ISSN: 0067-2904

Gentamicin Upregulates the Gene Expression of *hla* and *nuc* in *Staphylococcus aureus*

Rand Thair Abdulateef Alkhafajy*, Harith Jabbar Fahad Al-Mathkhury
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Received: 30/3/2022 Accepted: 17/7/2022 Published: 30/3/2023

Abstract

The current study aimed to detect the effect of gentamicin stress on the expression of *hla* (encodes hemolysin) and *nuc* (encodes nuclease) genes of *Staphylococcus aureus*. Fifty-eight isolates identified as *S. aureus* were isolated locally from different clinical specimens. Disk diffusion method was used to detect the resistance to *S. aureus*. The minimum inhibitory concentration (MIC) of gentamicin was estimated by broth microdilution method. *hla* and *nuc* genes were determined by polymerase chain reaction technique. The biofilm was evaluated using the microtiter plate method in the presence and absence of gentamicin at sub-MIC. The results showed that 18 (31%) and 40 (69%) *S. aureus* isolates were sensitive and resistant to gentamicin, respectively. All *S. aureus* isolates succeeded in forming biofilm. However, eight (13.79%), 28 (48.28%) and 22 (37.93%) isolates produced weak, moderate and strong biofilms respectively. In most isolates, gentamicin at sub-MIC decreased biofilm intensity. Due to gentamicin stress, the *hla* and *nuc* genes were upregulated in *S. aureus* biofilm.

Keywords: *Staphylococcus aureus*, *hla*, *nuc*, Biofilm, Gene expression, gentamicin

الجنتامايسين يرفع التعبير الجيني لجيني الهيمولايسين ونوكلياز في المكورات العنقودية الذهبية

رند ثائر عبد اللطيف الخفاجي*، حارث جبار فهد المذخوري
قسم علوم الحياة ، كلية العلوم ، جامعة بغداد، بغداد، العراق

الخلاصة

هدفت الدراسة الحالية إلى الكشف عن تأثير إجهاد الجنتاميسين في التعبير الجيني للهيمولايسين ونوكليازفي العنقوديات الذهبية.في هذه الدراسة تم التعرف على ثمانية وخمسين عزلة من المكورات العنقودية الذهبية معزولة محليًا من عينات سريرية مختلفة. تم أستعمال طريقة انتشار القرص للتحري عن مقاومة المكورات العنقودية الذهبية. تم تقدير التركيز المثبط الادنى من الجنتاميسين بطريقة التخفيف الدقيق للمرق. تم تحديد جينات*hla* و *nuc*بأستعمال تقنية تفاعل البلمرة المتسلسل. تم تقييم الغشاء الحياتي بأستخدام طريقة طبق المعايرة الدقيقة بوجود وغياب الجنتاميسين عند التركيز المثبط تحت الادنى. أظهرت النتائج الحالية أن 18 (31٪) و 40 (69٪) عزلة من المكورات العنقودية الذهبية كانت حساسة ومقاومة للجنتاميسين على التتابع. نجحت عزلات المكورات العنقودية الذهبية جميعها في تكوين الغشاء الحياتي. بينما أنتجت ثمانية عزلات (13.79٪) و 28 (48,28٪) و 22 (37.93٪) غشاء حياتيا ضعيفاومتوسطا وقويا على التتابع.

* Email: randthair89@gmail.com

في معظم العزلات خفض الجنتاميسين عند التركيز المثبط تحت الادنى من شدة الغشاء الحياتي. من الممكن ان نستنتج إن جينات *hla* و *nuc*أظهرت تعبيرا عاليا بسبب إجهاد الجنتاميسين.

Introduction

Staphylococcus aureus is one of the most well-known and widely dispersed bacterial pathogens causing each year an unknown number of simple skin infections as well as hundreds of thousands to millions of more dangerous and invasive infections. It is a common cause of pneumonia and other respiratory, surgical site, prosthetic joint and cardiovascular infections, as well as nosocomial bacteremia [1, 2].

The emergence and spread of antibiotic resistance among microbes have become a serious worry worldwide in the last decade. Resistant genetic determinants are frequently found on mobile elements and can be easily transmitted between hosts [3].

Biofilm formation is a significant pathogenicity component that protects bacteria from antimicrobial agents while also protecting them from host defense mechanisms [4]. Furthermore, biofilm-producing isolates persistence in the dairy environment assists in the spreading of virulence factors by allowing genetic material to be transmitted to other bacteria [5]. Biofilms are made up mostly of exopolysaccharide matrix, proteins and extracellular DNA (eDNA), as well as bacterial cells [6]. Exopolysaccharide intercellular adhesin is a non-protein [7] which improves bacterial adherence to diverse surfaces, a crucial step in the infection process [8].

S. aureus can escape host defenses by producing a wide range of virulence factors, including hemolysins, exotoxins, leukocidins, superantigens, capsules and secreted enzymes [9]. Some of these toxins, such as alpha hemolysin, a pore-forming cytotoxin that operates against a wide range of human cells, are encoded by the *hla* gene. The hemolytic, dermonecrotic and neurotoxic properties of this toxin contribute to its pathogenicity [10].

The staphylococcal nuclease, encoded by the nuc gene [11], is a thermostable nuclease that hydrolyzes DNA and RNA in host cells, causing tissue damage and staphylococci spread [12], and promoting microorganism escape when held by neutrophil extracellular traps [13]. Since decades till now the *nuc* gene has been regarded the gold standard for identifying *S. aureus* [14]. Furthermore, the *nuc* encoded staphylococcal thermonuclease is a biofilm inhibitor that destroys biofilm-associated eDNA [15].

Antibiotics of various classes may have varying impacts on bacterial morphology and virulence production [16]. As a result, antibiotics with sub-minimum inhibitory doses (sub-MIC) have a large impact on bacterial transcription and may act as signaling mediators rather than targeting microbe growth per se [17]. Gentamicin has been demonstrated to affect the gene expression of virulence determinants such as *hla* [18], *fnbA*, *fnbB* [19], *pslA* and *pelA* [20]. The present study aimed to assess the effects of gentamicin on expression of *hla* and *nuc* genes in *Staphylococcus aureus* isolated from clinical samples.

