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# Biofilm Shows Independency form Hemolysin Genes Arsenal in Methicillin Resistant *Staphylococcus Aureus*

**Nihad Taha Mohammed Jaddoa*, Harith Jabbar Fahad Al-Mathkhury**
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

**Abstract**

Normally, bacteria exposed to antibiotics at sub minimal inhibitory concentrations (MIC) inside the host. Therefore, the current study aimed to comprehend the association among hemolysins, biofilm, as well as gentamicin resistance in local MRSA isolates. Around 35 *Staphylococcus aureus* locally isolated from different clinical specimens were employed in this study. Methicillin resistance was detected via cefoxitin disk diffusion and *mecA* amplification methods. MIC of gentamicin was estimated by broth microdilution method. Hemolysin genes involving *hla*, *hlb*, *hld*, and *hlg* were determined using multiplex polymerase chain reaction (PCR) technique. Microtiter plate method was employed for biofilm assessment in the presence and absence of gentamicin at sub MIC. Moreover, atomic force microscopy technique was employed for confirming the effect of gentamicin on biofilm. The present findings revealed that methicillin resistant *S. aureus* (MRSA) constituted, nearly, 94.29% (33 isolates) of all *S. aureus* isolates. Around 12 (36.36%), four (12.12%), and 17 (51.51%) isolates were gentamicin-sensitive, intermediate, and resistant to gentamicin, respectively. *hla* and *hld* were located in 32 out 33 MRSA isolates. All MRSA isolates succeeded in forming biofilm; however, three (0.09%), 23 (69.69%), and seven (21.21%) isolates formed weak,moderate, andstrong biofilm, respectively. Gentamicin at sub MIC reduced the intensity of biofilm and the AFM confirmed this finding. In conclusion, very weak correlation linked the biofilm formation capacity and isolate MIC. On the other hand, possession of hemolysin genes seems has no correlation with biofilm formation. Nevertheless, gentamicin at sub MIC reduced the intensity of MRSA biofilm.

**Keywords**: MRSA, hemolysin, biofilm, gentamicin

# يظهر الغشاء الحياتي استقلالية عن ترسانة جينات الهيمولايسين في العنقوديات الذهبية المقاومة للمثيسلين

**نهاد طه محمد جدوع*، حارث جبار فهد المذخوري**
قسم علوم الحياة ، كلية العلوم ، جامعة بغداد، بغداد، العراق

**الخلاصة**

من الطبيعي ان تتعرض البكتريا الى مضادات الحياة عند التراكيز المثبطة تحت الدنيا داخل المضيف. لذلك هدفت الدراسة الحالية الى فهم الارتباط بين الهيمولايسينات و الغشاء الحياتي و كذلك مقاومة الجنتامايسين في عزلات محلية من العنقوديات الذهبية المقاومة للمثيسيلين. حوالي 33 عزلة محلية من العنقوديات الذهبية عزلت من مصادر سريرية مختلفة. تم التحري عن مقاومة المثيسيلين بطريقة انتشار قرص السيفوكسيتين و تضخيم جين *mecA*. تم الكشف عن جينات الهيمولايسنات متمثلة بـ *hla* و *hlb* و *hld* و

*Email: nihad.bio2015@gamil.com

*hlg* باستعمال تقنية تفاعل البلمرة المتسلسل المتعدد. واستعملت طريقة طبق المعايرة الدقيقة في تقييم الغشاء الحياتي بوجود و غياب الجنتامايسين عند التركيز المثبط تحت الأدنى. كما تم استعمال تقنية مجهر القوة الايونية في تأكيد تأثير الجنتامايسين في الغشاء الحياتي. بينت نتائج الدراسة الحالية ان العنقوديات الذهبية المقاومة للمثيسيلين (MRSA) شكلت حوالي 94.29% من مجموع العنقوديات الذهبية. تقريبا اظهرت 12 (36.36%) و أربعة (12.12%) و 17 (51.51%) عزلة حساسية و مقاومة متوسطة و مقاومة للجنتامايسين على التتابع. تم تحديد وجود *hla* و *hld* في 32 من 33 عزلة MRSA. نجحت عزلات MRSA جميعها في تكوين الغشاء الحياتي في حين كونت ثلاث (0.09 %) و 23 (69.69 %) و سبعة (21.21 %) عزلات غشاءا حياتيا ضعيفا و متوسطا و قويا على التتابع. خفض الجنتامايسين عند التركيز المثبط تحت الأدنى من شدة الغشاء الحياتي و قد أكد ذلك الفحص بمجهر القوة الايونية. كاستنتاج يمكن القول ان هناك ارتباطا ضعيفا ربط قدرة تكوين الغشاء الحياتي و التركيز المثبط الأدنى. و من جهة أخرى، يبدو ان امتلاك جينات الهيمولايسين ليس له علاقة بتكوين الغشاء الحياتي. مع ذلك فان الجنتامايسين عن التركيز المثبط تحت الأدنى قلل من كثافة الغشاء الحياتي في عزلات MRSA.

