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Original Article

Effect of Ultraviolet Light on the Expression of *icaD* Gene in *Staphylococcus aureus* Local Isolates in Iraq

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Abstract

The current study was designed to reveal the impact of ultraviolet (UV) light on polysaccharide intercellular adhesion of the *icaD* gene expression and its relation to biofilm as virulence markers in *Staphylococcus aureus*. In total, 37 isolates of *S. aureus* were gathered from various bacterial infection sources, and 26 *S. aureus* isolates were definite to *icaD* gene using the Polymerase Chain Reaction (PCR) technique. Prior to and after exposure to UV light, RNAs from the isolates were extracted which had strong biofilm formation by crystal violet staining assay, and they were then exposed to quantitative real-time PCR (qPCR). In vitro, the survival of isolates was evaluated after the exposition to several periods of the UV light power via counting the number of colonies. The results showed that the exposure to the UV light at 320 nm for 2, 4, 6, 8, and 10 min had inhibitory effects on the bacterial growth. The sub-lethal exposure time was 8 min. The rapid reduction of bacterial survival rates was associated with the increment irradiation time of UV power from 5 to 1.24 log10 CFU/ml. In general, down regulation of the *icaD* gene expression was decreased upon exposure to the UV light which was used as a physical agent for controlling the bacterial infection and biofilm formation.

Keywords: *Staphylococcus aureus*, UV light, *icaD* gene, real-time PCR

1. Introduction

Staphylococcus aureus is one of the highest opportunistic pathogens of humans. This bacterium regards the cause of several diseases and often causes serious problems through nosocomial infection in hospitals and medical devices which noticeably comprise biofilm formations. Moreover, the community-acquired infection has recorded the reason for sepsis and pneumonia (1). The importance of biofilm formation is the main reason for resistance to antibiotics and host immune response. Polysaccharide Intercellular Adhesion (PIA), cell surface protein, and DNA are the essential constituents of biofilms. The PIA is encoded via *ica* operon at the *Staphylococci* cell walls that are formed of A, D, B, and C genes and the regulator *icaR* gene (2). The increment of antibiotic resistance and the biofilm formation induced many researchers to investigate the influence of some biological, physical, and chemical factors as serious strategies or alternative treatments for antibiotics (3). One of the common physical antimicrobial agents is ultraviolet (UV) which is suited to the production of genetic mutations in microorganisms due to the small size of their cells which allow the radiation to attain the nuclei through the absorption of the radiation by nucleic acid (4).

The UV light is the electromagnetic radiance that has a range between 400nm and 100nm and holds a shortened wavelength than the notable light; however, it is more lengthy, compared to X-rays (5). Moreover, it can produce some chemical reactions and spurs fluorescent materials. It also has a big number of helpful uses in modernistic medicine for diagnostic and therapeutic goals. In addition, the decontamination of materials via UV radiation is applied for the inhibition of pathogenic microorganisms, such as viruses, bacteria, and fungi (6). This study aimed to determine the prevalence of the biofilm production of the *icaD* genes isolated from some *S. aureus* clinical isolates by Polymerase Chain Reaction (PCR). Subsequently, the biofilm production was evaluated via the crystal violet staining method, followed by the determination of the expression of the *icaD* gene influenced by different exposure periods of UV rays using the real-time PCR technique.

2. Materials and Methods

2.1. Clinical Samples Collection

A total of 37 isolates of *S. aureus* were collected from 70 clinical sources, such as burns, ear swabs, and blood of patients in Baghdad hospitals, Iraq. All specimens were examined with culture media using Mannitol salt agar as the primary identification depending on phenotypic and biochemical tests as described by Brooks, Butel (7). Afterward, they were confirmed by genotypic detection depending on certain housekeeping *recA* gene using the PCR technique.

