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Assessment of *ica A* and *D* Genes in Biofilm producers methicillin-resistant *Staphylococcus aureus* isolates

Mariam Khamees Abdrabaa[1]*, Rasmiya Abd Aburesha[2]
[1]*Ministry of Health, Baghdad, Iraq*
[2]*Department of Biology, College of Science, University of Baghdad, Baghdad Iraq*

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Abstract

This study investigates *in vitro* biofilm production. Presence of *ica A* and *D* genes in methicillin-resistant *Staphylococcus aureus* was evaluated for biofilm production by the microtiter plate method. Between December 2020 and October 2021, out of 215 clinical specimens were collected from patients with pulmonary fibrosis, pneumonia, bacteremia, chronic burns, deep wounds, urinary tract infection and catheterized patients. Out of which 45 MRSA isolates were identified by the susceptibility test utilizing cefoxitin and the occurrence of *mecA* gene for resistance for this antibiotic verified by polymerase chain reaction technique. A sensitivity test was conducted for five other antibiotics. All MRSA isolates were producers of biofilms but the formation of robust biofilms by 42% of MRSA isolates, 20% of isolates was intermediate and 38% of isolates weak. Formerly *ica A* and *D* genes, responsible for polysaccharide intracellular adhesin dependent biofilm formation were investigated in all MRSA isolates using the polymerase chain reaction technique. *ica A* were detect in 33 (73.3%) of the isolates and was lacking in 12 (26.6%) of the isolates. *ica D* gene was present in 38 (84.4%) isolates and was lacking in 7 (15.5%). However, the total number of isolates that contained *icaA* and *D* genes was 10 (22.2%). The most noteworthy finding was that the five weak isolates lacked any genes. Thus indicating that these isolates are capable of producing biofilm without the need for *ica* in order to make polysaccharide intracellular adhesin that means the isolates have an ability to form biofilm in *ica* independent biofilm mechanisms.

Keywords: MRSA, Biofilm production, *ica* A and *D*.

تقييم وجود جينات *icaA ,D* في عزلات المكورات العنقودية الذهبية المقاومة لمضاد الميثيسيلين

مريم خميس عبدربه[1]* , رسمية عبد أبو ريشة[2]
[1]وزارة الصحة ، بغداد، العراق
[2]كلية العلوم،قسم علوم الحياة ،جامعة بغداد ،بغداد، العراق

الخلاصة

تبحث هذه الدراسة في إنتاج الأغشية الحيوية مختبريا. حيث تم تقييم وجود جينات *ica A* و *D* في المكورات العنقودية الذهبية المقاومة للميثيسيلين المنتجة للأغشية الحيوية باستخدام طريقة اطباق المعايرة الدقيقة . بين ديسمبر 2020 وأكتوبر 2021 ومن أصل 215 عينة سريرية تم تحديد 45 عزلة بأنها عزلات مقاومة

*Email: Mariam.brees1102@sc.uobaghdad.edu

لمضاد الميثيسيلين باستخدام اختبار الحساسية. تم التحقق منها بأستخدام مضاد السيفوكسيتين ووجود جين *mecA*المسؤول عن مقاومة هذا المضاد الحيوي بواسطة تقنية تفاعل انزيم البلمرة المتسلسل. تم إجراء اختبار الحساسية لخمسة مضادات حيوية أخرى.جميع عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين كانت منتجة للأغشية الحيوية ولكن تكوين أغشية حيوية قوية كان بنسبة 42٪ من عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين وكانت 20٪ من العزلات متوسطة و 38٪ من العزلات ضعيفة التكوين للغشاء الحيوي . تم الكشف عن وجود جينات *ica A* و *D* ، المسؤولة عن تكوين الأغشية الحيوية المعتمدة على اللاصق المتعدد السكاريد داخل الخلايا في جميع عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين باستخدام تقنية تفاعل البلمرة المتسلسل. تم تحديد وجود جين*icaA* في 33 (73.3٪) من العزلات وعدم وجوده في 12 (26.6٪) من العزلات. وجد جين *icaD* في 38 (84.4٪) من العزلات وأفتقرت 8 عزلات منه بنسبة (17.7٪) لكن العدد الإجمالي للعزلات التي تحتوي على جينات *icaA and icaD* كان 10(22.2٪) عزلات.كانت النتيجة المثيرة للأنتباه هي وجود خمس عزلات لها القابلية على تكوين اغشية حيوية وتفتقر إلى وجود أي من جينات *icaA*and*D* ، مما يشير إلى أن هذه العزلات قادرة على إنتاج غشاء حيوي دون الحاجة إلى *ica* من أجل صنع اللاصق المتعدد السكاريد داخل الخلايا والذي يعني أن لديها القدرة على تكوين غشاء حيوي في آليات تكوين غشاء حيوي بطريقة مستقلة عن جينات *ica*.