## Introduction

*Staphylococcus aureus* is an important clinical pathogen, which causes a wide variety of infection for both humans and animals, fluctuating from mild to severe life-threatening infections[1]. *S. aureus* is recognized as a crucial agent associatedwith nosocomial infections leading toconsiderable morbidity and mortality among hospitalized patients. Partially, it is attributed to its ability to form biofilms on the surface of living tissues as well as indwelling medical devices[2].

In order to resist the host immune defenses,survive inside host tissues, and eventually establish an infection, *S. aureus* elaborates a widespreadarsenal of virulence determinants, such as extracellular enzymes and toxins (e.g. proteases, coagulases,hemolysins, leukocidins)[3].

The increasing incidence of multi-drug resistant *S. aureus* strains, particularly, methicillin-resistant *S. aureus* (MRSA) is a serious problematic issue in therapeutic strategies and simultaneously considered as a threatto boththe clinical settings and community[4].

Several microbiological studies have employed the Atomic force microscopy (AFM) to examine the bacterial cells, biofilm, and the effect of antibiotics on bacterial cells. Touhami*et al*. [5] used AFM for examining the growth and division in *S. aureus.* While Neethirajan and DiCicco [6]revealed that the fosfomycin inhibits cell division, and prevents the adhesion on the surface discouraging the biofilm attachment on methicillin-resistant*Staphylococcuspsudintermidius* by using AFM image analysis. The AFM also used to study microbial systems to provide a unique insight into their behavior and relationship with their environment [7].

The growing knowledge of bacterial virulence and pathogenesis highlights new horizons for the development of novel and alternative treatment choices by attenuating bacterial virulence involving biofilm[4]. Furthermore, the exposure of bacteria to antibiotics at sub MIC may usually take place during antibiotic therapy[8]. Hence, the present work aimed to understand the interconnection among hemolysins, biofilm, and gentamicin resistance in local MRSA isolates.

## Materials and Methods

### Microorganisms

Around 33 MRSA isolates were previously isolated form patients referring some hospitals in Baghdad. They were preserved at microbiology lab, Department of Biology, College of Science, University of Baghdad.

### Detection of MRSA by Cefoxitin Disk Diffusion method

All *S. aureus* isolates were tested by cefoxitin disk diffusion methodology using a 30 µg disk. A suspension of the isolate comparable to 0.5 McFarland standard and lawn culture was performed on Mueller-Hinton agar plate. Plates were incubated at 36ºC for 18hrs. Thereafter, diameter of inhibition zone was measured using a metric ruler. An inhibition zone diameter of ≤ 21 mm was reported as Methicillin-resistance and ≥22 mm was considered as methicillin sensitive [9].

**Determination of minimum inhibitory concentration (MIC) of Gentamicin**

Different concentrations of gentamicin (2 – 1024 µg/ml) were used to estimate the MIC following agar dilution method and the results were interpreted in accordance to the guidelines of Clinical Laboratory Standards Institute[9].

**Biofilm formation assay**

Quantification of biofilm formation by MRSA isolates was assessed as described by Atshan*et al*. [10]. In brief; each isolate was propagated in tryptic soy broth at 37°C for 24h; thereafter, bacterial culture was adjusted to McFarland standard no. 0.5. A volume (200 µl) of an isolate culture was added to three wells of sterile 96-well U shaped-bottom polystyrene microplates. All plates were covered with their lids and incubated under aerobic conditions at 37°C for 24h. Six wells filled with (200 µl) of bacteria-free tryptic soy broth were considered as a negative control. After incubation, all plates were gently washed thrice with distilled water, dried, and fixed at 65˚for 1 hr. Afterward, an aliquot (200 µl) of methanol was added to each well for 15 min. at room temperature. Subsequently, the plates were stained with 200 µl of 0.1% crystal violet solution for 15 min at room temperature. Excess stain was rinsed off by placing the plate under running tap water. After that, the plates were dried at 37°C for approximately 30 min to ensure they were completely dried. The adherent cells were resolubilized with 200 µl of absolute ethanol for 10 min. Eventually, optical density of each well was obtained at 630 nm using microplate reader (BioTek, USA). Classification of bacterial adherence summarized in Table-1 based on $OD_{630}$ values obtained for individual isolate of *S. aureus* wasused for the purpose of data simplification and calculation. After calculating the biofilm formation capacity for all tested isolates and negative controls, the cut-off value (ODc) was established. It is defined as three standard deviations (SD) above the mean OD of the negative control: ODc value was calculated for each microtiter plate separately. When a negative value was obtained, it presented as zero, while any positive value was an indicator for biofilm production.