Materials and Methods

Microorganisms

Staphylococci isolates were identified depending on the results of certain morphological features, microscopic examination, biochemical tests, Vitek-2 automated system and molecular methods. Previously 58 *S. aureus* isolates were collected from patients who had

been treated at several Baghdad hospitals. They were maintained at the University of Baghdad Microbiology lab, Department of Biology, College of Science.

Antibiotic Susceptibility Test

Disk diffusion method was used to test all *S. aureus* isolates using a gentamicin 10µg disk and a cefoxitin 30µg disk. An isolate suspension was compared to 0.5 McFarland standard and lawn cultures were performed on Mueller-Hinton agar (Neogen,USA) plates. These plates were incubated for 18-24 hours at 37°C. After that, a metric ruler was used to measure the diameter of the inhibitory zone. An isolate was interpreted as resistant, intermediate resistant or sensitive in accordance to the breakpoints described in CLSI [21]. Vitek-2 Compact (AST GP) was used for testing the sensitivity of the *S. aureus* isolates to vancomycin.

Determination of Gentamicinminimal Inhibitory Concentration

Broth micro dilution test and procedures, according to Andrews [22], were used to evaluate the lowest dosage of antibiotics that inhibited visible microorganism growth. According to CLSI [21], the results were compared to standard breakpoint values of sensitive (2 g/ ml), intermediate (4-8 g/ ml) and resistant (16 g/ ml).

Biofilm Formation Assay

The production of biofilms by *S. aureus* was measured using the method reported by Atshan *et al* [23]. All isolates were propagated in brain heart infusion broth (Himedia, India) for approximately 18 h at 37°C. Each isolate was diluted using tryptic soy broth (TSB) (Himedia, India) containing 1% glucose and mixed well via pipetting. A bacterial isolate suspension was adjusted to the McFarland No. 0.5 turbidity standard. A volume of an isolate culture (200µl) was inoculated in three wells of sterile 96-well U shaped-bottom polystyrene microtiter plates. The plates were covered with their lids and incubated at 37°C for 24 hours under aerobic conditions. After the incubation period, the planktonic cells were washed twice with deionized water to remove unattached bacteria, then shaking off the excess water by tapping plate on paper towels (filter paper) and air-dried. Each well was fixed for 20 minutes at room temperature with 200µl absolute methanol. In each well 200µl of 0.5% crystal violet was added for 15 minutes to stain the adhering cells. Excess dye was removed when the staining reaction was complete by repeated washing (2-3 washes) with distilled water. To ensure that the plates were thoroughly dry, they were left at room temperature for around 30 minutes. After that each well received 200µl of 95% ethanol for 10 minutes. Ethanol was used to dissolve the crystal violet dye that was bound to the adhering cells. The experiment was repeated three times with the absorbance of wells containing bacteria-free TSB acting as a negative control. By measuring the OD_{630} using a microplate reader, the quantity of crystal violet extracted by ethanol in each well was directly measured spectrophotometrically. For the purposes of data simplification and computation, Table 1 shows a class of bacterial adherence based on OD_{630} values obtained for various *S. aureus* isolates.

Table 1: Bacterial adherence classification by microtiter plate technique [23]

Mean OD_{630}	Biofilm Intensity
OD ≤ ODc*	Non-biofilm producer
ODc < OD ≤ 2ODc	Weak
2ODc < OD ≤ 4ODc	Moderate
OD > 4ODc	Strong

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

Effect of Gentamicin at sub-MIC on Biofilm Development

The biofilm formation analysis utilized the same methodology as the previous one, using gentamicin at sub-MIC levels in TSB. All plates were incubated for 24 hours at 37°C. The wells were then washed, dyed and read at 630 nm. Positive controls included adding 200µl of fresh bacterial suspension (gentamicin-free) (compatible to 0.5 McFarland standard).

Polymerase Chain Reaction Assay

DNA extraction

Genomic DNA was extracted from bacterial growth using the ABIOpure , USA extraction kit procedure. The DNA concentration and purity were determined using a Quantus fluorometer.

Detection of *hla*, *nuc* and *gyrB*

The presence of *hla*, *nuc* and *gyrB* of *S. aureus* was determined by employing the thermal cycler to amplify the isolated genomic DNA (Thermo Fisher Scientific, USA). Table 2 lists the primers required for amplification of *hla*, *nuc* and *gyrB* fragments. These primers came in a lyophilized package. According to the manufacturer's instructions, lyophilized primers were dissolved in 300µl of nuclease-free water to make a stock solution with a final concentration of 100 pmol/l. It was then kept in the deep freezer until employed in PCR amplification (Macrogen, Korea).

Table 2: Primers and their sequences used in the study

Primer Name		Sequence	Product Size (bp)	Reference
nuc	F	5`-GATTGATGGTGATACGGT-3`	274	[24]
	R	5`-CAAGCCTTGACGAACTA-3`		
hla	F	5`-TATTAGAACGAAAGGTACCA-3`	101	[25]
	R	5`-ACTGTACCTTAAAGGCTGAA-3`		
gyrB	F	5`-GGTGCTGGGCAAATACAAGT-3`	107	[26]
	R	5`-TCCCACACTAAATGGTGCAA-3`		

The extracted DNA and primers were combined with PCR master mix and vortexed to ensure homogeneous contents, yielding a PCR mixture with a final volume of 20µl. Ten micro liters of master mix, one micro liter of each primer and two microliters of DNA template made up the reactants. The capacity was then filled up to 20µl with sterile nuclease-free water. As shown in Table 3, the chosen PCR procedure was followed after multiple experiments. Electrophoresis on a 1.5% agarose gel was used to examine PCR results.

Table 3: PCR amplification program for *nuc*, *hla* and *gyrB* gene detection

Step	Temperature (°C)	Minute: Second	Cycles
Initial denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	[a]50, [b]60	00:30	
Extension	72	00:30	
Final extension	72	07:00	1

[a]*nuc*, [b]*hla* and[b]*gyrB*

Gene Expression

The tested isolates were selected after they were suspended in broth and incubated overnight at 37°C. Biofilm examination was conducted for the isolates according to the procedure previously mentioned with the microtiter plate, as well as the effect of gentamicin on *nuc*, *hla* and *gyrB* gene expression with the use of gentamicin-containing Mueller Hinton broth (Himedia, India) at sub-MIC as previously described, a similar protocol was followed.