2.2. Biofilm Formation Assay

Biofilm formation by *S. aureus* isolates was examined through crystal violet staining method as was described by Piechota, Kot (8). The absorbance values of >0.4 were regarded as strong producers, while <0.2 and 0.2-0.4 were considered weak and moderate producers, respectively.

2.3. Ultraviolet Light Exposure

S. aureus isolates were cultured on brain heart infusion broth (Himedia/India) and incubated at 37°C for 18-24 h under rotary conditions and standard turbidity (McFarland No. 0.5) (9). The contents were mixed well predated to standardize bacterial cell numbers which should be equivalent to 1.5×10^8 CFU/ml. The colony-forming units (CFU) were calculated for all isolates after sorting onto Mannitol salt agar (Oxoid/England) plates by counting the colonies on the surface of the media. In the next stage, the suspension of *S. aureus* was prepared by inoculating 5 ml of L-broth exposed to the UV light (UV transilluminator-FLX-20M/France) at 320 nm for 2, 4, 6, 8, and 10 min, respectively, in dark with a 11-cm distance between the UV source and bacterial suspension. After exposure, the bacterial suspension was collected immediately for each exposure and seeded onto Mannitol salt agar plates which incubated aerobically at 37°C overnight in dark to calculate the single colonies and detect the sub-lethal and lethal time of exposure.

2.4. Detection of the *icaD* Gene

Genomic DNA was isolated from *S. aureus* using DNA Extraction Kit (Promega, USA) according to the manufacturer's instructions. DNA concentration and purity of DNA were determined using a NanoDrop ND-1000. PCR amplification of the *icaD* was carried out in a DNA thermal cycler (Applied Biosystem, Singapore) with the following cycling program: Initial denaturation at 95ºC for 5 min, and 30 cycles of denaturation at 95ºC for 30 sec, annealing at 55ºC for 30 seconds, extension at 72ºC for 30 sec, and a final extension at 72ºC for 7 min. The primer sequences for *icaD* and *recA* genes are presented in table 1. The PCR products were detected via agarose gel (1.5%) electrophoresis which was stained through ethidium bromide.

2.5. RNA Extraction with TRIzol

RNA was isolated from bacterial isolates according to the protocol of TRIzol™ Reagent.

Table 1. Primers utilized in the study

Primers	Primer sequence (5-3)	PCR product (bp)	Reference
icaD	F- -ATGGTCAAGCCCAGACAGAG R- AGTATTTTCAATGTTTAAAGCAA	198	(22)
rec A	F-AAAGTTCAGGTAAGACGACAG	277	(23)

2.6. *icaD* Gene Expression by qRT-PCR

Total RNA was converted to complementary DNA (cDNA) by reverse transcriptase using WizScript™ RT FDmix Kit. Quantitative real-time PCR was performed using the QIAGEN real-time PCR System (Rotor-Gene Q, Germany) with qPCR software. The gene expression levels and fold change were determined by measuring the cycle threshold (Ct) utilizing the 2xqPCR Master Mix Kits components. *recA* reference gene was employed as an internal control to be used in calculating the ΔCT value. Table 1 tabulates the primer sequences for *recA*. All reaction mixtures were set up at 20µl reaction mixtures as follows: 10µl qPCR Master Mix, 1 µl (10 pmol) of each primer, and 4 µl of cDNA template; moreover, the volume was completed by Nuclease-Free Water.

The study protocol was conducted according to the thermic profile for *icaD* and *recA* genes: Initial denaturation at 95ºC for 5 min, and 40 cycles of denaturation at 95ºC for 15 sec, annealing at 60ºC for 60 sec, and melting curve analysis at 50-99°C for 2-5 sec/step. The relative expression ratios were calculated using the $2^{-\Delta\Delta Ct}$ method (10), and the Ct of the *recA* of each strain was regarded as the calibrator, where ΔCt represents the difference between the Ct of the gene studied and the Ct of the *recA* for each strain.

