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Detection of the Staphylococcus aureus icA gene Isolated from some clinical cases

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Abstract

One of the bacterial infections commonly found in the community, *Staphylococcus aureus*, is particularly important for its pathogenicity because of its virulence capacity to create biofilms. This study aims to detect biofilm production in clinical isolates from wounds and urine and compare the levels of the gene icaA, 107 clinical samples from wounds and urine for *Staphylococcus aureus* isolation were taken from patients between November and December 2022 in Iraq at the Alzahra Clinic, Al-Kindi Teaching Hospital, and Alwasti Hospital. The biochemical test technique, the (Analytical Profile Index) API Staph system, the vitek 2 compact system, and finally, the polymerase chain reaction by detecting the 16SrRNA used to identify the *S. aureus. S. aureus* isolates were tested for their capacity to generate biofilm using 96-well microtiter. a quantitative PCR assay was performed on 3 isolates with varying biofilm-building capacities. The results of this study show 29 isolated bacteria were identified as *S. aureus*. According to the results of the biofilm study, 20.7% of the isolates produced weak biofilm, 13.8% produced strong biofilm, and 65.5% had a moderate capacity to produce biofilm. IcaA gene expression was high (6.508) in strong biofilm producer S. aureus. The strongest biofilm-producing strain exhibited significantly greater levels of gene expression in comparison to the weak and intermediate isolates.

Introduction:

Staphylococcus aureus is a significant contributor to nosocomial and community-acquired infections, and it is also a primary source of a wide range of illnesses, including pneumonia, skin, and soft tissue infections[1]. A strain of staphylococci with varying degrees of virulence is one of the significant virulence factors of *S. aureus* that contributes to bloodstream infections and infections. This type of bacteria's capacity to create biofilms indicates its virulence. These microbes must adhere to surfaces and be embedded in different extracellular polymeric materials; biofilm is a functional component required for this. One of the final biofilm products created by *S. aureus* is thought to entail a complex mechanism known as the intracellular adhesion locus (ica)[2].

Extracellular polysaccharides (EPS) produced by bacteria during the development process, such as polysaccharides, nucleic acids, and proteins, stick to bacterial colonies to form an extracellular matrix (ECM) known as a biofilm, which is a term used to describe an organized bacterial community.[3]. As EPS and bacterial clusters interact, biofilm gains cohesion and viscoelasticity[4]. Thus, microbes are able to adhere to both biotic and abiotic surfaces. A significant factor in the development of chronic recurrent infection is pathogenic biofilm [5]. Currently, most experts concur that bacterial biofilms mediate more than 80% of chronic diseases.[6]. In medical settings*, Staphylococcus aureus* is common. It adheres to and stays on the tissues and internal medical equipment of the recipient. Infection of the epidermis and soft tissues, osteomyelitis, endocarditis, pneumonia, bacteremia, etc., may result from this [7]. Due to the biofilm that develops, which increases *S. aureus*'s antibiotic tolerance, these infections are challenging to treat [8].

The products of the icaADB and C gene locus, which in *Staphylococcus spp*. Generate the essential proteins required for the formation of PS/A and PIA, two types of polysaccharide intercellular adhesion that mediate cell aggregation and biofilm accumulation [9]. The biosynthesis of the PIA, whose main component is N-acetylglucosamine, and the biofilm accumulation phase development were both shown to be carried out by the ica-encoded genes, and both of these processes are critical to *S. aureus* invasiveness [10]. Numerous studies have demonstrated that the ica gene plays a crucial part as a virulence factor in staphylococcal infections [11].

All *S. aureus* isolates tested positive for icaA using the PCR method, according to several studies that showed the involvement of icaA in the formation of biofilms [12]. "The *ica* gene" Exopolysaccharide (EPS) synthesis within biofilms is controlled by a gene. This EPS promotes bacterial adhesion and shields bacteria from the body's defence mechanisms and antibiotic therapies [13]. It has been determined that the presence of the icaA gene does not significantly distinguish MSSA and MRSA strains of *S. aureus* [14]. This study aimed to detect biofilm production in clinical isolates from wounds and urine and compare the levels of the gene *icaA*, which is involved in the formation of slime layers, in isolates that produce different amounts of biofilm. (strong, moderate, weak).

