Gentamicin Modulates the Gene Expression of HLA in Methicillin Resistance Staphylococcus Aureus Biofilm

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Abstract

Objective: The present work was undertaken to investigate the impact of sub inhibitory concentration of gentamicin on hla gene expression in methicillin resistant Staphylococcus aureus isolates. Methods: The bacterial isolates used in this study represent 33 MRSA strains, previously isolated from patients visiting several hospitals in Baghdad. Gentamicin, vancomycin, and oxacillin MIC were determined using broth dilution method. Microtiter plate method was adopted to investigate the biofilm forming capacity. Alpha hemolysin was detected by culturing MRSA isolates on rabbit blood agar. Furthermore, hla gene was detected in MRSA isolates using conventional PCR technique; while, qRT-PCR method was performed to assay the hla expression in planktonic and biofilm cells in presence and absence of gentamicin. Results: the present results demonstrated that 12 (36.36%) isolates were gentamicin resistant; whereas, all isolates were resistant to oxacillin and sensitive to vancomycin. Out of 33 MRSA, 3, 23, and 7 isolates formed a weak, moderate, and strong biofilm, respectively. Phenotypically, 30 isolates produced alpha hemolysin on rabbit blood agar plates; nevertheless, hla gene was located in 29 isolates. Of considerable interest, the addition of gentamicin significantly (P < 0.05) reduced the hemolysis activity; while, insignificant fold change (less than two) of hla gene was observed in all tested isolates in the presence of sub MIC of gentamicin (16 µg/ml). Conclusion: gentamicin upregulated the hla gene expression in biofilm cells; hitherto, this increment was isolate specific.

Keywords: HLA, MRSA, Biofilm, Gene expression, Gentamicin

Introduction

Regularly, bacteria may exist as sessile communities known as biofilms that are outstanding structures controlled by a genetically programmed developmental process. Biofilms are accountable for approximately 65% of bacterial infections, moreover[1], cells in biofilms are 1000-fold more resistant than their counterpart cells in the planktonic form, an issue initiating a challenge in treatment of this bacterial pathogen [2]. Interestingly, biofilm of S. aureus grown in the presence of gentamicin developed an increment in biomass in order to reduce the interactions between bacterial surfaces and the antibiotic [3].

Principally, S. aureus elaborate plentiful exotoxins, comprising a group of polypeptides able of causing damage to the plasma membrane of host cell. These polypeptides embrace pore-forming toxins among them; leucocidins, hemolysins, and the phenol soluble modulins. α-Hemolysin is the most well-categorized S. aureus virulence determinants. Once it binds the surface of the target cell, monomers of α-hemolysin start to assemble into a homoheptamer, creating a pore and eventually resulting in a necrotic death [4].

In vitro findings demonstrated that antibiotics alter the hla expression. For instance, aminoglycosides, clindamycin, and macrolides downregulate the alpha toxin production, while beta-lactam antibiotics highly increased and fluoroquinolones slightly increased its level of production. Nevertheless, cell-free supernatants of S. aureus strains treated with nafcillin were significantly more toxic than untreated
culture supernatants, using rat model [5]. Given that MRSA are highly resistant to a wide variety of antibiotics [6-9], targeting virulence determinants might be very interesting new possibility for drug development.

Taking together, the impact of gentamicin on the gene expression of α-hemolysin of S. aureus planktonic and biofilm cells was the prime aim of this study. Given that, a considerable shortage in literature investigating the gene expression of hla in biofilm lifestyle.

**Materials and Methods**

**Microorganisms**

The bacterial isolates used in this study represent 33 MRSA strains, previously isolated from patients visiting several hospitals in Baghdad. They were preserved at microbiology lab, Department of Biology, College of Science, University of Baghdad.

**Determination of Minimum Inhibitory Concentration (MIC)**

Broth dilution method was applied for MIC determination. Double serial dilutions (2 to 1024 μg/ml) of gentamicin, vancomycin, and oxacillin were prepared form stock solution (20 mg/2ml) in a microtiter plate using Mueller Hinton broth as diluent. The MIC for each bacterial isolates was interpreted as the lowest concentration showing no visible growth. The results were compared with standard break points values according to CLSI [10].