RNA Extraction

RNA was extracted from biofilm cells before and after gentamicin treatment using TRIzolTM Reagent according to the manufacturer's instructions (Promega, USA).

The concentration of extracted RNA or cDNA was determined using a Quantus fluorometer (Promega, USA) to determine the quality of samples for downstream applications. 199µl of diluted QuantiFlour Dye were combined with 1µl of RNA or cDNA. RNA concentration measurements were taken after a 5-minute incubation period at room temperature in a dark environment.

The extracted RNA and primers were combined with qPCR master mix (Promega, USA) and were vortexed to ensure homogeneous contents, resulting in a qPCR mixture with a final volume of 10µl. Five micro liters of master mix and 0.5 micro liters of each primer were used in the reactants, while the RNA volume was 2µl, 0.25 RT and 0.25 $Mgcl_2$. The capacity was then filled up to ten liters with sterile nuclease-free water. After multiple experiments the chosen qRT-PCR procedure was followed, as shown in Table 4.

Table 4: q RT-PCR program

Step	Temperature (°C)	Minute: Second	Cycles
Reverse transcription (RT). Enzyme activation	37	15:00	1
Initial denaturation	95	10:00	
Denaturation	95	00:15	40
Annealing	[a]50, [b]60	00:30	
Extension	72	00:30	

[a]*nuc*, [b]*hla* and [b]*gyrB*

Relative quantitation was used to determine expression levels. The difference in cycle thresholds (Ct) and fold changes between the treated groups and the calibrators for each gene were evaluated [27]. The *gyrB* values were used to normalize the data. A fold change of less than two was considered insignificant [28]. With temperatures ranging from 72°C to 95°C at 0.3°C/s, a melting curve was formed.

Statistical Analysis

All experiments were performed in triplicate and data was expressed as mean and standard deviation. The effect of study variables on biofilm was tested using the T test. These statistical analyses were done using SPSS version 26 software. The differences were considered significant when $P<0.05$.

Results

Identification of *Staphylococcus aureus*

All isolates were identified using macroscopic and microscopic examination, biochemical tests. Vitek-2 was used to to complete the identification of *S. aureus*.

Antibiotic Susceptibility Test

In the present study 40 (69%) *S. aureus* isolates exhibited resistance to gentamicin. The results also clarified that 40 (69%) of *S. aureus* isolates were resistant to cefoxitin.
Vancomycin susceptibility was tested by Vitek -2 compact system for *S. aureus* isolates. The result showed that 57 (98%) of *S. aureus* isolates were sensitive to vancomycin and only in one isolate (2%) an intermediate resistant was noticed.

Minimal Inhibitory Concentration

The MIC was estimated for all gentamicin-resistant *S. aureus*. In the present study the results of disk diffusion test found that 69% of *S. aureus* isolates were resistant to gentamicin while 31% were only sensitive. However, most isolates (70%) had an MIC of 32 µg/ml. Whereas the lowest frequent MIC was recorded in 2.5% of isolates as is illustrated in Figure1.

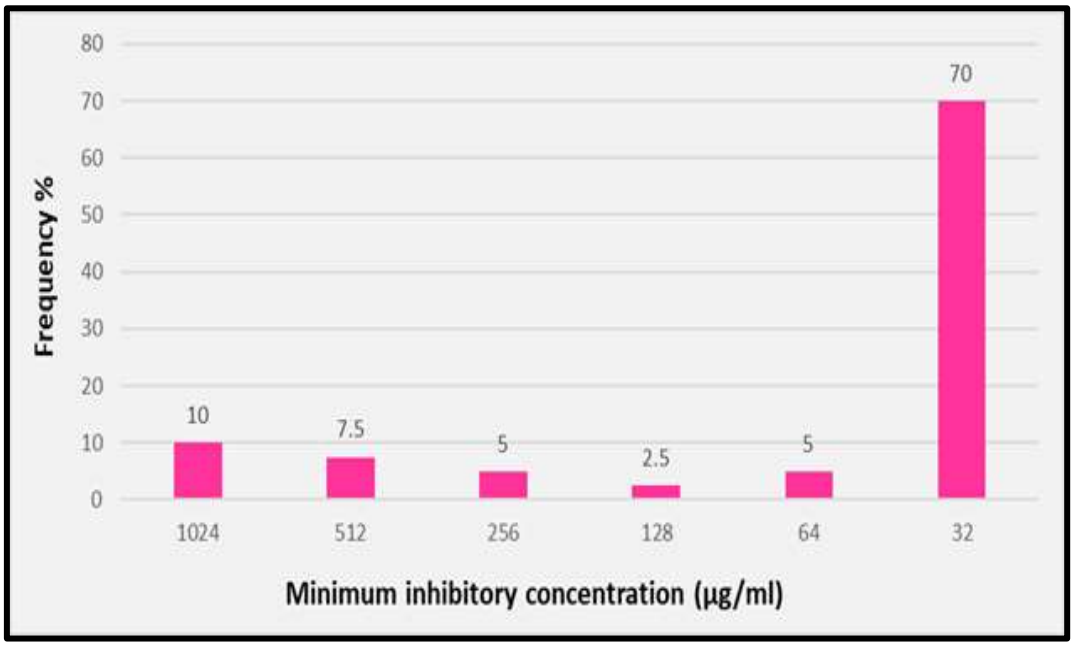

Figure 1: Frequency of *Staphylococcus aureus* in accordance to minimum inhibitory concentration of gentamicin.

Biofilm of *Staphylococcus aureus*

The absorbance at 630nm was measured using a microplate reader to evaluate biofilm intensity. As a result absorbance readings were found to be proportional to the thickness of the biofilm formed by the isolates in question. In the current study, according to the limits presented in Table 5, eight (13.79%), 28 (48.28%) and 22 (37.93%) isolates produced weak, moderate and strong biofilms respectively (Figure2).

