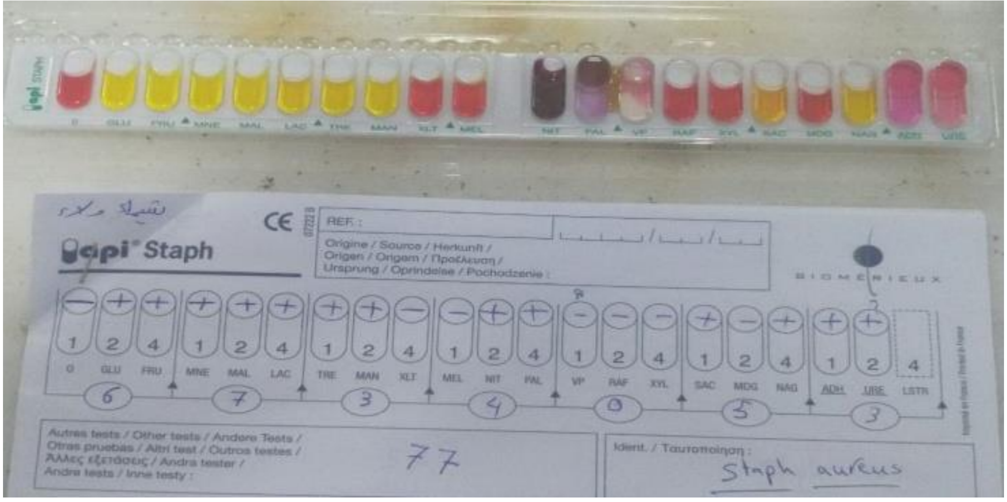


Figure 4-API staph identification strip.

2. Biofilm formation assay

The present study revealed that all isolates were able to produce biofilm but they ranged between strong, moderate and weak producers (Figure-5). It was observed that 46.15% of the isolates were strong biofilm producers, 46.15% had moderate ability to produce biofilm, and 7.70 % were weak producers. This finding agrees with a local study, performed by Muhammad, which involved studying biofilm forming capacity in methicillin-resistant *S. aureus* [22], which showed that 100% of the isolates were able to form biofilm.

In 2017, another local study, performed by Saleh and Khalaf, revealed that 15% of *S. aureus* isolates were weak biofilm producers, whereas 15% were moderate and 70% were strong [23].

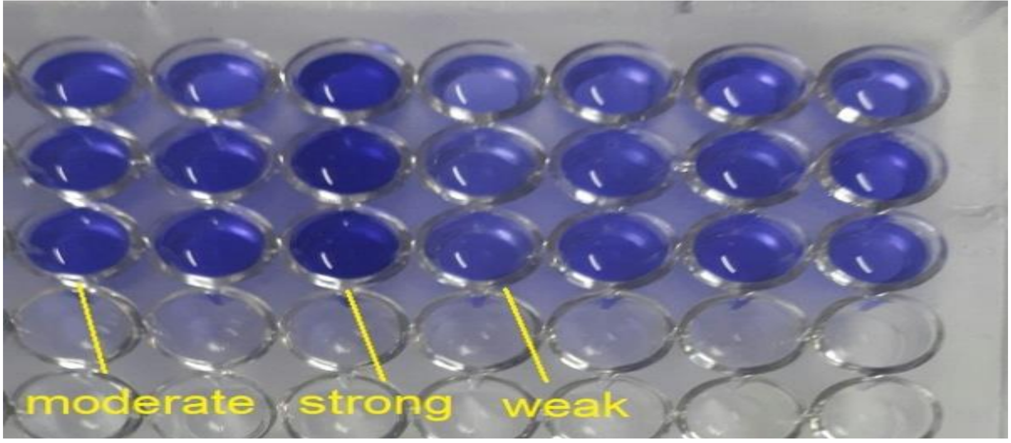


Figure 5-Biofilm formation by *Staphylococcus aureus* isolates.

3. Detection by molecular techniques

- Polymerase chain reaction for 16SrRNA

The results showed that 9 isolates were identified as *S. aureus*, depending on this molecular technique. The PCR products of the isolates were visualized on agarose gel, as clarified in Figure-6.

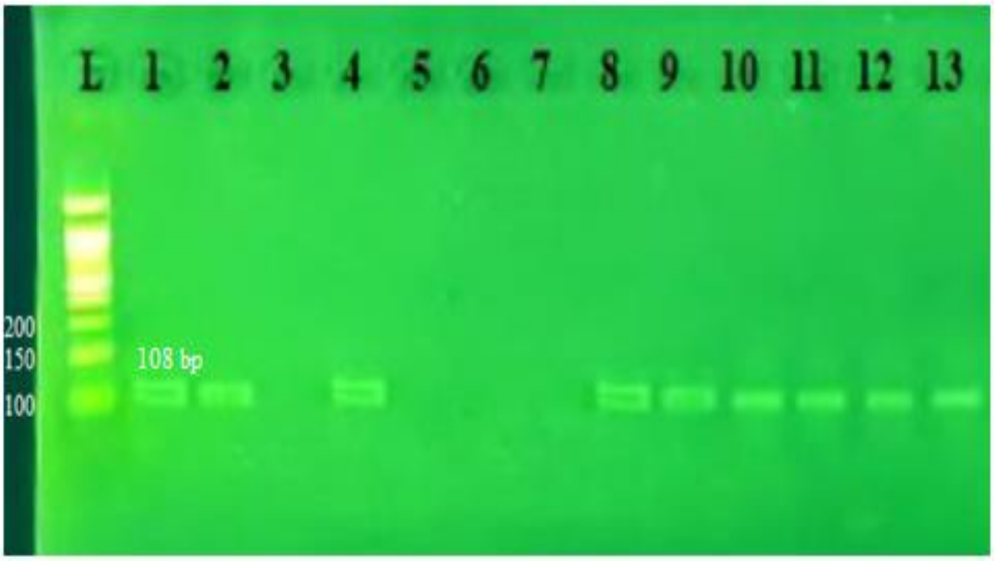


Figure 6-Visualization of *Staphylococcus aureus 16S rRNA* gene by 1.0% agarose gel, stained with red safe stain. The shown bands are representative of PCR product (108 bp) with 50bp DNA ladder.

