Influence of Photodynamic Antimicrobial Chemotherapy on Multi-resistance *Staphylococcus aureus* by using Low Power Laser Light Diode with Methylene Blue.

Fattma A. Ali (PhD)[1],**Susan Ibrahim (**M.Sc.)[2]and
Sevan Hassan Bakir(M.Sc)[3]

Abstract

Background: Methicillin resistant *Staphylococcus aureus* (MRSA) causes serious infection of hospital-acquired patient. In recent years photodynamic inactivation (PDI) has been proposed as a therapy for a large variety of localized infection caused by MRSA.

Objective: To assess the effect of photosensitizer low power laser light diode with methylene blue in the reduction the viability of *Staphylococcus aureus*(MRSA).

Patients and Methods: Thirty five isolates of Staphylococcus aureus bacterial were isolated and identified using standard methods(Beta hemolytic in blood agar and mannitol fermentation) and Vitek2 system from(16 urine,8 wound, 7 pus, 4 eye) during the period from January 2014 to November 2015. The bacterial isolates were taken from Rizgary Teaching Hospital in Erbil city. All isolated were tested susceptibility to antibiotic test by Vitek2system against 8 antibiotics, Methicillin resistance susceptibility test (MRSA) by using Cefoxitin and Oxacillin disk , production of Biofilm by using Congo red agar methed,with explore photodynamic inactivation of MRSA by using Methylene blue (MB) in combination with diode laser (red 650 nm, 5 mW) at different time of exposure.

Results: We obtained in this study (35) isolates of Staphylococcus aureus isolated from (urine, wound, pus, blood, eye), (45.7%) isolates mltiresistance showed the resistance to more than 4 antibiotics from different classes, in our results also the isolated bacteria showed the percentage resistance of Cefoxitin was highest 35 (100%), resistant for Oxacillin was 21 (60%),thus all 35 clinical strains of Staphylococcus aureus isolates 100% were considered to be MRSA. Furthermore the investigation showed that (22%) black (complete) to slightly black on published congo red agar in which considered to be positive in forming biofilm and (78%) red color on published congo red agar in which considered to be negative in forming biofilm. We achieved in vitro study to explore photodynamic inactivation of methicillin resistant Staphylococcus aureus bacteria by using Methylene blue (MB) in combination with diode laser (red 650 nm, 5 mW). After one hour of laser illumination of bacteria we achieved a reduction more than half numbers of each isolated of MRSA.

Conclusion: Reduction in number of colony-forming units by the Laser (physicochemical properties of photosensitizers) combination with Methylene blue dye treatment of S. aureus which have a great effective to destroy the microorganism.

Key words: *S. aureus* (MRSA), Methylene blue (MB) in combination with diode laser.
Corresponding Author:fattmaabeer@yahoo.com
Received: 30th January 2017
Accepted: 12th March 2017

[1]Department of Medical Microbiology - College of Health Sciences-Hawler Medical University-Erbil-Iraq.
[2]Department of physic - College of Health Sciences-Hawler Medical University-Erbil-Iraq.
[3]Radiology department - Erbil Medical Technical Institute- Erbil Polytechnic University-Erbil-Iraq.

Introduction

*Staphylococcus aureus*is a major pathogen that can cause various forms of diseases varying from simple to life-threatening infection in human population [1]. Antibiotic resistance in *S. aureus* has an adverse effect on healthcare management of infections. In response to the increasing rate of antibiotic resistance in *S. aureus*, there are four general mechanisms that microorganisms use to fend off attack by antimicrobial agents. These are limiting uptake of the drug, modification of the drug target, inactivation of the drug, and active efflux of the drug. Resistance to β-lactam group of antibiotics in S. aureus is mediated through a variety of β-lactamases or the expression of low-affinity penicillin binding protein PBP2a. The chromosomally mediated penicillin binding protein 2a initiates resistance to methicillin which confers a low affinity for all β -lactams and other unrelated group of antibiotics, thereby limiting choice for treatment[2].

