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EFFECT OF PHYSICAL MUTAGENESIS ON THE ABILITY OF *STAPHYLOCOCCUS AUREUS* IN STAPHYLOKINASE PRODUCTION

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ABSTRACT

This study included collecting isolate *S. aureus*, a total of (200) clinical samples were collected from two clinical sources were (100) samples of urine and (100) samples from skin infections. Staphylokinase production by *S.aureus* carried out , ability of (54) isolates *S.aureus* were tested to staphylokinase production in selective medium plasma agar and the results showed all isolates were produced staphylokinase in different levels and appered zone of lysis on plasma agar and chose efficient producing isolate was (*S.aureus* A15) for further experiments. Enhanced staphylokinase production by UV mutated (*S.aureus* A15) achieved after exposure fresh culture of *S.aureus* A15 for UV irradiation (254nm) for 30 second under sterile conditions and the results appeared two mutated isolates were more production comparison with wild type. Genomic DNA of the all isolates *S.aureus* before and after mutagenesis were extracted by boiling method, and amplification of *Sak* gene by PCR carried out by specific primers and size of staphylokinase gene was about 400bp.

KEYWORDS: *S. aureus*, staphylokinase, *S.aureus* A15.

INTRODUCTION

Staphylococcus aureus is the most important human pathogen causing skin and tissue infection, deep abscess formation and both in the hospital setting and as community infections, gives rise to a diverse spectrum of diseases ranging from minor cutaneous and wound infections to life-threatening conditions such as sepsis, endocarditis, septic arthritis, and osteomyelitis (Lijnen *et al*., 1991; Shagufta *et al*., 2014).

In every year most of the death were leaded by cardio and cerebrovascular disorders around the world. In Europe and united states, most of the deaths and disabilities were leaded by thrombotic diseases, where a thrombus develops in circulatory system can cause vascular blockage (Moussa, 2012). New generation thrombolytics plays an important role in the treatment of thromboembolic disorders which leads to the death. Staphylokinase is an ideal fibrin specific plasminogen activator, converts a precursor, plasminogen to active enzyme, plasmin to dissolve the blood clot during thrombolytic therapy and it also can to destroy the normal components of haemostatic system which leads to life threatening consequence and also death (Kotra *et al*., 2013). Staphylokinase (SAK) is 136 amino acid extra cellular protein produced during the late exponential growth phase by lysogenic strains of *S. aureus*. SAK is a profibrinolytic agent that forms a 1:1 stoichiometric complex with plasminogen, which after conversion to plasmin, activates other plasminogen molecules to plasmin (Mouss, 2012). Staphylokinase has very important activity as a thrombolytic agent, So in modern studies, overproduction of microbial enzyme by induced mutagenesis were carried out. So it is used as thrombolytic agent due to its activity and cheap cost as compared to another thrombolytic agents. Overproduction of staphylokinase achieved after exposure *S. aureus* to the mutagenesis by UV irradiation (Mohanasrinivasan *et al*., 2014) .The aim of this study was to determined the effect of Physical Mutagenesis on the ability of *Staphylococcus aureus* in staphylokinase production.

MATERIALS AND METHODS

Isolation of *S. aureus* from clinical samples

In order to isolate *S. aureus*, a total of 200 clinical samples were collected from hospitals in Baghdad governorate during the period between November and December 2013 , by taking swabs from urine and skin infections from patients attends Al-Imam Ali hospital , Al-Sader hospital and Baghdad teaching hospital . All samples were then cultured in nutrient broth medium and incubated at 37 ^{0}C for 34 hours . After incubation, serial dilutions for each sample were carried out, then 100 µl from the appropriate dilution was spread on blood agar and mannitol agar medium and incubated at 37 ^{0}C for 34 hours (Lemaire,2008) . Biochemical tests for identification of *S. aureus* were carried out according to (Harlly and Prescott, 2002).