**Biofilm Formation Assay**

Quantification of biofilm formation by S. aureus was assessed as previously described by Atshan et al. [11]. In brief; 200 μl of a fresh bacterial growth (using tryptic soy broth) comparable to McFarland standard no.0.5 were added to each well of sterile 96-well U shaped-bottom polystyrene microplates. Thereafter, the plates were covered with their lids and incubated under aerobic conditions at 37°C for 24 h.

Each bacterial isolate was tested in triplicate. Wells with bacteria-free were considered as a negative Control. After incubation, supernatants were removed from each well and biofilm were gently washed twice with normal saline, dried and fixed at 65°C for 1 hr. Subsequently, the plates were stained with 200μl 0.1% crystal violet solution for 15 min at room temperature. Excess stain was rinsed off by placing the plate under running tap water. Thereafter, the plates were dried in a 37°C incubator for approximately 30 min to ensure they were completely dry. Afterward, the adherent cells were resolubilized with 200μl absolute ethanol for 10 minute. Finally, the optical density of each well was obtained at 630nm using microplate reader.

Classification of bacterial adherence summarized in Table 1 based on OD<sub>630</sub> values obtained for individual isolate of S. aureus were used 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 (OD<sub>c</sub>) was established. It is defined as three standard deviations above the mean OD of the negative control; OD<sub>c</sub> 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.

**PCR Study**

**Culture and DNA Extraction**

Strains were grown on brain heart infusion broth at 37°C overnight. Genomic DNA used as target for PCR assays was extracted by using G-spin™ total DNA extraction kit (Intron, Korea). The DNA concentration and purity were determined using Nanodrop (ACTGene, USA).

**Identification of HLA gene**

The primers HLA-1 (5’-CTG ATT ACT ATC CAA GAA ATT CGA TTG-3’) and HLA-2 (5’-CTT TCC AGC CTA CTT TTT TAT CAG T-3’) were used to amplify a 209-bp hla fragment. Amplification was carried out on a Master thermocycler gradient PCR (Eppendorf) under the following conditions: an initial 5-min denaturation step at 94°C; followed by 35 cycles of 1.25 min of denaturation at 94°C, 1.25 min of annealing at 62°C, and 1.25 min of extension at 72°C; and a final extension step at 72°C for 10 min. PCR products were analyzed by electrophoresis through 1.5% agarose gel [12].
Gene Expression

![Box Plot of Biofilm Formation by MRSA Isolates](image)

**Figure 1:** Box plot of biofilm formation by MRSA isolates. Boxes range from the 25th to 75th percentile and are intersected by the median line. Asterisks denote the mean. Whiskers extending below and above the box range represent the maximum and minimum values, respectively. Outliers are indicated as individual data points.

### RNA Extraction from *S. Aureus* Isolates

The RNA was extracted from planktonic and biofilm cells of MRSA isolates; which was firstly cultivated in blood agar media to enhance the gene expression (Koohari et al., 2017) by Qeasy™ plus CTB RNA Extraction Mini Kit (Intron, Korea).

### Quantitative Reverse Transcription-PCR

In order to assess the gene expression of *hla* gene, results were normalized using 16SrRNA. Primers of these genes were listed in Table 2.

RT-qPCR was performed using Toptical thermocycler (Analytikjena, Germany) and undertaken on the KAPA SYBER FAST ONE-STEP qPCR kits (Kapa Biosystems, USA) using SYBR green fluorescent dye.

The reaction mixture consisted of SYBR Fast qPCR master mix (10 µl), forward and reverse primers (0.5 µl), dNTP (0.5 µl), 1 µl KAPA RT mix (Reverse transcriptase), RNA template (6 µl) and nuclease free water (1.5 µl). Moreover, after several trials thermocycler protocol was optimized and the resultant protocol is listed in Table 3. Melting curve was obtained with temperatures ranging from 60°C to 95°C with a 1°C increase in temperature every one second.