Emergence of multi-antibiotic-resistant strains including *S. aureus* infection (MRSA) are becoming more critical, since chronic infection due to biofilm growth, renders antimicrobial agents and the host immune response ineffective in clearing these biofilms [3]. Community of cells, attached to a substratum embedded in a matrix of extracellular polymeric substance exhibit altered growth, gene expression, and protein production phenotypes can cause major medical and economic sequel [4].

Photodynamic therapy (PDT) has emerged as a therapeutic option for the treatment of infectious diseases. This therapy consists of the activation of a photosensitive dye with light of an appropriate wavelength, consequently resulting in the production of reactive oxygen species, such as free radicals and singlet oxygen. These reactive oxygen species can damage DNA and the cell membrane, resulting in the leakage of cell components, inactivation of transport systems and cell death [5].

Although only experimental stages are known up to present, there are remarkable results in killing by photodynamic inactivation of germs which generate several types of infections. For example, in vitro studies of photodynamic inactivation of *S. aureus* and *Escherichia coli* using different photosensitizers (polylysine-ce6-conjugates, octacatioic Zn (II) phtalocyanine, methylene blue, toluidine blue O, deuteroporphyrin, hematoporphyrin derivative) and light radiation with wavelength of 650 nm, 660 nm, 675 nm or 632.8 nm have shown a 90% reduction in bacterial viability [5,6].

Some studies showed that the photodynamic treatment has induced not only lethal effect but also decrease in virulence of bacteria [7].

The aim of the present study was to evaluate the effect of photodynamic antimicrobial chemotherapy on many *S. aureus* strains isolated from different human clinical body sites.

Patients and Methods

Isolation of Microorganisms

Staphylococcus aureus bacterial isolates were isolated from(16 urine,8 wound, 7 pus, 4 eye) during the period from January 2014 to November 2015. The bacterial isolates were taken from Rizgary Teaching Hospital in Erbil city/ Iraqi Kurdistan. Then isolates were inoculated on blood and macConkeys and Mannitol Salt agar plates by direct streaking method after that incubation overnight at 37Co gar plates [8].

Identification of microorganism

Bacteria isolated as a pure colonies were examined microscopically by using Grams stain technique,identification tests included cultural,morphology, physiology characteristics of each bacterial isolates and Vitek 2 compact were done[8].

Antimicrobial susceptibility testing

All 35 isolates were used for this test using Disc diffusion method (Kirby-Bauer method) by culturing them on Mueller Hinton agar the sensitivity disks that were used in this study containing conventionalantibiotics(TetracyclinTE(10µg) ,GentamycinCN(10µg),C,Nitrofirantoihn F (100µg),ErythromycinE(15µg),TobromycinT OB(10mg), and Trimethoprim Sulfamethoxazole SXT (25 µg)), was carried out according to the Clinical and Laboratory Standard Institute guidelines (CLSI) [8].

Methicillin resistance susceptibility test (MRSA) using Cefoxitin disc diffusion and Oxacillin disc diffusion methods

This test was determined using Cefoxitin 30 micrograms and Oxacillin 1 microgram as recommended by CLSI. Bacteria inculated onto Mueller-Hinton agar together with Oxacillin disk and Cefoxitin disk and the plate was incubated at 37ºC for 24 hour. For Oxacillin disk diffusion tests regarding *Staphylicoccus aureus* was considered positive when were ≥ 13mm as susceptible, (11-12) mm was considered intermediate, and ≤ 10 mm was considered resistance, regarding using Cefoxitin disk was considered positive when were ≥ 20 mm as susceptible and ≤ 19 mm was considered negative as resistance [9].

Biofilm Formation test was considered positive whenCongo red agar method

Pramodhini *et al*., [10] have described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium. CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo Red indicator 8 g/L. First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121ºC for 15 minutes) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55ºC. CRA plates were inoculated with test organisms and incubated at 37ºC for 24 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.

Laser experimental part

Bacteria: Three isolates of *S. aureus* (MRSA) used in this study were selected for Laser part(S16 from urine and S33 from pus and S 35 from wound)resistance for all antibiotics used in our study and biofilm production. Bacteria culture saved at nearly 4○C during the experiment and every month re-cultured.