Material and method

Collection and isolation of samples

In the two months between November and December 2022, 107 samples in total were gathered. These samples consisted of 45 wound swabs and 62 midstream urine samples. The samples were obtained at the Al-Wasti, Al-Kindi, and Alzahraa health centres. Every sample was raised in Brain Heart Infusion Broth (BHIB) before being kept for 24 hours at 37°C. Every sample was injected into the blood and Mannitol salt agar. To validate the biochemical characterization of the isolates, the VITEK-2 Compact System (BioMérieux, Marcy l'Etoile, France) was used. Later, it was kept at -20 °C in glycerol stock.

Biofilm formation by Microtiter plates method

In 96-well polystyrene microtiter plates, 180 L of BHIB with 0.1% glucose and 20 L of bacteria were added to quantify the amount of biofilm development. The dishes were then incubated for 24 hours at 37°C. The experiments were done in triplicates and involved three

PBS washes, Crystal Violet staining for 14 minutes, 200 L of Glacial Acetic Acid for 10 minutes, and biofilm production measurement for optical density (O.D.). The following describes how the results were arrived at: OD630 (bacteria) equals biofilm and OD630 (bacteria) - OD630 when divided by three. (control). Strains that produce biofilm were given a value of strong, moderate, or weak. The following scores were in red: Strong: O.D.>4xODc, Moderate: 2xODcOD4xODc, and Weak: ODcOD2xODc[15].

Isolation and identification of *S. aureus*

Staphylococcus aureus was isolated from blood and mannitol agars[12]. The occurrence of hemolysis on blood agar and the change in the color of the mannitol agar from pink to yellow indicated the positive result of the test (fermentation of mannitol sugar). This test was performed to distinguish fermented from non-fermented mannitol staphylococcus bacteria [13]. Catalase, oxidase, and coagulase were performed for identification and then confirmed with API Staph and VITEK2 COMPACT system.

Biofilm formation by Microtiter plates method

The plate was the standard tissue culture plate method as described by (14). Based on the optical density obtained, biofilm formation was determined using pre-sterilized polystyrene 96 well. Bacterial isolates were inoculated using a loop in tubes containing 2ml of Brain-Heart infusion broth (BHI) with 1% glucose, incubated at 37°C for 24 hrs and each flat well of the microplate was filled with 200µL of this final solution. Microplates were covered with lids and incubated at 37°C for 24hrs. The medium in wells was removed and washed two times with sterile phosphate buffer solution (PBS) and then inverted to blot and let to dry. Microplates were stained by adding 200µL of 0.1gm/100ml crystal VB violate to each well incubated for 20 minutes which was then washed two times with PBS and inverted to blot and dried. Finally, 200 µL of acetone: ethanol 99% was added to each well, waiting for about 10 min then the results were read at 630nm by an ELISA reader (15).

DNA extraction

The genomic DNA purifying kit was created using the PrestoTM Mini gDNA Bacteria Kit (GBB004 GBB100/101 GBB300/301 gene aid/Thailand). The meticulously selected staphylococcal isolates were cultured overnight, and then DNA was extracted using the manufacturer's recommended methods. Table 1, showed the primers used and Table 2, lists the PCR conditions with time required.

Table 1: primers used in this research.

1. Primer name	2. Primer sequence 5′→3′	3. Amplicon size bp	4. Reference
5. 16SrRNA-F	6. AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG	7. 108	8. Martineau et al 1998
9. 16SrRNA-R	10. CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	11. 108	12. Martineau et al 1998

Table 2: The optimum condition of gene detection

Steps	No. of cycles	Time	Temperature
Initial Denaturation	1	3 min	95ºc
Denaturation	35	30 sec	95ºc
Anneling	35	30 sec	78ºc
Extension	35	25 sec	72ºc
Final Extension	1	3 min	72ºc

Statistical Analysis

All the results of the current study were subjected to statistical analysis, and the SPSS version (17) program was used for this purpose. The Chi-square test and the one- and multiple-variance analysis test were applied according to the least significant difference (LSD), and the probability level value was less than <0.05.

