How to Cite:
Hashim, M. H., & AlKhafaji, M. H. (2022). Molecular detection of biofilm coding genes in Staphylococcus aureus. *International Journal of Health Sciences*, *6*(S4), 4672–4681. https://doi.org/10.53730/ijhs.v6nS4.9162

Molecular detection of biofilm coding genes in Staphylococcus aureus

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***Abstract*---**In accordance with epidemic COVID-19, the elevated infection rates, disinfectant overuse and antibiotic misuse what led to immune suppression in most of the population in addition to genotypic and phenotypic alterations in the microorganisms, so a great need to reevaluate the genetic determinants that responsible for bacterial community (biofilm) has been raised. A total of 250 clinical specimens were obtained from patients in Baghdad hospitals and streaked on Mannitol salt agar medium. The results revealed that 156 isolates appeared as round yellow colonies, indicating that they were mostly identified as *Staphylococcus aureus* from 250 specimens. The antibiotic resistance pattern of the isolates for methicillin 37.17% (n=58), Amoxicillin-Clavulanate 58.9% (n=92), chloramphenicol 6.4% (n=10), Tetracyclin 62.8% (n=98), ceftriaxone 53.8% (n=84), Ciprofloxacin 6.4% (n=10), Gentamicin 42.3% (n=66), levofloxacin 28.2% (n=44), Penicillin 33.3% (n=52). The results demonstrated that 49 isolates were multidurg resistance. The biofilm formation ability of MDR was detected and total of 120 *S. aureus* isolates (76.92 %) were found to be adherent to varied degrees. Only fifty isolates (32.05% of the total) were classified as strong biofilm producers. Twenty-three (14.75%) were moderate producers, and forty seven isolates (30.12%) were found to be weak producers. A total of 36 isolates (23.08%) exhibited no biofilm production. Molecular detection of four biofilm coding genes *icaA, icaD, icaR and eno* was applied using doublex PCR technique. The current study revealed that 72%, 78%, 70% and 84% of isolates that carried *icaA, icaD, icaR and eno* genes respectively, *eno* is the predominant in the Iraqi isolates

***Keywords*---**Staphylococcus aureus, biofilm, multi drug resistant.

Introduction

Staphylococcus aureus is a common human pathogen that can colonize a variety of tissues and cause serious disease in both immune-compromised and otherwise healthy people. The persistence of biofilm infections induced by this bacterium species, even in the face of intensive treatment intervention, is of special concern. Multiple drug resistant (MDR) *S. aureus* is one of the most common causes of serious nosocomial infections, with the gastrointestinal tract serving as a major source of transmission [1].

Septicemia, meningitis, pneumonia, and endocarditis are among the illnesses that range in severity from mild to severe life-threatening infections. Infection puts a burgeoning stress on health-care resources, as well as rising morbidity and mortality. [2]. Several virulence factors are made by *S. aureus*, including biofilm, leukocidins, hemolysins, proteases, exfoliative, enterotoxins, toxins, and immunemodulatory factors[3]. The key molecule responsible for intercellular adhesion in Staphylococci is the Polysaccharide Intercellular Adhesion (PIA) protein. The *ica* gene locus' products are responsible for PIA biosynthesis, which includes a *icaA* and *icaD* (Nacetylglucosamine transferase), a PIA deacetylase (*icaB*), a *icaC* (putative PIA exporter) and a *icaR* (regulatory gene) [4]. A number of regulatory proteins and environmental factors affect the expression of the *ica* gene locus. *icaA* and *icaD* have been exposed to play significant roles in biofilm establishment among the *ica* genes. The *icaA* and *icaD* genes control the ability of *S. aureus* strains to produce biofilm by facilitating the synthesis of PIA, implying that the *ica* locus would be a good target in the treatment of implantation infections [5].

Methods

Bacterial isolates

A total of 250 specimen were collected from different hospitals of Baghdad from November 2020 to May 2021, including nasal swab , blood, wound, burn, ulcer and urine. To isolate *S. aureus*, all collected specimens were cultured on selective and differential media. Biochemical characteristics such as colony morphology on blood agar medium, mannitol salt agar medium for mannitol fermentation, and slide and tube coagulase tests were used to identify the isolated bacteria. The identification of isolates was confirmed by vitek2 compact system and PCR.