Assay of staphylokinase production

Heated plasma agar plate assay method described by Pulicherla *et al*. (2011) was used to detect staphylokinase production and thrombolytic activity of each bacterial isolate. This was

performed by inoculating 5ml of nutrient broth medium in test tubes with 50µl of fresh culture of each bacterial isolate and incubated at 37^{o}C until the optical density of growth medium was 0.3 (OD_{600} = 0.3) , then test tubes were centrifuged at 5000 rpm for 10 minutes. Aliquot of 25 µl of culture filtrate (supernatant) was taken and loaded into wells done in plasma agar plates and incubated at at 37 ^{o}C for 24 hours. Formation of zone of hydrolysis around wells indicates a positive result.

mutagenesis by UV irradiation

Mutagenesis by UV irradiation was carried out according to David *et al*. (2005). This was achieved first by propagating *S.aureus* in 100 ml of lauria broth at 37 ^{o}C to cell optical density ($O.D_{600}$) of 0.4. Cells were then harvested by centrifugation at 5000 rpm for 10 minutes, and re-suspended in sterile phosphate buffer (pH 7.0) . Portion of cell suspension (8ml aliquout) was transferred to sterile petridish and radiated with UV-light (254 nm) for six different periods (5, 10, 15, 20, 25, 30) second under sterile conditions, then 0.1 ml of irradiated cell suspension was taken after each period, diluted serially and 100 µl of appropriate dilution was plated on nutrient agar medium. Plates were then incubated over night at 37ºC to determine the viable count and survivals of *S. aureus*. Bacterial cells subjected to the dose at which 90% of the cells were killed (LD90) were considered mutants and were screened for their ability in staphylokinase production as in item.

Genomic DNA extraction

Genomic DNA of *S.aureus* was extracted according to boiling method described by klingenberg *et al*.(2004). Aliquot of 1 ml of bacterial fresh culture was spined at 8000 rpm for 5 minutes. Pelleted cells were re-suspended in 10 ml of TE buffer and heated to boiling at 100 ºC for 10 minutes, then left to cool and centrifuged at 8000 rpm for 5 minutes. Supernatant containing genomic DNA was used for amplification staphylokinase gene by polymerase chain reaction (PCR).

Amplification of staphylokinase (*Sak*) gene

In this study, conventional and gradient PCR were used to amplify *Sak* gene in mutant and non-mutant isolates of *S.aureus* in staphylokinase production using specific primer for amplification: These primers were supplied by (Alpha DNA Company) in lyophilized form, and were dissolved in free nuclease distilled water to give final concentration of 100 picomole /µl according to the recommendations of manufacturer company, then 10 picomole

/µl of each primer was prepared by adding 10µl of primer stock solution to 90µl of free nuclease distilled water , mixed by vortexing and stored at -20˚C until use . The primers were:

Forward primer: 5'- CGCGGATCCTCAAGTTCATTCGAC-3'

Reverse primer: 5'- GAATCTAGACCCAAGCTTTTTCCTTTCTATAACAAC-3'

The conditions of polymerase chain reactions indicated in (table 1) were optimized at different annealing temperatures between 50 an 60 ˚C by using gradient thermo cycler.

Table (1): Conditions for amplification of staphylokinase gene

Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
95 ˚C for 5 min.	35	94 ˚C for 1min.	51 ˚C for 1mins.	72 ˚C for 1 min.	72 ˚C for 10 min.
			52 ˚C for 1min.	72 ˚C for 1min.	72 ˚C for 10 min.
			53 ˚C for 1 min.	72 ˚C for 1 min	72 ˚C for 10 min.

PCR products were analyzed on agarose gel (1%) using horizontal electrophoresis unit, gel was immersed in 0.5X TBE buffer, then samples were loaded into the wells of the gel . Electrophoresis was carried out for one - two hours at 50V. After electrophoresis gel was stained with 10µl of ethedium bromide stock solution . DNA bands were visualized by using U.V transilluminator at 365 nm. in presence of 1500bp DNA ladders marker . (Maniatis *et al*., 1982).