**Table 1**-Classification of bacterial adherence by tissue culture plate method [10]

| **Mean $OD_{630}$** | **Biofilm intensity** |
|---|---|
| OD ≤ ODc* | Non–adherent |
| 2ODc> OD >ODc | Weak |
| 4 ODc> OD > 2ODc | Moderate |
| OD >4 ODc | Strong |

*Cut off value (ODc) = average OD of negative control + (3 *Standard Deviation).

**Effect of gentamicin at sub MIC on biofilm formation**

The same protocol was used for the biofilm formation assay, which previously mentioned (ten isolates were chosen which exhibited MIC 32µg/ml). However, tryptic soy broth contained gentamicin at subMIC. The plates were incubated at 37ºC for 24h. After that, all wells washed, stained, and read at 630 nm. Positive controls were performed as well by adding 200 µl of gentamicin-free fresh bacterial suspension (compatible to 0.5 McFarland standard).

**Atomic force microscopy (AFM)**

The isolate SA31, which exhibited the highest biofilm intensity, was chosen for investigating the effect of gentamicin at sub MIC on biofilm using AFM technique. The same protocol was followed for the biofilm formation assay which previously mentioned, except for staining step that was skipped and then sends to examination by AFM (Unico, USA).

**Polymerase chain reaction assay**

**DNA extration**

DNA was extracted from all *S. aureus* isolates using G-spin DNA extraction kit (Intron, Korea). The DNA concentration and purity were determined using Nanodrop instrument.

**Detection of *16SrRNA* and *mecA***

The presence of *16SrRNA* of *S. aureus* and *mecA* were detected by amplifying the extracted genomic DNA using the Master thermocycler gradient PCR (Eppendorf, Germany).

Primers needed for amplification a fragment of *16SrRNA* and *mecA* are listed in Table-2. These primers were provided in a lyophilized form, dissolved in sterile nuclease-free water to prepare 100

pmol/µl as a final concentration in accordance to the manufacturing company instructions, and stored in the deep freezer until used in PCR amplification.

**Table 2-**The primers and their sequences used in the conventional PCR

| Target gene | Primer sequences (5´-3´) | Amplicon size (bp) | Reference |
|---|---|---|---|
| *16SrRNA* | SA1-AATCTTTGTCGGTACACGATATTCTTCACG | 108 | [11] |
| | SA2'-CGTAATGAGATTTCAGTAGATAATACAACA | | |
| *mecA* | MRS1 -TAGAATGACTGAACGTCCG | 154 | [12] |
| | MRS2 -TTG CGATCAATGTCCGTAG | | |
| *hla* | F-CTGATTACTATCCAAGAAATTCGATTG | 209 | [13] |
| | R-CTTTCCAGCCTACTTTTTTATCAGT | | |
| *hlb* | F-GTGCACTTACTGACAATAGTGC | 309 | |
| | R-GTTGATGAGTAGCTACCTTCAGT | | |
| *hld* | F-AAGAATTTTTATCTTAATTAAGGAAGGAGTG | 111 | |
| | R-TTAGTGAATTTGTTCACTGTGTCGA | | |
| *Hlg* | F-GTCAYAGAGTCCATAATGCATTTAA | 535 | |
| | R-CACCAAATGTATAGCCTAAAGTG | | |

All PCR reaction tubes were prepared in 25 µl as a final volume. Reactants included five microliters of PCR premix (Taq PCR Master Mix), one microliter of each primer; while DNA template volume was 1.5 µl. Thereafter, the volume was completed up to 25 µl with sterile nuclease-free water. Afterward, the mixture was vortexed gently and the PCR tubes were centrifuged briefly in order to obtain good mixing. The adopted PCR protocol was followed after several trials as it is demonstrated in Table-3.

**Table 3-**The optimal conditions for detection of *16SrRNA* and *mecA* genes

| Phase | T (°C) | Time | No. of cycles |
|---|---|---|---|
| Initial Denaturation | 94 | 3 min. | 1 |
| Denaturation | 94 | 30 sec. | 35 |
| Annealing | 58 | 30 sec. | |
| Extension | 72 | 30 sec. | |
| Final Extension | 72 | 10 min. | 1 |

**Multiplex PCR for the detection of hemolysin genes**

PCR assay was performed in a multiplex pattern in order to amplify different fragments of genes under study in a single tube for detecting *hla*, *hlb*, *hld* and *hlg* genes.