4. Expression of *ica*A gene in biofilm producing *S. aureus* isolates

Expression levels of *icaA* gene that is involved in biofilm formation were investigated by quantitative PCR (qPCR) for three *S.aureus* isolates (Sr10, Sr77 , Sr57) selected based on different degrees of biofilm formation (strong, moderate , weak, respectively) , isolated from wounds. Table-7 indicates that the expression of *icaA* gene was significantly higher (6.508) in isolate sr10 that had strong biofilm formation when compared with the isolates sr57 and sr77 that had weak and moderate biofilm formation (1.231 and 6.508), respectively. According to the results, there was a highly significant difference (P<0.01) in the expression of sr10 compared with sr57 and sr77.

Table 7-*icaA* expression of *s. aureus* isolates with variable biofilm-forming abilities.

S. aureus isolates	Biofilm characteristics	*IcaA* gene expression
Sr57	Weak	1.231 ± 0.07 b
Sr77	Moderate	1.624 ± 0.11 b
Sr10	Strong	6.508 ± 0.52 a
P-value		0.00294
a and b indicate having with the different letters in same column differed significantly**,** ** (P<0.01).		

Regarding to the results above, it has been noticed that there is an association between the levels of *icaA* gene expression and biofilm formation ability.

In a previous study, Crawford *et al* [24] studied *icaA* expression in association with biofilm activity, as compared to that at logarithmic and stationary phases, in two strains of *staphylococcus pseudintermedius*. They confirmed that the expression of *icaA* was significantly higher in the biofilm condition compared to logarithmic and stationary phases. These findings are not surprising given the role of this gene the in formation of polymer intercellular adhesion (PIA). Also, this gene was shown to be expressed at a higher level in the stationary phase as compared to the logarithmic phase. This study supports the statement that cell conditions affect the expression of biofilm genes and that *icaA* can be expressed under different growth phases at different levels [25].

The *icaA* gene regulates the production of exopolysacharide (EPS) in biofilms. This EPS enforces the adhesion of the bacteria and can serve as a shelter against the host immune system and antibiotics treatment [26]. Biofilm is a society of microorganisms were microbial cells adhere on a living or non-living surfaces with the production of PIA [27]. There are different genes responsible for the expression of biofilm. The ica operon is mainly involved in the production of capsular polysaccharides upon activation. The deletion of ica genes (ABCD) eliminates the ability to produce PIA and form biofilm *in vitro* [28]. A previous study investigated the influence of forces of staphylococcal adhesion to different biomaterials on *icaA* gene expression in *S. aureus* biofilms [26]. The results revealed that *IcaA* gene expression decreased as adhesion forces on the surface increased in line with the level of PNAG production, but the expression levels of *icaA* elevated after 3-6 hours. It should be explained that the adhesion force causes a nano-scale cell wall deformation and membrane stress that act as a mechanism of signaling the organism to enter its adhering state [26] .The nature of the first layer of cells that adhere on the surface is different from that of the next layer of cells that will interact and accumulate with the previously present cells to form biofilm. PIA is the primary determinant promoting accumulation phase of adhesive interactions between bacterial cells of biofilm. It is normal to observe that the expression level of *icaA* is decreased as adhesion force is increased in the first hour and then elevated after 3hours; this period (1-3 hours) can be described as the time required for the bacteria to adapt to the surface environment [26]. The nature of bacterial cell plays a role in the expression levels of genes that are involved in the synthesis of exopolysacharides. Weakly adhering bacteria retain the planktonic phenotype [26]. It may be suggested that the isolates with weak biofilm formation ability could be more considered as planktonic and these isolates display low expression level of *icaA*.

In another study published in 2018 [25], it was confirmed that the expression of *icaA* gene was significantly higher under biofilm conditions when compared to that under planktonic condition for the same isolates of methicillin resistant *Staphylococcus aureus*. Furthermore, the same study compared the expression levels of *icaA* gene in weak and strong biofilm producer isolates and the results showed that the expression levels were higher in strong biofilm producers compared with the weak ones [25]. This findings comes in agreement with the results of this study that revealed that the expression of *icaA* gene in the strong biofilm producer isolates was higher than that of the weak ones. The major difference between strong and weak biofilm producers arise from the differences in their metabolic activity levels [29].

Conclusions

S. aureus isolates were found to be more prevalent in wound samples than those of urinary tract infections .All isolates were able to produce biofilm and the tissue culture plate assay revealed that 46.15% of the isolates were strong biofilm producer, 46.15% had moderate ability. and 7.70 %were weak. The analysis of the gene expression of *icaA* by using real time PCR assay revealed a highly significant difference in the expression level between strong biofilm producing isolates and weak and moderate ones.

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