

Inactivation of Staphylococcal Virulence Factors Using a Diode Laser light-Activated Antimicrobial Agent

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Abstract

Total of 120 samples were collected from different body sites and lesions for patients from both sexes. There were 78 isolates (65%) *Staphylococcus aureus* from them 37 isolates were Methicillin-resistant *Staphylococcus aureus* (MRSA). Each isolate was exposed to diode laser 632nm wavelength with 300μM of methylene blue (MB) at 458.6 watt/cm² for 15 minutes, both phenotypic and genotypic changes in virulence factors and the antibiotic-resistance were evaluated before and after irradiation with laser light. It was observed that the effect of sub-lethal dose on the antibiotic sensitivity was isolate-dependent. In general, results showed large variations in the susceptibility of antibiotic. Exposure to diode laser increased resistance to Vancomycin, in contrast increased sensitivity to Methicillin, Ciprofloxacin, and Norfloxacin. Also Results showed decreases the activity of β-haemolysin, with 33 (90.3%) isolates of *S. aureus* in comparison to the control as shown in blood agar method and haemolytic titration assay, but there was no effect on thermonuclease enzyme after irradiation. Detection of three genes represented in MRSA isolates by a confirmatory test was carried out using PCR technique. The results of the PCR amplification of *mecA* gene noted that it was present in 27(72.2%) *S.aureus* isolates and 14(83.3%) isolates showed reduction in β-toxin production after exposure to laser light, with no altered or deficiency in thermonuclease gene (*nuc*).

Keywords: Irradiation, antibiotic susceptibility, beta-haemolysin, Methylene blue, diode laser

الملخص

شملت الدراسة مئة وعشرين عينة تم الحصول عليها من مواقع مختلفة من الجسم لكلا الجنسين. عزلت منها ثمان وسبعين عزلة (65%) بكتيريا المكورات العقدوية الذهبية، وتم تحديد سبعة وثلاثين عزلا على أنها بكتيريا المكورات العقدوية الذهبية المقاومة للميثيسلين (MRSA). تم تعريض كل عزلا بكتيرية لضوء الليزر ذو الطول الموجي 632 نانومتر، وبتركيز 300 مايكرومول من المتصحسن الضوئي المثنين الأزرق (MB) وبجرعة 458.6 واط / سم² وبזמן تعريض خمس عشرة دقيقة، تم تقييم التغيرات على عوامل الضراوة والحساسية لمضادات الحيوية من خلال فحص الأنماط المظهرية والجينية لها قبل وبعد التعرض لضوء الليزر. لوحظ إن تأثير لجرعة مادون القتل على حساسيتها لمضادات الحيوية كانت تعتمد على العزلة. وبشكل عام تباينت بشكل واضح نتائج الحساسية لمضادات الحيوية للتتحسين الضوئي. التعرض لضوء الليزر زاد من مقاومة العزلات البكتيرية لفانкомايسين بالمقابل زيادة في حساسية العزلات البكتيرية للمكورات العقدوية الذهبية للميثيسلين، سايبروفلوكاساسين ونورفلوكاساسين ومن خلال التحليل باستخدام وسط غراء الدم الأساس (Base Blood Agar) وبطريقة قياس المعياري لهيمولايسين، لوحظ نقصان في إنتاج بيتا هيمولايسين مقارنة بالعزلات القياسية، حيث لوحظ أن 33 عينة من المكورات العقدوية الذهبية وبنسبة 90.3% كما أظهرت النتائج إن التعرض لضوء الليزر لم يمتلك أي تأثير على ثبات انتيم النيوكليز للحرارة. أجري تشخيص تأكيدى لثلاث جينات موجودة في عزلات بكتيريا المكورات العقدوية الذهبية المقاومة للميثيسلين (MRSA) باستخدام تقنية تفاعل سلسلة البمررة، حيث اعطت نتائج الكشف عن وجود جين المقاومة للميثيسلين في 27 عينة بكتيرية وبنسبة 72.2%، و 14 عينة بكتيرية وبنسبة 83.3% اظهرت نقصان في قابلية إنتاج بيتا هيمولايسين بعد التعرض لضوء الليزر دون أي تغير أو نقصان بالنسبة لثبات انتيم النيوكليز للحرارة.