Table 5: Limits of *Staphylococcus aureus* biofilm intensity

Mean OD_{630}	Biofilm Intensity
OD ≤ 0.045	Non-biofilm producer
0.045 < OD ≤ 0.09	Weak
0.09 < OD ≤ 0.18	Moderate
OD > 0.18	Strong

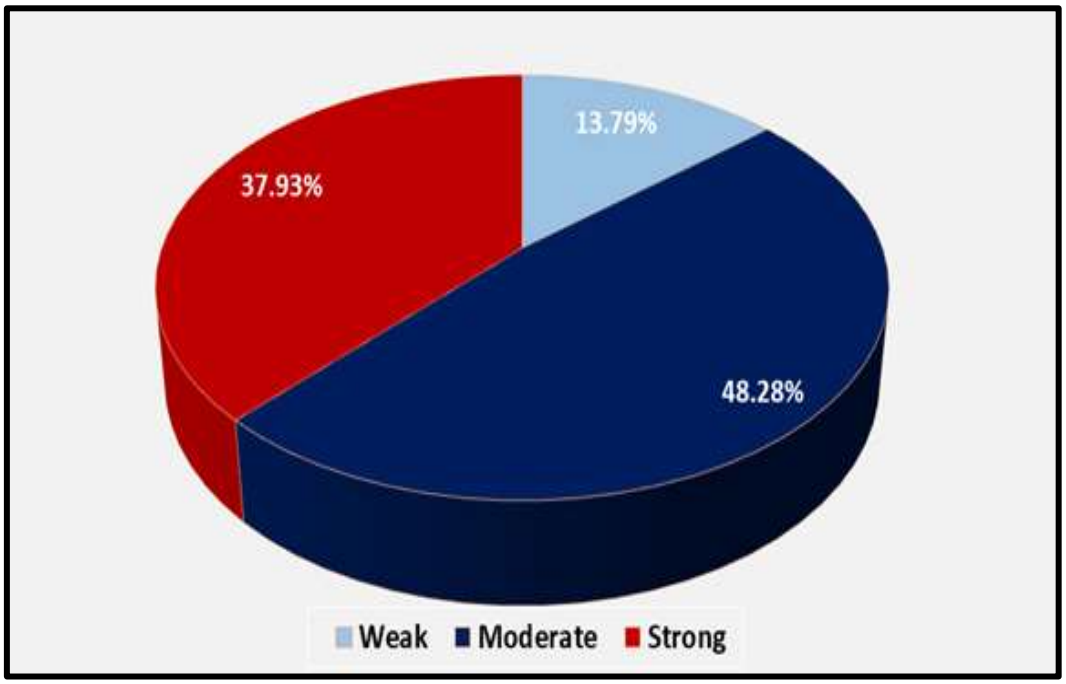

Figure 2: Distribution of *Staphylococcus aureus* biofilm in accordance to intensity (n= 58).

Effect of Gentamicin on *Staphylococcus aureus* Biofilm Formation

The ability of *S. aureus* isolates to form biofilm under the stress of gentamicin at sub-MIC was measured by microtiter plate reader at 630nm. Figure 3 depicts the mean and median biofilm intensity before treatment with gentamicin (0.206 and 0.149 respectively) hade significantly ($P < 0.001$) reduced down to (0.070 and 0.067 respectively). Moreover, the data set before and after treatment with gentamicin is right-skewed (skewness = 1.623 and 1.330, respectively) which indicates that the most biofilm intensity values before and after treatment with gentamicin are below the mean.

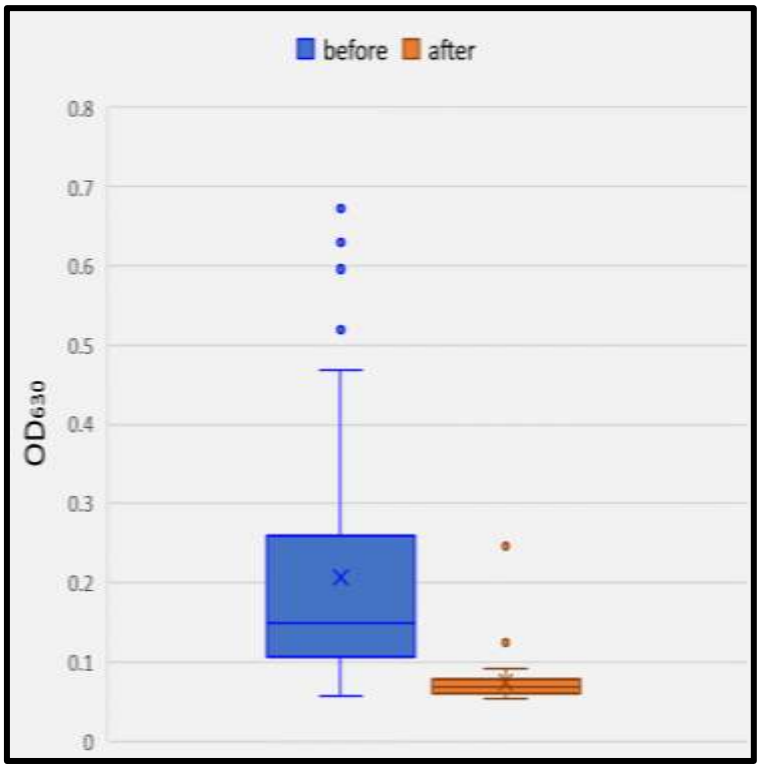

Figure 3: Box plot diagram of gentamicin effect *Staphylococcus aureus* biofilm. Boxes range from the 25th to 75th percentile and are intersected by the median line. Asterisk denotes the mean. Whiskers extending below and above the box range represent the maximum and minimum values respectively. Outliers are indicated as individual data points. T test = 8.4 × 10^{-9}.

Detection of gyrB, *hla* and *nuc* genes

PCR method was used to validate the identification by amplification of a fragment of *gyrB*, *hla* and *nuc* genes in five *S. aureus* selected isolates. The result showed that all isolates contained these genes as portrayed in Figure 4.

