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The effect of antimicrobial photodynamic therapy on the expression of biofilm associated genes in *Staphylococcus aureus* strains isolated from wound infections in burn patients

Hassan Mahmoudi[a], Maryam Pourhajibagher[b], Mohammad Yousef Alikhani[a,*], Abbas Bahador[c,**]

[a] Department of Microbiology, Hamadan University of Medical Sciences, Hamadan, Iran
[b] Dental Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran
[c] Oral Microbiology Laboratory, Department of Microbiology, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Purpose: Burn patients are particularly susceptible to microbial infection. *Staphylococcus aureus* causes burn wound, impetigo and cellulitis. Although sub-lethal antimicrobial photodynamic therapy (aPDT) would not result in microorganism killing, it can considerably influence microbial virulence factor.

Methods: Twelve methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) isolated from burns patients. To determine the sub-lethal dose of aPDT, 12 clinical isolates of *S. aureus* photosensitized with 100 μg ml $^{-1}$ toluidine blue O (TBO) and irradiated by light emitting diode (LED) with a wavelength of 630 ± 10 nm and energy densities of 52.0, 104.1, and 156.2 J/cm^2, then bacterial viability was measured. The effects of sub-lethal aPDT on the expression levels of *ica ABCD* and *ica R* genes were assessed by quantitative Real-time PCR (qRT-PCR) method.

Result: Fifty and 100 μg ml^{-1} of TBO significantly reduced the mean cell survival in the MRSA (2.5 – 3 $\log_{10}$) and MSSA (2.75–3.1 $\log_{10}$) isolates. The average expression levels of *icaA*, *ica B*, *ica C*, and *ica D* in the MRSA and MSSA isolates were decreased by (12, 14, 11, and 9) and (13, 14.5, 12, and 9.5) fold change, respectively ($P < 0.05$). However, the expression of *ica R* gene was decreased by 6 and 8 folds change in MRSA and MSSA, respectively.

Conclusion: The potential of TBO-mediated aPDT could reduce the expression of *ica ABCD* as important genes involved in biofilm formation and *ica R* gene as a repressor of the *ica* operon. Therefore, the use of aPDT agents as a complementary therapy in wound infections of burn patients is recommended.

1. Introduction

Burn wounds are often infected by multidrug-resistant (MDR) bacteria like *Staphylococcus aureus* [1]. Colonization with methicillin-resistant *S. aureus* (MRSA) raises the risk of bacteremia, septicemia, and serious clinical problems comprising the loss of skin grafts in burn patients [2]. Burn patients are particularly susceptible to bacterial infection, such as burn wound impetigo; burn wound cellulitis, and types of invasive microbial infections, due to impaired skin barrier function and the accompanying reduction in cell-mediated immunity [3,4]. Injured host tissue is also a risk factor for expanding the biofilm-associated infections [5]. On the one hand, microbial biofilm production is considered as a major virulence factor, since the biofilm functions as a barrier to antimicrobial agents and the host immune system that helps maintained the bacterial colonization [6,7].

One of the pathways of biofilm formation of *S. aureus* isolates is facilitated by the polysaccharide intercellular adhesin (PIA) encoded by the *ica* operon [8,9]. These data would propose that the *ica* locus could potentially be an important target in the therapy of infections. It is necessary to study the changes in the expression of ICA genes due to the direct effect of these genes on the development of biofilms and the pathogenesis of S. aureus in order to achieve a strategy to combat this phenomenon. Conventional antimicrobial treatments usually are unsuccessful in destroying biofilms, which leads to persistent infection [10]. Due to resistance to penicillin derivatives, cephalosporins, monobactams, and carbapenems, the glycopeptide antibiotic

* Corresponding author at: Department of Microbiology, Hamadan University of Medical Sciences, Hamadan, Iran.
** Corresponding author at: Department of Microbiology, Tehran University of Medical Sciences, Tehran, Iran.
E-mail addresses: Hassanmahmoudi24@gmail.com (H. Mahmoudi), mphb65@yahoo.com (M. Pourhajibagher), alikhani@umsha.ac.ir, y.alikhani.phd@gmail.com (M.Y. Alikhani), abahador@sina.tums.ac.ir (A. Bahador).

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vancomycin has remained as last line of defense against Gram-positive cocci [11,12].

Thus, in the search for novel antibacterial strategies, the use of aPDT agents has developed as a hopeful candidate. aPDT includes the interplay of visible light and a photosensitizer agent which under photoactivation produce short lived cytotoxic species in site [13,14]. A very favorable feature for aPDT is the potential for inactivation of virulence factors, especially secreted proteins, by reactive oxygen species [15]. Two oxidation mechanisms are involved in the inactivation of the target cells. The type I path comprise electron/hydrogen atoms-transfer reactions from the photosensitizing agent triplet state with the contribution of a substrate to generate free radical ions. The type II path involves energy transition from that triplet state to molecular oxygen to produce singlet oxygen ($^1O2$) [12]. Inactivation of membrane enzymes and receptors is also probably due to type I reactions at the cytoplasmic membrane [12]. The production of reactive cytotoxic species leads to irreversible damages to the molecular cell components or its destruction [12]. As well as, aPDT influences the expression of virulence factors, causing their degradation [16]. Consequently, it would be favorable for a therapy targeting pathogenicity to inactivate various virulence factors. Therefore, as many such strategies do not have direct antimicrobial action, the use of a therapy method that combines antibacterial activity with decreases of pathogen's virulence potential, would be beneficial [17].

The biological activity of certain virulence factors produced by some species of Gram-negative bacteria has been shown to be successfully reduced by aPDT action. The inhibitory influence of red laser light and the photosensitiser (PS) TBO on virulence factors from *Escherichia coli*, *Pseudomonas aeruginosa* and *Porphyromonas gingivalis* has previously been confirmed [18,19]. One of the unique features of our study with other studies in this field was the use of *S. aureus* clinical strains isolated from wound infections in burn patients with different antibiotic resistance patterns. Commonly, aPDT is used for treatment of localized infections because the tissues should be accessible for exposure to PS. However the effect of aPDT on expression of staphylococcal biofilm-associated genes has not yet been studied, It was hypothesized that aPDT could be reduces the expression of biofilm-associated genes. Therefore the aim of the current study was to evaluate the effects of aPDT on expression of biofilm-associated genes in *S. aureus* strains isolated from wound infections in burn patients.

2. Materials and methods

2.1. Specimen collection from burn patients

This study was approved by ethical committee of the Hamadan University of Medical Sciences (License No: IR.UMSHA.REC.1396.31). The flowchart of the experiment was shown in Fig. 1. In current study, microbiological wound swabs were collected from 95 patients with clinical signs and symptoms of burn wound infection in Burn Intensive Care Unit (BICU), Besat Hospital of Hamadan University of medical Sciences, Iran, between March to August 2017. The swabs were collected by the attending physicians and obtained from deep areas of the burns before any cleaning [20]. Samples were cultured on nutrient agar, Mac-Conkey agar and Blood agar plates (Merck Co, Germany) and incubated at 37 °C for 18–24 h. Isolation and identification of microorganisms was done according to the standard procedures [21].