RESULTS AND DISCUSSION

Isolation and Identification of *S.aureus*

In this study , and in order to isolate *S. aureus*, a total of 200 clinical samples were collected from three hospitals (Baghdad teaching hospital, Al-Imam Ali hospital and Al-Sadder hospital) includes urine samples (100 samples) and another 100 samples collected from skin infections. All sample were then cultured in nutrient broth medium and incubated at 37 ^{0}C for 24 hours. After incubation , serial dilution for each sample were carried out , then 100 µl from the appropriate dilution was spread on blood agar and mannitol agar medium and incubated at 37 ^{0}C for 24 hours to select *S.aureus* according to Lemaire (2008).

Results showed that bacterial isolates grown on blood agar were large, round, creamy white colonies, smooth translucent area surrounding the colonies as a result of β-haemolysin production (Morello *et al*., 2006). While on mannitol salt agar medium, which was also

considered a selective and differential medium for *S. aureus* , because it contains 7.5 % salt and phenol red as an indicator. On this medium, *S. aureus* appeared golden yellow surrounded with large yellow zone, round, smooth, raised, mucoid and glistening and color of medium was changed from pink to yellow due to ferment the mannitol and acid production (Atlas *et al*., 1995).

Identification of isolate were confirmed by VITECK-2 tests. The identified isolates of *S.aureus* from skin infections and urinary tract infections were distributed as in table (2). Results showed the there are 35 isolates obtained from samples collected from skin infections, while only 19 isolates were obtained from samples collected from urinary tract infections. A study by Al-Marjani *et al*.(2015) identify 60 isolates of *S.aureus* from wound infections.

Table (2): Distribution of *S.aureus* isolates collected from clinical sources.

Clinical Sample	Total samples	Number of isolates	Percentage (%)
Skin infections	100	35	35
Urinary tract infections	100	19	19
Total	200	54	27

In other study carried by Nandita and Stanley (2014), they collected 101 samples from urine infections and they identify 23 isolates of *S.aureus* from total samples in a percentage of 22.7 % . *S. aureus* is known to cause urinary tract infections (UTI), and have virulence factors that gives the resistance to opsonisation and immune response of host and in enhancing the resistance against antibiotic therapy. Moreover being deficient in adequate information about antimicrobial susceptibilities, an inappropriate use of antimicrobial agents is causing spread of resistance among microorganism causing urinary tract infections (Shah and Kumar, 2014). In locally study by Al -Marjani and Hadi(2013), 86 Staphylococcus isolates from urine, blood and swaps from different hospitals in Baghdad, 64.1% of were identified as methicillin resistant *Staphylococcus spp.*

Staphylokinase production

Staphylokinase is one of the important virulence factors produced by *S.aureus* to resist human defense by interact with α-Defensins, a peptide secreted by host polymorphonuclear cells provides antimicrobial protection mediated by disruption of the integrity of bacterial cell wall. The biological consequence of this interaction was an almost complete inhibition of the bacteriocidal effect of α-Defensins (Tao *et al*., 2004). Further more staphylokinase interacts

with plasminogen and converted into proteolytic enzyme plasmin, which staphylokinase digested fibrin clots (Chen *et al*., 2013; Yerasi *et al*., 2014). In this study, ability of staphylokinase production by local isolates of *S.aureus* was examined by using well diffusion method. Results showed that all of the 54 isolates were able to produce staphylokinase on plasma agar medium according to formation of zone of hydrolysis around each well containing culture filtrate of each bacterial isolate. Results also showed that these local isolates differ in their abilities in staphylokinase production due to differences in size of zone of hydrolysis around each well. Results indicated in table (3) showed that the zones of hydrolysis were ranged between 25 mm and 36 mm. Hence the most efficient isolate in staphylokinase production was *S.aureus* A15 isolated from skin infections because the size of zone of hydrolysis around the well containing its culture filtrate was 36 mm.