الكلمات المفتاحية: التعرض للأشعاع، الحساسية لمضادات الحيوية، بيتا هيمولايسين، المثنين الأزرق، لضوء الليزر

Introduction

Photodynamic therapy is a promising new strategy for the treatment of superficial skin infections. The quickly growing resistance of Methicillin resistant *Staphylococcus aureus* (MRSA) to conventional antimicrobial agents and the limitation of antibiotic treatment for these diseases is that even after successful killing of the infecting organism, secreted virulence factors may still be present and cause significant damage to host tissues [1].

Photosensitization technique is one of the recent alternative strategies to prevent and treat infection that witness a great development since the invention of laser [2]. Also it's one of the substituent's for antimicrobials that may fail in bacterial killing. This method to kill bacteria offers several advantages over the conventional antimicrobial agents. First, development of resistance to the photochemically generated free radicals responsible for bacterial killing would be unlikely. Second, there will be no need to maintain high concentrations of the photosensitizer, and lastly, the necessary contact time of the photosensitizer would be no more than few minutes [3].

Since *S. aureus* had acquired resistance to penicillin, semi synthetic penicillin derivative and a wide variety of antimicrobials, which became a common problem in the entire world. Antibiotics resistances are attributed to many mechanisms such as decrease the permeability of outer membrane, efflux system, alteration of target site, alteration of metabolic pathways, or secretion of modifying enzymes [4].

MRSA strains became increasingly endemic, then rather more appearance of new resistance against Vancomycin further aggravate the problem [5]. A therapeutic option for the treatment of antimicrobial treatments is the photodynamic therapy. It's consist in visible light to excite the photosensitizer to generate reactive oxygen species, such as singlet oxygen and superoxide that are toxic to cells because can damage DNA and the cell membrane, resulting in the leakage of cell components, inactivation of transport systems and cell death [6], selection of an effective photosensitizer is essential for the success of the technique. As well as being non-toxic to humans, the ideal photosensitizer needs to absorb the light at the compatible wavelength and has to produce high excitation efficiency [3]. Actually, methylene blue (MB) was photosensitizer used clinically for antimicrobial treatments because the low toxicity of this dyes to human cells, plus their ability to produce high quantum yields of singlet oxygen [7].

The main objective of this study is to assess the effects of diode laser on *Staphylococcus aureus* (MRSA); their resistance pattern, its virulence factors, its genes content, furthermore viability of *S. aureus* to the local therapeutic agents with presence of methylene blue.

Materials and methods

Isolation and identification of *Staphylococcus aureus*.

Total of 120 samples were collected from different body sites and lesions of patients from both sexes. Several biochemical tests were carried out to identify *S. aureus*. The suspected isolates were identified by using Mannitol Salt Agar tests (Mast diagnostic, U.K), Catalase tests. Then, DNase and tube coagulase tests were done to increase the efficiency of detection of *S aureus*. The strains were finally confirmed by API Staph System (bioMérieux, France).

Determination of Haemolysins Production

All 37 *S. aureus* (MRSA) isolates were first cultivated in Brain Heart Infusion agar (Mast diagnostic, U.K) and incubated at 37°C overnight. The strains were passed on blood agar base supplemented with 5-7% (v/v) human erythrocytes, and incubate at 37°C for 24h to show its ability to produce beta toxin. A positive result was indicated by the formation of a clear zone of haemolysis (β -haemolysis). Also by haemolytic titration assay the haemolytic titre for each sample was determined as the reciprocal of the highest dilution giving rise to lysis [8].

Antibiotic sensitivity

The antibiotic sensitivity test was done before and after exposure process to diode laser using the standard disc diffusion method in accordance with the recommendations of Clinical and Laboratory Standards Institute [9]. The results of the susceptibility testing were classified into three categories :" (S) susceptible, (I) intermediate and (R) resistant". Sensitivity testing were done for: Cefotaxime (CTX) 30 μ g, Ciprofloxacin (CIP) 5 μ g, Norfloxacin (NOR) 10 μ g and Vancomycine (VA) 30 μ g.

Methicilline sensitivity test (5 μ g MET disc) was performed by the disk diffusion method, referred to the Clinical and Laboratory Standard Institute [9].