2.2. Isolation and identification of S. aureus

Identification of *S. aureus* was performed by standard microbiological methods included Gram staining, growth on mannitol salt agar, catalase, DNase and coagulase tests and *nuc* polymerase chain reaction (PCR) [21,22]. To maintain the quality of data every sample was processed in triplicates and every result was cross checked by the principal investigator and the coinvestigator.

2.3. Identification of MRSA

All *S. aureus* isolates for which the cefoxitin minimum inhibitory concentration (MIC) of was $\leq 8\,\mu g\,ml^{-1}$ were classified as MRSA. Methicillin resistance was determined by the presence of the mec *A* gene by PCR as described previously [23] (Table 1). *S. aureus* ATCC 33591 was included as positive control.

2.4. Detection of biofilm related genes (ica A, ica B, ica C, ica D, ica R)

The distribution of biofilm encoded genes in 12 different *S. aureus* isolates with ability biofilm formation was examined by the multiplex PCR method. The primer sets for biofilm encoded genes were previously described as shown in Table 1 [24,25]. The reaction mixture of PCR for detection of *ica ABCDR* genes were 25 µl in total volume containing 12.5 µl of master mix, 0.5 µl of (10 pmol) each forward and reverse primers, 2 µl of genomic DNA, and 9.5 µl of distilled water (dH_2O). The PCR performed with initial denaturation at 95 °C for 5 min and followed for 40 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s and elongation at 72 °C for 20 s. The final elongation was at 72 °C for 5 min. The *S. aureus* ATCC 25923 reference strain was included as positive control and sterile water as negative control for the PCR assays.

2.5. Light source

The light-emitting diode (LED) (FotoSan 630 nm LAD, CMS dental, Denmark) at wavelengths of 630 ± 10 nm of emission and output intensity of 2000 mW/cm^2 with energy densities of 52.0, 104.1, and 156.2 J/cm^2 was utilized as light source.

2.6. Photosensitizer

A stock solution of TBO as a photosensitizer was prepared by dissolving the TBO powder (Sigma-Aldrich) in sterile distilled water to a concentration of $400\,mg\,ml^{-1}$ and filtering it through a 0.22 µm membrane filter (GVS, USA). The dye solution was kept at 4 °C in the dark [26].

2.7. Determination of sub minimum inhibitory concentration (sMIC) of TBO

A sub-lethal dose of TBO on *S.aureus* (MRSA, MSSA, *S. aureus* ATCC 25923*)* was defined as a sMIC, which was determined according to the method described in the previous study [27]. Briefly, rows of a round-bottom 96-well microtiter plate(TPP, Trasadingen, Switzerland) wells were filled with 100 µl of Brain Heart Infusion broth (BHI) (Merck Co, Germany) Then, 100 µl of $400\,\mu g\,ml^{-1}$ TBO was added in the first well and diluted two-fold step-wise from column one to column ten. 100 µl of MRSA and MSSA suspension with a concentration of 1×10^6 colony-forming unit(CFU) ml^{-1} was transferred to each well. Thus, the final concentration of bacterial cell in each well was 5×10^5 CFU ml^{-1}, and the final concentration of TBO was the range of 100–0.19 µg ml^{-1}. Two columns 11 and 12 were used as a negative control (BHI broth without bacterial cells) and a positive control (bacterial suspension in the absence of TBO), respectively. The microtiter plates were incubated for 5 min in dark condition at room temperature (25 ± 2 °C). 10 µl of each well were cultured in a BHI agar plate (Merck, Darmstadt, Germany) and incubated for 24 h at 37 °C. The bacteria colonies growing on the BHI agar surface were counted.

2.8. Determination of sub-lethal dose of LED

In this way, 200 µl of the free-floating both MSSA and MRSA strains in the planktonic suspension at the final concentration of 1×10^6 CFU ml^{-1} was placed inside a round-bottom 96-well microtiter plate. The LED in continuous mode was applied with an output power of