Results and discussion

Isolation of *S. aureus*

In this study from 107 patients total samples were separate to 62 UTI samples and 45 Wound swabs, at p-value 0.101 as shown in Table (3). 29 isolates belong to Staph. aureus was obtained, figure (1). This study investigated the influence of forces of staphylococcal adhesion to different biomaterials on *icaA* gene expression in S. aureus biofilms., this study agreement with [16]., also agree with [1], refer to *S. aureus* isolates were found to be more prevalent in UTI samples than those of wound and All isolates were able to produce biofilm. the reason is due to the abundance of bacteria in UTI

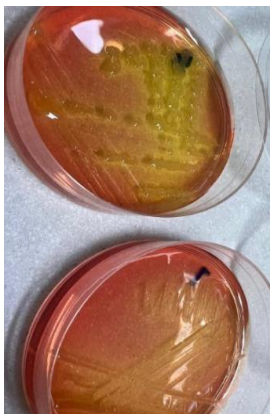

Fig. 1 *Staphylococcus aureus* bacteria on Mannitol salt agar at 37˚C for

Table 3: Distribution of sample study according to Source

Source	No.	Percentage
UTI	62	57.9%
Wound infection	45	42.1%
Total	107	100%
Chi-square- χ^2	---	2.701 NS

(P-value)	(0.101)

NS: Non-Significant.

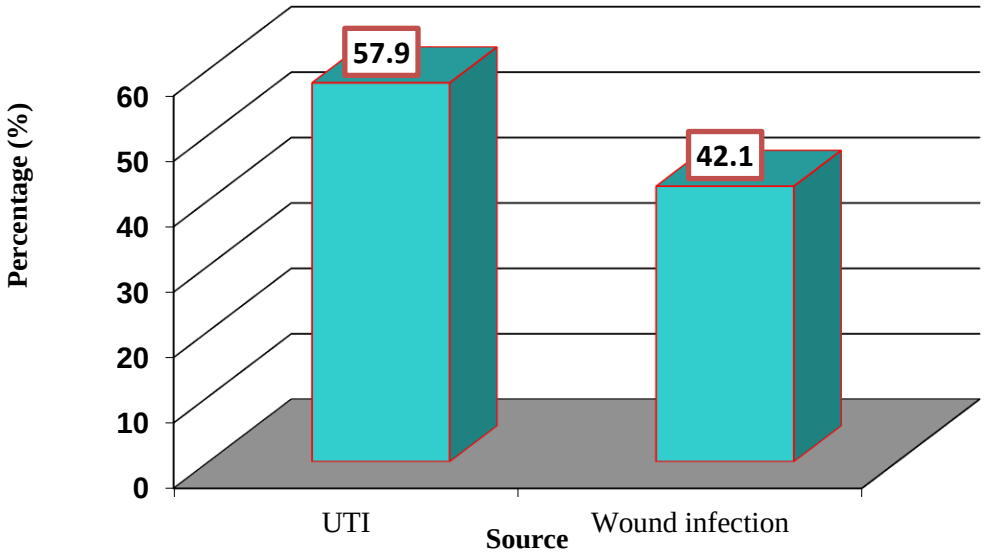

Fig. 2 Shows the distribution of the sample analysis by source.

This study agrees [18] refer to *S. aureus* isolates are the main factor that creating disease Infection is in hospitals and societies around the world, and tens of thousands of people die from this infection every year. consistent with research done in Iraq that examined the occurrence of community-acquired UTIs and the development of methicillin-resistant *S. aureus* biofilms in hospitals. This is significant per the National Institute of Diabetes. [17].

Quantitative of biofilm formation by Micro-titer plate method

The current research found that although all 29 isolates were capable of producing biofilm, their levels of production varied from strong to moderate to weak. The findings from 29 isolates revealed that only 4 isolates (13.8%) produced strong biofilms, while 19 isolates (65.5%) produced moderate biofilms and 6 isolates (20.7%) produced weak biofilms.