2.7. Statistical Analyses

Analysis of the gene expression was performed using the Pfaffl method (Relative quantification) (Folding=2-ΔΔCT). Additionally, the Statistical Analysis System (2012) program was utilized to notice the influence of variance features in the study. T-test was used to clarify the significant differences between treated and untreated isolates with UV light under probability levels of <0.05. Furthermore, the Chi-square test was applied to signify the contrast among percentages in this study (under 0.05 and 0.01 probabilities).

3. Results and Discussion

In total, 70 clinical samples were isolated from various patients in different units in hospitals in Baghdad. The results included 37 positive samples of swabs diagnosed as *S. aureus* from the following different sources: burns (n=21), ear (n=9), and blood (n=7). Mannitol salt agar was used for specific isolation of *S. aureus*, and the yellow colony indicated a Mannitol salt fermenting (11). Confirmed isolates were identified as *S. aureus* by the PCR technique using *recA* as the housekeeping gene.

The results of biofilm formation indicated that all tested strains could form biofilms (100%). This result was similar to the findings that have been reported by the European countries (8, 12). The levels of biofilm formation (n=3) have been determined as strong (n=12; 32.43%), moderate (n=19; 51.35%), and weak (n=6; 16.21%). The distribution of biofilm formation with sources of *S. aureus* isolates is given in table 2. In a study conducted by Karmakar, Jana (13), strong (32%) and moderate (68%) biofilm formations were revealed; however, the results of a study performed by Shahmoradi, Faridifar (14) showed strong (6%), moderate (30%), and weak (64%) strains of *S. aureus* in biofilm formations. This indicated a significant decrease, compared to the results in the current study, which can be attributed to the biofilm growth style of *S.*

aureus that is firmly controlled through the combination of genetic factors. Host immune responses versus persistent biofilm infections are inefficient and cause chronic diseases (15).

Table 2. Sources and rates of the biofilm formation in *S. aureus*

Isolation source	Number of isolates	Strong %	Moderate %	Weak %	Chi-Square (χ^2)
Burns	21	8 (38.08%)	12 (57.14%)	1 (4.76%)	11.48 **
Ear	9	2 (22.22%)	4 (44.44%)	3 (33.33%)	7.25 **
Blood	7	2 (28.57%)	3 (42.85%)	2 (28.57%)	5.02 *
Total	37	12 (32.43%)	19 (51.35%)	6 (16.21%)	10.63 **

* (P≤0.05), ** (P≤0.01).

The detection of the *icaD* gene in the examined isolates was confirmed via the amplification of the specific fragments (Figure 1). Totally, 26 out of 37 *S. aureus* isolates were *icaD*-positive (70.27%) with high significance for all sources, specifically for burn isolates. The spreading of the *icaD* biofilm genes with the levels of biofilm formation in *S. aureus* isolates is presented in table 3. The results are in line with the findings of a study conducted by Marques, Santos (16) who found that 95% of the isolates were *icaD*-positive.

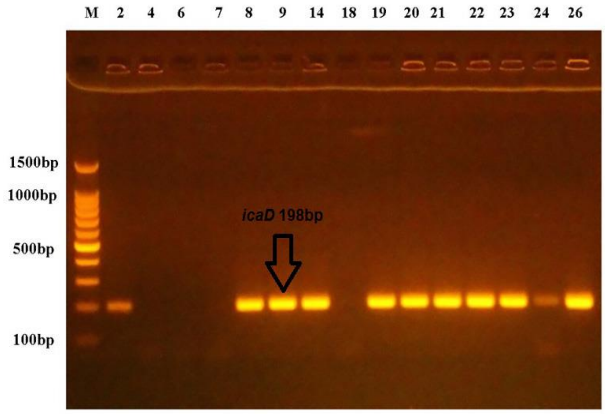

Figure 1. Agarose (1.5% w/v) gel electrophoresis of the PCR amplification of the *icaD* gene (198bp) (Lane M, 100 pb)