1 - Introduction

Staphylococcus aureus is one of the most significant pathogens in humans that has the ability to infect nearly every organ in a human body and causes debilitating illnesses. Chronic infections necessitate prolonged antibiotic therapy and can have a significant influence on the patient's quality of life [1, 2].

Methicillin-resistant *Staphylococcus aureus* is a common cause of both in and outside the health care system illnesses. The acquisition of the *mecA* gene, which produces a modified penicillin-binding protein, results in methicillin resistance in *S. aureus* strains (PBP2a). MRSA, is a common source of hospital and community acquired infections as these infections are resistant to the entire class of beta-lactam medicines, and they are also difficult to treat. MRSA strains, associated with hospitals, are frequently multidrug resistant, leaving only low efficiency antibiotics such as vancomycin available for therapy [2,3]. As with many other strains of *S. aureus*, toxins and adhesion proteins are among the virulence factors generated by MRSA. MRSA biofilms are a prevalent virulence feature that contributes significantly to the etiology of life threatening illnesses. The capacity of bacteria to build biofilms is connected with pathogenicity and a variety of chronic bacterial illnesses [4]. MRSA is regarded as an alarming development in the healthcare settings due to the major problems it creates. Strain is capable of forming biofilms on polymer surfaces and may also play a significant influence in the development of staphylococcal infections [5]. A crucial complicating factor is the capacity of bacteria to produce biofilms which is a dynamic architecturally complicated multilayered cellular matrix. Biofilms are three-dimensional mosaic microbial consortia that cling to and build on implant surfaces via extracellular polymers known as glycocalyx [6, 8].The expression of polysaccharide intracellular adhesion (PIA) is required for biofilm development. Polysaccharide intracellular adhesin is transcribed by the *ica*-genes operon products (intercellular adhesion) on chromosome [9, 10]. The operon contains the *icaA, D, B, C* genes, as well as the *icaR* gene, which serves as a regulator and is expressed in the reverse direction transcriptionally. When this operon is activated, it creates these proteins: Ica A, Ica D, Ica B, and Ica C which are required for the production of PIA [11, 12].

The aim of current study was the detection of biofilm formation in MRSA isolates and assessment of intracellular adhesin *ica A* and *D genes* in all MRSA biofilm producers isolates.

2 - Materials and Methods

2.1 Specimen collection

Between December 2020 and October 2021, 215 specimens (wound, urine catheter, pus swabs, blood, urine and sputum specimens) were collected in sterilized containers from hospitals throughout Baghdad, Iraq, including Baghdad Teaching Hospital, Teaching Laboratories of Medical City's, AL-kindy Teaching Hospital and Abn AL-Nafees Hospitals.

2.2 Isolation of Staphylococci

Depending on laboratory protocols, *Staphylococcus aureus* diagnosis has been achieved in all clinical samples using Vitek2 system and GP diagnostic kit. For checking on mannitol fermentation and hemolysis type, all samples, were smeared separately on mannitol salt agar and blood agar, and incubated aerobically (37°C for 24 hours) [13].

2.4 Identification of MRSA

Suspensions of all *S. aureus* isolates were prepared and cultured on Mueller-Hinton agar medium containing 2% sodium chloride and cefoxitin antibiotics (30 µg/ml) by using disc diffusion method. These isolates were then incubated for 24 hours at 37°C. The inhibition zones of growth were determined using the Clinical Laboratory Standards Institute (CLSI 2021) guidelines. A polymerase chain reaction technique (PCR) was then used to detect the *mecA* gene [14].

2.5 Antibiotic sensitivity test

The disk diffusion method was used to evaluate antibiotic susceptibility, as recommended by CLSI [13]. gentamycin (10 µg), ciprofloxacin (5 µg), pencillin G (10U), vancomycin (30 µg), and chloramphenicol (30µg) were all evaluated as antimicrobial disks [14].