All the primers that were selected for detecting hemolysin genes are listed in Table- 2. These primers were provided in a lyophilized form, in order to give a final concentration of 100 pmol/µl. They were dissolved in sterile nuclease-free water as instructed by the manufacturer, and stored in the deep freezer until used in PCR protocol.

PCR reactant mixture included five microliters of PCR premix (Taq PCR Master Mix), one microliter of each primer. Lastly, template DNA (1.5 µl) was added. Sterile nuclease-free water was used to complete the volume up to 25 µl. After several trials, the PCR conditions were followed and summarized in Table-4. PCR products were visualized in 1.5% agarose.

**Table 4-**The optimal conditions for the detection of the hemolysin genes

| Phase | T (°C) | Time (min) | No. of cycle |
|---|---|---|---|
| Initial Denaturation | 94 | 5 | 1 |
| Denaturation | 94 | 1.25 | 35 |
| Annealing | 62 | 1.25 | |
| Extension | 72 | 1.25 | |
| Final Extension | 72 | 10 | 1 |

**Statistical analysis**

Means of triplicate were statistically analyzed using ANOVA, T test, and $LSD_{0.05}$. P value less than 0.05 was considered significant.

**Results**

PCR technique was adopted to confirm the identification via amplification of a fragment of *16SrRNA*gene for 35 *S. aureus* isolates. The result showed that all these isolates harbored this gene.

**Detection of methicillin resistance**

The cefoxitin sensitivity test was done for all *S. aureus* isolates. The results showed that 33 isolates (94.29%) out of 35 *S. aureus* were MRSA. Methicillin resistance was also detected molecularly as well in all cefoxitin-resistant through the amplification of *mecA* gene. Markedly, the result revealed that *mecA* gene was located in all cefoxitin-resistant *S. aureus* isolates.

**Determination of MIC**

Findings of this work clarified that 12 (36.36%), four (12.12%), and 17 isolates were gentamicin-sensitive, intermediate, and resistant to gentamicin, respectively. Interestingly, 16 isolates (94.1%) out of the 17 resistant isolates resisted ≥ 32 µg/ml of gentamicin whereas only one isolate (5.88%) exhibited MIC 256 µg/ml, hence sub-MIC (16 µg/ml) was used for further experiments.

**Biofilm forming capacity**

In order to estimate biofilm intensity, absorbance was determined at $630_{nm}$ by microplate reader. Given that, absorbance values are corresponding to the degree of biofilm thickness that formed by the isolates in question. The obtained results were categorized into four groups (viz., non-biofilm producer, weak, moderate, and strong) based on limits summarized in Table- 1. The present study demonstrated that out of 33 MRSA, three isolates formed a weak biofilm, 23 (69.69%) isolates formed a moderate biofilm, whereas seven isolates formed a strong biofilm.

**Effect of of gentamicin at sub MIC on biofilm formation**

Findings of the present work (Figure-1) clarified that the gentamicin at sub MIC was effective against all bacterial biofilms under test. Yet, this effect differs from one isolate to another. Obviously, the biofilms were significantly ($P< 0.05$) reduced in isolates S13, S15, S16, S27, S31, S107 and S115. Furthermore, this reduction was insignificant ($P< 0.05$) among the isolates S16, S20, and S109.