Microtiter plate method

The Microtiter plate method was showed as earlier described [6]. Nutrient broth supplemented (180 µl) with glucose 1% was inoculated into the wells of a microtiter plate. Each bacterial culture 20 µl added to 96-well of polystyrene microtiter plates with a turbidity of 0.5 McFarland standard. After a 24-hour incubation at 37°C, the wells were poured and rinsed three times with sterile saline phos-phate buffer. The wells were then treated with methanol (20 minutes) and crystal violet 0.1 percent (15 minutes). The stained wells were washed and dried at room temperature. The crystal violet dye was dissolved in 0.2 ml 99 % ethanol per well, and the plates' optical density (OD) was measured using an

ELIZA reader at 590 nm (A590). Each assay was carried out three times. To determine background OD, nutrient broth medium was used as a negative control. The OD cut-off was then calculated as the average OD of the negative control plus 3* standard deviations (SD) of the negative control. Each microtiter plate's OD cut-off value was calculated separately. The absorbance of the crystal violet stained adherent cells was used to calculate and characterize biofilm development by isolates.

Table 1. Classification of biofilm development abilities

Cut-off value	Biofilm formation capabilities
OD ≤ ODc	Negative
ODc < OD≤ 2*ODc	Weak
2 * ODc < OD	Moderate
OD > 3 * ODc	Strong

*c: control

Antimicrobial susceptibility test

According to the guidelines of Clinical and Laboratory Standards and As described in a previous study Antimicrobial susceptibility test was done [7]. Different disks were used in AST, including Amoxicillin-Potassium Clavulanate, Vancomycin, chloramphenicol, methicillin, Tetracyclinen, ceftriaxone, Ciprofloxacin, Gentamicin, levofloxacin, Penicillins.

Molecular experiments

DNA extraction

DNA was extracted from *S.aureus* isolates using genomic DNA extraction kit [8]. and the extracted DNA samples were characterized to ensure their purity and quality using qubit 4.0 assay and the results revealed that all the extracted DNA samples were pure with a concentration of more than 88 ng/µl.

Quantitation of DNA by Qubit 4.0

The assay favors double-stranded DNA (dsDNA) over RNA and works well with samples ranging in concentration from 10 pg/L to 100 ng/L. The test is carried out at room temperature, and the signal lasts three hours. Salts, free nucleotides, solvents, detergents, and protein are among the common impurities tolerated by the assay.

- The Qubit® working solution was obtained by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS buffer.
- A volume of 190 µL of Qubit® working solution was added to each tube designated to be a standard, and then 10 µL of each provided standard solution was added to the same tubes, which were then vortexed..
- A total of 197 µL of the Qubit® working solution was added to each sample tube, followed by 3 µL of sample.

- All of the components were vortexed and incubated for 3 minutes at room temperature.
- To create a concentration curve, standards tubes were inserted into the Qubit instrument.
- To read the concentration of dsDNA in each sample, tubes for samples were added one by one.

Primers

Multiplex PCRs were utilized to amplify the genes *icaA, icaD, icaR*, and *eno*. For these genes, specific primers were designed, as well as PCR thermal profiles as listed in table 2.

Table 2. Primers used for the amplification of genes in *S. aureus*

Name	Sequence 5'-3'	Product Size (bp)	Tm (℃)
icaR-F	TACTTTCTTCCACTGCTCCAA	465	56.9
icaR-R	CAGAGAAGGGATATGACGGTA		56.1
eno-F	TAATGGTGGTTCTCACTCAGA	332	56
eno-R	CTTCGAACTTGCTGTAGTCAT		56
icaA-F	CGTTGTCTAATGTTCTTGCAC	635	55.8
icaA-R	TAGTAATACTTCGTGTCCCCC		56.3
icaD-F	AGCACTTATCGCTATATCGTG	233	55.5
icaD-R	CTCTTCCTCTCTGCCATTTTT		55.9

F: Forward primer R: Reverse primer

Reaction mixture

A final volume of 20 µL, a mixture of extracted DNA, primers, and PCR master mix was produced, which included 5 µL of PCR Green master mix, 1 µL of each primer, and 2 µL of template DNA. Sterilized de-ionized distilled water was used to complete the volume by first adding the De-ionized water, then the primers, and finally the DNA template. The mixture was mixed, and a negative control containing all of the material except the template DNA was created by substituting distilled water for the template. PCR reaction tubes were placed in a thermocycler PCR instrument, and the DNA was amplified, as shown in (Tables 3, 4).