Table 3. Relationship between biofilm formation and *icaD* genes in *S. aureus*

Levels of biofilm formation		*icaD* gene
Strong	(n=12)	11 (91.66%)
Moderate	(n=19)	14 (73.68%)
Weak	(n=6)	1 (16.66%)
Total	(n=37)	26 (70.27%)

On the other hand, some researchers concluded that the biofilm formation of *staphylococci* was linked to the existence of both *icaA* and *icaD* genes which caused catheter-related and nosocomial infections (17). The results of the current study showed that genotypic and phenotypic detection of the *icaD* gene was related to the biofilm production ability among the clinical strains of the *Staphylococcus* isolates.

3.1. UV Light Exposure

A total of 11 isolates of *S. aureus* with strong biofilm formations and *icaD* gene-positive were exposed to the UV light (320 nm) for 2, 4, 6, 8, and, 10 min, respectively, and the results showed a decrease in the counts of the colony with a reduced range approximately from 5 to 1.24 log10 CFU/ml (Figure 2).

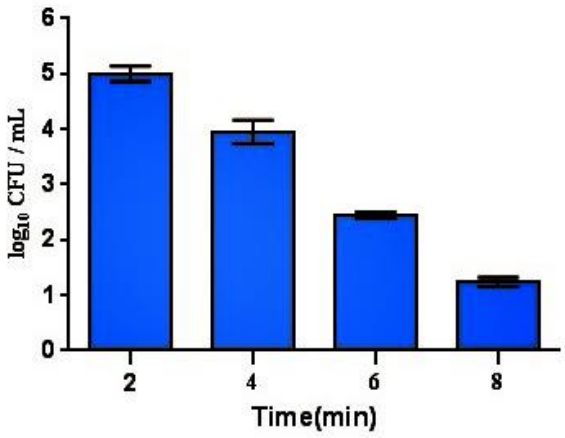

Figure 2. Counts (log cfu/mL) for *S. aureus* samples after the UV light treatment

These results proposed that the counts of the colony were successfully reduced with an increase in the exposure time from 2 to 10 min. In this study, an 8-min

exposure to the UV was chosen as the time given to the least number of colonies to calculate the CFU (sub-lethal) and was selected for the analysis of the mRNA expression of these isolates. Teixeira, Bicalho (18) noticed the logarithmic discount in bacterial colony counts for the total bacterial count in *Escherichia coli*, *Staph. aureus*, and *Streptococcus spp.* as 5.2, 1.2, and 0.2, respectively. Moreover, the UV light was operative in deactivating nosocomial pathogens developed by biofilms (19).

3.2. Real-Time PCR Quantification of the *icaD* Expression

The total genomic RNA extracted from 11 positive isolates of the *icaD* gene which had a strong ability to form biofilm in vitro and the levels of *icaD* gene expression before and after the UV treatment at an exposure period of 8 min were evaluated in highly precise conditions using TRIzoL with a ready kit. The amplification was logged as Ct value indicating that high Ct values point to decreases in the gene expression, while the decrement of the Ct value shows a rise in gene expression (10). The housekeeping gene was used in molecular studies due to the fact that its expression stays stable in cells under inquiry and different conditions (20). These small variations in the Ct values among our study groups (treated and untreated with UV) render the *recA* gene as a useful normalizing gene. The mean Ct values for the *icaD* in the untreated group with UV were from 23.89 to 28.72 as shown in figure 2 and 3 and table 4. In addition, the mean Ct values for the *icaD* in the treated group with UV were from 29.34 to 31.86 as shown in figure 4 and table 4.