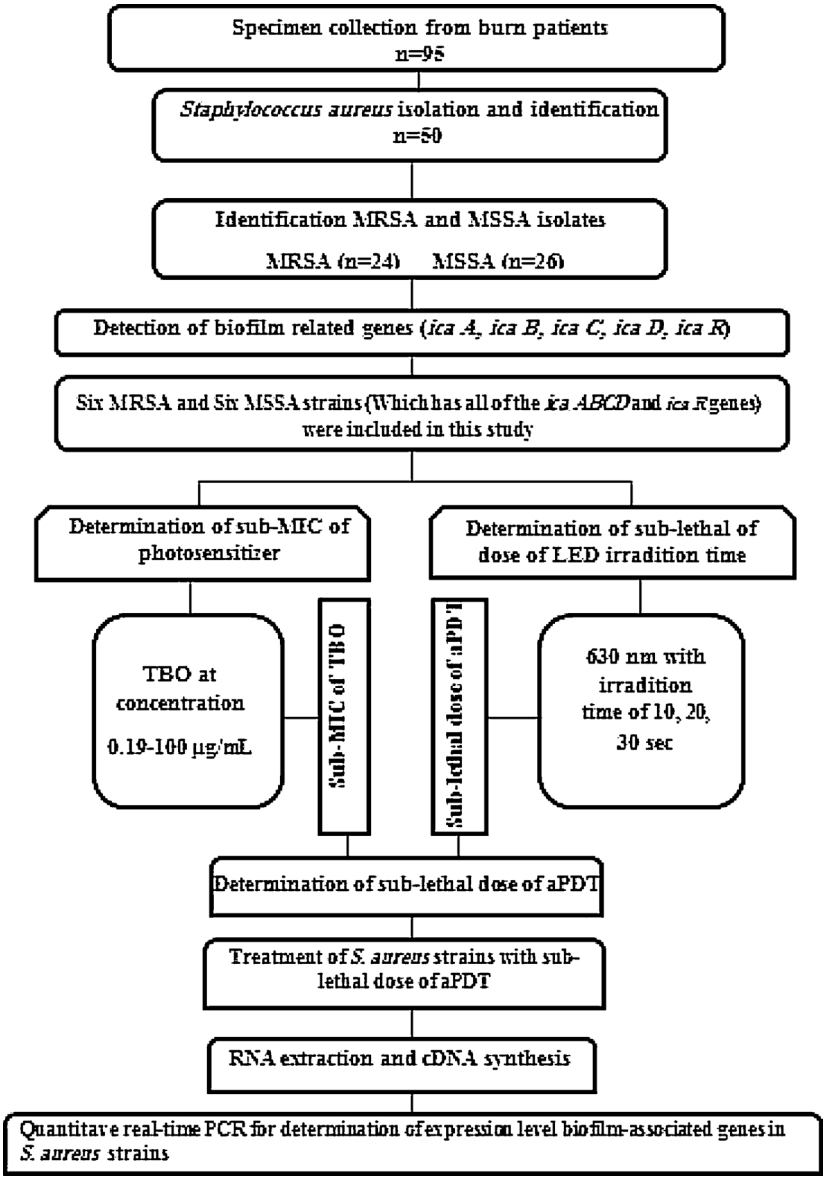

Fig. 1. Flowchart of the experimental steps.
Abbreviations; sub-MIC, sub-lethal minimum inhibitory concentration, aPDT, antimicrobial photodynamic therapy, TBO, toluidine blue O, LED, light-emitting diode, cDNA; complementary DNA, MRSA; Methicillin-resistant *Staphylococcus aureus,* MSSA; Methicillin-resistant *Staphylococcus aureus.*

Table 1
The primers used in this study.

Genes	Nucleotide sequence of primers (5' —— 3')	Reference PCR annealing Temperature (°C)	Amplicon size (bp)	References
ica A-F	GAGGTAAAGCCAACGCACTC	60*	151	[24]
ica A-R	CCTGTAACCGCACCAAGTTT			
ica B-F	ATACCGGCGACTGGGTTTAT	60	211	[24]
ica B-R	TTGCAAATCGTGGGTATGTGT			
ica C-F	CTTGGGTATTTGCACGCATT	60	209	[24]
ica C-R	GCAATATCATGCCGACACCT			
ica D-*F*	ACCCAACGCTAAAATCATCG	60	140	[24]
ica D-*R*	GCGAAAATGCCCATAGTTTC			
ica R-F	ATCTAATACGCCTGAGGA	55	205	[25]
ica R-R	TTCTTCCACTGCTCCAA			
mecA-F	GTAGAAATGACTGAACGTCCGATAA	60	310	[23]
mecA-R	CCAATTCCACATTGTTTCGGTCTAA			
16S rRNA-F	GGGACCCGCACAAGCGGTGG	60	191	[24]
16S rRNA-R	GGGTTGCGCTCGTTGCGGGA			

* Optimized PCR annealing temperature condition for all the genes at 60 °C.

52.0–156.2 J/cm^2 for 10, 20, and 30 s with energy densities of 52.0, 104.1, and 156.2 J/cm^2, respectively.

2.9. Sub-lethal doses of aPDT against S. aureus isolates

After determining the sub-lethal doses of LED and sub-MIC of TBO in the previous sections, the *S. aureus* isolates were exposed to the sub-lethal dose of aPDT. The sub-lethal dose of aPDT (sPDT) was determined as the lowest concentration of PS with the shortest irradiation time of LED in the last well which showing growth. 100 µl of 2 × BHI broth was added to each well of the 96-well microtiter plate and 100 µl of TBO and at 2 × MIC was serially diluted two fold to 1/8 MIC. Finally, 100 µl of *S. aureus* (MRSA, MSSA, *S. aureus* ATCC 25923*)* suspension at final concentration of 1.5 × 10^5 CFU ml^{-1} was added to each well. The microtiter plate was kept in the dark for 5 min at room temperature and exposed to a sub-lethal dose of LED irradiation time. For all experiments, one column contained the positive (growth) control and one column was not inoculated and served as the sterility control. The untreated *S. aureus* isolates which did not receive any treatment was used as a control group.

2.10. RNA preparation and complementary DNA synthesis

Total RNA of 12 clinical *S. aureus* isolates and control groups (untreated) were extracted using FavorPrep™ Total RNA mini kit (FAVORGEN Biotech Corp. Co., Korea), as described by the manufacturer. The purity of the RNA was estimated by calculating the absorbance ratio ($A_{260/280}$) with a nanoDrop spectrophotometer (Thermo Scientific NanoDrop™.US).

The extracted RNA was electrophoresed in 1.5% agarose gel to confirmation of their integrity. Afterwards, genomic DNA was eliminated by RNase-free DNase I treatment (Thermo Scientific GmbH). Before synthesis of cDNA, normalizing of RNA concentrations were performed. cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (GeneAll Biotechnology Co., Korea) with random primers, as recommended by the manufacturer's instructions. The cDNAs were used for the quantification of mRNA levels biofilm encoding genes by qRT-PCR with the Roche Light cycler® 96 Real-Time PCR Detection System and Software (Life Science, Roche Molecular Systems, Inc).

Quantitative real-time (qRT) PCR

The qRT-PCR assay was performed in a 20 µl reaction mixture containing 10 µl RealQ Plus 2x Master Mix Green, High ROX (Ampliqon, Denmark), 2 µl cDNA, 0.5 µl of (10 pmol) each forward and reverse primers (Table 1), and 6 µl of sterile distilled water, under the thermal cycling conditions: 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Negative control (contained the reagents of the qRT-PCR reaction but lacked cDNA), and DNA sample were included in each run as a positive control. The expression levels of biofilm-associated genes were calculated relative to the calibration sample an endogenous control *16S rRNA* to normalize the sample input. The changes in the expression level of target gene were analyzed by using the method adopted by Livak and Schmittgen [28].

2.11. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's tests were used to assess the differences between the relative quantities of the *ica ABCD* and *ica R* genes expressions in MRSA and MSSA strains under the treatments. All the experiments were done in triplicate, and P-values lower than 0.05 were considered statistically significant.

3. Results

3.1. Sub-lethal dose of LED and sub-MIC of TBO

According to the results of our study, 50 and 100 µg ml^{-1} of TBO significantly ($P < 0.05$) reduced the mean cell survival in the MRSA (2.5$\log_{10}$ - 3$\log_{10}$) and MSSA (2.75 $\log_{10}$ - 3.1$\log_{10}$) isolates (Fig. 2). Whereas there was no statistically significant reduction when the concentration of TBO was increased from 0.19 to 25 µg ml^{-1} ($P > 0.05$, Fig. 3). Therefore, the sub-MIC for *S. aureus* evaluated with TBO was 25 µg ml^{-1}.

3.2. Determination of sub-lethal dose of LED

According to Fig. 3, our assays revealed that the cell viability of *S. aureus* isolates was significantly reduced 40.8% after 30 s of LED exposure ($P < 0.05$). There was no significant difference in the count of *S. aureus* cells in the 10 and 20 s LED irradiation times ($P > 0.05$). Therefore, the sub-lethal dose of LED irradiation time against *S. aureus* strains was 20 s with an output energy density of 104.1 J/cm^2.