Expression levels were quantified using relative quantitation. The difference in cycle thresholds (ΔCt) and fold changes evaluated between the treated groups and calibrators of each gene. These values were normalized to 16SrRNA expression using 2^ΔΔCt method. Fold change less than 2-fold was considered insignificant [13].

### Measuring the Hemolytic Activity of Alpha Hemolysin

A bacterial culture was prepared from a 24 hrs. old Brain hearth infusion broth and concentration was adjusted to McFarland standard no.0.5. Gentamicin was added at sub MIC to the activated bacterial suspension and incubated for 24 hrs. at 37°C.

A volume of one ml of Rabbit blood was aspirated using a sterile syringe and the RBC were washed by normal saline thrice, centrifuged at 3000rpm for 5 minutes. Thereafter, the supernatant was discarded. Afterward one ml of washed and packed rabbit RBCs were added to nine ml of the bacterial suspension prepared in step a, incubated at 37°C for 1 hrs.in a water bath and gently mixed every 15 minutes.
At the end of incubation period, absorbance was read at 600nm. The control consisted of rabbit RBC and D.W.; while the normal saline solution served as a blank. The lysis percentage for each sample was calculated by using following formula:

\[
\% \text{ lysis} = \frac{(\text{OD}_{600 \text{ test}} - \text{OD}_{600 \text{ blank}})}{(\text{OD}_{600 \text{ control}} - \text{OD}_{600 \text{ blank}})} \times 100
\]

Same protocol was followed to assess the effect of gentamicin on hemolysis activity by incubating the bacterial suspension with gentamicin at sub MIC at 37°C for 24 hr.

**Statistical Analysis**

Data were presented as mean ± standard deviation. Means were compared with each other by ANOVA, LSD, and T test. Differences were considered significant when \( P < 0.05 \).

### Table 1: Classification of bacterial adherence by tissue culture plate method [11]

<table>
<thead>
<tr>
<th>Mean OD_{450}</th>
<th>Biofilm intense</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD ≤ ODc*</td>
<td>Non–adherent</td>
</tr>
<tr>
<td>2ODc &gt; OD &gt; ODc</td>
<td>Weak</td>
</tr>
<tr>
<td>4 ODc &gt; OD &gt; 2ODc</td>
<td>Moderate</td>
</tr>
<tr>
<td>OD &gt;4 ODc</td>
<td>Strong</td>
</tr>
</tbody>
</table>

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

### Table 2: The primers and their sequences used in the real time PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hla</td>
<td>F: TATTAGAACGAAAGGTACCA</td>
<td>Goldsworthy, 2008</td>
</tr>
<tr>
<td></td>
<td>R:AACGTACCTAAAGGCTGAA</td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>SA1-AATCTTTTGTCGTACACGCCATTTCTTCACG</td>
<td>Martineau et al., 1996</td>
</tr>
<tr>
<td></td>
<td>SA5-CGTAATGAGATTTCAGTAGATAATACAAC</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3: q RT-PCR protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcripion</td>
<td>42</td>
<td>5 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Enzyme inactivation</td>
<td>95</td>
<td>3 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>3 seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>59</td>
<td>20 seconds</td>
<td>40</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>20 seconds</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4: Effect of gentamicin on rabbit erythrocytes hemolysis by *S. aureus* supernatants.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Without gentamicin</th>
<th>With gentamicin</th>
<th>T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hemolysis (%)</td>
<td>SD</td>
<td>Hemolysis (%)</td>
</tr>
<tr>
<td>S6</td>
<td>98.40</td>
<td>4.88</td>
<td>84.65</td>
</tr>
<tr>
<td>S13</td>
<td>90.15</td>
<td>1.65</td>
<td>72.60</td>
</tr>
<tr>
<td>S15</td>
<td>89.95</td>
<td>4.81</td>
<td>79.06</td>
</tr>
<tr>
<td>S16</td>
<td>88.00</td>
<td>5.86</td>
<td>74.60</td>
</tr>
<tr>
<td>S20</td>
<td>93.35</td>
<td>4.72</td>
<td>71.45</td>
</tr>
<tr>
<td>S27</td>
<td>87.05</td>
<td>1.65</td>
<td>82.38</td>
</tr>
<tr>
<td>S31</td>
<td>88.00</td>
<td>3.00</td>
<td>73.40</td>
</tr>
<tr>
<td>S115</td>
<td>86.50</td>
<td>6.50</td>
<td>70.70</td>
</tr>
<tr>
<td>S109</td>
<td>92.35</td>
<td>5.65</td>
<td>77.10</td>
</tr>
<tr>
<td>S107</td>
<td>98.30</td>
<td>4.81</td>
<td>71.85</td>
</tr>
</tbody>
</table>

