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Photo Response of Locally Isolated Methicillin-Resistant *Staphylococcus aureus* to Q-switched Nd:YAG Laser In Vitro Study

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Abstract: This prospective study investigates the prevalence of methicillin-resistant *S.aureus* (MRSA) in burn unit of Al-Kindy Iraqi hospital, their susceptibility to antibiotics and bactericidal effect of near infrared light from high powered 1064nm Nd: YAG laser and green light 532nm from SHG Nd: YAG laser using various energy densities on these bacteria. Twenty four clinical isolates of *S.aureus* out of sixty four examined patients with sever burn ulcers.MRSA was associated with 50% of *S.aureus* infections .Results of antimicrobial susceptibility revealed that MRSA were multidrug resistant. After laser treatment of non MRSA with Nd:YAG with wavelength of 1.064nm, 4mm beam diameter, energy density of 0.636 kh/cm^2 and 180sec exposure time, the diameter of inhibition zone increased for almost of the used antibiotics beside some isolates became sensitive or intermediate except for penicillin (G). For MRSA isolates no observable change in the sensitivity against the antibiotics after 180sec irradiation was observed, except for erythromycin and gentamycin which changed from resistant to intermediate, while vancomycin changed from resistant to sensitive. An inhibitory effect of Nd: YAG laser on MRSA was noticed at all energy densities.Simillarly SHG Nd: YAG laser with chosen energy densities was inhibitory for MRSA .A noticeable decrease in MRSA growth corresponding to 92% was obtained by using SHG Nd: YAG with 0.955J/cm^2 energy density, with 3HZ repetition rate and 1260 pulses. Our results demonstrated that methicillin resistant *S.aureus* can be successfully killed by Q-switched Nd: YAG laser. This effectiveness of Q-switched Nd: YAG in vitro suggests it would be effective in human cases of MRSA infections, and particularly in patients with skin burn infections.

***Keywords*:** MRSA, Q-switched Nd: YAG laser, burn infections

Introduction

Methicillin resistant Staphylococci are becoming an increasing problem in burn units around the world (Zetola *et al.*, 2005). Burns that are caused by a variety of non-mechanical sources including chemicals, electricity, heat, sunlight or nuclear radiation are damaged to skin (Mayhall , 2003.).Burns cause the destruction of the derma-epidermal barrier, facilitating bacterial contamination, and burned tissues are a good culture medium from where germs can reach blood stream and generate systemic infection (Bollero *et al.,* 2003).The use of endo- tracheal tubes, ventilation support, surgical drainage in the treatment of burn infections, multiplying colonization by nosocomial germs (Alekseev *et al.*,1999).The medical and nursing staff can be also responsible for cross contamination between patients, they are an involuntary vehicle for germs (Doebbeling *et al.* ,1992).Burn infection is problematic, bacteria and fungi are the most common pathogens in this regard (Church *et al.*,2006).Gram –positive bacteria are one of

the first to colonize burns, followed by gram-negative. *S.aureus* (75%), *P.aeruginosa* (25%) and various coliform bacilli (5%) represent a relevant percentage of pathogens isolated from burn wounds (Stefano *et al.*,2006). Half of burn patients commonly infected with methicillin are resistant to *Staphylococcus aureus* (Bollero *et al* ., 2003). The use of systemic antibiotics as an adjustment in the treatment of periodontal disease has been necessary.

However, overuse of antibiotics has been a major culprit in the production of drug resistant organisms as exemplified by the threat of MRSA and VRSA world wide(Hiramatsu *et al.*,1997.,Tenover *et al.*,1998 .,Bae *et al.*,2004., Hiramatsu *et al.*,2004.,). New therapeutic approaches are urgently needed to combat such multiple –antibiotic resistant bacteria. Soon after the invention of laser on 1960, lasers were described as a solution in search of a problem laser is unlikely to induce resistance in microorganisms. Bactericidal effect of different lasers on gram positive and gram negative bacteria were demonstrated by distinct authors world wide (Bertoloni *et al.*, 1993, DeSimone *et al.*, 1999).