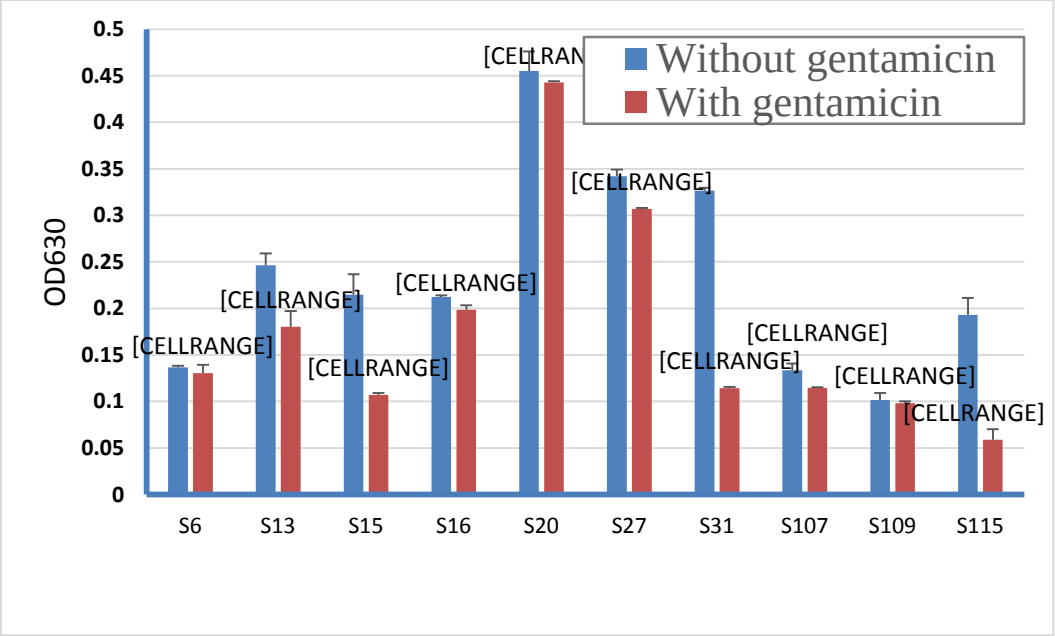


**Figure 1**-Effect of gentamicin on biofilm forming capacity. Error bars denote to standard deviation. Asterisks represent significance differences (*; P< 0.05, **; P< 0.01)

**Atomic force microscopy examination of MRSA biofilm**

The AFM image depicted in Figure-2 enlightens that the gentamicin inhibits biofilm formation which appeared as a reduction in tower heights (from 326.6 nm down to 25.9 nm). Moreover, the average of roughness analysis revealed that gentamicin treatment reduced the roughness average from 5.78 nm down to 76.2 nm (Figure-3).