Laser and photosensitiser

The laser that was used in this study is diode laser (Red Beam Laser Pointer Pen 632nm, Ge-As diode lasers, china), with output power equals to 100 mW.with 3 Volt DC power supply. The beam diameter was adjusted to 5mm. Phenothiazinium dye (Methylene blue) at a concentration of 300 μ M was used for photosensitization of the *Staphylococcus aureus* strains. The MB prepared as a stock solution by

dissolving 0.00711g of (MB) powder in 100ml distilled water to obtain (20mM). pH was fixed at 7 (MB) was kept in a dark place after sterilization and filtration by using 0.22µm Millipore filter paper. Determination of diode Laser sub-lethal time at 15min and effective concentration of MB with 300µM, this results were obtained by previous experiments [10]. Irradiation was applied to all 37 clinical *S. aureus* plus 10 samples of *S. aureus* as control.

Irradiation procedure:

Bacterial strain and culture condition

In this experiment 37 *S. aureus* isolates a methicillin-resistant strain (MRSA) obtained from various clinical samples were used. The isolates were inoculated into 5 ml of brain heart infusion broth (Mast diagnostic, U.K) and incubated at 37°C for 24 hours in a shaking incubator at 200 rpm. Cultures were centrifuged and resuspended in an equal volume of PBS (0.5M, pH=7.4) and the optical density was adjusted to 0.05 at 600nm, corresponding to approximately 1×10^7 colony forming units (CFU) per ml. The bacterial suspension of each isolate was divided into 2 parts. First part of *S. aureus* suspension was remaining without exposure to laser light, 100 µl of the second part of *S. aureus* suspension put in Eppendorf tube and exposed to laser light with MB [9]. Then irradiated and non-irradiated suspension was spread on brain heart infusion agar and incubated at 37° C for overnight, then tested for their sensitivity to antibiotics and β-haemolysin production.

Genotypic Characterization

DNA was extracted from 74 (irradiated and non-irradiated) *S. aureus* isolates plus ten clinical *S. aureus* isolates obtained from Rasheed [11], used as positive controls. Using Genomic DNA Mini kit supplemented by the manufacturing company (Geneaid "presto", Korea). The obtained Chromosomal DNAs were used as templates for all PCR experiments. The concentration of DNA was assessed Spectrophotometrically. The genetic determinants for the virulence factors *mecA*, *hlb* and *nuc* were investigated using monoplex PCR.

PCR amplification of *mecA*, *hlb* and *nuc* genes encoding pathogenic factors

PCR was performed using: Verti 96 wells Thermal Cycler ABI: applied Biosystem Company, Singapore, with Green Mastermix (Promega), using primers and cycling parameters from publications listed in Table (1).

Table (1): The name, sequence, product size and reference of primers used in this study

Target	gens	Primer	Nucleotide sequences (5' —————→ 3')	Product Size bp	Reference
HLB	<i>hlb</i>	HLB-F	GTCGCACTTACTGACAATAGTGCG	309	[12]
		HLB-R	GTTGATGAGTAGCTACCTTCAGT		
TNase	<i>nuc</i>	NUC-F	CGATTGATGGTGATACGGTT	280	[13]
		NUC-R	ACGCAAGCCTTGACGAACAAAGC		
Methicillin resistance	<i>mec A</i>	<i>mecA</i> -F	GTGGAATTGGCCAATACAGG	1339	[14]
		<i>mecA</i> -R	TGAGTTCTGCAGTACCGGAT		

The primers were supplied by the Alfa DNA Company (Montreal, Quebec, Canada) Alfa DNA company protocol was adopted for primer resuspension, by bringing the final concentration of primers to 10 pmol/µl with TE buffer, and stored at -20°C until being used.

A reaction mixture containing 5µl (approximately 25ng) of template DNA, 2µl (20pM concentration) of each PCR primer, 5.5µl of Nuclease free water and 12.5µl of Green Mastermix (Promega USA) in a total of 25µl. The amplification conditions were: initial denaturing step of 5min at 94°C, followed by 30 cycles; each consist of 1min at 94°C annealing 1min at 60°C for *mecA*, 62°C for *hlb* and 55°C for *nuc*. And extension at 72°C for 1min and final extension at 72°C for 10min. The amplified products were analyzed by electrophoresis with 2% agarose gel followed by ethidium bromide staining and UV-transilluminator visualization.

Results & Discussion

Bacterial Identification

Out of 120 isolates, seventy-eight (65%) were identified as *S. aureus* and forty-two related to other *Staphylococcus* spp. 37 isolates of them were methicillin-resistant (MRSA).

Antibiotics Sensitivity

The results of effect of the exposure to laser light on antibiotics sensitivity of 37 isolates of *S. aureus* (MRSA) were including in table (2) which showed that the isolates resistant to methicillin before laser

irradiation exposure, become sensitive to it with percentage of 21.6%, in contrast the isolates that sensitive to Vancomycin become resistant to it with percentage of 32.43%.