\( P \) value = 0.00077 - 1.96 \times 10^{-6}\)

LSD_{0.05} = 1.96 \times 10^{-6}\)

SD denotes to standard deviation.
### Results and Discussion

#### Determination of MIC

Findings of this work revealed that 12 (36.36%), four (12.12%), and 17 isolates were gentamicin-sensitive, intermediate, and resistant to gentamicin, respectively. Interestingly, 16 isolates (48.48%) out of the 17 resistant isolates resisted ≥ 32 µg/ml of gentamicin whereas only one isolate (3%) exhibited MIC 256 µg/ml, hence sub-MIC (16 µg/ml) was used for further experiments. Other antibiotics, vancomycin and oxacillin, were tested as well; the result showed that all MRSA isolates were resistant to oxacillin and sensitive to vancomycin depending on the breakpoints of CLSI (2016).

Belbase et al. [14] reported that the highest rates of susceptibility of MRSA isolates were seen toward vancomycin (100%) followed by tetracycline and chloramphenicol (97.2%), while 61.2% of MRSA isolates were susceptible to gentamicin and all isolates were resistant to penicillin and cefoxitin. Johan and Murugan [12] 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.

#### Biofilm Formation

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 [11].

The microtiter plate assay was considered as a standard test for the detection of biofilm formation [15]. Therefore, in this study, the ability of *S. aureus* biofilm producing isolates was evaluated by using pre-sterilized 96-well polystyrene microtiter plates.

After the exclusion of the outlier value (OD$_{630}$ 0.345) MRSA isolates exhibited a wide spectrum of biofilm-forming capabilities ranging from OD$_{630}$ 0.069 to OD$_{630}$ 0.2. Moreover, the nine isolates exhibited levels of biofilm formation comparable to the median (OD$_{630}$ 0.125) values. Four isolates were located at the first quartile (OD$_{630}$ 0.124) and another four isolates at the third quartile (OD$_{630}$ 0.173) as well (Fig. 1).

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**Table 5**: Gene expression of *hla* in MRSA planktonic cells in the presence of gentamicin at sub MIC; 16 µg/ml

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Gene expression</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without gentamicin</td>
<td>With gentamicin</td>
</tr>
<tr>
<td>SA15</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>SA16</td>
<td>0.018</td>
<td>0.023</td>
</tr>
<tr>
<td>SA20</td>
<td>0.076</td>
<td>0.125</td>
</tr>
<tr>
<td>SA13</td>
<td>0.001</td>
<td>0.045</td>
</tr>
<tr>
<td>SA27</td>
<td>0.002</td>
<td>0.011</td>
</tr>
<tr>
<td>SA31</td>
<td>0.004</td>
<td>0.005</td>
</tr>
<tr>
<td>SA6</td>
<td>0.007</td>
<td>0.25</td>
</tr>
<tr>
<td>SA115</td>
<td>0.0001</td>
<td>0.002</td>
</tr>
<tr>
<td>SA109</td>
<td>0.0006</td>
<td>0.611</td>
</tr>
<tr>
<td>SA107</td>
<td>0.002</td>
<td>0.048</td>
</tr>
</tbody>
</table>