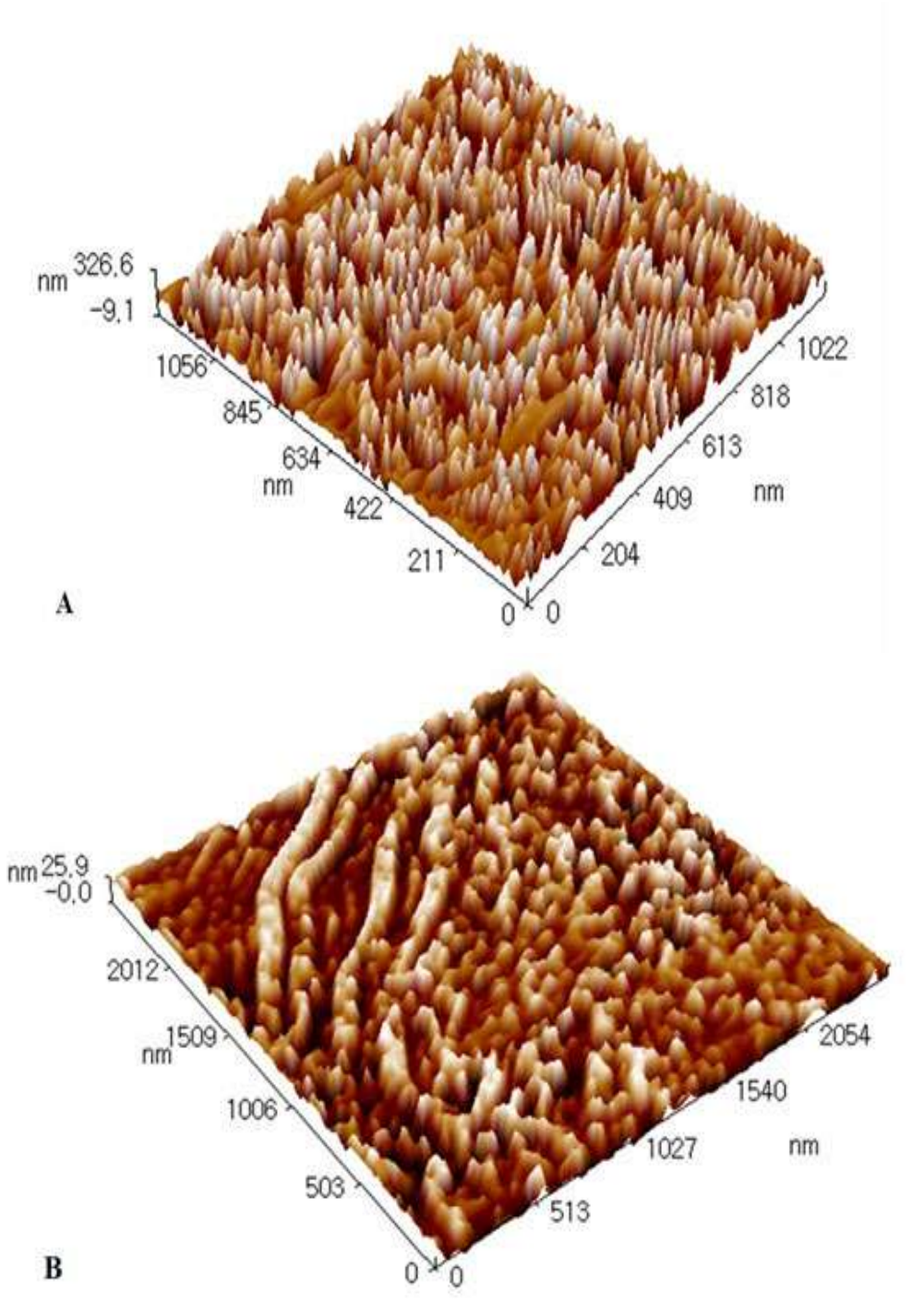


**Figure 2-**Atomic force micrograph of methicillin-resistant *S. aureus* biofilm. A) without and B) with gentamicin

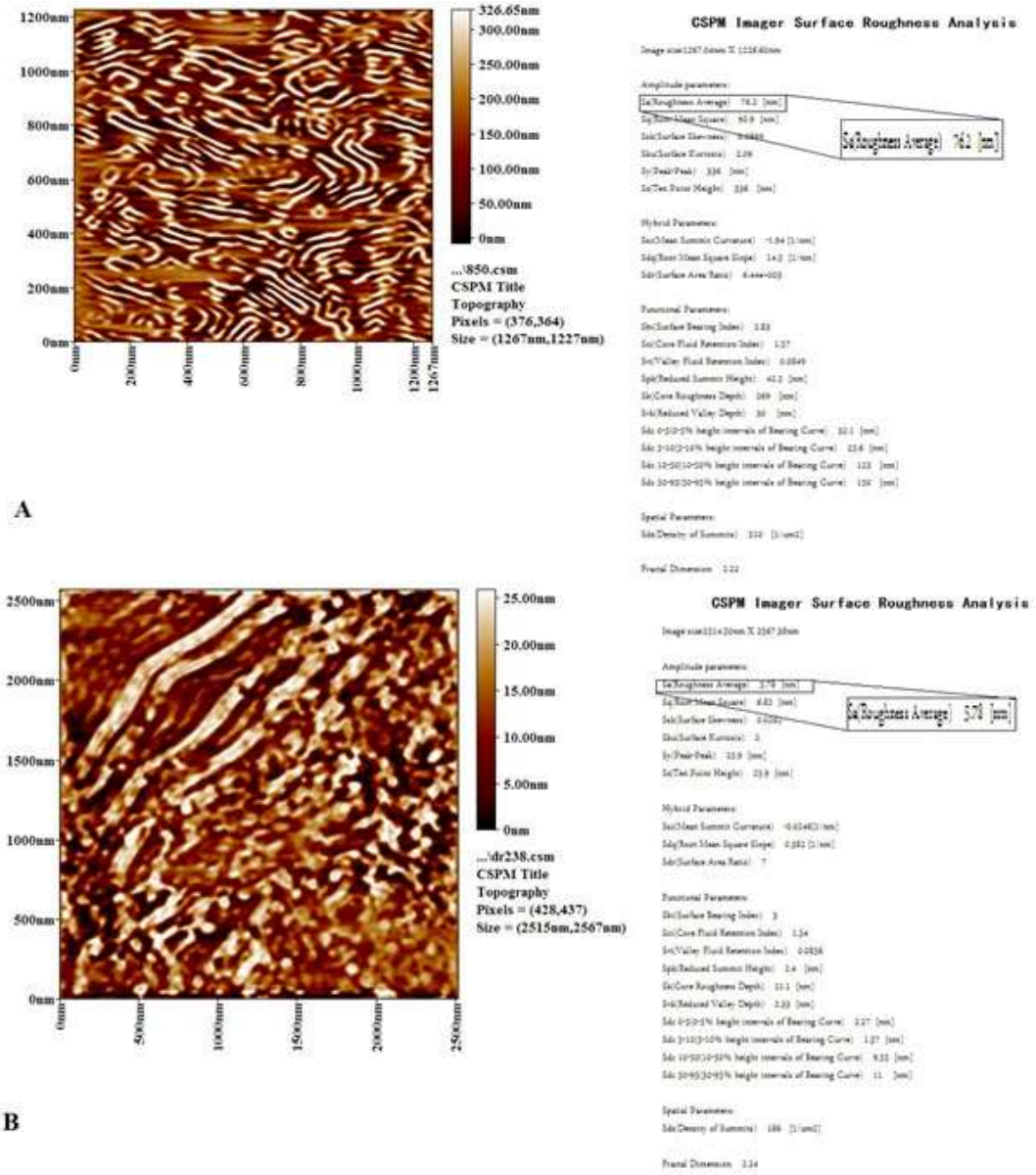


**Figure 3-**Atomic force micrograph of *S. aureus* biofilm roughness analysis before (A) and after (B) gentamicin treatment.