Table 3. Program used to amplify the *icaA* and *eno* genes

Step	Temperature	Time	Cycle

	(°C)		
Initial denaturation	95	3min	35
Denaturation	95	1min	
Annealing	55.8	40sec	
Extension	72	40sec	
Final Extension	72	5min	

Table 4. Program used to amplify the *icaD and icaR* genes

Step	Temperature (°C)	Time	Cycle
Initial denaturation	95	3min	35
Denaturation	95	1min	
Annealing	56.1	40sec	
Extension	72	40sec	
Final Extension	72	5min	

Agarose gel electrophoresis

After PCR amplification, 1.5 % agarose gel electrophoresis was adopted to confirm the presence of amplification. A 1.5 percent agarose gel was prepared for the determination of PCR specificity by dissolving 1.5 gm of agarose powder in 100 ml of 1X TBE buffer, boiling, then cooling to 50-60oC, mixing in 5µL of bromide dye, agarose was poured out into the gel jar to prevent bubble formation, and the gel was cooled to 20 degrees Celsius.. When the agarose gel was poured, many wells were carefully formed with a comb about 5-10 mm distant from the gel's end on one side; The comb was carefully removed after final solidification, and the jar was placed in the electrophoresis tank. Six µL of the 100 bp DNA ladder were placed in the agarose electrophoresis gel's first left well or middle well, followed by 10 µL of each PCR product in the remaining wells. The electrophoresis tank was then sealed with its specific top, and the electric current was adjusted (70 volt for 1.5 h). Gel documentation system was used to visualize the redsafe stained bands in the gel.

Results

Isolation and identification of *Staphylococcus aureus*

Two hundred and fifty clinical specimens were streaked on Mannitol salt agar medium from patients attending hospitals in Baghdad. One hundred fifty-six isolates appeared as round yellow colonies and were thus identified as *S. aureus*. Mannitol salt agar medium is used for the isolation of staphylococcal species from different sources either clinical or environmental samples. Given that, its high content of sodium chloride reached about 7.5%, Most other bacterial species will be inhibited, preventing the growth of it except staphylococci. *S. aureus* ferments

mannitol and produces yellow zones in reddish agar due to the production of fermentation acids that contribute in lowering the pH of the medium converting the color of phenol red to yellow [9]. This test differentiates it from *S.epidermidis*, which forms colonies with red zones. *S.aureus* show small, round, yellow colonies on the surface of Mannitol salt agar [10]. Isolates that gave a positive result in mannitol fermentation were submitted to further identification tests; Gram stain, coagulase, production, Oxidase and Catalase and the results were summarized in Table (5). Cells of this bacterium appeared by Gram staining as Gram-positive cocci exhibited in clusters, paired or single.

Table 5. Cultural, biochemical and microscopical properties of *S. aureus* (n=156)

Id	Test	Result
1	Mannitol fermentation	100% positive with yellow colonies
2	Gram stain	100% Gram positive cocci
3	Catalase	100% positive
4	Oxidase	100% negative
5	Coagulase	100 % positive

Biofilm formation

A total of one hundred and twenty (76.92%) of the *S. aureus* isolates tested were establish to be adherent to varying degrees. Only fifty isolates (32.05%) were defined as strong biofilm producers; Twenty-three (14.75%) of the clones were moderate producers, and Fourty seven isolates (30.12%) were found to be weak producers. A total of 36 isolates (23.08%) exhibited no biofilm production