3.3. Effect of sub-lethal dose of aPDT on MRSA and MSSA strains

TBO-mediated aPDT at a sub-lethal dose using 25 µg ml^{-1} concentration with 20 s of irradiation time at an energy density of 104.1 J/cm^2 showed no significant reduction in cells count of *S. aureus* (MRSA and MSSA) isolates when compared with the control groups (untreated isolates; $P > 0.05$; Fig. 4).

3.4. Effect of sub-lethal dose of aPDT on biofilm-associated genes expression

The RNA integrity and primers specificity are shown in Fig. 5A and B, respectively. The average total RNA concentration was > 400 ng µl^{-1} + 52 SD among all samples. Also, in Fig. 5B, the presence of a single curve for each primer implied the formation of a single product (i.e., specificity of primers for target genes). The amplified products of qRT-PCR were visualized after separation on an agarose gel, showing all primer pairs resulted in amplification of a single product (Fig. 5C).

The effect of sub-lethal dose of aPDT on the expression levels of the biofilm-associated genes and *16S rRNA* gene were evaluated for all of the isolates after exposed to LED exposure. Expression of the *16S rRNA* gene was also measured as an internal control. The expression level of *ica A, B, C, D* and *ica R* genes of clinical isolates of *S. aureus* significantly reduced after exposure to TBO-mediated sub-lethal dose aPDT compared with untreated *S. aureus* isolates ($P < 0.05$, Fig. 6). Expression levels of all *ica* genes has a significantly relationship compared with gene reference ($P < 0.05$). The average expression levels of *ica A*, *ica B*, *ica C*, *ica D* and *ica R* in the MRSA strain treated by TBO-mediated sub-lethal dose aPDT was decreased by 12, 14, 11, 9 and 6 fold change, respectively ($P < 0.05$). However, the expression level of *ica ABCD* and *ica R* in the MSSA strain treated by TBO-mediated sub-lethal dose aPDT was decreased by 13, 14.5, 12, 9.5, and 8 fold change, respectively ($P < 0.05$). The *ica B* gene expression was more than other genes in the MSSA isolates treated by aPDT.

4. Discussion

S. aureus is considered as an important human pathogen due to multiple virulence factors such as surface proteins, extracellular toxins and enzymes [29]. One of these virulence factors in *S. aureus* is the biofilm formation, because the microorganisms that establish in a burn wound biofilm basically differ from planktonic population [30]. The microorganisms in terms of biofilm growth conditions are 1000 times more tolerant and / or resistant to antimicrobial agents compared to planktonic growth conditions [31,32].

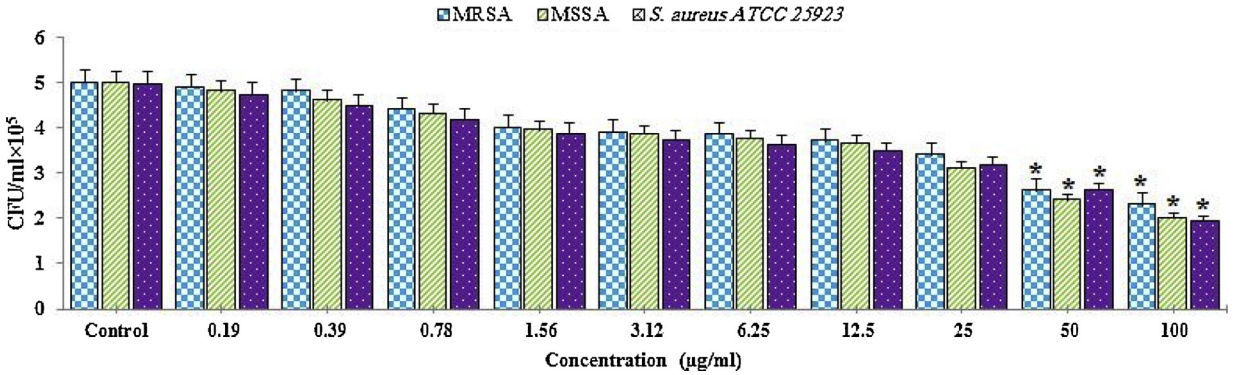

Fig. 2. Cell survival of TBO against *S. aureus* strains CFU ml^{-1}.*significantly different from the control group (no treatment), $P < 0.05$.

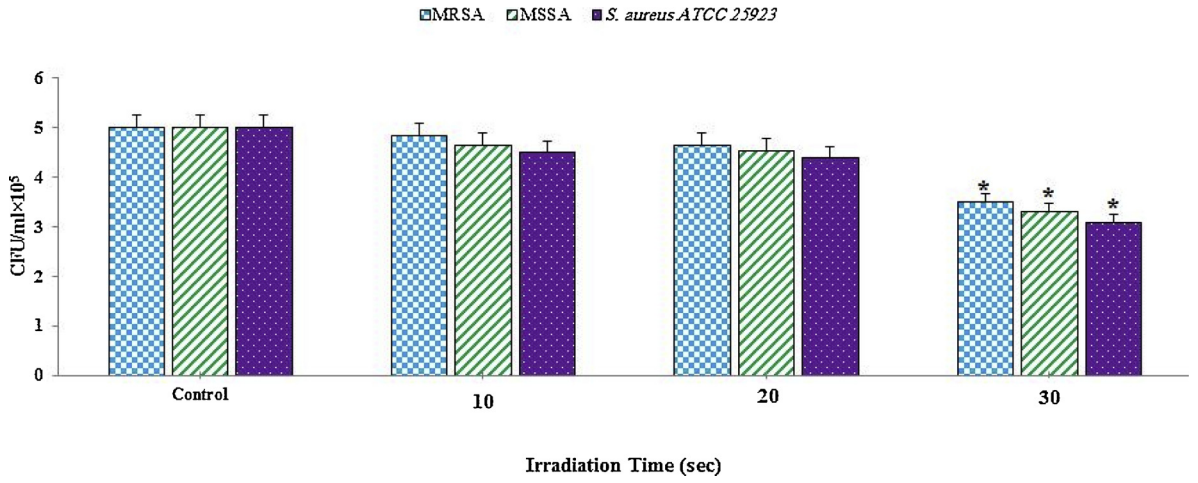

Fig. 3. LED irradiation at wavelength 630 nm against *S. aureus* strains,* significantly different from the control (no treatment) $P < 0.05$.

TBO, a phenothiazinium salt, is a moderately effective cationic PS causing damage to the Gram-negative and -positive bacteria cell membrane without the need for membrane-permeabilizing agents [18]. TBO is clearly demonstrated to be safe to the human normal tissues and cells [18,33,34]. Oxidative damage to cytoplasmic membrane, membrane proteins, and bacterial enzymes by $^1O_2$ have also reported using TBO [19,35].