**Amplification of hemolytic genes by multiplex PCR technique**

In regard to the detection of hemolysin genes; alpha, beta, gamma and delta for each *S. aureus* isolate, amplification of fragments of these genes were performed by PCR technique in the multiplex pattern.

The present results (Table-5) clarified a presence of *hla* and *hld* genes (coded for α- and δ-hemolysins, respectively) in nearly all (100%) MRSA isolates with the exception of one isolate. Whereas, *hlb* (coded for β- hemolysin) was located in12 (36.36%) isolates. What's more, one isolate has *hlg* (coded for γ- hemolysin)

**Table 5**-gentamicin MIC, hemolysins, biofilm intensity of MRSA isolates

| Isolate code | MIC of Gentamicin (µg/ml) | hla | hlb | hld | hlg | Biofilm intensity |
|---|---|---|---|---|---|---|
| **SA2** | **4** | + | - | + | - | **M** |
| **SA5** | **4** | + | - | + | - | **M** |
| **SA6** | **32** | + | - | + | - | **M** |
| **SA9** | **32** | + | - | + | - | **M** |
| **SA13** | **32** | + | - | + | - | **M** |
| **SA15** | **32** | + | + | + | - | **M** |
| **SA16** | **32** | + | + | + | - | **M** |
| **SA20** | **32** | + | + | + | - | **S** |
| **SA22** | **8** | + | - | + | - | **M** |
| **SA23** | **4** | + | - | + | - | **M** |
| **SA24** | **4** | + | + | + | - | **W** |
| **SA25** | **8** | + | - | + | + | **M** |
| **SA26** | **32** | + | - | + | - | **M** |
| **SA27** | **32** | + | - | + | - | **S** |
| **SA29** | **32** | + | - | + | - | **M** |
| **SA30** | **2** | + | - | + | - | **S** |
| **SA31** | **32** | + | - | + | - | **S** |
| **SA33** | **8** | + | - | + | - | **M** |
| **SA34** | **32** | + | + | + | - | **S** |
| **SA35** | **2** | + | - | + | - | **M** |
| **SA37** | **4** | + | - | + | - | **M** |
| **SA38** | **32** | + | - | + | - | **W** |
| **SA41** | **32** | + | + | + | - | **M** |
| **SA50** | **2** | + | + | + | - | **M** |
| **SA56** | **2** | + | + | + | - | **M** |
| **SA60** | **256** | + | - | + | - | **M** |
| **SA66** | **8** | + | - | + | - | **M** |
| **SA70** | **2** | + | + | + | - | **S** |
| **SA95** | **2** | + | + | + | - | **M** |
| **SA101** | **2** | - | + | - | - | **S** |
| **SA107** | **32** | + | + | + | - | **M** |
| **SA109** | **32** | + | - | + | - | **W** |
| **SA115** | **32** | + | - | + | - | **M** |

**MIC; minimum inhibitory concentration, +; present, -; absent, W; weak, M; moderate, S; strong.**

## Discussion

Rapid and accurate detection of MRSA is an important role of clinical microbiology laboratories to avoid treatment failure. Cefoxitin is a more potent inducer of *mecA* regulatory system than penicillins [14], accordingly, it is being recommended for the detection of MRSA when using disk diffusion test.
This result (94.29%) is in agreement with many local studies. Mohammed [15] reported that the incidence of MRSA is 95%. Karam and Al-Mathkhury [16] demonstrated that 80% of *S. aureus* were identified as MRSA. Upon the result of Al-Dahbi and Al-Mathkhury [17], MRSA covered 94.3% among *S. aureus* isolates. Nevertheless, Muhammad and Al-Mathkhury [18] reported in a study accomplished in Al-Sulaimania city that MRSA covered 68% of all *S. aureus* isolates.
Cefoxitin disk diffusion methodology is highly accepted technique for detecting methicillin resistance in *S. aureus* isolates by many international reference organization dealing with antibiotic resistance such as CLSI. Interestingly, detection of MRSA by this method was not influenced by temperature deviations between 35˚C and 36˚C whereas at 37˚C one false result was obtained [19].