2.6 Detection of the MRSA isolates ability to formation of biofilm

1. The media for biofilm development was Tryptic soy broth enriched with an extra 1% glucose.
2. Biofilm inoculums were prepared by diluting bacteria cultivated in broth 1:100 in TSB mixed with 1% glucose and then dropping 200 µl into each well. The negative control wells were filled with broth (200 µl TSB containing 1% glucose per well). Each strain replicated at least three replicates, then a lid was placed over a inoculation plate and incubated aerobically 24 hours at 35–37°C under static circumstances.

3 - The contents of the wells were decanted and rinsed well three to four times. With 300 µl Phosphate buffered saline. The plates should then be drained inverted and fixed with 150 ml methanol.

4. Stained was performed by using 150 µl of crystal violet at room temperature for 15 minutes, rinsed, and dried at room temperature.
5. Dye was resolubilized by adding 150 µl of 95% ethanol, covered with a lid, and let at room temperature for 30 minutes without any shaking.
6. Each well optical density was calculated at 570 nm by the reader of microtiter plate as interpreted according to Stepanović *et al* [12].

2.7 Genomic DNA Extraction

Bacterial DNA extraction was performed in accordance with the manufacturer's instructions, using an Easy Pure® Genomic DNA Extraction Kit (TRANSgen, China).

2.8 Polymerase Chain Reaction assay

All MRSA isolates were screened molecularly for *mecA* ,*icaA* and *D* genes using the PCR. The amplification protocol for *icaA* and *D* included an initial denaturation step at 94 °C for 5

minutes, followed by 30 cycles of denaturation at 94 °C for 60 seconds, annealing at 55 °C for 60 seconds, and elongation at 72 °C for 60 seconds. Electrophoresis of the PCR product in a 2 % agarose gel dyed with red stain was used to evaluate them [15,16]. The primers and product sizes of the PCR amplification result are reported in Table 1.

Table 1: The primers sequences used in PCR for detection of *mecA, icaA* and *icaD*

Primer Name	Sequence 5' → 3'	Size (bp)	References
***mecA*-F**	TCCAGATTACAACTT CACCAGG	162	**[15]**
mecA-R	CCACTTCATATCT TGTAACG		
***ica A*-F**	ACACTTGCTGG CGCAGTCAA	188	[16]
***ica A*-R**	TCTGGAACCA ACATCCAACA		
***ica D*-F**	ATGGTCAAGCCC AGACAGAG	198	[16]
***ica D*-R**	AGTATTTTCAA TGTTTAAAGCAA		

2.9 *Statistical Analysis*

The Statistical Analysis System- SAS (2012) was used to analyze the effect of different factors in in this study parameters Chi-square test was used to significant compare between percentage (0.05 and 0.01probability) in this study [17].

3 - *Result*

3. 1 Staphylococci and MRSA Isolation and Identification

215 clinical specimens were obtained from the urinary tract infections patients, cystic fibrosis patients, pneumonia patients, bacteremia patients, chronic burns, deep wounds and catheterized patients. Only 155(72%) isolates grew on blood agar and mannitol salt agar, which was deemed as a selective, differential medium for *Staphylococcus* [18]. Staphylococci from a total of 155 was distributed in chronic burns swabs 35 (22.5%), followed by deep wounds swabs 33 (21.2%), urine from UTI patients 32 (20.6%), sputum from cystic fibrosis patients 27 (17.4%), catheterized patients swabs 19 (12.2%), and blood bacteremia patients 9 (5.8%). Coagulase testing was performed to differentiate between Staphylococci that generate coagulase enzyme (CoPS) and those that do not (CoNS), with the results indicating that 66 (42.8%) isolates were CoPS and 89 (57.4%) isolates were CoNS. All CoPS isolates grew on skim milk agar and produced a white colony from culture characteristics and biochemical testing. All isolates were recognized as *S.aureus*, as shown in Table 2.

Table 2: The biochemical tests of *S.aureus* isolates.

Biochemical test	Results of 66 CoPS isolates
Mannitol salt agar	100% yellow colonies

Blood agar	100% golden yellow colonies (alpha and beta hemolysis)
Milk agar (pigment of colony)	100% White colonies
Catalase	100% Babbles (+)
Oxidase	100% No Purple color (-)
Coagulase	100% (+)
Motility	100% (-)

(+): positive; (-): Negative

Finally the confirmation test was conducted by VITEK2 system, with a probability that 95% belonged to *Staphylococcus aureus.*
To screen methicillin-resistant isolates of *S. aureus*, the antibiotic disc diffusion method was tested on all 66 isolates. The test was conducted using an antibiotic disc containing cefoxitin (30 µg/disc) because to its excellent sensitivity for detecting the *mecA* gene [19, 20]. MRSA was identified using the standard PCR. The presence of the *mecA* gene was confirmed molecularly when a 162bp band emerged on a 2% agarose gel. This finding complements our phenotypic work identifying MRSA using the cefoxitin disc diffusion method, in which 45 (68.1%) isolates possessed the *mecA* gene was confirmed with PCR. Primers listed in Tables 1 were used to initiate the amplification of genes.