The results of this study show that, qRT-PCR assay revealed a significant decrease in *ica ABC,* and *ica R* genes expression in the all *S. aureus (*MRSA, MSSA *and S. aureus* ATCC 25923*)* isolates following treated by sub-lethal dose aPDT ($P < 0.05$). Given the fact that biofilm production depends on *ica ABCD* operon and *ica* locus encoded the proteins necessary for the synthesis of polysaccharide intercellular adhesin (PIA) and capsular polysaccharide/adhesion (PS/A), and PIA is required for attachment and biofilm production [30]. A study showed that mutation in the *ica* genes of *S. aureus* defective in biofilm formation and PIA making [36]. Consequently, inactivation virulence factors through interference in the expression of genes of the *ica ABCD* operon by PDT may be able to reduce pathogenesis and biofilm formation of *S. aureus* in hosts [37]. Therefore, the aPDT effectiveness on virulence factors is extremely significant probably during the infection. The accurate mechanism of inhibition of these virulence factors has not yet been determined; nevertheless, it is possible that the reactive oxygen species (ROS) formed during photosensitization can damage to the proteins structure, thereby disrupting their function [37].

All strains of *S. aureus* were shown to be susceptible to lethal aPDT by regimens tested, a significantly high reduction in bacterial counts was observed between *S. aureus* strains treated by aPDT compared to control group (untreated bacteria ($P < 0.05$). The results of our study demonstrate that it is possible to reduce the number of MRSA and MSSA isolated from burn patients. TBO at 100 µg ml^{-1}, 156.2 J/cm^2, attained 3.1 log killing for MSSA strain; 3 log killing for MRSA, and 2 log killing for *S. aureus* (ATCC 25923). Our results shown, *S. aureus* strains isolated from wound infection is more sensitive to aPDT-mediated killing than *S. aureus* ATCC 25923. The reason for different activity against wild-type *S. aureus* strains and *S. aureus* (ATCC 25923) is unknown, but may be due to different genetic backgrounds of isolates.

These findings demonstrate that PDT is not only effectual in the inactivation of microorganisms but as well as considerably influence microbial pathogenicity through changes in gene expression of the virulence factors. To our knowledge, this is the first report on the effects of aPDT with TBO, as a photosensitizer, on the expression genes involved in biofilm formation in *S. aureus* strains isolated from wound infections in burn patients following a sub-lethal TBO concentration, as low as 25 µg ml^{-1}. The mean expressions of *ica A, B, C, D* and *ica R* in MRSA and MSSA were significantly downregulated to approximately 12, 14, 11, 9, 6 and 13, 14.5, 12, 9.5, 8 fold, respectively. Pourhajibagher at al., evaluated the expression of *rgp A* gene in *P. gingivalis* following a sub-lethal doses of aPDT by TBO as PSs. They showed that TBO suppressed the *rgp A* gene expression, which can reduce pathogenicity of *P. gingivalis* isolates in endodontic infection [38]. In the other study in assessing of expression of *rcpA* gene in *Aggregatibacter actinomycetemcomitans* in response to sub-lethal doses of aPDT by ICG as PSs, concluded that *rcpA* gene downregulated to 6-fold which can decrease the biofilm formation activity in *A. actinomycetemcomitans* [39]. Chiniforush et al., in evaluating the efficacy of aPDT on the expression of *esp* gene as a major virulence factor for biofilm formation in *Enterococcus faecalis*, showed that the sub-lethal dose of aPDT with methylene blue suppressed the expression of *esp A* approximately 4-fold [40].

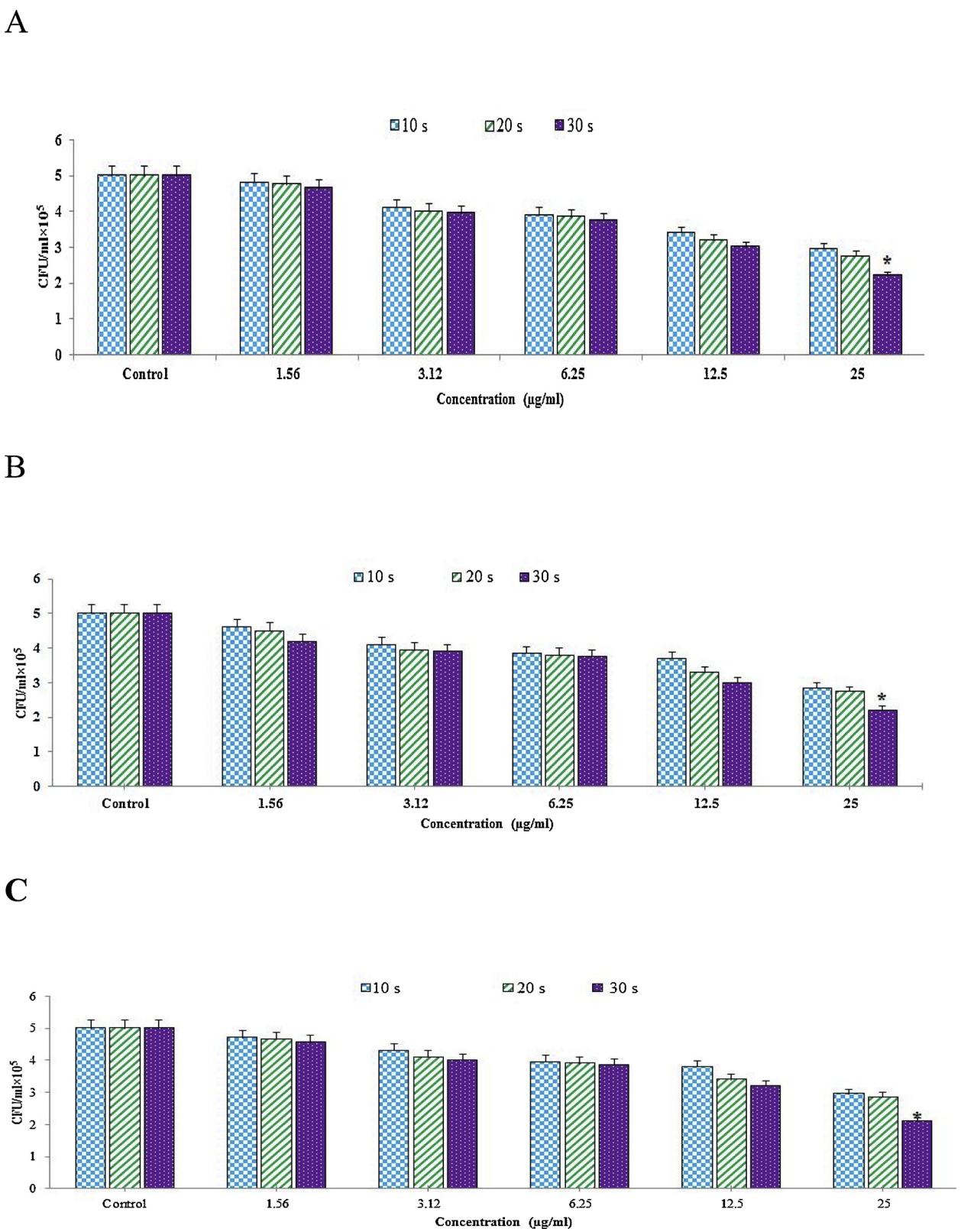

Fig. 4. Effect of sub-lethal dose of aPDT based on TBO on A: MRSA, B: MSSA, and C: *S.aureus* ATCC 25923 strains $CFUml^{-1}$, *significantly different from the control, $P < 0.05$.