On the other hand, the isolates that were resistant to Cefotaxime before laser irradiation become within the sensitivity range after laser irradiation with percentage of 51.35%, and also there was isolates within the sensitivity range of Ciprofloxacin before laser, become more sensitive after irradiation, and the isolates of the *S. aureus* that resistance to Norfloxacin before laser irradiation, become sensitive to it with percentage of 16.2%.

Table (2): Effect of exposure of *S. aureus* to Diode laser on sensitivity of antibiotics in percentage value (%)

Antibiotic Disk Changing in Results	Methicilline (MET)	Ciprofloxacin (CIP)	Vancomycin (VA)	Norfloxacin (NOR)	Cefotaxime (CTX)
No change	78.378%	51.351%	62.162%	75.675%	40.54%
R → S	21.621%	21.621%	2.7%	16.216%	2.7%
S → R			32.432%		
R → I					51.351%
I → S		27.027%	2.7%	8.108%	
S → I					2.7%
I → R					2.7%
Total	100%	100%	100%	100%	100%

S = Sensitive, I = Intermediate, R = Resistant

Results showed a slight changes in the susceptibility of most isolates to antibiotics as demonstrated in Figure(1). The obtained data let to demonstrate that irradiation by diode laser light have limited impact on bacterial sensitivity to antibiotics.

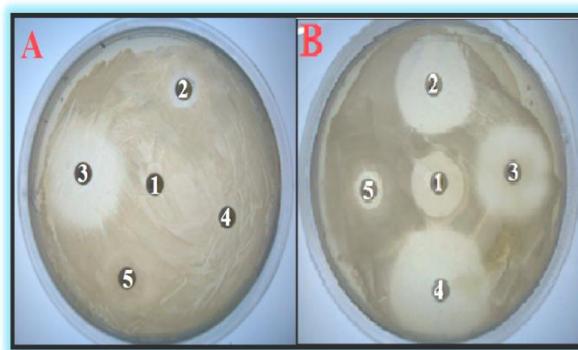


Fig. (1): Sensitivity of *S.aureus* isolate (No23) to antibiotics (A) before irradiation, (B) after laser irradiation, (1) = Methicilline, (2) = Ciprofloxacin, (3) = Vancomycin, (4) = Norfloxacin and (5) = Cefotaxime.

Changes in sensitivity of bacterial isolates to antibiotics after treatment with diode laser may be due to the combination effect of laser and antimicrobial agent making the bacterial cell more sensitive to these agents. In addition, increasing of bacterial sensitivity to antibiotics after irradiation may be as a result of many mechanisms, which may be induced, such as cell wall, protein synthesis, membrane function, nucleic acid, and metabolic processes inhibitions [15].

Efflux mechanisms have been recognized as important components of microbial resistance of MRSA to various classes of antibiotics. NorA efflux pump as one of the multidrug resistance pumps (MDRs) has the ability to expel a variety of structurally diverse compounds [16].

The nucleolus organizer region (NOR) or nucleolar organizer is a chromosomal region around which the nucleolus forms. This region is the particular part of a chromosome that is associated with a nucleolus after the nucleus division. The region contains several tandem copies of ribosomal DNA genes.

Also these changes in sensitivity of bacteria may be due to the changing in bacterial pumping systems (efflux pump) that mainly responsible on bacterial resistance or sensitive to antibiotics [17]. Failure of the bacteria to use the alternative mechanism of bacterial resistance such as; alteration in the protein

target, over production of target and production of specific enzymes which chemically modify specific antibiotic could be as suggested by Kujawa et al., [18].

β-Haemolysin (Sphingomyelinase) of *S. aureus*.

Irradiation was applied using the same conditions, resulted in decrease the activity of β-haemolysin, with 33 (90.3%) samples of *S. aureus* that revealed to reduction in toxin production in comparison to control as shown in blood agar method assay in Figure (2).

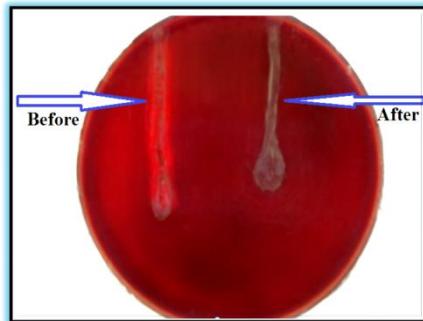


Fig. (2): Photograph of Blood Agar with 5% human erythrocytes and Streaks of *S. aureus* growth, producing β-haemolysin (before irradiation); and decrease in activity of β-haemolysin (after irradiation).