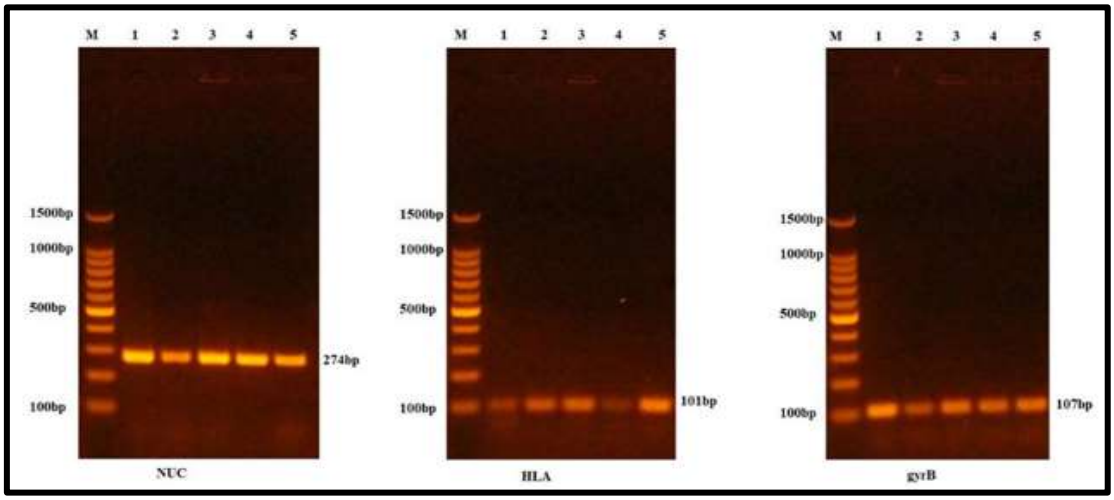

Figure 4: Agarose gel electrophoresis of *gyrB*, *hla* and *nuc* genes of *S. aureus*. M:100bp DNA ladder, lanes 1-5 represent *S. aureus* isolates S1, S2, S3, S4 and S5 accordingly. The electrophoresis was run in 1.5% agarose gel, TAE 1X, 150 volt for 45 min stained with Eth.Br.

Gene Expression

***hla* and *nuc* Expression in *Staphylococcus aureus* under Gentamicin Stress**

The results summarized in Tables 6 and 7 revealed that *hla* and *nuc* were upregulated in *S. aureus* biofilm due to gentamicin stress. The fold change of *hla* and *nuc* gene expression level in biofilm cells ranged from 29.5 to 247.3 and 2.5 to 1383.8 accordingly.

Table 6: Gentamicin effects on *hla* gene expression in *Staphylococcus aureus* biofilm

Sample	Before Gentamicin			After Gentamicin			DDCT	Folding
	gyrB	*hla*	DCT	*gyrB*	*hla*	DCT		
T_S1	22.05	22.25	0.2	20.55	15.87	-4.68	-4.88	**29.5**
T_S2	32.11	30.05	-2.06	30.93	24.73	-6.2	-4.14	**17.6**
T_S3	26.25	26.57	0.31	24.17	18.78	-5.39	-5.7	**52.1**
T_S4	20.06	21.02	0.95	18.96	11.96	-7	-7.95	**247.3**
T_S5	26.32	25.1	-1.22	24.3	13.82	-10.48	-9.26	**611.9**

DCT=Delta cycles threshold, DDCT=Delta Delta cycles threshold

Table 7: Gentamicin effects on *nuc* gene expression in *Staphylococcus aureus* biofilm

Sample	Before gentamicin			After gentamicin			DDCT	Folding
	gyrB	*nuc*	DCT	*gyrB*	*nuc*	DCT		
T_S1	22.05	28.51	6.454682	20.55	18.57	-1.98	-8.43	**345.2**
T_S2	32.11	30.83	-1.28381	30.93	28.35	-2.58	-1.29	**2.5**
T_S3	26.25	31.83	5.57	24.17	24.91	0.74	-4.83	**28.5**
T_S4	20.06	26.68	6.613161	18.96	15.14	-3.82	-10.43	**1383.8**
T_S5	26.32	29.8	3.476774	24.3	17.57	-6.73	-10.2	**1179.7**

DCT= Delta cycles threshold, DDCT= Delta Delta cycles threshold

Discussion

Several structural and secreted virulence factors play a role in S. aureus infections which are multifactorial and depend on bacterial adherence and biofilm formation (29).

When using of antibiotic sensitivity test, 40 (69%) *S. aureus* isolates exhibited resistance to gentamicin. This result was approximately parallel with Ahmed and Al Mossaw [30] who showed that 60% of isolates were gentamicin resistant. In a local study, in Duhok by Abdullah [31] showed that 59 (39.07%) of *S. aureus* isolates from UTI were gentamicin-resistant. The inactivation of antibiotics by aminoglycoside-modifying enzymes encoded by genetic elements is the main mechanism of resistance to aminoglycosides [32]. *S. Aureus* isolates resistance to cefoxitin is in agreement with some other local studies such as Abed and Hamim [33] who showed that 70% of *S. aureus* isolates were resistant. According to Muhammad and Al-Mathkhury [34] in Sulaimania city 68% of *S. aureus* isolates were resistant to cefoxitin. The sensitivity test of *S. aureus* isolates to vancomycin showed that 57 (98%) *S. aureus* isolates were sensitive to vancomycin. The local study by Jaddoa and Al-Mathkhury [35] illustrated that all MRSA isolates (100%) sensitive to vancomycin. Another

study by Adhikari *et al*. [36] stated that vancomycin sensitivity was observed in all MRSA isolates.

The lowest concentration of an antibiotic that will prevent visual growth of a bacterium during an overnight incubation period, is called as the minimum inhibitory concentration [22]. In the present study 70% isolates had an MIC of 32 μg/ml. Whereas the lowest frequent MIC was recorded in 2.5% of isolates and (5%) had an MIC of 256 μg/ml. Jaddoa and Al-Mathkhury [35] showed that out of the 17 resistant isolates, 16 (94.1%) were able to withstand 32 g/ml of gentamicin, whereas just one isolate (5.88%) had a MIC of 256 g/ml.