Photosensitizer PS: Methylene blue powder purchased from a chemical suppliers of accepted degree of purity. Methylene blue is Soluble in water and Stoke solution. The solution prepared in dark at 4 oC and warped by aluminium foil to avoid exposure to light [11].

Light source: Continuous wave (CW) diode laser, 5 mW, 650 nm (red). Laser light wave length selection is according to selection roles which states that the wave length should be with absorption spectrum of the dye.

Preparation of suspension of microbial cells: Spectroscopic analyses

Spectroscopic analysis using UV-spectrophotometer performed in Medical Researches Center at Howler University. The absorption curves of MB solution alone taken for selection role consecrations. Also, absorption curves of bacteria samples taken to know the amount of declining numbers of bacteria [11].

Incubation with Methylene blue: According to previous publish studies and during our experiment, the best MB concentration achieves killing more numbers of bacterial cells was 10µM for1mL bacteria

sample (10µM:1mL). Three *S. auras* (MRSA)S16 and S33 and S35 cultured samples incubated at 37 o C with MB about 15 minutes. In order to prevent bacteria-Methylene blue samples from room light, samples warped with aluminum foil directly after preparation to ensure the dye still effective until the laser irradiation.

Light delivery: Four samples of 1mL taken from the three incubated samples (bacteria- Methylene blue) into test tubes. Spectroscopic analyses achieved before and after laser irradiation. Laser head has fixed vertically in about 10 cm from samples. The irradiation times were: 10, 20, 40, 60 minutes. Other samples irradiated without incubation with Methylene blue (MB free) as (control) in order to comparison and to know the influence of the PS dye Methylene blue on bacteria numbers [11].

Statistical analysis

We analyzed the data by SPSS version (23) software were measured and the paired sample t-test was used to compare these means. P- values<0.05 were considered as significant. The research was approved by ethical committee at Hawler Medical University/ College of Health Science.

Results

Table (1) shows that *S. aureus* was isolated from (5) different body sites and were predominate in urine and pus and wound specimens and all of them produced beta hemolysis in blood agar and yellow colony on mannitol salt agar (manittol fermentation) also the susceptibility pattern of *S. aureus* strains against 8 antibiotics is presented in the same table. All these sample were detected by Vitek 2 compact system, the results show that most of *S. aureus* isolates were multiresistant (resistant to three classes or more of antibiotics) and the antibiotic used in. our study (Tetracyclin, Gentamycin, Nitrofirantoihn, Erythromycin, Tobromycin, and Trimethoprim Sulfamethoxazole, Oxacillin and Cefoxitin), our result refer that (3) isolates (8.5%) showed the resistance to all antibiotics (8) and about (16) isolates (45.7%) showed the resistance to more than 4 antibiotics from different classes.

Table (1): Distribution of *Staphylococcus aureus* in relation with antibiotics resistance.

Isolated pathogen	Type of specimens	No. of antibiotics resistance
S1	wound	6
S2	wound	0
S3	wound	2
S4	wound	٢
S5	pus	٠
S6	wound	٦
S7	urine	٥
S8	pus	٤
S9	pus	٣
S10	wound	٣
S11	urine	٦
S12	urine	٢
S13	urine	٦
S14	blood	٣
S15	urine	٣
S16	urine	٨
S17	urine	٣
S18	eye	٥
S19	eye	٦
S20	eye	٢
S21	eye	١
S22	urine	٣
S23	blood	٤
S24	wound	٧
S25	urine	١
S26	urine	٦
S27	urine	٣
S28	urine	٣
S29	urine	١
S30	urine	٢
S31	urine	٢
S32	urine	٤
S33	pus	٨
S34	blood	٧
S35	wound	٨

S= *Staphylococcus aureu*

The results of present study exhibited no significant difference statistically regarding number of antibiotic resistant with isolated pathogen (P>0.05).

Table (2) shows the percentage of MRSA by disk diffusion test, the percentage of resistant to Cefoxitin was the highest 35 (100%) among the resistant types of S.aureus and for Oxacillin was 21 (60%) .

Table (2): The incidence of MRSA by using two methods (OX) and (COF) in *Staphylococcus aureus* isolated from different clinical samples.