Based on the results of the current study and other studies mentioned above, it can be concluded that aPDT can be used as a high-performance tool for modulating the virulence factors of gram-positive and negative bacteria. However, there are studies consistent with the present study that confirms our results. The study conducted by Tubby et al., showed to inhibit the expression of sphingomyelinase, α-hemolysin and staphylococcal V8 protease [37]. Also in another study by Tubby et al., showed that the biological activity of lipopolysaccharides from *E. coli* and proteases from *P. aeruginosa* were successfully reduced by TBO mediated aPDT [37]. Previous report have shown that TBO-

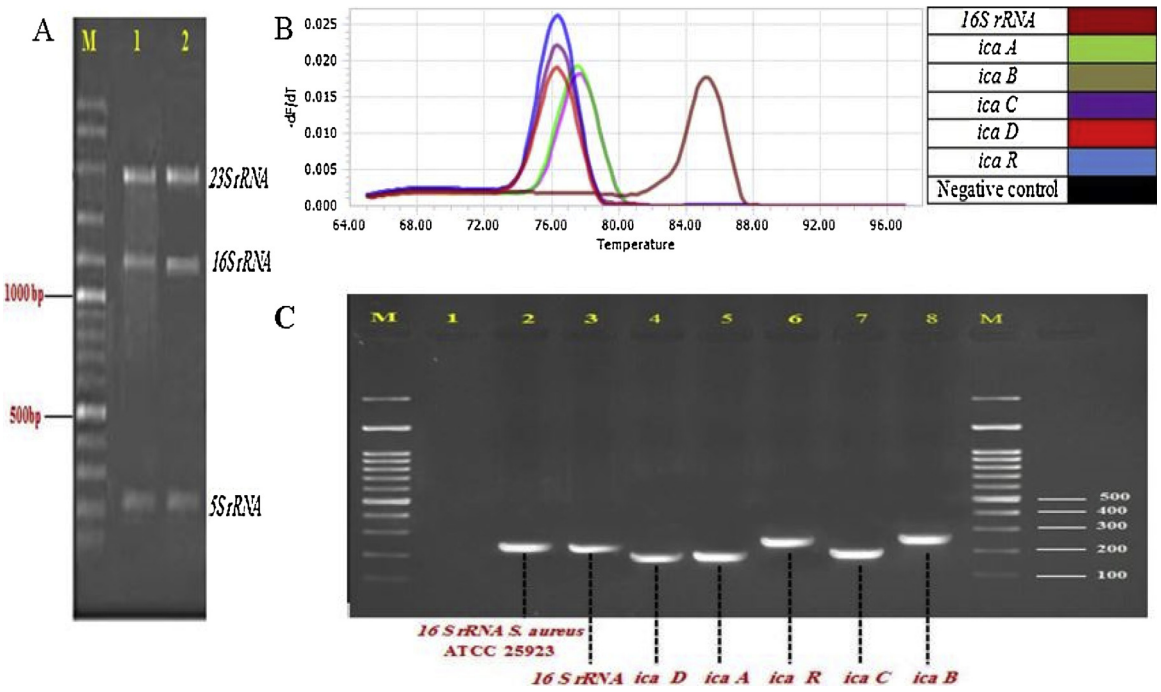

Fig. 5. Confirmation of specificity of primers and RNA integrity. (A) Agarose gel (1.5%) electrophoresis of extracted RNA: lane 1 and lane 2 extracted RNAs and lane M; ladder 100 bp. (B) The melting curve profiles generated following real-time amplification to assess potential primer–dimer artifacts and nonspecific PCR product. (C) End amplified products of qRT-PCR were visualized after separation on an agarose gel, showing all primer pairs resulted in amplification of a single product of lane 1 (negative control), lane 2 (*16 S rRNA*, *S. aureus* ATCC 25923), lane 3 (*16 S rRNA*), lane 4 (*ica D*), lane 5 (*ica A*), lane 6 (*ica R*), lane 7 (*ica C*), lane 8 (*ica B*), M: molecular weight marker.

aPDT at a sub-lethal TBO inhibit the activities of the protease, lipase, staphylococcal α-hemolysin, and enterotoxin type B produced by community-associated MRSA stains [41].

Because of aPDT can damage the cytoplasmic membrane can comprise leakage of cellular contents or inactivation of membrane transport systems and enzymes [12,42]. Considering the *ica* operon are located in the membrane section, this increases the importance of using aPDT [12,24]. Bartolomeu et al., showed that, the effect of aPDT on catalase activity, β-hemolysis, lipases, deoxyribonuclease, enterotoxins, and coagulase production were effective in *S. aureus* strains. So that, the results indicated that the expression of some external virulence factors is reduced by aPDT [17]. Several other studies in this field have showed that the oxidative damage caused by aPDT treatment affects the expression of functional proteins involved in metabolic activities, cell division, and oxidative stress responses [16,43]. Oxidative damage by $^1O_2$ to cytoplasmic membrane, membrane proteins and bacterial enzymes have also been showed using TBO [19,35].

Based on the results, aPDT affects the expression of virulence factors, also causing their degradation. One of the main advantages of aPDT is the nature of the photoinactivation process and development of resistance to aPDT is very unlikely. Other advantages of aPDT, it is a local treatment and thus does not provoke systemic effects in humans, no aPDT resistant bacteria have been reported, and low-cost light sources are available [41]. Finally, only a limited number of genes were examined. To test this idea, aPDT could be further evaluated in an animal models. Further investigation is needed to target other genes (e.g., *agr* operon, *sig B* and *sar*) responsible for biofilm formation in *S. aureus* to find out the complete mechanism involved in reduction of *S.*