**Table 6**: Gene expression of *hla* in MRSA biofilm cells in the presence of gentamicin (sub MIC; 16 µg/ml)

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Gene expression</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without gentamicin</td>
<td>With gentamicin</td>
</tr>
<tr>
<td>SA15</td>
<td>9.524 × 10$^-5$</td>
<td>0.0002</td>
</tr>
<tr>
<td>SA16</td>
<td>1.140 × 10$^-3$</td>
<td>0.0001</td>
</tr>
<tr>
<td>SA20</td>
<td>0.0002</td>
<td>0.0008</td>
</tr>
<tr>
<td>SA13</td>
<td>0.0005</td>
<td>0.008</td>
</tr>
<tr>
<td>SA27</td>
<td>1.504 × 10$^-5$</td>
<td>0.002</td>
</tr>
<tr>
<td>SA31</td>
<td>0.0002</td>
<td>0.006</td>
</tr>
<tr>
<td>SA6</td>
<td>0.004</td>
<td>0.011</td>
</tr>
<tr>
<td>SA115</td>
<td>2.377 × 10$^-5$</td>
<td>0.002</td>
</tr>
<tr>
<td>SA109</td>
<td>0.0001</td>
<td>0.006</td>
</tr>
<tr>
<td>SA107</td>
<td>1.660 × 10$^-6$</td>
<td>8.176 × 10$^-6$</td>
</tr>
</tbody>
</table>
In order to estimate biofilm degree, absorbance was determined at 630\textsubscript{nm} by microplate reader. Given that, absorbance values represented the degree of the biofilm thickness that formed by the studied isolates on the surface of the microtiter well. 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 declared 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.

This result is an agreement with other studies of MRSA biofilm formation, Moghadam et al. [16] stated the majority of MRSA strains (62.5\%) were moderate biofilm producers, but this results were incompatible with study published by Karam and Al-Mathkhury [8] 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 \textit{S. aureus} biofilm-protected bacteria from host defense mechanism and shows resistance to standard antibiotic therapy [17].

\textbf{Alpha-hemolysin Phenotyping}

\textit{Staphylococcus aureus} produced at least four different cytolytic toxins called alpha, beta, gamma, and delta hemolysins [18]. Since the erythrocytes were the first type of cell used for assaying the cytolytic activity. The hemolytic assay is still the simplest and most convenient one [19].

Phenotypic detection of alpha-hemolysin was carried out based on lysis zone of each MRSA isolate on triplicate plate of blood agar base supplemented with 5\% rabbit blood after 24 h incubation at 37\°C the plates were examined and the isolate was considered hemolytic if complete lysis of red blood cells surrounding the colony was observed.

Gray and Kehoe [20] cloned the gene that encoded alpha hemolysin (\textit{hla}) from \textit{S. aureus}. This toxin is under control of accessory gene regulator (\textit{agr}) and therefore made during the late exponential phase of growth in batch culture. Some strain contains \textit{hla} gene but does not produce a detectable alpha toxin [21].

To show the hemolysis activity rabbit blood agar was used for this reason the rabbit erythrocytes are extraordinary susceptible to hemolysis by alpha toxin at least 100 time more than other mammals and 1000 times more than human erythrocytes [22] for the reason that the rabbit blood cells contain 1200-5000 toxin binding site [23].

Phenotypically, the present work found that 30 out of 33 MRSA isolates developed beta hemolysis on rabbit blood agar plates, such result indicates that these isolates have produced alpha hemolysin. Furthermore, some MRSA isolates may developed mutation(s) in structural gene, which prevented the protein from being translated or due to the environmental factor, influenced alpha-hemolysin expression. Ohlsen et al. [24] revealed that number of environmental factor impact on alpha toxin production, the expression of \textit{hla} reached maximum at 42\°C in mid-exponential phase, high osmolarity repressed \textit{hla} transcription whereas CO\textsubscript{2} stimulate activity, oxygen was essential for expression and growth on solid media was found to be more conductive to expression of the gene.

\textbf{Molecular Studies}

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

\textbf{Amplification of HLA Gene by Multiplex PCR Technique}

In regard to the detection of \textit{hla} gene for each \textit{S. aureus} isolate, amplification of a fragment of these genes were performed by PCR technique. The present results clarified a presence of \textit{hla} in nearly all MRSA isolates exception of one isolate. Ariyanti \textit{et al.} [25] demonstrated that \textit{hla} and \textit{hld} genes were widely distributed among \textit{S. aureus} isolated from human. Likewise, in a study from southern Iran observed the \textit{hla} and \textit{hld} were present in all isolates, while \textit{hlb} gene was found in 40\% of human isolates; however, gamma hemolysin was not detected at all [26]. Shukla \textit{et al.} [27] and Kateete \textit{et al.} [28] reported that the percentage of \textit{hla} in MRSA isolates were 100\%. Moreover, they stated that the existence of hemolytic genes in MRSA...
isolates are important for their pathogenicity.