Isolated pathogen	MRSA (OX)	MRSA (COF)
S1	R	R
S2	S	R
S3	S	R
S4	S	R
S5	S	R
S6	R	R
S7	R	R
S8	R	R
S9	S	R
S10	S	R
S11	R	R
S12	S	R
S13	R	R
S14	R	R
S15	R	R
S16	R	R
S17	R	R
S18	S	R
S19	R	R
S20	S	R
S21	S	R
S22	S	R
S23	S	R
S24	R	R
S25	S	R
S26	R	R
S27	R	R
S28	S	R
S29	R	R
S30	R	R
S31	R	R
S32	R	R
S33	R	R
S34	R	R
S35	R	R

The results which revealed no significant difference regarding number of resistant to Cefoxitinand Oxacillin with isolated pathogen (P>0.05).While in table (3) shows the results of biofilm forming bacteria, among 35 S. aureus isolates (100%) MRSA was (22%) black on published congo red agar considered to be positive in forming biofilm and (78%) red color on published congo red agar considered to be negative in forming biofilm as in figure 1 and 2.

Table (3): Screening of Staphylococcus aureus isolates for production of biofilm by using Congo red agar method.

No. of specimens	Biofilm results
S1	Medium
S2	Medium
S3	Medium
S4	Medium
S5	Medium
S6	Strong
S7	Strong
S8	Medium
S9	Medium
S10	Medium
S11	Medium
S12	Medium
S13	Strong
S14	Medium
S15	Medium
S16	Strong
S17	Medium
S18	Medium
S19	Strong
S20	Medium
S21	Medium
S22	Medium
S23	Strong
S24	Strong
S25	Medium
S26	Strong
S27	Medium
S28	Medium
S29	Medium
S30	Medium
S31	Medium
S32	Medium
S33	Strong
S34	Strong

The results raveled no significant difference statistically regarding biofilm forming with bacteria isolated pathogen ($P>0.05$).

We choose three isolates of MRSA with MDR (S16, S33, S35) and they were produced biofilm and were resistant to all antibiotics we use and the isolates obtained from urine (S16), pus (S33) and wound (S35).

The absorption spectrum of Methylene blue was examined to make sure that the laser light wave length is within Methylene blue absorption band (selection role). Figure (1) shows Methylene blue highest absorption in region (630nm - 675nm). This result

confirms the correct choice of red laser diode (650 nm) in this study.

We can discuss the irradiation time effect on killing bacteria by checking figures (3) Samples divided according to irradiation times (10, 20, 40, 60 minutes) to four samples for each bacteria culture sample. Slight reduction observed on surviving bacteria fraction in samples that irradiated with times 10 and 20 minutes. At 40 minutes, a significant decline on bacteria surviving fraction. The time 60 minutes achieved highest reduction on surviving fraction. The curve of free MB bacteria samples of same figure was almost constant during the four times, this confirm PS effect and its rule in PDI. The spectroscopic analyses of incubated bacteria with Methylene blue demonstrate reduction in numbers of bacteria to more than half of original number. Free Methylene blue bacteria still relatively constant during laser application, figure (4).

Our results reveled significant difference statistically regarding the groups submitted to PACT with different time, comparison with control group significant increasing reduction ($P<0.01$), it was observed.