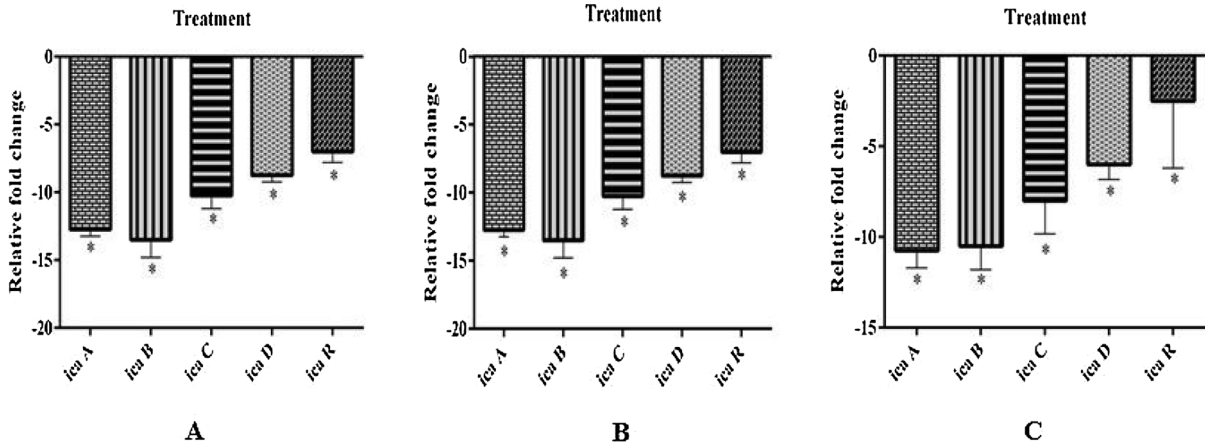

Fig. 6. mRNA expression levels of *ica A, B, C, D* and *ica R* were determined by qRT-PCR and normalized by *16S rRNA* expression following sub-lethal dose of aPDT with TBO. *(*A); MRSA strains. (B) MSSA strains. C; *S. aureus* ATCC 25923.

Abbreviations: MRSA, methicillin-resistant *S. aureus;* MSSA, Methicillin-susceptible *S. aureus;* TBO, toluidine blue O.

aureus biofilm formation.

5. Conclusion

The results presented in this investigation have shown that TBO-mediated aPDT is impressive in reducing the potency of genes associated with biofilm formation in *S. aureus* isolates. aPDT could provide a supplemental in the treatment of wound and tissue infection, and burn patients may benefit from combined treatments.

Conflict of interest

The authors declare that the research reported here was conducted in the absence of any commercial or financial relationships that could constitute potential conflicts of interest.