Bacterial biofilm production is important for their survival in the host and has been identified as a crucial virulence factor in the development of severe chronic illnesses [37]. The results of biofilms were in disagreement with the local study performed by Hatem *et al*. [38] who showed 47.7% of the isolates produced a strong biofilm, 38.6% produced moderate biofilms, and 13.6% had weak biofilm. However, the results were close to Mohammed and Radif [39] who showed that 46.15 % of the isolates produced strong biofilm, 46.15 % produced moderate biofilm and 7.70 % produced weak biofilm. The differences in the results connected to the accessory gene regulatory (agr) quorum sensing system's activity. This mechanism changes biofilm formation in *S. aureus*.

Antibiotics with sub-MIC have an effect on the ultrastructure and antigenicity of bacteria, as well as their adhesion to epithelial cells. Other research shows that antibiotic sub-MIC play an important role in gene mutation, causing the hypermutable state, inducing various gene transfer mechanisms including transposition and conjugation, and promoting enzyme-catalysed activities [40]. Several investigations found that many antibiotics, with the exception of gentamicin and some other antibiotics, caused biofilm development [41]. Previously several drugs have already been shown to induce biofilm development [42]. Antibiotics, according to Bleich *et al*. [43], can both elicit and stimulate biofilm formation.

The heptameric pore-formation of *hla* play an important role in the pathogenicity of *S. aureus* by destroying a variety of host cells [44]. A local study by Saleem [45] demonstrated *hla* in 114 (95%) *S. aureus* isolates from different clinical sources. Another study by Motamedi *et al*. [46] showed that 11 (7.97%) isolates of *S. aureus* from different clinical sources revealed *hla* gene. The *nuc* gene has long been regarded as the gold standard for identifying *S. aureus* and it is still being used today [47]. Ibraheem and Al-Mathkhury [48] reported that only 60.7% of *S. aureus* harboured *nuc* gene. Another study by Andrade *et al*. [49] showed that the *nuc* gene was found in 67 (48.9%) out of 137 isolates, with only 35 being *S. aureus*.

The results revealed that *hla* and *nuc* were upregulated in *S. aureus* biofilm due to gentamicin stress. Likewise Jaddoa and Al-Mathkhury [35] showed that the gentamicin, at sub-MIC, increased the level of *hla* expression in all tested biofilms.

Antimicrobial sub-MIC can impact the expression levels of bacterial toxins and components involved in colonization and invasion, according to growing evidence [50]. Several antibiotic classes were studied for their impact on *S. aureus* toxin generation and host immunological response. They showed that sub-MIC of ribosome-targeting antibiotics and cell wall active chemicals cause *S. aureus* virulence factor expression to be contradictory, with the former resulting in reduced production and the latter increasing the expression. Such research is crucial for the development of effective treatment regimens for *S. aureus*

infections that improve patient outcomes. In *S. aureus*, agr locus tightly controls the expression of important virulence components by encoding a two-component signalling mechanism. Types I to IV are the four primary agr groupings of *S. aureus* isolates [51]. When the agr system is activated, the bacteria transforms from a sessile colonizer to an invasive and aggressive pathogen [52].

Conclusion

In the present study the gentamicin, at sub-MIC, upregulated the gene expression of *hla* and *nuc* in biofilm of *S. aureus*.

References

[1] J.P. Rasigade, O. Dumitrescu and G. Lina, "New epidemiology of *Staphylococcus aureus* infections, " *Clinical Microbiology and Infection*, vol.20, pp.587-8, 2014.

[2] S.Y. C. Tong, J.S. Davis, E. Eichenberger, T.L. Hollandand V.G. Fowler Jr, " *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management, " *American Society for Microbiology*, vol. 28, no.3, pp.603–661, 2015.

[3] V.K.C. Pérez, G.M. da Costa, A.S. Guimarães, M.B. Heinemann, A.P. Lage and E.M.S. Dorneles, "Relationship between virulence factors and antimicrobial resistance in *Staphylococcus aureus* from bovine mastitis, " *Journal of Global Antimicrobial Resistance*, vol.22, pp.792–802, 2020.

[4] H.C. Flemming, J. Wingender, U. Szewzyk, P. Steinberg, S.A. Rice and S. Kjelleberg "Biofilms: An emergent form of bacterial life, "*Nature Reviews Microbiology*, vol.14, pp. 563–575, 2016.

[5] B. Turchi, F. Bertelloni, F. Marzoli, D. Cerri, S. Tola, E. Azara, C.M. Longheu, R. Tassi, M. Schiavo, G. Cilia and F. Fratini, "Coagulase negative Staphylococci from ovine milk: Genotypic and phenotypic characterization of susceptibility to antibiotics, disinfectants and biofilm production, " *Small Ruminant Research*, vol.183, p.106030, 2020.

[6] H. Buttner, M. Dietrich and H. Rohde, "Structural basis of *Staphylococcus epidermidis* biofilm formation: Mechanisms and molecular interactions, " *Frontiers in Cellular and Infection Microbiology*, vol.5, no.14, 2015.

[7] C.R. Arciola, D. Campoccia, S. Ravaioli and L. Montanaro, "Polysaccharide intercellular adhesin in biofilm: Structural and regulatory aspects, " *Frontiers in Cellular and Infection Microbiology*, vol.5, p. 7, 2015.

[8] C. Heilmann, "Adhesion Mechanisms of Staphylococci, " *Advances in Experimental Medicine and Biology*, vol.715, pp.105–123, 2011.

[9] S. Dehbashi, H. Tahmasebi, B. Zeyni and M. Arabestani, "the relationship between promoter dependent quorum sensing induced genes and methicillin resistance in clinical strains of *Staphylococcus aureus*, " *Journal of Zanjan University of Medical Sciences and Health Services*, vol.26, pp.75 87, 2018.

[10] D. Oliveira, A. Borges and M. Simões, "*Staphylococcus aureus* toxins and their molecular activity in infectious diseases, " *Toxins*, vol.10, no.6, p.252, 2018.

[11] K.H. Schleifer and J.A. Bell, P.D. Vos, G.M. Garrity, D. Jones, N.R. Krieg, W. Ludwig, F.A. Rainey, K.-H. Schleifer, W.B. Whitman, Eds, Family VIII-Staphylococcaceae fam. Nov, "*In Bergey's Manual of Systematic Bacteriology*, " 2nd ed, Springer: New York, NY, USA, vol. 3, pp. 392–421, 2009.