Table (3): Staphylokinase production by *S.aureus* isolates expressed by size of zone of hydrolysis around wells on plasma agar medium after incubation at 37 ^{0}C for 24 hours

Symbol	Source of isolation	Diameter of zone (mm)
A 15	Skin infection	36
A 31	Skin infection	36
A 43	Urinary tract infection	36
A 34	Skin infection	35
A49	Urinary tract infection	35
A 27	Skin infection	34
A 35	Skin infection	34
A 53	Urinary tract infection	34
A 4	Skin infection	33
A 7	Skin infection	33
A 17	Skin infection	33
A 26	Skin infection	33
A 40	Urinary tract infection	33

These results are in consonance with other studied achieved by Pulicherla *et al*. (2011) and Yerasi *et al*. (2014), Shagufta *et al*. (2014), who detect staphylokinase production by *S.aureus* isolated also from skin infections and obtaining clear zones of hydrolysis on plasma agar medium after an overnight incubation at 37 ^{0}C .

The heated plasma agar plate assay was considered a specific and accurate method for determining the Staphylokinase (SAK) activity. The observation revealed that staphylokinase producing *S.aureus* grown overnight at 37 ^{0}C on heated plasma agar medium causing the formation of clear fibrinolytic zones as a result of sak gene expression and Staphylokinase production (Pulicherla *et al* ., 2011; Subathra *et al* ., 2012).

Mutagenesis of *S.aureus* 15

This study was first aimed to enhance the ability of *S.aureus* A15 in staphylokinase production by mutagenesis with UV irradiation to induce genetic mutation alters staphylokinase production and obtaining higher productivity mutants of *S.aureus* A15. Mutagenesis was carried out by subjecting fresh culture of *S.aureus* A15 to UV ray for 5,10,15,20,25 and 30 seconds. Results shown indicates that LD90 was reached after 30 seconds of irradiation under UV ray. Survival of irradiated bacterial cells obtained after 30 seconds of irradiation were selected and screened to detect their ability in staphylokinase production on plasma agar medium. As indicated in table (4), it was found that only tow mutants out of forty survived mutants exhibits an increase in staphylokinase production compared with the productivity of wild-type. Among them *S.aureus* A15-M1 was the most efficient mutant in staphylokinase production because the diameter of hydrolysis zone on plasma agar medium was 38 mm in comparison with other higher producer mutants and wild-type as shown in table (4). On other hand, 38 mutants showed lower staphylokinase production due to decrease in the diameter of zones of hydrolysis.

Table (3) Ability of *S.aureus* 15 mutants in staphylokinase production after subjection to LD90 of UV irradiation.

Mutant No.	Diameter of hydrolysis zone (mm)
S.aureus A15-M1	38
S.aureus A15-M2	30
S.aureus A15-M3	33
S.aureus A15-M4	37
S.aureus A15-M5	29
S.aureus A15 Wild-type	36

These results are in consonance with those obtained by Mohanasrinivasan *et al*. (2014) who mentioned that the production of Staphylokinase from *S. aureus* was increased after subjection to UV-ray using different exposure times and radiation intensities and increase thrombolytic activity hence the mutated strain produced staphylokinase which can be considered as a good thrombolytic agent. Several studies were carried out to achieve overproduction of different important enzymes by mutagenesis with UV-ray . Penicillin G acylase which is very important enzyme in β-lactam antibiotics synthesis was produced with over expression after exposure of *E.coli* to UV irradiation (Rubina *et al* ., 2010). In other study carried out by Surendar *et al*. (2013), it was found that there is a significant effect of UV irradiation on amylase production from *Bacillus subtilis* to achieve overproduction of this