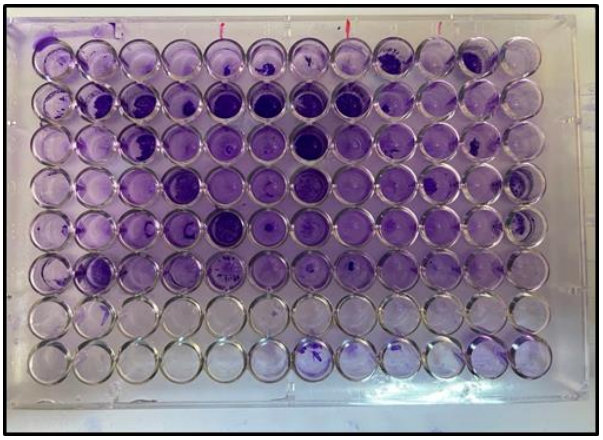

Figure 1. Micro-titter plate for detecting biofilm

Antibiotic susceptibility test

Vancomycin resistance was found in all of the isolates. The antibiotic resistance pattern of the isolates for Amoxicillin-Potassium Clavulanate 58.9% (n=92), chloramphenicol 6.4% (n=10), methicillin 37.17% (n=58), Tetracyclinen 62.8%

(n=98), ceftriaxone 53.8% (n=84), Ciprofloxacin 6.4% (n=10), Gentamicin 42.3% (n=66), levofloxacin 28.2% (n=44), Penicillins 33.3% (n=52). The results demonstrated that 49 isolates were multidurg resistance.

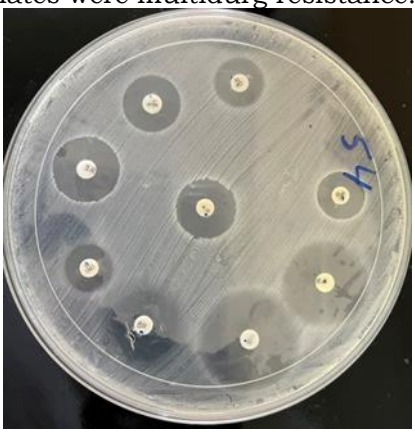

Figure2. Antibiotic Susceptibility Patterns of S.aureus Isolates

Detection of *icaA, icaD* and *icaR* and *eno* genes by PCR

multiplex PCR technique was used to confirm the presence of *icaA, icaD and icaR* and *eno*. The presence of genes was identified by existence of single band at a given molecular weight (635 bp, 233 bp, 465bp and 322 bp for *icaA, icaD* and *icaR and eno* respectively) of marker that be used as in Figures 3 and 4. The current study revealed that 72%, 78%, 70% and 84% of isolates that carried *icaA, icaD, icaR and eno* genes respectively.

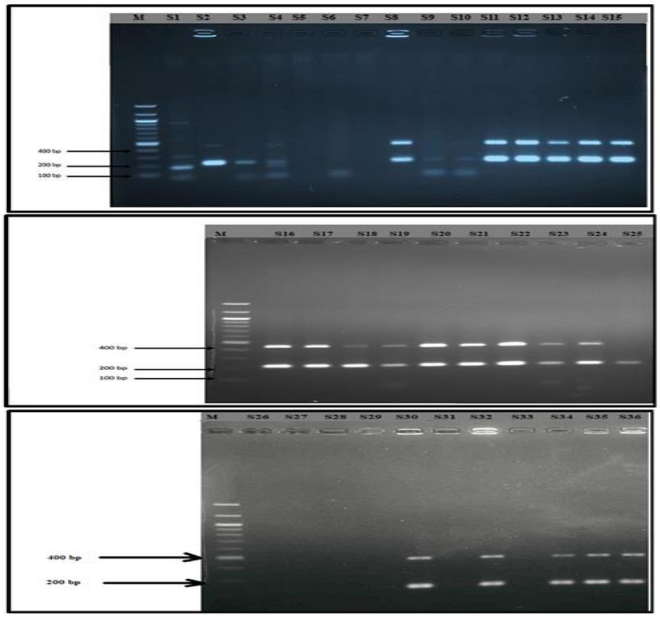

Figure 3. Agarose gel electrophorphoresis of douplex product of of *icaD* (465bp) and *icaR* (233 bp), 1.5% agarose, red safe stain, TBE buffer, 75 volt, 1hr