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References

[1] F. Saint-Aignan Cedex, Antibiofilm Peptide development for clinical and industrial applications Fany Reffuveille, J. Postdoc. Res. 25 (June) (2015) 29–35.
[2] E.A. Montazeri, A.D. Khosravi, A. Jolodar, M. Ghaderpanah, S. Azarpira, Identification of methicillin-resistant Staphylococcus aureus (MRSA) strains isolated from burn patients by multiplex PCR, Burns 41 (3) (2015) 590–594.
[3] A.D. Khosravi, H. Hoveizavi, Z. Farshadzadeh, The prevalence of genes encoding leukocidins in Staphylococcus aureus strains resistant and sensitive to methicillin isolated from burn patients in Taleghani Hospital, Ahvaz, Iran, Burns 38 (2) (2012) 247–251.
[4] E.A. Montazeri, A.D. Khosravi, A. Jolodar, M. Ghaderpanah, S. Azarpira, Identification of methicillin-resistant Staphylococcus aureus (MRSA) strains isolated from burn patients by multiplex PCR, Burns 41 (3) (2015) 590–594.
[5] H. McCarthy, J.K. Rudkin, N.S. Black, L. Gallagher, E. O'Neill, J.P. O'Gara, Methicillin resistance and the biofilm phenotype in Staphylococcus aureus, Front. Cell. Infect. Microbiol. 5 (2015) 1.
[6] B. Diemond-Hernández, F. Solórzano-Santos, B. Leaños-Miranda, L. Peregrino-Bejarano, G. Miranda-Novales, Production of icaADBC-encoded polysaccharide intercellular adhesin and therapeutic failure in pediatric patients with staphylococcal device-related infections, BMC Infect. Dis. 10 (1) (2010) 68.
[7] S. Stepanović, D. Vuković, V. Hola, G.D. BONAVENTURA, S. Djukić, I. Ćirković, et al., Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci, Apmis 115 (8) (2007) 891–899.
[8] H.-S. Joo, M. Otto, Molecular basis of in vivo biofilm formation by bacterial pathogens, Chem. Biol. 19 (12) (2012) 1503–1513.
[9] O'Gara JP. ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus, FEMS Microbiol. Lett. 270 (2) (2007) 179–188.
[10] S. Wu, T. Yang, Y. Luo, X. Li, X. Zhang, J. Tang, et al., Efficacy of the novel oxazolidinone compound FYL-67 for preventing biofilm formation by Staphylococcus aureus, J. Antimicrob. Chemother. 69 (11) (2014) 3011–3019.
[11] G. Jori, S.B. Brown, Photosensitized inactivation of microorganisms, Photochem. Photobiol. Sci. 3 (5) (2004) 403–405.
[12] A. Tavares, C. Carvalho, M.A. Faustino, M.G. Neves, J.P. Tomé, A.C. Tomé, et al., Antimicrobial photodynamic therapy: study of bacterial recovery viability and potential development of resistance after treatment, Mar. Drugs 8 (1) (2010) 91–105.
[13] P. Soria-Lozano, Y. Gilaberte, M. Paz-Cristobal, L. Pérez-Artiaga, V. Lampaya-Pérez, J. Aporta, et al., In vitro effect photodynamic therapy with differents photosensitizers on cariogenic microorganisms, BMC Microbiol. 15 (1) (2015) 187.
[14] H. Mahmoudi, A. Bahador, M. Pourhajibagher, M.Y. Alikhani, Antimicrobial photodynamic therapy: an effective alternative approach to control bacterial infections, J. Lasers Med. Sci. 9 (3) (2018) 154–160.
[15] M.R. Hamblin, T. Hasan, Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem. Photobiol. Sci. 3 (5) (2004) 436–450.
[16] E. Alves, M.A. Faustino, M.G. Neves, A. Cunha, J. Tome, A. Almeida, An insight on bacterial cellular targets of photodynamic inactivation, Future Med. Chem. 6 (2) (2014) 141–164.
[17] M. Bartolomeu, S. Rocha, Â Cunha, M. Neves, M.A. Faustino, A. Almeida, Effect of photodynamic therapy on the virulence factors of Staphylococcus aureus, Front. Microbiol. 7 (2016) 267.
[18] N. Kömerik, M. Wilson, S. Poole, The effect of photodynamic action on two virulence factors of gram-negative bacteria, Photochem. Photobiol. 72 (5) (2000) 676–680.
[19] S. Packer, M. Bhatti, T. Burns, M. Wilson, Inactivation of proteolytic enzymes from Porphyromonas gingivalis using light-activated agents, Lasers Med. Sci. 15 (1) (2000) 24–30.
[20] British Columbia Provincial Nursing Skin and Wound Committee. Procedure: Swab for Culture & Susceptibility (C & S) in Suspected Wound Infection, (2015), pp. 1–6 https://www.picnet.ca/wp…/Procedure-Wound-Culture-and-Susceptibility-June-2015.
[21] P. Tille, Bailey & Scott's Diagnostic Microbiology-E-Book, Elsevier Health Sciences, 2015.
[22] H. Mahmoudi, M.R. Arabestani, S.F. Mousavi, S. Ghafel, M.Y. Alikhani, Study of polymorphism spa gene (encoding protein A) of Staphylococcus aureus in clinical isolates and nasal carriers, Tehran Univ. Med. J.TUMS Publ. 73 (1) (2015) 24–30.
[23] H. Mahmoudi, M.R. Arabestani, S.F. Mousavi, M.Y. Alikhani, Molecular analysis of the coagulase gene in clinical and nasal carrier isolates of methicillin-resistant Staphylococcus aureus by restriction fragment length polymorphism, J. Glob. Antimicrob. Resist. 8 (2017) 41–45.
[24] S.S. Atshan, M.N. Shamsudin, A. Karunanidhi, A. van Belkum, L.T.T. Lung, Z. Sekawi, et al., Quantitative PCR analysis of genes expressed during biofilm development of methicillin resistant Staphylococcus aureus (MRSA), Infect. Genet. Evol. 18 (2013) 106–112.
[25] D. Yu, L. Zhao, T. Xue, B. Sun, Staphylococcus aureus autoinducer-2 quorum sensing decreases biofilm formation in an icaR-dependent manner, BMC Microbiol. 12 (1) (2012) 288.
[26] L. Beytollahi, M. Pourhajibagher, N. Chiniforush, R. Ghorbanzadeh, R. Raoofian, B. Pourakbari, et al., The efficacy of photodynamic and photothermal therapy on biofilm formation of Streptococcus mutans: an in vitro study, Photodiagnosis Photodyn. Ther. 17 (2017) 56–60.
[27] M. Pourhajibagher, N. Chiniforush, S. Shahabi, R. Ghorbanzadeh, A. Bahador, Sub-lethal doses of photodynamic therapy affect biofilm formation ability and metabolic activity of Enterococcus faecalis, Photodiagnosis Photodyn. Ther. 15 (2016) 159–166.
[28] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method, Methods 25 (4) (2001) 402–408.
[29] S. Arvidson, K. Tegmark, Regulation of virulence determinants in Staphylococcus aureus, Int. J. Med. Microbiol. 291 (2) (2001) 159–170.
[30] S.O. Moghadam, M.R. Pourmand, F. Aminharati, Biofilm formation and antimicrobial resistance in methicillin-resistant Staphylococcus aureus isolated from burn patients, Iran, J. Infect. Dev. Countries 8 (12) (2014) 1511–1517.
[31] M. Pourhajibagher, N. Chiniforush, A. Monzavi, H. Barikani, M.M. Monzavi, S. Sobhani, et al., Inhibitory effects of antimicrobial photodynamic therapy with curcumin on biofilm-associated gene expression profile of Aggregatibacter actinomycetemcomitans, J. Dent. (Tehran, Iran) 15 (3) (2018) 169.
[32] M. Berlanga, R. Guerrero, Living together in biofilms: the microbial cell factory and its biotechnological implications, Microb. Cell Fact. 15 (1) (2016) 165.
[33] L. Nastri, G. Donnarumma, C. Porzio, V. De Gregorio, M. Tufano, F. Caruso, et al., Effects of toluidine blue-mediated photodynamic therapy on periopathogens and periodontal biofilm: in vitro evaluation, Int. J. Immunopathol. Pharmacol. 23 (4) (2010) 1125–1132.
[34] X. Luan, Y. Qin, L. Bi, C. Hu, Z. Zhang, J. Lin, et al., Histological evaluation of the safety of toluidine blue-mediated photosensitization to periodontal tissues in mice, Lasers Med. Sci. 24 (2) (2009) 162–166.
[35] M. Bhatti, A. MacRobert, S. Meghji, B. Henderson, M. Wilson, A study of the uptake of toluidine blue O by Porphyromonas gingivalis and the mechanism of lethal photosensitization, Photochem. Photobiol. 68 (3) (1998) 370–376.
[36] N.K. Archer, M.J. Mazaitis, J.W. Costerton, J.G. Leid, M.E. Powers, M.E. Shirtliff, Staphylococcus aureus biofilms: properties, regulation, and roles in human disease, Virulence 2 (5) (2011) 445–459.
[37] S. Tubby, M. Wilson, S.P. Nair, Inactivation of staphylococcal virulence factors using a light-activated antimicrobial agent, BMC Microbiol. 9 (1) (2009) 1–10.
[38] M. Pourhajibagher, R. Ghorbanzadeh, A. Bahador, Investigation of arginine A-specific cysteine proteinase gene expression profiling in clinical Porphyromonas gingivalis isolates against photokilling action of the photo-activated disinfection, Lasers Med. Sci. 33 (2) (2018) 337–341.
[39] M. Pourhajibagher, N. Chiniforush, S. Shahabi, S. Sobhani, M.M. Monzavi, A. Monzavi, et al., Monitoring gene expression of rcpA from Aggregatibacter actinomycetemcomitans versus antimicrobial photodynamic therapy by relative quantitative real-time PCR, Photodiagnosis Photodyn. Ther. 19 (2017) 51–55.
[40] N. Chiniforush, M. Pourhajibagher, S. Parker, S. Benedicenti, S. Shahabi, A. Bahador, The effect of sub-lethal photodynamic therapy on the expression of Enterococcalsurface protein (esp) encoding gene in Enterococcus faecalis: quantitative Real-time PCR assessment, Photodiagnosis Photodyn. Ther. (2018) 30160–30161.
[41] S.-P. Tseng, W.-C. Hung, H.-J. Chen, Y.-T. Lin, H.-S. Jiang, H.-C. Chiu, et al., Effects of toluidine blue O (TBO)-photodynamic inactivation on community-associated methicillin-resistant Staphylococcus aureus isolates, J. Microbiol. Immunol. Infect. 50 (1) (2017) 46–54.
[42] F.P. Imray, D.G. MacPhee, The role of DNA polymerase I and the rec system in survival of bacteria and bacteriophages damaged by the photodynamic action of acridine orange, Mol. Gen. Genet. MGG 123 (4) (1973) 289–298.
[43] E. Alves, A.C. Esteves, A. Correia, Â Cunha, M.A. Faustino, M.G. Neves, et al., Protein profiles of Escherichia coli and Staphylococcus warneri are altered by photosensitization with cationic porphyrins, Photochem. Photobiol. Sci. 14 (6) (2015) 1169–1178.