3.2 Microtiter plate method

Forty five MRSA clinical isolates obtained from 66 *S. aureus* isolates, were utilized to identify biofilm formation by using the polystyrene microtiter plate method as described by Stepanović *et al* [12]. 45 (100%) MRSA isolates were biofilm producers, of them 19 (42%) isolates had a robust biofilm, 9 (20%) isolates had a moderate biofilm, and 17 (38%) isolates had a weak biofilm, and none of them had any attachment.

3. 3 Antibiotic Susceptibility test

Susceptibility test of five different antibiotics (ciprofloxacin, chloramphenicol, penicillin G, gentamycin, and vancomycin) was determined for MRSA isolates using the method of disc diffusion as recommended by the National Committee for CLSI (2021), where inhibition zone was measured using a ruler [14].

The results indicated that all MRSA isolates were resistant to penicillin, also 60% of the isolates were resistant to gentamycin, while 42%, of the isolates were ciprofloxacin resistant. On the other hand all isolates were sensitive to vancomycin and 95% of them were sensitive to chloramphenicol as shown in Table 3.

Table 3: Distribution of antibiotic resistance among MRSA isolates

Antibiotic	Percentage of resistance isolate; R	Percentage of moderate isolate; I	Percentage of sensitive isolate ;S	Chi-Square (χ^2-)

CN	27(60%)	4(8.88%)	14(31.11%)	12.61 **
CIP	19(42.22%)	7(15.5%)	19(42.22%)	9.892 **
PEN	45(100%)	0	0	15.00 **
VAN	0	0	45(100%)	15.00 **
CHL	1(2.22%)	1(2.22%)	43(95.55%)	14.97 **
Chi-Square (χ^2)	16.498 **	4.927 *	15.033 **	----

*** (P≤0.05), ** (P≤0.01). CN(gentamycin), CIP(ciprofloxacin), PEN(penicillin), VAN(vancomycin), CHL(chloramphenicol)**

This investigation discovered significant differences in antibiotic resistance between isolates, with high significant differences in gentamycin and ciprofloxacin resistance between strong, moderate and weak biofilm former isolates. The number of isolates that showed multidrug resistance are shown in Table 4.

Table 4: Number of multidrug resistance isolates

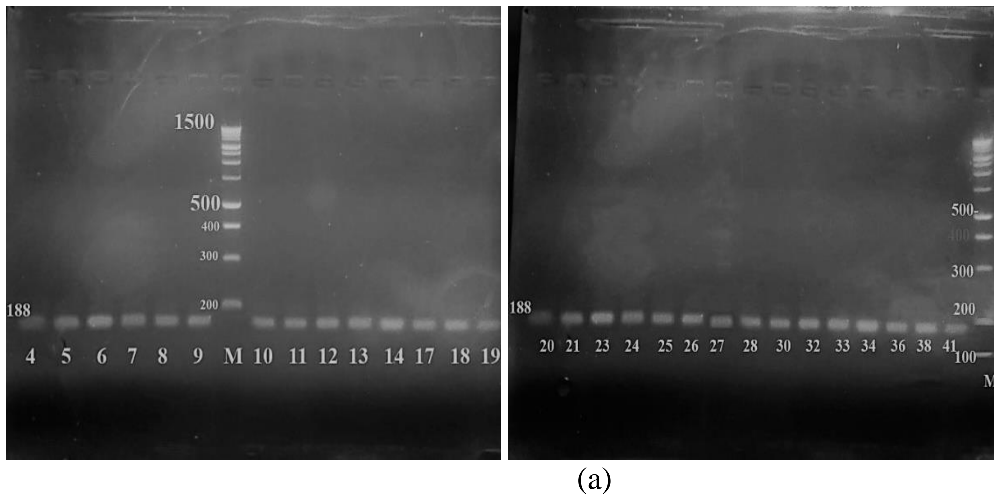
Number of Antibiotics were resistant by isolates	NO.(%)
2 or less	17(37%)
3	10(22%)
4	16(36%)
5 or more	2(5%)
Total	45(100%)

3.4 Identification of Intracellular adhesion (icaA and D genes)

Conventional PCR was employed to screen for the *icaA* and *D* genes responsible for PIA production involved in biofilm formation.