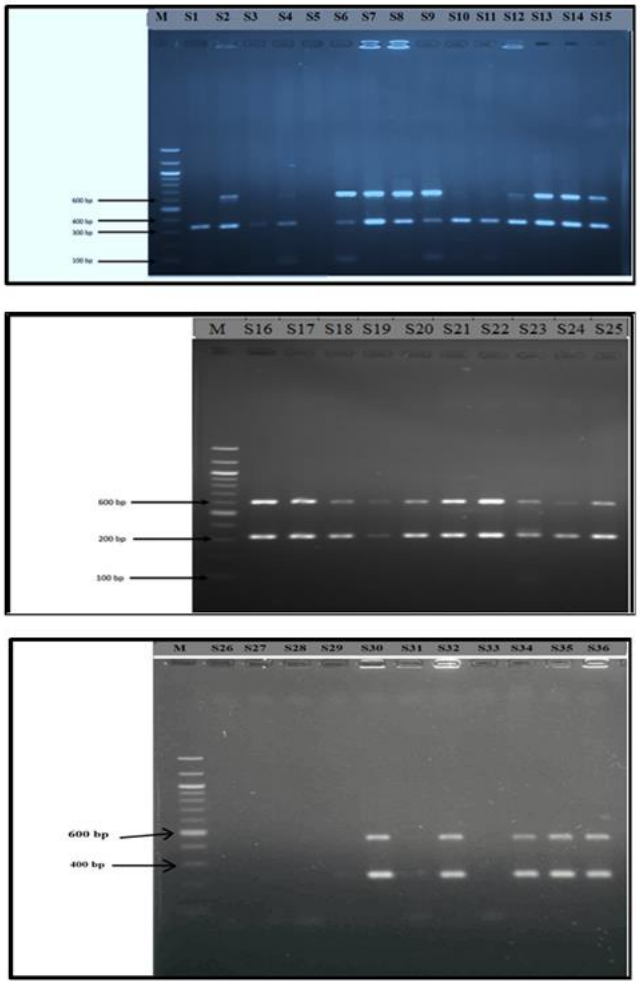

Figure 4. Agarose gel electrophorphoresis of douplex product of *icaA* (635bp) and *eno*(332bp), 1.5% agarose, red safe stain, TBE buffer, 75 volt, 1hr, M: DNA lader,

Discussion

The ability of *S. aureus* to produce biofilm, which occurs through the expression of *ica* and *eno* genes, is an important factor in infectivity. The current study is the first of its kind to look at the expression of biofilm-forming genes in clinical isolates of *S. aureus* from Iraq. An earlier study from Egypt looked at the expression of virulence genes in *S. aureus* biofilm forming strains subjected to antibiotics [12,13]. The findings of our study show some genes responsible for biofilm production are present to varying degrees in clinical isolates. As shown in Table 6, there was a significant relationship between *ica* and *eno* gene expression and biofilm production. The results of the MTP assay and the polymerase chain reaction assay for biofilm production were largely consistent.

In *S. aureus and S. epidermidis*, the *ica* genes *icaA* and *icaD* have been demonstrated to be important in biofilm production [14]. It's important to note

that both of these genes have been found in *S. aureus* biofilm-producing strains. More research is needed to help develop biomaterials and physical electrical barriers that will help inhibit bacterial colonization, as well as novel therapeutic intervention options. However, other studies demonstrated that the presence of the ica genes did not always correlate with biofilm production. De Silva et al. [15] reported that only 59% of *S. epidermidis* strains positive for the ica operon were biofilm producers by CRA method. In correlating also to MTP method, Cafiso *et al.* [16] demonstrated that 83.3% of the ica-positive isolates produced biofilm by both methods, while Yazdani et al. [17] reported that only 54% and 52% of ica-positive strains were also positive by CRA and MTP methods, respectively. Cafiso *et al.* [16] also proposed that the product of *icaR* gene (a regulator gene which seems to function as a repressor) [17] could influence transcription of the ica operon. Nevertheless, irrespective of ica genes expression, ica-positive isolates should be considered to be potential biofilm producers [18].

Acknowledgements

We would like to thank biology department, university of Baghdad to support this study. Also, we thank all participants for their collaboration during sample collection.

Conflict of Interest

The authors declare that there is no conflict of interest

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