In a local study, the survival percentages of *S.aureus* and *E.coli* bacterium isolated from urinary tract infections, septicemia, wound and burn infections were gradually decreased after irradiation with He Ne laser (in the presence of Toluidin Blue O) until reaching the bacterial death times which were 5 minutes for *S.aureus* and 6 minutes for *E coli* , all *S.aureus* and *E.coli* isolates loosed their ability to produce β-lactamase enzymes and the inhibition zone diameter for almost of the used antibiotics increased ,beside some isolates became sensitive especially after 2 min of laser exposure time(Al-Dulaymi,2005). In another study (AL-Derajy, 2009) the increase in inhibition zones for antibiotic was observed in both oxacillin resistant and oxacillin sensitive *S.aureus* using 805nm diode laser with (2.5W) power and (4.9W/cm^2) power density at (15, 25) min exposure time and (100µg/ml) indocyanine green dye (ICG). A decrease in growth of bacteria was also observed at different dye concentrations using different exposure times. The purpose of this study was to investigate the effect of Q-switched Nd: YAG laser (1064 nm), and second harmonic generation SHG Nd: YAG laser 532nm on viability and resistance pattern of locally isolated methicillin resistant *Staphylococcus aureus* (MRSA) and non MRSA from burn units of Iraqi hospital.

Materials and Methods

Bacterial Samples

Samples were collected from forty six patients with sever skin burn ulcers from burn unit of Al-Kindy Iraqi hospital from 17th February 2009 till 21st April 2009 using a sterile cotton swab. Samples were grown on blood agar, and mannitol salt agar, incubated at 37C^o for 24h and identified according to Bergey's manual for systemic bacteriology (Sneath *et al.*, 1986)using different morphological and biochemical tests.Api 20 Staph kit was used to confirm *S.aureus* diagnoses. The pure cultures were subculture on nutrient agar slant and preserved in the refrigerator at 4C°until required for the study (Chessbrough, 2000).

Antibiotic Susceptibility Test

Antimicrobial activity of panel of antibiotics was investigated using disk diffusion test. Bacterial strain cultures were grown on tryptic soya broth incubated at 35C^o and adjusted to match 0.5 McFarland turbidity standard, usually 2-6 hours and spread on Muller –Hinton agar plate using a sterile cotton swabs. The following antibiotic disks: ampicillin /sulbactum (10µg/disc), cephalothin (30µg/disc), ciprofloxacin(5 µg/disc), clindamycin (2 µg/disc),erythromycin (15 µg/disc), oxacillin (1 µg/disc), penicillin G (10U), tetracylin (30 µg/disc), trimethoprim /sulfamethoxazole (1.25/23.75 µg/disc), vancomycin (30 µg/disc), gentamycin (10 µg/disc), and Refampin (5 µg/disc) were applied and the plates were incubated at 35C^o for 16hr, 24 hours for oxacillin and vancomycin.On the next day, plates were read by taking measurement of the zone inhibition. Results were recorded and graded as resistant (R) or sensitive (S) according to antibiotic zone diameter interpretive chart recommended by (NCCLS, 2002).

Identification of MRSA

For determination of methicillin resistant isolates of *S.aureus*, agar dilution method was used. Sample of *S.aureus* stock 100µl was inoculated into 2ml of tryptic soya broth,and incubated at 37C. From the cultures ,100µl portion were plated on tryptic soya agar containing methicillin 25mg/l.Resistance was confirmed by the presence of colonies on the surface of methicillin plates after incubation for 24h at 37C(NCCLS,2000).

Laser Apparatus

The laser used in this study was Q-switched Nd: YAG laser (1064nm), SHG Nd: YAG laser with Potassium Titanium Phosphate (KTP) crystal producing green light at wavelength of 532nm (Diamond Beauty, China).

Procedure

S. aureus and MRSA bacterium were grown aerobically on nutrient agar plate overnight at 37C^{o}. Isolated colonies (4-5) were transferred to a test tube containing 4ml brain heart infusion broth ,and incubated at 37C for 18h.Bacteria was harvested by centrifuging the broth culture at 3500 r.p.m. for 15 minutes ,washed twice with phosphate buffer saline and diluted in the same buffer .The turbidity of the obtained suspension was adjusted to an absorbance 0.5 McFarland standard at 600nm corresponding to 1.5×10^{8} bacterial cell/ml(Gorbach *et al.* ,1998).

The effect of 1064nm Nd:YAG laser and 532nm green light from SHG Nd:YAG laser was studied on both isolates .Bacterial solution 1ml volumes growth was transferred into sterilized eppendroff tubes, irradiation was performed using different energies (80,100,120) mJ for three irradiation time's 180sec, 300sec and 420sec, and 3HZ repetition rate.