1 - Detection of *icaA* gene

Among total 45 MRSA isolates determined that these genes is present in 33 (73.3%) of MRSA isolates and 12 (26.6%) of MRSA isolates lacked it. The occurrence of the *icaA* gene was screened using 2% agarose gel electrophoresis stained with gel stain, electrophoresed at 70 volts for 1.5 hours, and photographed using an ultraviolet (UV) Trans illuminator as shown in Figures (1a,b and 3).

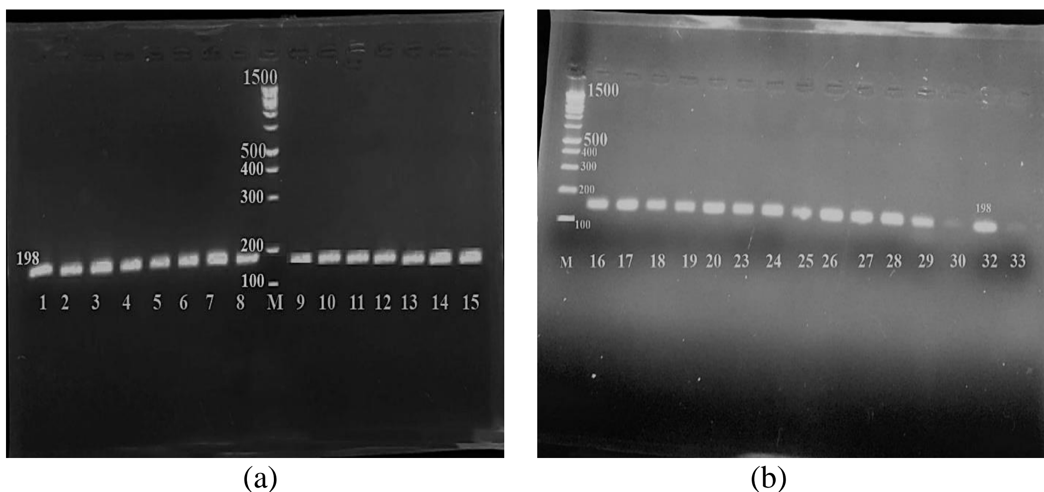


(a) (b)

Figure 1: Gel electrophoresis of amplified PCR product of *ica A* gene (188bp) of MRSA isolates in monoplex pattern, 2% agarose stained with gel stain, TBE buffer (1x), 70 volt for 1.5 hrs. M: DNA ladder marker (100 bp), (a) 4-19: MRSA isolates,(b) 20,21,23-28,30,32-34,36,38: MRSA isolates

2 - Detection of *icaD* gene

The investigation found that this gene was present in 38 (84.4%) of MRSA isolates and was lacking in 8 (17.7%). The *icaD* gene was validated by electrophoresis on 2% agarose gel stained with gel stain, electrophoresed at 70 volt for 1.5 hours, and photographed under UV trans illuminator as shown in Figures (2a, b and 3).



(a) (b)

Figure 2: Gel electrophoresis of amplified PCR product of *icaD* gene (198bp) of MRSA isolates in monoplex pattern, 2% agarose stained with gel stain, TBE buffer (1x), 70 volt for 1.5 hrs. M: DNA ladder marker (100bp), (a) 1-15: MRSA isolates, (b) 16-20, 23-30, 32-33 MRSA isolates.