**Effect of Gentamicin on Hemolytic Activity in MRSA Isolates**

To detect the effect of gentamicin on hemolytic activity in MRSA isolates, gentamicin was added at a subinhibitory concentration to overnight cultures of MRSA.

The results summarized in Table 4 illustrated that the isolated culture supernatants had highly significant (P< 0.001) variability in hemolysing rabbit erythrocytes either with or without the addition of gentamicin. However, isolates S115 and S6 scored the lowest (86.5% ± 6.50) and highest (98.4% ± 4.88) hemolysis activity in the absence of gentamicin, respectively. Furthermore, the addition of gentamicin significantly (P< 0.05) reduced the hemolysis activity; nonetheless, isolates S115 and S6 scored the lowest (70.70% ± 3.50) and highest (84.65% ± 4.35) hemolysis activity in the presence of gentamicin, respectively.

**Real Time-qPCR**

The MRSA isolates chosen for the *hla* expression study were characterized by 1) Exhibition of beta hemolysis on RBA and 2) have similar minimum inhibitory concentration of gentamicin (32 µg/ml).

**Effect of Gentamicin on the Expression of HLA in MRSA Isolates**

Quantitative real-time PCR was carried out using SYBR green through a one-step RT-PCR method in order to determine the effect of sub MIC of gentamicin on the expression of *hla* gene in planktonic and biofilm cells of MRSA isolates. Concerning planktonic state, the results summarized in Table 5 showed a variation in *hla* gene expression among MRSA isolates ranging from 0.0001 to 0.076; whereas in the presence of gentamicin *hla* expression fluctuated from 0.005 to 0.611 at sub MIC of 16 µg/ml. Thence, the fold change ranged from 0.001 to 0.785.

Nevertheless, insignificant fold change (less than two) of alpha toxin gene was noticed in all tested isolates in the presence of sub MIC of gentamicin (16 µg/ml). Markedly, phenotypic hemolysis was significantly reduced by the addition of gentamicin, hence it can be concluded that gentamicin did not affect alpha-hemolysin production at transcriptional level by blocking mRNA synthesis, perhaps, it exerts its effect on later processes (for instance, translation stage or biosynthesis pathway), given that gentamicin is a potent protein inhibitor works irreversibly by binding the 30S subunit of the bacterial ribosome, interrupting protein synthesis.

The antibiotics are capable of modifying cellular processes of bacteria when they are incorporated into culture media at sub MIC [29]. Yet, Drummond et al. [30] demonstrated that not all antibiotics exhibit the same effect and this depending on pathogen and antibiotic pair under investigation. Ohlsen et al. [24] and Bernardo et al. (2004) stated that inhibitors of protein synthesis at sub MIC significantly inhibit the production of virulence factors (including α-hemolysin) in *S. aureus*.

Crosswise, inhibitors of cell wall synthesis, upregulate the expression of α-hemolysin, enterotoxins, and TSST-1 via a stimulatory effect on biosynthesis of exoprotein [31]. Some methicillin-resistant *S. aureus* isolates produced up to 30-fold more alpha-toxin in the presence of 10 mg of methicillin per ml than in its absence [24]. Same authors also reported a very remarkable observation opposing other investigators as they found glycopeptide antibiotics had no effect and fluoroquinolones slightly stimulated *hla* expression.

Also, Worlitzsch et al. [32] stated that gentamicin decreased the level of *hla* expression. Furthermore, Otto et al. [33] explored the effect of antibiotics on *pvl*, *hla* and *spa* mRNA level and they found the sub MIC of clindamycin decrease PVL, Hla and SpA production in all tested strains. Upon the previous reports, the antibiotic selected to treat infections caused by *S. aureus* should not only partake antimicrobial activity but must develop a capability to suppress the expression of virulence factors released by killed bacteria or those at stress [34].