This foundation supported the local research conducted by Muhammad[18], which examined the ability of *S. aureus* isolates to form biofilm using the microtiter plate technique. The results indicated that all strains of S. aureus exhibited the ability to form biofilms. In a study conducted by Saleh and Khalaf 2017[19] it was found that 15% of S. aureus isolates exhibited the formation of weak biofilms, while another 15% displayed the production of moderate biofilms, and the remaining 70% demonstrated the formation of robust biofilms. Given that most isolates have a mediocre capacity for biofilm production, this foundation disagreed with the findings obtained. The major difference between strong and weak biofilm producers arises from the differences in their metabolic activity levels.

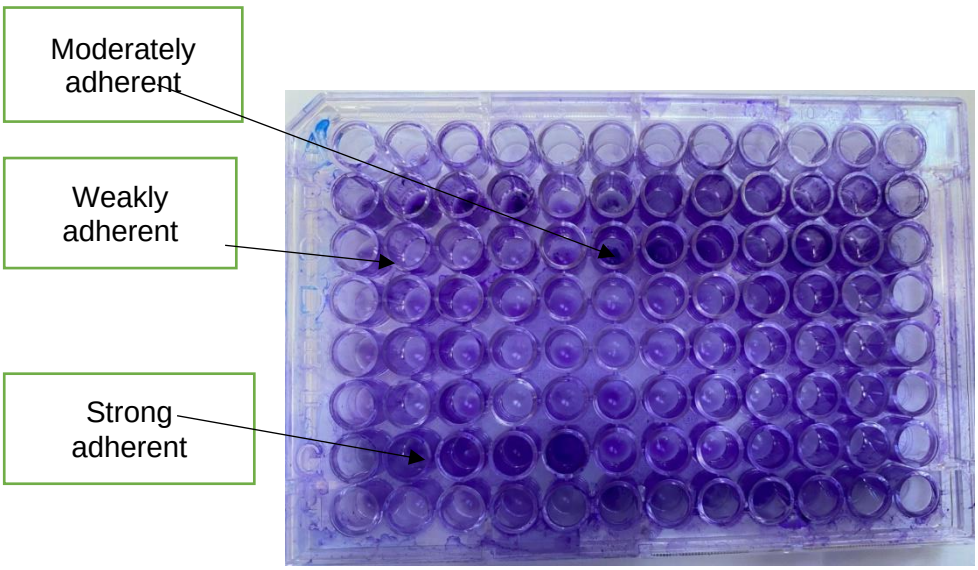

Fig. 3 Biofilm detection by Micro-titer plate method

Detection of 16SrRNA by Polymerase chain reaction (PCR)

The method was used to prepare isolates from overnight cultures used to extract the genomic DNA of bacteria. Nanodrop calculated the DNA's purity and quantity. The content varied between 36.3 and 134.7 ng/l, and the purity was between 1.6 and 1.9. Most people agree that DNA is pure if the number is between 1.8 and 2.0. The presence of contaminants such as protein, phenol, or other substances that strongly absorbed light at or near 280 nm may be determined if the ratio was considerably lower than the suggested ratio[20]. For *S. aureus* isolates, the PCR method was used to amplify a 16S rRNA gene fragment and validate the identification.

Based on this molecular method, the results revealed that all 29 of the isolates were correctly identified as *S. aureus*. To determine the PCR product's size, which was 108 bp, the isolates' PCR products were observed on an Agarose gel as outlined. (Figure 4).