The other phenotypic test included oxacillin disk diffusion, oxacillin screen agar, oxacillin agar dilution method, oxacillin MIC test, and CHOMagar, detection of MRSA by these phenotypic assays are easy to perform and reasonably low-cost methods for detecting methicillin resistance. Yet, the

precise detection of MRSA via phenotypic tests is affected by inoculum size variations, incubation intervals, and pH [20]. Consequently, such phenotypic methods are not entirely dependable.

Infections due to MRSA are an accumulative problematic issue worldwide, both outside and inside clinical settings. It is clinically and epidemiologically important for laboratories to be able to differentiate MRSA from MSSA. Not only for choosing appropriate antibiotic therapy for the individual patient, but also for control of MRSA transmission [21]. Misdiagnosis of MRSA lead to failure treatment and spread of infection with these resistant strains. This means that disk diffusion testing by using cefoxitin disk is so far superior to most of the currently recommended phenotypic methods and is now an accepted method for the determination of MRSA by many authors and related centers including CLSI [19]. Swenson *et al*. [22] have demonstrated that the results of the cefoxitin disk diffusion test correlate better with the presence of *mecA* than do the results of disk diffusion tests using oxacillin

Belbase *et al*. [23] reported that MRSA isolates exhibited high susceptibility (100%) to vancomycin followed by tetracycline and chloramphenicol (97.2%), while 61.2% of MRSA isolates developed susceptibility to gentamicin and all isolates were resistant to penicillin and cefoxitin. Johan and Murugan [24]observed that 65.56% of MRSA were resistant to gentamicin and 100% of MRSA sensitive toward vancomycin, which assures this drug to be an efficient choice for treatment.
The biofilms production can be a marker of virulence; which can be detected phenotypically, there are several methods for the detection of biofilm formation, but the most widely used assay is the microtiter plate method [10].The microtiter plate assay was considered as a standard test for the detection of biofilm formation [25]. Therefore, in this study, the biofilm forming capacity of *S. aureus* was assayed using pre-sterilized 96-well polystyrene microtiter plates. This result is in agreement with other studies of MRSA biofilm formation, Moghadam *et al*. [26] stated the majority of MRSA strains (62.5%) were moderate biofilm producers, but these results were incompatible with study published by Karam and Al-Mathkhury [16] as they revealed that out of 16 MRSA, 12 formed weak biofilm; while only four isolates developed mild biofilm. Biofilm formation is a defense mechanism of *S. aureus* biofilm-protected bacteria from host defense mechanism and shows resistance to standard antibiotic therapy [27]. Paara-Ruzi *et al*. [28] demonstrated that the low level of clarithromycin could inhibit the biofilm formation process of *S. aureus.*

Biofilm-forming bacteria has lower sensitivity to antibiotics in comparison with planktonic cells. Such sensitivity could be attributed to the exopolysaccharide structure and reduced metabolic activity [29]. Nevertheless, there are different mechanisms shed light on the impact of antibiotics at sub MIC on biofilm formation, for example, quorum sensing inhibition and regulation of gene expression; which responsible for the intracellular adhesion [30].

Gentamicin reduced biofilm intensity in 70% of MRSA isolates, which was illustrated by AFM in terms of height of biofilm towers and roughness for the tested isolate. Nonetheless, there was very weak inverse correlation between antibiotic resistance and biofilm intensity (r = -0.04). Generally, antibiotics reduced biofilm formation; however, several studies showed that the antibiotics could significantly induce biofilm formation depending on antibiotics class and the bacterial strain [31]. A study accomplished by Majidpour *et al.*[8] demonstrated when MRSA isolates exposed to sub MIC of azithromycin and vancomycin could significantly induce the biofilm formation at least of two isolates, and such as these results may cause an adverse effect on the course of treatment. Similarly, Ozturk *et al*. [32] indicated that some antibiotics may induce the biofilm formation.

This study demonstrated the dominance of *hla* and *hld* over other hemolysins genes. Interestingly, these two genes were cofounded together in approximately all isolates, seemingly, this association needs to be investigated carefully. Moreover, very weak correlation was found between presence of hemolysin genes and biofilm intensity (r = 0.18). Ariyanti *et al*. [33] demonstrated that *hla and hld* genes were widely distributed among *S. aureus* isolated from human. Likewise, in a study from southern Iran observed the *hla* and *hld* were located in all isolates, while *hlb* gene was found in 40% of human isolates; however, gamma hemolysin was not detected at all [34].

**Conclusion**

Findings of this study revealed a very weak correlation between the biofilm formation capacity and isolate MIC. This weak correlation also linked the possession of hemolysin genes and biofilm formation. Markedly, gentamicin at sub MIC reduced the intensity of MRSA biofilm.

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