Noticeably, Qiu et al. [35] suggested that thymol significantly inhibited *hla* via inhibition of *agrA* transcription. Nonetheless, the mechanisms by which *S. aureus* regulates the expression of virulence determinants are objectively complicated and comprise an interactive, hierarchical regulatory cascade among the products of the *sar*, *agr*, and other determinants.
Consequently, they assumed that the decline in the production of virulence factor may partly be influenced by thymol-induced inhibition of the Agr system. The similar interpretation adopted by Mun et al. [34] to explain the decline in hla expression by concentration below the optimal of punicalgin.

Of considerable interest, limited work has been carried out scrutinizing the effect of sub MIC of antibiotics on virulence determinants expression in bacterial biofilm; consequently, the present study focused on the effect of exposing MRSA biofilm to gentamicin at subMIC on the expression of hla gene.

Concerning biofilm state, the results listed in Table 6 showed a variation in hla gene expression among MRSA isolates ranging from $1.66 \times 10^6$ to 0.004; whereas in the presence of gentamicin, the expression of hla fluctuated from $8.176 \times 10^6$ to 0.011 at sub MIC of 16 $\mu$g/ml.

Obviously, the expression of hla gene in biofilm cells of MRSA increased at least four-fold (in a range extended up to 164.27-fold) in the presence of gentamicin at sub MIC than its absence. Upon such finding, it can be suggested that gentamicin acted as an inducer and upregulated the hla gene expression in biofilm cells; hitherto, this increment was isolate specific.

Biofilm is subjected to chemical signaling mechanism called quorum sensing, which allows bacterial cells to alter their genetic expression, in biofilm the expression of genes coding for the adhesion become less important so these genes repressed and other genes such as virulence genes are activated. S. aureus relies upon quorum sensing to establish itself.

Cell density control of staphylococcal virulence is mediated by an octapeptide auto inducer; this chemical sensor is released by staphylococcal cells, all of which have surface receptor detect it. The more bacteria at the site releasing the auto inducer, the higher its local concentration and the more surface receptor are stimulated. At predetermined concentration, the cell initiates an intracellular mechanism to repress the expression of adhesion gene and activate those able to facilitate invasion, these include agr group of the gene, which allow the transcription of the gene responsible for encoding a variety of toxins such as an alpha toxin [36].

What's more, gentamicin at sub MIC has been found to promote the biofilm formation [33]. On contrary, Henry-Stanley et al. [37] pointed out to bactericidal concentrations of gentamicin and streptomycin inhibited the formation of biofilm when the S. aureus isolates were incubated in diluted or one-fold tryptic soy broth supplemented with 0.2% glucose (TSBg). However, authors demonstrated that biofilms of S. aureus were not inhibited when they incubated in three-fold TSBg.

Moreover, they stated that this nutrient-dependent aminoglycoside susceptibility is not restricted to biofilm development only, nonetheless, planktonic cells incubated in three-fold TSBg containing gentamicin revealed antibiotic resistance. Interestingly, these findings seemed specific for aminoglycosides as biofilm formation was inhibited in diluted and concentrated growth media in the presence of bactericidal concentrations of ampicillin and vancomycin.

In the present work, albeit the hla expression by biofilm cells was less than that of planktonic cells when gentamicin was added the fold change was much higher in biofilm cells than in planktonic cells.

It is well established that bacterial cells in biofilm state are up to 1000 time more resistant to antibiotics than their counterpart planktonic cells of the same species [38], on the other hand, Oliveria et al. [39] reported that exposing MRSA biofilm to an antibiotic stress triggered switching on virulence determinants such as hla gene allowing bacterial cells to elaborate the alpha toxin.

Biofilm matrix, per se, could be altered due to the presence of sub MIC of particular antibiotics. Data presented by Schilcher et al. [40] proposed that sub MIC of clindamycin modified the ability of S. aureus to form biofilms and shifted the nature and composition of the biofilm matrix toward rich eDNA content.
References