enzyme more than productivity of wild type. Study on biosynthesis of alkaline protease by mutagenized culture of *Bacillus pumilus* was carried out by Muhammad *et al* . (2010) and achieved overproduction of this enzyme after exposure to UV irradiation. Genomic DNA of the (54) isolates *S.aureus* and two mutatnts (*S.aureus* A15-M1*S.aureus* A15-M2) were extracted by boiling method and considerd as a templet DNA for amplification of staphylokinase gene. The purity of genomic DNA defined as the ratio of the absorbance at 260nm and 280nm (260 /280 ratio) was 1.7 which refers to pure DNA. It was clear that purity of DNA ranged between 1.8 to 2.0 due to the 260 /280 ratio that will vary somewhat with the relative amounts of G/C and A /T in the DNA sample (Nazina, 2001). On the other hand results also showed that the concentration of DNA was 50 µg /µl. From this DNA concentration, aliqoutes of DNA were taken and used for amplification of *Sak* gene. Amplification of Sak gene by PCR technique was carried out for all *S.aureus* isolates and two mutatnts (*S.aureus* A15-M1*S.aureus* A15-M2). Forward primer contain site restriction for *BamHI* , while reverse primer contain site restriction of *XbaI* . Reaction products of gradient PCR were analyzed on (1%) agarose gel to identify the amplified Sak gene in presence of 1500 bp DNA ladder marker . Results of amplification shown in figure (1) represents that there is a DNA fragment of about 400 bp obtained after electrophoresis on agarose gel.

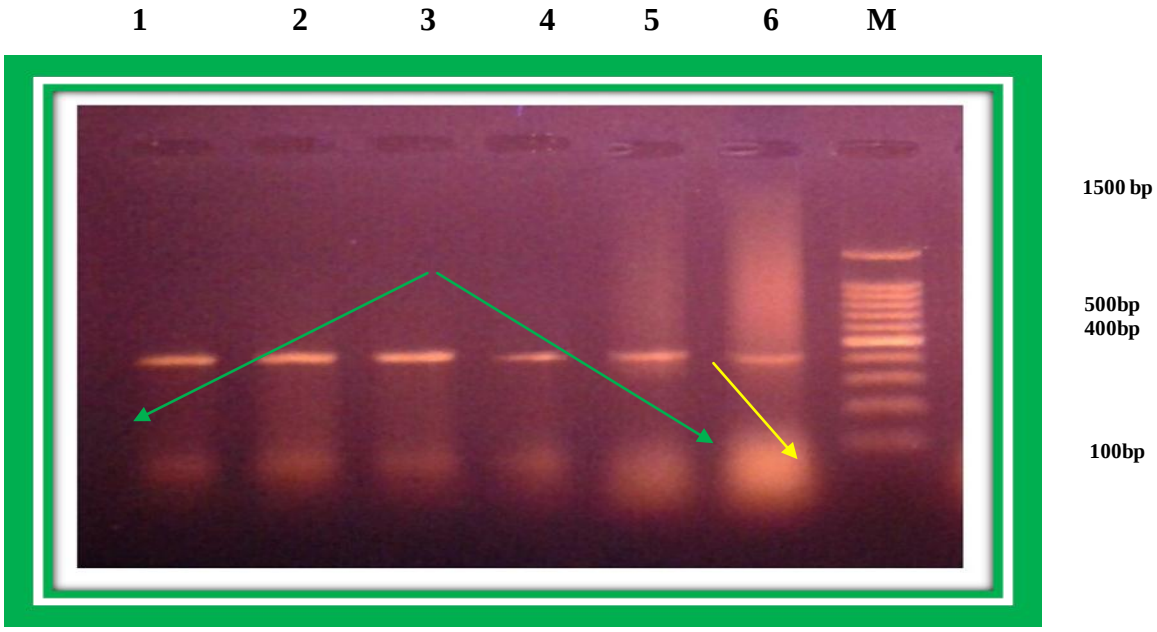

Figure (1) : Gel electrophoresis for amplified staphylokinase gene on agarose gel (1%) , 50V for 1 hour.

Lane (1 – 6) : *S.aureus* isolates (A15,A15-M1,A31,A34,A43,A49).

(M) : DNA ladder (1500 bp) .

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