The gene, named *hlb*, is chromosomally located and encodes a 330 amino acid polypeptide with a predicted molecular weight of 35kDa [19].

Accessory gene regulator alleles (*agr* I-IV) and β-haemolysin (*hlb*) that to be associated with mobile genetic elements, the *hlb* gene is a known insertion site for toxin-carrying bacteriophage, which inserts itself into the *hlb* gene, rendering it inactive upon lysogeny. Lysogeny refers to the fusion of the bacteriophage nucleic acid with that of the host bacterium, potentially integrating genetic material for transmission to daughter cells during subsequent cell division [20]. Products of genes under the control of the *agr* system which disrupt eukaryotic cell membranes. The staphylococcal accessory regulator known as *SarA* binds to conserved promoter regions known as ‘*Sar* boxes’ for transcriptional regulation [21].

After irradiation, several *S.aureus* *hlb* mutant was constructed. This is achieved by using allelic replacement cassettes in mutagenesis process [22].

Mutations in *sarA* or *agr* were resulted in reduced hemolytic activity. MB (photosensitizers) and light leads to mutation with altered hemolytic activity, the genes of regulator β-toxin expression was may be affected due to the insertion in this gene [23]. β-haemolysin is the most susceptible of the virulence factor under test, perhaps due to the nature of its amino acid composition, which may leave it more vulnerable to attack by reactive oxygen species [22].

Thermonuclease of *S. aureus*

All irradiated *S. aureus* have the ability to produce DNase that cleaved DNA into subunits composed of nucleotides (Oligonucleotides).This result was revealed that the exposure to diode laser had no effect on TNase and no significant difference was observed in *S. aureus* isolates treated by light irradiation as illustrated in Figure (3).

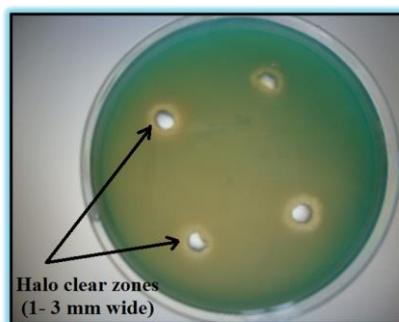


Fig. (3): Photograph of Thermonuclease (TNase) activity on DNase agar and formation of halo clear zones (1to 3 mm wide) was surrounding each test well (The arrow).

Results of Polymerase Chain Reaction (PCR) Techniques

Detection of *mec A*, *hlb* and *nuc* genes

Monoplex polymerase Chain Reaction (PCR) was done for the detection of three genes in MRSA isolates. The results of the PCR amplification of *mecA* gene noted that it was present in all the 37 *S. aureus* isolates samples (100%) before irradiation whereas 27 (72.2%) *S. aureus* isolates after exposure to laser with a PCR product size of 1339 bp Figure (4).

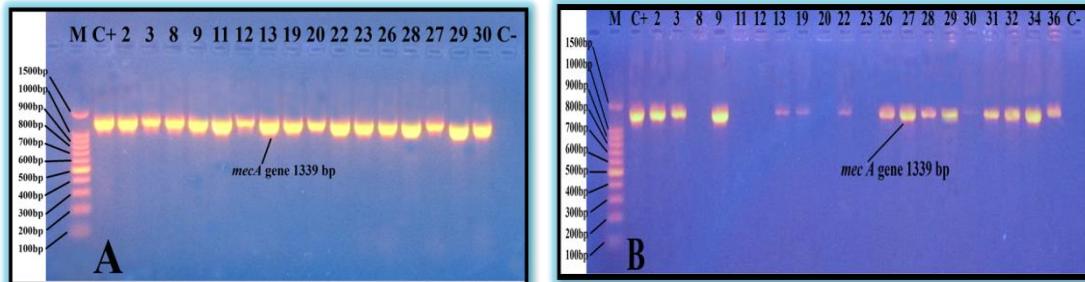


Fig. (4): Agarose gel electrophoresis (2%agarose, 5 V/cm²) PCR results with primer for *mecA* gene. M: Molecular size marker; lane C+, positive control; lane 2-30 1339 bp band obtained with DNA from MRSA ; lane C-, Negative control,(A) represented PCR results before irradiation,(B) after irradiation.