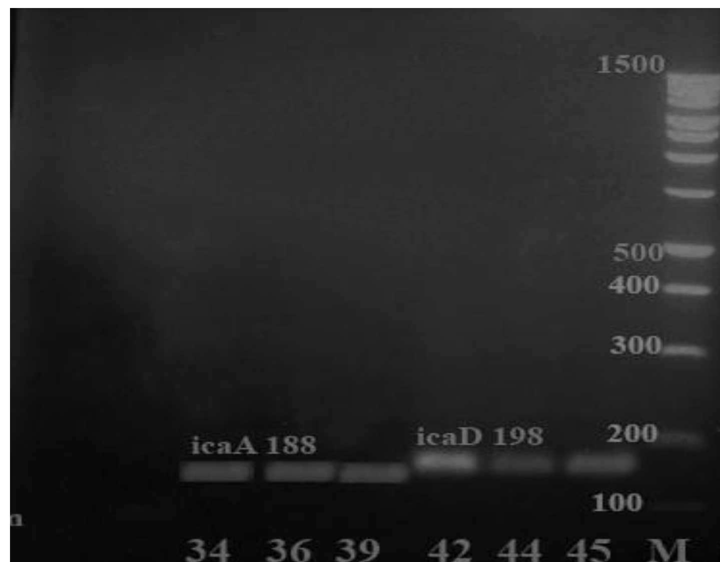


Figure 3: Gel electrophoresis of amplified PCR product of *icaA and D* gene (188,198bp) of MRSA isolates in monoplex pattern, 2% agarose stained with gel stain, TBE buffer (1x), 70 volt for 1.5 hrs. M: DNA ladder marker (100bp), 34, 36, 39,42, 44, 45: MRSA isolates.

The most noteworthy finding was that the five weak biofilm former isolates (22, 31, 35, 37, 40) lacked any genes.

4 - Discussion

Bacterial biofilms can provide a serious health risk to patients requiring catheterization. The development of biofilms is affected by a combination of factors such as the environment, nutrition availability and most importantly, the presence and expression of biofilm-associated genes. It has been demonstrated that biofilm formation plays a vital role in the pathogenicity of MRSA and the generation of multidrug resistant (MDR) strains were characterized as those that were resistant to more than three types of antibiotics classes [21, 22]. Chloramphenicol has demonstrated significant action against MRSA and is anticipated to play a critical role in the treatment of MRSA infections, offering a cost effective substitute to more expensive, newer antimicrobials in resource-scarce countries. Chloramphenicol has shown substantial antimicrobial activity against MRSA *in vitro* and is expected to play a vital role in the treatment of MRSA infections, providing a cost effective replacement to newer, highly expensive antibiotic in resource-constrained countries [23, 24].

Moghadam*et al*. [25] discovered that 97.5% of MRSA isolated from burns, which are the most common dangerous source of infections, are biofilm producers. MRSA infections impact immune-compromised, long-term hospitalized, and severely ill people disproportionately. Antibiotic resistance among nosocomial isolates of MRSA exacerbates the situation and presents a significant therapeutic challenge for hospital acquired infections in general [25].

The spread of antimicrobial resistance among staphylococci species in Iraq has posed a significant challenge to the medical community, and unfortunately, treatment failures in MRSA infections are increasing due to a lack of adequate information about antimicrobial resistance, particularly glycopeptide resistance, among endemic Staphylococci. This knowledge is necessary for the proper treatment of individuals with staphylococci infections, It is the third most frequently encountered cause of bacteremia and the second most frequently encountered cause of urinary tract infections [7].

Salina *et al*. [26] just conducted a study that there was no association between the production of *icaA* and *D* genes and the development of biofilms, indicating that the correlation is ambiguous and requires clarification [26].

The most noteworthy finding was that the five weak isolates lacked any genes from the *ica* genes, indicating that these isolates are capable of producing biofilm without the need for *ica* in order to make PIA meaning that they have an ability to form biofilm in *ica* independent biofilm mechanisms. It is proposed that these biofilm developer isolates utilized other systems for biofilm formation that have been found, including the protein A, the surface proteins G and C, and the fibronectin-binding proteins [9].

Azmi *et al*. [11] reported that 100% of MRSA clinical isolates in Palestine were *icaA* and *D* positive while Arciola *et al*. [27] found *icaA* and *D* in 61% of S. *aureus* isolates. The first two genes, *icaA* and *icaD*, are essential for exopolysaccharide production. The *icaA* gene product is a transmembrane protein containing a N-acetylglucosaminyl transferase enzyme that catalyzes the formation of poly-N acetyl glucosamine polymer. It has been established that the *icaD* gene product is required for the most optimal enzymatic activity of the *icaA* gene product [25]. Some isolates contain two gene and show strong ability to form biofilm whereas other isolates show weak ability with also two genes [27].

5 - *Conclusion*

Although all of the MRSA isolates in the current study have been capable of generating biofilms. The occurrence of *icaA* and *D* genes was not always related with *in vitro* production of biofilm. Then, the capacity of five isolates to produce biofilms in the lack of *icaA* and *D* genes emphasizes the importance of additional genetic study on biofilm formation pathways independent of *ica* locus gene.

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