Figure (1): MRSA

Figure (2): Biofilm producing bacteria

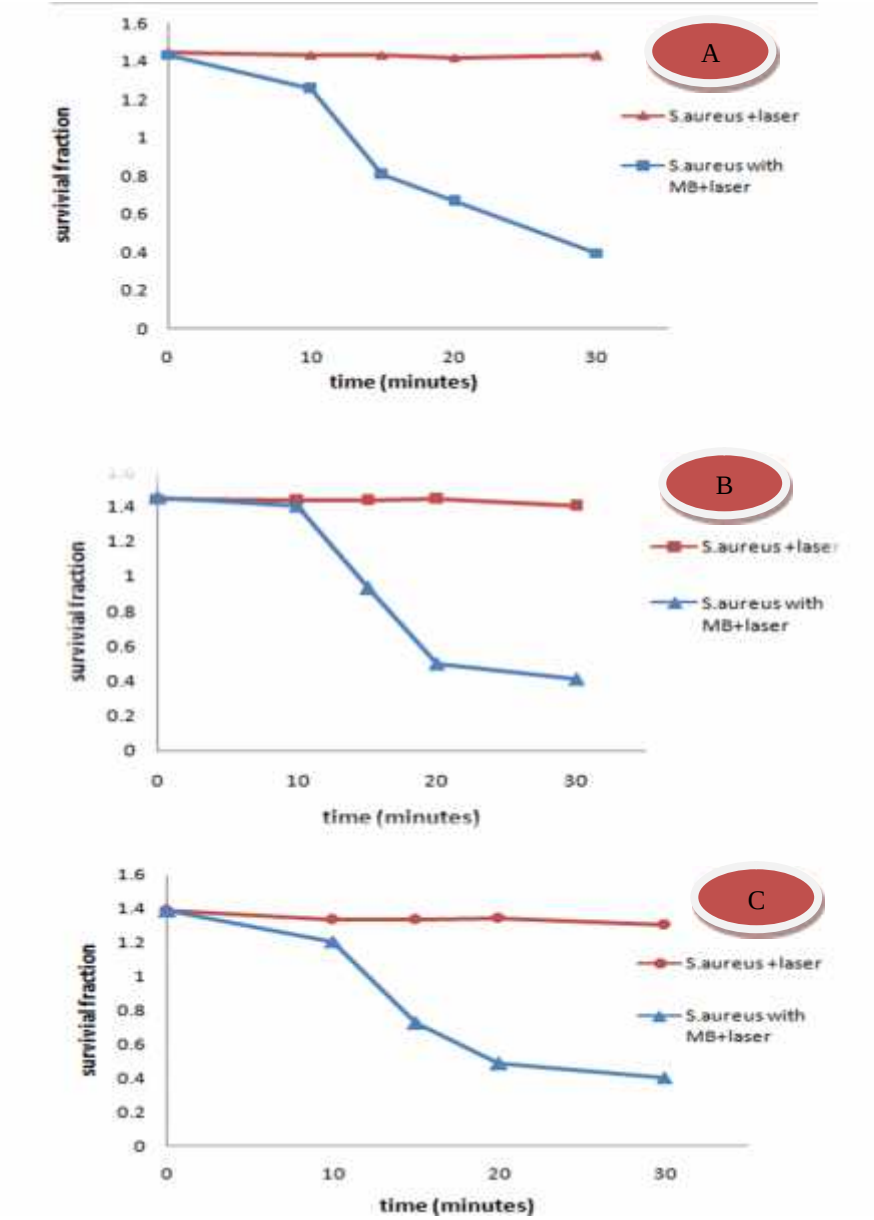

Figure (3): Chart explain reduction behavior of *S.aureus* bacteria in PDI.

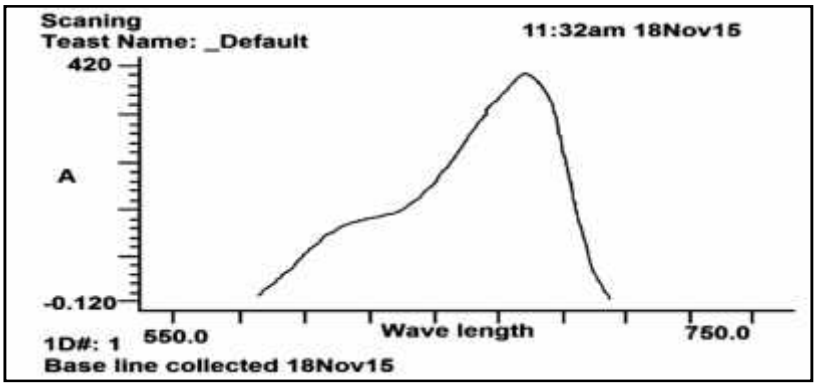

Figure (4):MB absorption curve.