At the end of irradiation, 1ml from unirradiated (control) and irradiated bacterial cells were serially diluted with phosphate buffer saline, 0.5ml from each dilution were plated in duplicate nutrient agar plates. The number of colonies found after 24h incubation at 37C^{o} were counted.

Irradiation of Bacteria for Susceptibility Test

Aliquots (1ml) of MRSA and non MRSA suspension (1.5×10^{8} cfu/ml) transferred to a sterile ependorff tubes were exposed to a measured amount of laser light for 180sec. Control ependorff tubes were not exposed to the light source.

Following exposure to light , the suspension from each ependorff was plated onto Muller-Hinton agar by a sterial cotton swab ,drug disks were applied and placed invertely in an incubator at 35-37C^{o}for 18 hr. On the next day, plates were read by taking measurement of the zone inhibition after irradiation.

Results

Twenty four clinical isolates of *S.aureus* out of forty six samples burn ulcer specimens were identified according to the following results: the colonies of these bacteria were golden yellow colonies (cream white to orange) on nutrient agar. On blood agar, hemolysis produced surrounding transparent zone.

In mannitol-salt agar changed the color of phenol from red to yellow, they were catalase positive, coagulase positive. On Kligler iron agar, yellow colonies were produced indicates acid production and its ability ferment and use a variety of carbohydrates as energy source.

The results of Api20 staph system came to ensure the biochemical identification of *S.aureus* .After staining with gram stain ,they are purple under the light microscope which is denoted as gram-positive .The isolates of *S.aureus* bacterium were individual cocci or in pairs irregular clusters ,spherical cells . Methicillin resistant *S.aureus* was detected in 12 (50%) out of the 24 examined patients implicated with *S.aureus* by methicillin agar screen test. Susceptibility of the bacterial isolates was performed against 12 different antibiotics.

The data obtained showed that all *S.aureus* isolates are sensitive to ampicillin/sulbactam, oxacillin and erythromycin. While the resistant rate of *S.aureus* isolates against penicillin was 100%, cephalothin 32.62%, ciprofloxacillin 59.25%, clindamycin64.96%, gentamicin 48.14% and 50.34% for refampin.All *S.aureus* isolates were sensitive to vancomycin .

Methicillin resistant *S. aureus* isolates were sensitive to trimethoprin/sulfamethoxazole, resistant to all antibiotics, except for gentamicin and refampin the resistance rate was 80% and 67.3% respectively. Resistance to vancomycin was observed in only one isolate, but three of the isolates were vancomycin intermediate, this made this bacteria as a great threat in burn units. Two selected isolates of bacteria (*S.aureus* and methecillin/vancomycin resistant *S.aureus*) were exposed to laser light from Nd: YAG and SHG Nd: YAG laser.

Figure (1) shows a noticeable reduction in the viability of non MRSA at different energy densities and times after irradiation with Nd: YAG and SHG Nd: YAG lasers. Each experiment was repeated three times and the average values are shown in all graphs. Colony counting method was used to evaluate cell growth which is accurate more than optical density measure-

ment, since the latter cannot show the decrease in cell population, because it measures the turbidity of cell suspension and is not able to recognize the living cell from dead cells.

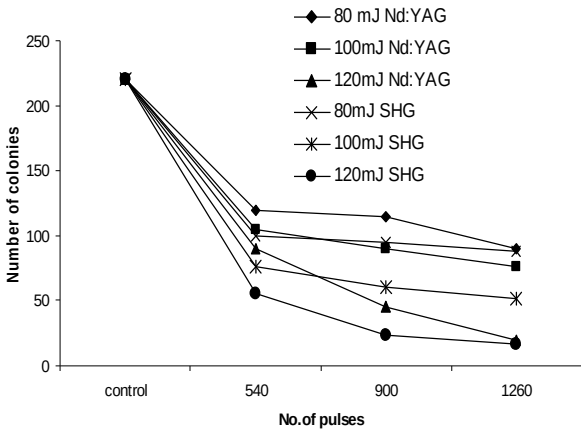

Fig. (1): Growth curves of control and irradiated non MRSA in different energy doses with Nd: YAG and SHG Nd:YAG lasers.