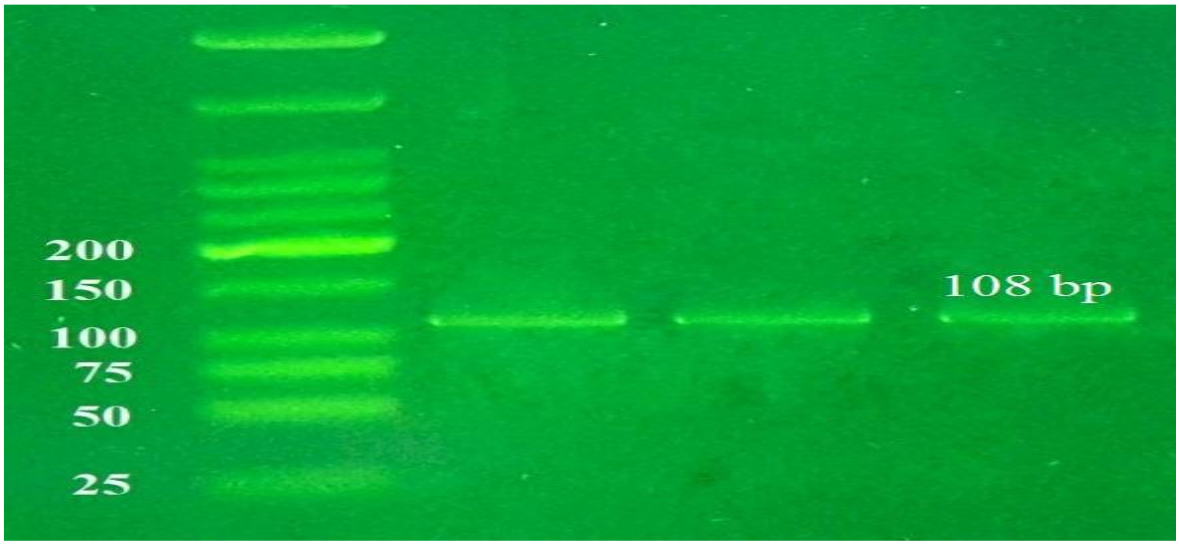

Fig. 4 1.0% agarose gel visualization of the *S. aureus* 16S rRNA gene labeled with red safe stain. The bands on show correspond to the 108 bp PCR product with a 50 bp DNA ladder**.**

According to Table (4), compared to isolates15 and isolate22, which had weak and moderate biofilm formation, isolate6 had substantially higher (6.508) expression of the *icaA* gene. Three *S. aureus* isolates (6, 15, and 22) chosen based on varying levels of biofilm formation (strong, weak) were subjected to a quantitive PCR assay (qPCR) to measure the expression levels of the icaA gene, which is implicated in biofilm formation. (weak producer). Table 4 shows a highly significant variation between isolate 6's expression and isolates 15 and 22 at (P<0.01).

Table 4: *S. aureus* strains with different biofilms exhibit icaA expression**.**

S. aureus isolate	**Biofilm formation**	**icA gene expression**
Isolate 15	Weak	1.231 ± 0.07 b
Isolate 22	Moderate	1.624 ± 0.11 b
Isolate 6	Strong	6.508 ± 0.52 a
LSD Value		1.373 **
P-value		0.00294**
The difference between means with distinct letters in the same column was significant, ** (P 0.01).		

According to Table (4), When compared to isolates 15 and 22, which had weak and moderate biofilm formation, isolate6's expression of the icaA gene was considerably higher (6.508). Three *S. aureus* isolates (6, 15, and 22) chosen based on varying levels of biofilm formation (strong, weak) were subjected to a quantitive PCR assay (qPCR) to measure the expression levels of the icaA gene, which is implicated in biofilm formation (strong, moderate and weak producer). (Table 4) shows a highly significant difference between isolate6's expression and isolate15's and isolate22's at (P<0.01).

"The ica gene" Extracellular polysaccharides (EPS) are produced by biofilm under the control of a gene. This EPS promotes bacterial adhesion and shields bacteria from the body's