Discussion

Our result refer that (3) isolates (8.5%) showed the resistance to all antibiotics (8) and about (16) isolates (45.7%) showed the resistance to more than 4 antibiotics from different classes, our results similar to Jasim *et al*., [12] from Baghdad and also Naditaand Ishaya, [13] from Nigeria they showed that S.aureus isolates isolated from clinical source different sites in body have been shown multidrug resistance for many antibiotics, Also our results our results similar to finding of to Jasim *et al.* [12]. In that the MRSA have been more prevelance among isolates of *S. aureus* isolated from cinical samples further more Muder *et al.* Who found that most of *S. aureus* isolates was MRSA isolated from urinary tract infection patients with urinary tract catheterization[14]. These results exhibited no significant difference statistically regarding number of antibiotic resistant with isolated pathogen (P>0.05).

Although, the power used 5mW was adequate to achieve bacterial reduction. This illustrates that 5mW is within threshold power to achieve wanted chemical interaction and produce reactive oxygen. This kind of laser interactions classified also power laser interaction.

The percentage of resistant to Cefoxitin was the highest 35 (100%) among the resistant types of *S. aureus* and for Oxacillin was 21 (60%). The organism's resistance to these agents might be due to the easy availability of the antimicrobial agents in this environment which usually leads to their frequent and in discriminate use in UTIs Okeke *et al*.[15]. Also the truth of using awidespread of antibiotics without physician permission was lead to increase these resistance to antibiotics had been recognized among medical field [16]. The results revealed no significant difference statistically regarding number of resistant to Cefoxitinand Oxacillin with isolated pathogen (P>0.05).

35 *S. aureus* isolates (100%) MRSA was (22%) black on published congo red agar considered to be positive in forming biofilm and (78%) red color on published congo red agar considered to be negative in forming biofilm. and our results similar to the finding of Mariana *et al*., from Malaysa [17]. The ability of *S. aureus* to form biofilm on implanted medical devices or damaged host tissue is also virulence factor for this pathogen, especially in health care's where antibiotic usage is high and such biofilm formation represents a survival mechanism for the bacteria [3].

There was no statistically significant difference between the biofilm forming bacteria and according to isolated pathogen (P>0.05).

It was found a direct effect of dye concentration on surviving bacteria fraction by changing concentration between 1µM to15 µM for each1mLof bacteria. The best concentration achieves killing largest number of bacteria was 10 µM. Concentrations which more and less 10 µM were ineffective. Low concentrations were insufficient to produce imported amount of oxygen. The higher concentration than 10 µM may create opaque medium resulting in blocking a portion of laser beam through the sample.

Studies carried out by Zeina *et al*. It was examined the effects 630, 660, 810 and 905nm of low-intensity laser irradiation delivering radiant exposure of 1-50 J/cm2 on three species of bacteria in vitro, including *Staphylococcus aureus*, the authors concluded that the response photobiological a microorganism exposure to monochromatic light depends directly on the parameters of irradiation (wavelength, intensity and dose) [18]. 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. Actually, methylene blue (MB) are photosensitizer used clinically forantimicrobial treatments

because the low toxicity of these dyes to human cells, plus their ability to produce high quantum yields of singlet oxygen.

The time 60 minutes achieved highest reduction on surviving fraction. The curve of free MB bacteria samples of same figure was almost constant during the four times, this confirm PS effect and its rule in PDI. Present results were similar to the finding of the Dimitrov *et al*.,[19] from Bulgaria in which concluded that were a great reduction of *S. aureus* with the presence of Methylene blue at 40 and 60 minute which have a great effective to destroy the microorganism, irradiation of Staphylococcus aureus with diode laser in Zn-phtalocyanines with Methylene blue for 12 and 20 minutes, there were a great reduction of S. aureus.The use of Methylene blue for 5 minutes and of Porphyrin for 5, 12 and 20 minutes weren't very effective to destroy the microorganism. Our results reveled significant difference statistically regarding the groups submitted to PACT with different time, comparison with control group significant increasing reduction ($P<0.01$), it was observed.

On these results we can concluded that photodynamic therapy with Methylene blue is an effective treatment for MRSA, but it is difficult to be optimized without an appropriate animal model or in vivo studies.

References

[1] Barber, M. Methicillin resistant staphylococci" Journal of Clinical Pathology, 1961;14: 385–393.