Table 4. Folding of *icaD* gene expression

Untreated Isolates					Treated Isolates					T- test
Is. No.	**Means Ct of *icaD***	**Means Ct of *recA***	**ΔCt**	**Folds=2 $^{-\Delta\Delta Ct}$**	**Means Ct of *icaD***	**Means Ct of *recA***	**ΔCt**	**ΔΔCt**	**Folds=2 $^{-\Delta\Delta Ct}$**	
S2	27.37	30.46	−3.09	1.0	30.94	32.33	−1.39	1.7	0.30	0.437 *
S8	25.63	29.03	−3.4	1.0	29.34	31.98	−2.64	0.76	0.59	0.392 *
S9	27.25	29.11	−1.86	1.0	30.30	30.69	−0.39	1.47	0.36	0.389 *
S14	28.15	30.32	−2.17	1.0	31.86	30.94	0.92	3.09	0.11	0.791 *
S19	26.23	28.01	−1.78	1.0	32.64	31.95	0.69	2.47	0.18	0.436 *
S20	26.08	28.12	−2.04	1.0	31.81	30.57	1.24	3.28	0.12	0,.447 *
S21	23.89	29.27	−5.38	1.0	30.41	31.92	−1.51	3.87	0.06	0.367 *
S22	29.08	29.43	−0.35	1.0	30.92	30.89	0.03	0.38	0.76	0.253 NS
S23	28.20	28.38	−0.18	1.0	30.73	30.54	0.19	0.37	0.77	0.366 NS
S24	26.83	29.07	−2.24	1.0	30.99	30.82	0.17	2.41	0.18	0.429 *
S26	28.72	28.81	−0.09	1.0	31.58	30.99	0.59	0.68	0.62	0.371 *

* ($P \leq 0.05$)

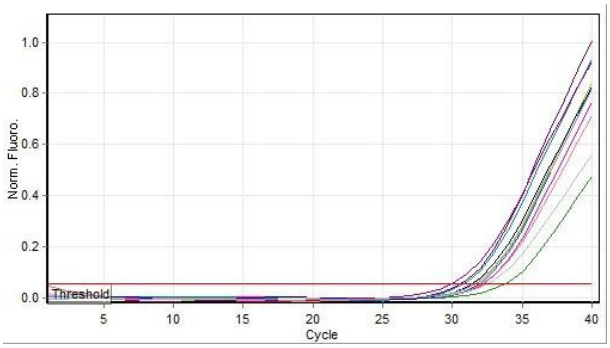

Figure 3. *icaD* in the treated amplification plots by qPCR (Ct values ranged)

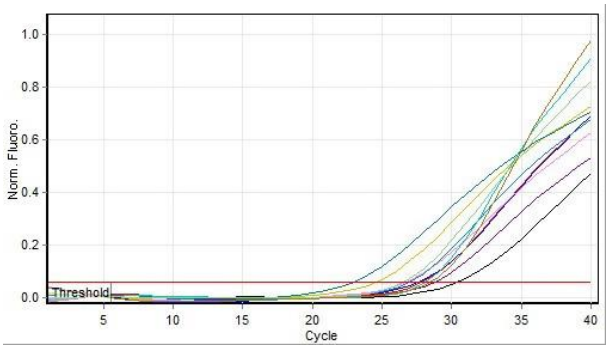

Figure 4. *icaD* in the untreated amplification plots by qPCR (Ct values ranged)

The transcriptional levels of the *icaA* gene for the strong biofilm-producer strains treated with UV were significantly lower than the expression levels of the untreated isolates (P≤0.05), especially for S14, while no significant differences were recorded in S22 and S23. The UV kills microorganisms by the formation of pyrimidine dimers in RNA and DNA that can overlap with the transcriptional and replication process (21). The conventional application of UV light is in biological safety cabinets although the newest uses of UV power have been expanded to the killing of microorganisms that cause food poisoning or water contamination.

Authors' Contribution

Study concept and design: M. H. A.
Acquisition of data: S. H. M.
Analysis and interpretation of data: Z. H. S.
Drafting of the manuscript: A. W. A.
Critical revision of the manuscript for important intellectual content: A. A. A.
Statistical analysis: S. H. M.
Administrative, technical, and material support: S. H. M.

Ethics

The present study was approved by the Ethics Committee of the University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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