defence mechanisms and antibiotic therapies.[13]. In research, the effect of staphylococcal adhesion forces on the expression of the icaA gene in *S. aureus* biofilms was examined. The findings showed that icaA gene expression dropped with increasing surface adhesion forces and PIA production, but it returned to normal after 3-6 hours. The process by which the adhesion force signals the organism to enter its adhering state should be viewed as causing nanoscale cell wall deformation and membrane stress[13]. The succeeding stratum of cells that will interact and coalesce with the original stratum to establish a biofilm exhibits dissimilarities from the initial stratum of cells that cling to the surface. The primary role of (PIA), which facilitates the accumulation phase of adhesive connections among bacterial biofilm cells, is anticipated to result in a drop in the expression level of care as the adhesion force grows during the initial hour, followed by an increase after three hours. This time frame can be used to explain how long it takes for bacteria to adjust to their surroundings on the surface in (1-3 hours)[13]. Different exopolysaccharide-producing genes are expressed at different levels depending on the kind of bacterium cell. The study conducted by Mathur et al., found that 57.8 % of *staphylococcal* clinical isolates demonstrated a biofilm-positive phenotype, and 14.47 % of these isolates exhibited significant biofilm development [21]. Gad et al. showed a greater rate of biofilm development, with 83.3% of *S. aureus* isolated from urinary tract catheterized patients producing biofilm during the MTP assay[22]. This indicates that biofilm production is more prevalent. The ability of *S. aureus* isolates to form biofilms has been linked to the presence of ABCD genes, according to the findings of several investigations. The investigation found that all of the *ica* genes were present in 12 *S. aureus* isolates, accounting for 38.7% of the total. Similar to our research findings, several other studies have demonstrated that the ica genes were found in every single *S. aureus* isolate [23, 24]. The observed disparity among different studies could perhaps be attributed to variations in the bacterial origins, including genetic characterization, source of isolation, and environmental conditions.

Conclusions

All *S. aureus* isolates examined in this investigation exhibited the ability to create biofilms. A high level of biofilm efficiency was formed by 13.8% of the samples, 65.5% of the samples produced an intermediate level of biofilm, and 20.7% of the samples produced a low level of biofilm, according to the tissue culture plate assay. because of its effectiveness. There is a moderate potential to generate biofilms in the majority of the isolates. In accordance with the findings of the quantitative PCR analysis for icaA gene expression, the robust biofilm-producing strain exhibited significantly greater levels of gene expression in comparison to the weak and intermediate isolates.

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الكشف عن جين icA Staphylococcus aureus المعزول من بعض الحالات السريرية

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الخلاصة:
تعد المكورات العنقودية الذهبية ذات أهمية خاصة بسبب قدرتها على تكوين الأغشية الحيوية. هدفت هذه الدراسة إلى الكشف عن إنتاج الأغشية الحيوية في العزلات السريرية من الجروح والادرار ومقارنة مستويات الجين icaA. تم أخذ 107 عينة سريرية من الجروح والادرار لعزل المكورات العنقودية الذهبية من المرضى بين نوفمبر وديسمبر 2022 في العراق في عيادة الزهراء، مستشفى الكندي التعليمي، ومستشفى الواسطي. تم تشخيص اعتمادا على الاختبار البيوكيميائي، ونظام API Staph (مؤشر الملف التحليلي)، ونظام vitek 2 المدمج، وأخيرًا، تفاعل البوليميراز المتسلسل عن طريق الكشف عن SrRNA16 المستخدم للتعرف على المكورات العنقودية الذهبية. تم اختبار قدرة عزلات المكورات العنقودية الذهبية على توليد الأغشية الحيوية باستخدام عيار 96 ميكرومتر. تم إجراء اختبار كمي PCR على ثلاث عزلات ذات قدرات بناء الأغشية الحيوية المختلفة.اشارت النتائج اهذه الدراسة الى التعرف على 29 نوع من البكتيريا المعزولة على أنها المكورات العنقودية الذهبية . حسب نتائج دراسة الأغشية الحيوية فإن 20.7% من العزلات أنتجت غشاء حيوي ضعيف، 13.8% أنتجت غشاء حيوي قوي، و65.5% كانت ذات قدرة متوسطة على إنتاج الأغشية الحيوية. كان التعبير الجيني IcaA مرتفعًا (6.508) في منتج الأغشية الحيوية القوية للمكورات العنقودية الذهبية. أظهرت أقوى السلالة المنتجة للأغشية الحيوية مستويات أعلى بكثير من التعبير الجيني مقارنة بالعزلات الضعيفة والمتوسطة.