[2] Jean SS, Hsueh PR. High burden of antimicrobial resistance in Asia. International Journal of Antimicrobial Agents. 2011; 37(4): 291–295.

[3] OGara JP, Humphreys H. *Stphylococcus* epidermidis biofilms: Importance and implications. J Med Microbiol.2001; 50:582-587.

[4] Sakoulas G, Moellering Jr RC. Increasing antibiotic resistance among Methicillin-Resistant *Staphylococcus aureus* strains.Clin Infect Dis. 2008 Jun; 46 Suppl 5:S360-7.

[5] Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci. 2004; 3(5):436-50.

[6]Høiby,N,BjarnsholtT,Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. Int J Antimicrob. 2010;35,322–332.

[7] Nitzan Y, Gozhansky S, Malik Z. Effect of photoactivated hematoporphyrin derivative on the viability of *Staphylococcus aureus*. Curr Microbiol. 1985; 8(5): 279-284.

[8] Wayne PA. Clinical and laboratory standards institute. Performance standard for antimicrobial susceptibility testing.15th ed. Informational Supplement.CLSI / NCCLS M .2005;100- S 15.

.[9] Rahbar M, Safadel N. Evaluation of Cefoxitin Disk Diffusion Test for Routine Detection of Methicillin-resistant Staphylococcus aureus. Iranian Journal of Pathology. 2006; 1 (4): 145-148.

[10] Pramodhini S, Niveditha S, Umadevi S, Kumar S and Stephen S. Antibiotic resistance pattern of biofilm-forming uropathogens isolated from catheterized patients in Pondicherry, India. Australasian Mdical Journal. 2012; 5 (7): 344-348.

[11] Huang L, Tianhong D, Michael RH. Antimicrobial Photodynamic Inactivation and Photodynamic Therapy for Infections" Methods Mol Biol. 2010; 635: 155–173.

[12] امنة نصيف جاسم، هدى سهيل عبدو و شيماء عواد عبد . الحمل الانفي للعنقوديات الذهبية المقاومة للمثيسلين Methicillin-Resistant Staphyloccus aureus (MRSA) في الطلاب الجامعيين الاصحاء . ص ٢٥-٣٠: مجلد جامعة النهرين. المجلد ١٥: العدد (٤). ٢٠١٢.

[13] Nandita D, Ishaya PI. Sensitivity profile of Staphylococcus aureus isolated obtained from patients with urinary tract infection in Kaduna, Metropolis, Kaduna, Nigeria. J Int Curr Microbiol App Sci. 2014; 3:(9): 8-16.

[14] Robert RM, Carole B, John DR, Marilyn MW, Asia O, Janet ES *et al.* Isolation of Staphylococcus aureus from the Urinary Tract: Association of Isolation with Symptomatic Urinary Tract Infection and Subsequent Staphylococcal Bacteremia. J Infectious Diseases Society of America. 2006: (42).

[15] Okeke, IN, Lamikanra A, Edelman R. Socioeconomic and behavioral factors leading to acquired bacterial resistance to antibiotics in developing countries. Emerg Infect Dis. 1999; 5: 18.

[16] Millar MR, Walsh TR, Linton CJ, Zhang S, Leeming JP, Bennett PM. Carriage of antibiotic – resistant bacteria by healthy children. J Antimicrob Chemother. 2001; 47: 605- 610.

[17] Mariana NS, Salman SA, Neela V, Zamberi S. Evaluation of modified Congo red agar for detection of biofilm produced by clinical isolates of methicillin–resistance Staphylococcus aureus. J African Journal of Microbiology Research. 2009; 3(6):330-338.

[18] Zeina, B, Greenman J, Purcell WM, Das B. Killing of cutaneous microbial species by photodynamic therapy. Br J Dermatol. 2001; 144(2): 274–278.

[19] Dimitrov SI, Dogandhiyskal V, Kalchinov V, Belcheval M, Mantareva V, *et al.* Susceptibility of *S. aureus* to Methylene Blue Haematoporphyrin, Phtalocyanines Photodynamic Effects. J. of IMBA. 2010; 16(4).