[12] Y. Hu, J. Meng, C. Shi, K. Hervin, P.M. Fratamico and X. Shi, "Characterization and comparative analysis of a second thermonuclease from *Staphylococcus aureus*,"*Microbiological Research*, vol.168, no.3, pp.174–182, 2013.

[13] E.F. Kenny, A. Herzig, R. Krüger, A. Muth, S. Mondal, P.R. Thompson, V. Brinkmann, H.V. Bernuth and A. Zychlinsky, "Diverse stimuli engage different neutrophil extracellular trap pathways, " *eLife*, vol.6, 2017.

[14] J.-A. McClure, D. L. J. Zaal, J. M. Conly, K. Zhang, and S. S. Richter, "Novel multiplex PCR assay for detection of chlorhexidine-quaternary ammonium, mupirocin, and methicillin resistance genes, with simultaneous discrimination of *Staphylococcus aureus* from coagulase-negative Staphylococci, " *Journal of Clinical Microbiology*, vol.55, no.6, pp.1857-1864, 2017.

[15] M. R. Kiedrowski, J. S. Kavanaugh, C. L. Malone, J. M. Mootz, J. M. Voyich, M. S. Smeltzer, K. W. Bayles and M. Otto, "Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*,"*Plos One*, vol.6, no.11, p.e26714, 2011.

[16] O. Dumitrescu, P. Choudhury, S. Boisset, C. Badiou, M. Bes, Y. Benito, C. Wolz and G. Lina, "Beta lactams interfering with PBP1 induce Panton-Valentine leukocidin expression by triggering *sarA* and rot global regulators of *Staphylococcus aureus*, " *Antimicrobial Agents and Chemotherap*, vol.55, no.7, pp.3261–71, 2011.

[17] N. A. Khan, S. Ahmed, I. H. Farooqi, I. Ali, V. Vambol, F. Changani, M. Yousefi and A. H. Khan, "Occurrence, sources and conventional treatment techniques for various antibiotics present in hospital wastewaters: A critical review, " *Trends in Analytical Chemistry*, vol.129, p.115921, 2020.

[18] N. T. M. Jaddoa, and H. J. F. Al-Mathkhury, "Gentamicin modulates the gene expression of *HLA* in methicillin resistance *Staphylococcus aureus* biofilm, " *Journal of Global Pharma Technology*, vol.10, no. 7, pp.61-70, 2018.

[19] H.T. Ibrahim, "The Dual Role of Gentamicin in Gene Expression of Fibronectin Binding Proteins in *Staphylococcus Aureus*, " *Journal of Global Pharma Technology*, Vol. 10, no.11, pp.756-766, 2018.

[20] M. A. R. H. AL-Sheikhly, L. N. Musleh and H. J. F. Al-Mathkhury, "Gene expression of *pelA* and *pslA* in *Pseudomonas Aeruginosa* under gentamicin stress, " *Iraqi journal of Science*, vol.61, no.2, pp.295–305, 2020.

[21] CLSI, "Performance Standards for Antimicrobial SusceptibilityTesting, " M100, 30th edition, *Wayne*, vol.40, no.1, 2020.

[22] J. M. Andrews, "BSAC standardized disc susceptibility testing method, " *Journal of Antimicrobial Chemotherapy*, vol48, no.1, pp.43-57, 2001.

[23] S. S. Atshan, M. Nor Shamsudin, Z. Sekawi, LT. Lung, R. A. Hamat, A. Karunanidhi, A. Mateg Ali, E. Ghaznavi-Rad, H. Ghasemzadeh-Moghaddam, JS. Chong Seng, J.J. Nathan and CP. Pei, "Prevalence of adhesin and regulation of biofilm-related genes in different clones of *Staphylococcus aureus*, " *Journal of Biomedicine and Biotechnology*, vol.6, pp. 1-10, 2012.

[24] A.-M. Costa, I. Kay, and S. Palladino, "Rapid detection of *mecA* and *nuc* genes in Staphylococci by real-time multiplex polymerase chain reaction, " *Diagnostic Microbiology and Infectious Disease*, vol.51, no.1, pp.13-17, 2005.

[25] M.J.H. Goldsworthy,"Gene expression of Pseudomonas aeruginosa and MRSA within a catheter-associated urinary tract infection biofilm model, "*biohorizons*, vol.1, no.1, 2008.

[26] M. Pruneau, G. Mitchell, H. Moisan, E. Dumont-Blanchette, C.L. Jacob and F. Malouin,"Transcriptional analysis of antibiotic resistance and virulence genes in multiresistant hospital-acquiredMRSA,"*FEMS Immunol Med Microbiol*, vol.63, pp.54-64, 2011.

[27] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔct Method,"*Methods*, vol.25, no.4, pp.402-8, 2001.

[28] J. P. Rasigade, A. Moulay, Y. Lhoste, A. Tristan, M. Bes, F. Vandenesch, J. Etienne and O. Dumitrescu, "Impact of sub-inhibitory antibiotics on fibronectin-mediated host cell adhesion and invasion by *Staphylococcus aureus*, "*BMC Microbiol*, vol.11, no.263, 2011.

[29] T.D. Scherr, C.E. Heim, J.M. Morrison and T.Kielian,"Hiding in Plain Sight: Interplaybetween Staphylococcal Biofilms and Host Immunity, " *Front Immunol*, vol.5, pp.1-7, 2014

[30] M.E. Ahmed and M.T. Al Mossaw, "Production of purified MRSAcin from local clinical strain of methicillin-resistant *Staphylococcus aureus* and its applicability as antimicrobial, " *Pharmacy & Pharmacology International Journal*, vol.5, no.6, pp.219–224, 2017.

[31] I.M. Abdullah, "Multiple drugs resistance among urinary tract Infection patients in Duhok City – Kurdistan Region–Iraq, " *Duhok Medical Journal*, vol.13, no.1, pp.22-31, 2019.

[32] N. Perumal, S. Murugesan and P. Krishnan, "Distribution of genes encoding aminoglycoside-modifying enzymes among clinical isolates of methicillin-resistant Staphylococci, " *Indian Journal of Medical Microbiology*, vol.34, no.3, pp.350-2, 2016.