The results before irradiation showed that 17 samples of *S. aureus* isolates with percentage 45.94% of total thirty seven were capable to detect of *hlb* gene encoded for β-haemolysin, whereas after irradiation only three of these samples were capable to detect *hlb* gene Figure (5). On the other hand 83.3% (14 samples) showed reduction in toxin production.

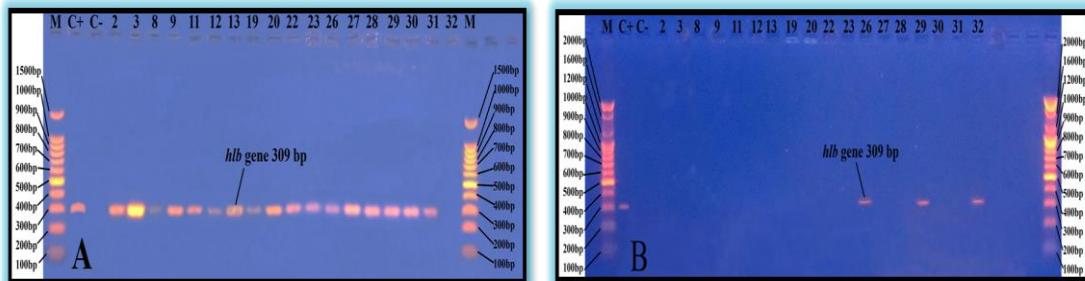


Fig. (5): Agarose gel electrophoresis (2%agarose,5 V/cm²) PCR amplicon of gene encoding for *hlb* gene.Lane M:100-bp-molecular-size DNA ladder; lane 2-32 : 309 bp amplicon; lane C+ Positive control: lane C- negative control, (A) represented PCR results before irradiation,(B) after irradiation.

Moreover, Figure (6), showed presence of 280 bp band which was related to the amplification of a specific fragment of *nuc* gene responsible for thermonuclease gene with no change between before and after irradiation.

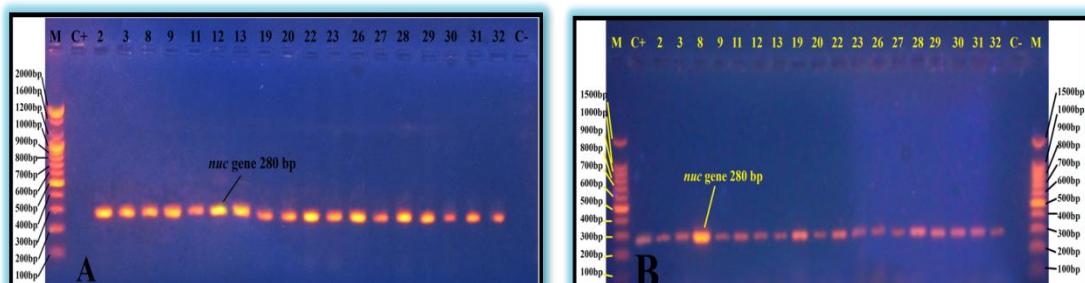


Fig. (6): The PCR products were run in a 2% agarose gel to determine the quantity of the amplicon, TBE buffer (1X), 5V/CM², 90 min.). (M): The DNA molecular weight marker (100 bp ladder). ; Positive amplification of 280 bp for *nuc* gene, lane (C+) Positive control. Lane (C-). (A) Represented PCR results before irradiation, (B) after irradiation

The exposure of *mecA* gene in *S. aureus* by MB-PDI is significantly less in the case of the strain over expressing the drug efflux pump comparing with *hlb* gene in wild-type *S. aureus* strain. In the case of

the NorA mutant, there was still some effectiveness observed when was irradiated. This observation correlates with the fact that additional efflux systems are present in *S. aureus* that have not yet been extensively studied or characterized, including NorB, NorC, and AbcA [24].

S. aureus also secrete β-toxin, sphingomyelinase encoded by the *hlb* gene [19]. The site of mutation is uncertain, but termination, frame shift, or deletion of the *hlb* structural gene, MB (photosensitizers) and light leads to mutation with altered in nucleotides sequences consequently to make difficult the progress of binding specific primer. The *nuc* gene which is specifically used to detect *S. aureus* was present in all isolates with no altered or deficiency after irradiation.

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