[33] F.S. Abed and S.S. Hamim, "Genetic Evaluation for the Proposed role of *Staphylococcus aureus* in Burn Patients, " *Medico-legal Update*, vol.21, no. 3, 2021.

[34] H. A. A. U. Muhammad and H. J. F. al-Mathkhuri, "the prevalence of methicillin resistant *Staphylococcus aureus* and methicillin resistant *Staphylococcus epidermidis* in Al-Sulaimania City, " *Iraqi Journal of Science*, vol 55, no.2A, pp.386-393, 2014.

[35] N.T.M. Jaddoa and H.J.F. Al-Mathkhury, "Gene expression of *hla* in methicillin resistant *Staphylococcus aureus* under gentamicin stress, " *Journal of Global Pharma Technology*, vol.10, no.07, pp.61-70, 2018.

[36] R. Adhikari, N. Dutt Pant, S. Neupane, M. Neupane, R. Bhattarai, S. Bhatta, R. Chaudhary and B. Lekhak, "Detection of Methicillin Resistant *Staphylococcus aureus* and Determination of Minimum Inhibitory Concentration of Vancomycin for *Staphylococcus aureus* Isolated from Pus/Wound Swab Samples of the Patients Attending a Tertiary Care Hospital in Kathmandu, Nepal, " *Canadian Journal of Infectious Diseases and Medical Microbiology*, ID2191532-2191532, 2017.

[37] B. Parrino, D. Schillaci, I. Carnevale, E. Giovannetti, P. Diana, G. Cirrincione and S. Cascioferro, "Synthetic small molecules as anti-biofilm agents in the struggle against antibiotic resistance, " *European Journal of Medicinal Chemistry*, vol.161, pp.154–178, 2019.

[38] Z.A. Hatem, S.A. Jasim and Z.H. Mahdi, "Phenotypic and Genotypic Characterization of Antibiotic Resistance in *Staphylococcus aureus* Isolated from Different Sources, " *Jundishapur Journal of Microbiology*, vol.14, no.4, p.e115221, 2021.

[39] S.W. Mohammed and H.M. Radif, "Detection of *icaA* Gene Expression in Clinical Biofilm-Producing *Staphylococcus Aureus* Isolates, " *Iraqi Journal of Science*, vol.61, no.12, pp.3154-3163, 2020.

[40] D. Wojnicz, and S. Jankowski, "Effects of subinhibitory concentrations of amikacin and ciprofloxacin on the hydrophobicity and adherence to epithelial cells of uropathogenic *Escherichia coli* strains, " *International Journal of Antimicrobial Agents*, vol.29, no.6, pp.700-4, 2007.

[41] S. Hemati, N. Sadeghifard, S. Ghafurian, F. Maleki, Z. Mahdavi, A. Hassanvand, H. Valadbeigi, S. Hemati and V. Hatami, "The association of biofilm formation and sub-minimal inhibitory concentrations of antimicrobial agents, "*J Bas Res Med Sci*, vol.3, no.1, pp.26-30, 2016.

[42] N. Kashef, M. Akbarizare and S. K. Kamrava, "Effect of sub-lethal photodynamic inactivation on the antibiotic susceptibility and biofilm formation of clinical *Staphylococcus aureus* isolates, "*Photo diagnosis and Photodynamic Therapy*, vol.10, no. 4, pp.368-373, 2013.

[43] R. Bleich, J. D. Watrous, P. C. Dorrestein, A. A. Bowers and E. A. Shank, "Thiopeptide antibiotics stimulate biofilm formation in Bacillus subtilis, "*Proceedings of the National Academy of Sciences of the United States of America*, vol.112, no.10, pp.3086-91, 2015.

[44] D. Grumann, U. Nubel and B. M. Broker, "*Staphylococcus aureus* toxins--their functions and genetics. Infect, " *Infect Genet Evol*, vol.21, pp.583-592, 2014.

[45] A. J. Saleem, "High frequency of hemolysin associated genes among *Staphylococcus aureus* clinical isolates in Iraq, "*Journal of Global Pharma Technology*, vol.9, no.12, pp.62-68, 2017.

[46] H. Motamedi, B. Asghari, H. Tahmasebi and M. R. Arabestani, "Identification of Hemolysin Genes and their Association with Antimicrobial Resistance Pattern among Clinical Isolates of *Staphylococcus aureus* in West of Iran, " *Advanced Biomedical Research*, vol.7, no.1, p.153, 2018.

[47] J.A. McClure, J. Zaal DeLongchamp, J.M. Conly, K. Zhang, "Novel Multiplex PCR Assay for Detection of Chlorhexidine- Quaternary Ammonium, Mupirocin, and Methicillin Resistance Genes, with Simultaneous Discrimination of *Staphylococcus aureus* from Coagulase-Negative Staphylococci, " *Journal of Clinical Microbiology*, vol.55, no.6, pp. 1857–1864, 2017.

[58] H. T. Ibraheem, and H. J. F. Al-Mathkhury, "Staphylococcal nuclease removes *Escherichia coli* and *Klebsiella pneumoniae* previously adhered to uroepithelial cells, " *Iraqi Journal of Science*, vol.56, no. 1B, pp. 367-378, 2015.

[49] N.C. Andrade, M. Laranjo, M.M. Costa and M.C. Queiroga, "Virulence Factors in Staphylococcus Associated with Small Ruminant Mastitis: Biofilm Production and Antimicrobial Resistance Genes, "*Antibiotics*, vol.10, p.633, 2021.

[50] E. Hodille, W. Rose, B.A. Diep, S. Goutelle, G. Lina and O. Dumitrescu, "The role of antibiotics in modulating virulence in *Staphylococcus aureus*, " *Clinical Microbiology Reviews*, vol.30, no.4, pp.887–917, 2017.

[51] B. Shopsin, B. Mathema, P. Alcabes, B. Said-Salim, G. Lina, A. Matsuka, J. Martinez and B. N. Kreiswirth, "Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians, " *American Society for Microbiology*, vol.41, no.1, pp.456–9, 2003.

[52] T.A. Thompson and P.D. Brown, "Association between the agr locus and the presence of virulence genes and pathogenesis in *Staphylococcus aureus* using a Caenorhabditis elegans model, " *International Journal of Infectious Diseases*, vol.54, pp.72–6, 2017.