The effect of different energies on the viability of MRSA and the population of cells decreased as the irradiation energy increased as shown in Figure (2). the effect of different energy densities of both lasers at 420 sec on both isolates of bacteria is shown in Figure (3) .From above results all energy densities of Nd:YAG and SHG Nd:YAG showed inhibitory effect on *S.aureus* and MRSA ,but for both isolates ,inhibitory effect on the growth was achieved with SHG Nd:YAG laser at (0.796 and 0.955) J/cm^2 for 300 and 420 seconds.

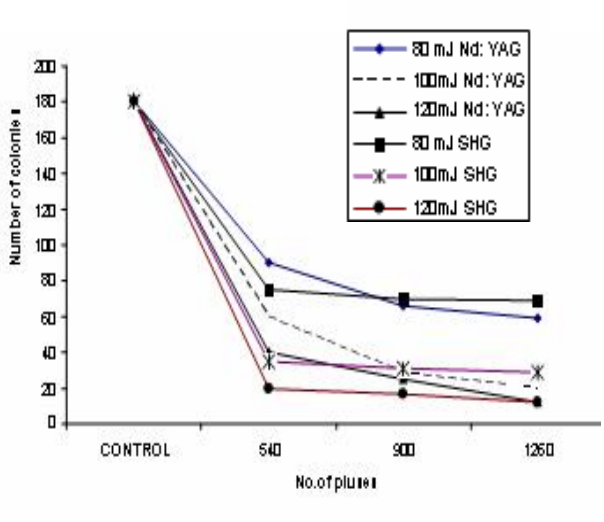

Fig. (2): Growth curves of control and irradiated MRSA using different energy doses with Nd: YAG and SHG Nd: YAG lasers

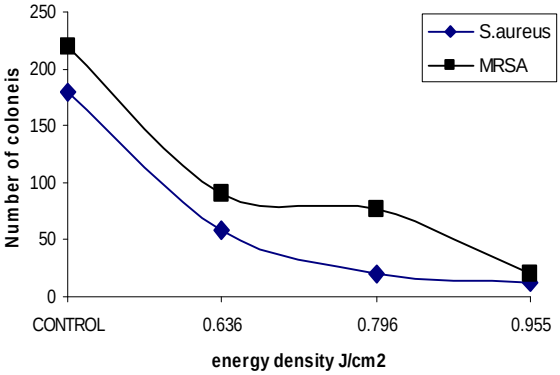

Fig. (3): The effect of different energy densities of Nd: YAG irradiation on both Bacteria are shown by colony counts.

Results of antibiotic sensitivity test after irradiation of *S.aureus* isolate with laser light from Nd:YAG 1.064nm with 4mm beam diameter and energy density equal 0.636 at 180sec exposure time illustrated increasing sensitivity of *S.aureus* to all of used antibiotics The resistant isolates to some antibiotics became sensitive or intermediate except for penicillin (G) as shown in Table(1).

Table (1): The effect of Nd: YAG laser on the sensitivity of *Staphylococcus aureus* to the antibiotics

Isolate	Antibiotics	Laser irradiation	
		0 sec Inhibition zone (mm)	180sec Inhibition zone (mm)
S.aureus	Ampicillin /sulbactum	16(S)	19(S)
	Cephalothin	13(R)	18(S)
	Ciprofloxacin	13(R)	17(I)
	Clindamycin	12(R)	22(S)
	Erythromycin	23(S)	24(S)
	Oxacillin	15(S)	17(S)
	Penicillin G	23(R)	25(R)
	Tetracyclin	20(S)	22(S)
	Trimethoprin-Sulfamethoxazole	17(S)	20(S)
	Vancomycin	20(S)	21(S)
	Gentamycin	10(R)	14(I)
	Refampin	15(R)	19(I)

The effect of laser light on MRSA sensitivity to antibiotics was shown in table2 .The results of this table show that there was no observable change in the sensitivity of MRSA against the

antibiotics after 180sec irradiation, except for erythromycin and gentamycin which changed from resistant to intermediate, while for vancomycin it changed from resistant to sensitive.

Table (2): The effect of Nd: YAG laser on the sensitivity of methicillin-resistant *Staphylococcus aureus* to the antibiotics

Isolate	Antibiotics	Laser irradiation	
		0 sec Inhibition zone (mm)	180sec Inhibition zone (mm)
Methicillin resistant S.aureus (MRSA)	Ampicillin /sulbactum	7(R)	9(R)
	Cephalothin	10(R)	12(R)
	Ciprofloxacin	8(R)	11(R)
	Clindamycin	10(R)	13(R)
	Erythromycin	11(R)	15(I)
	Oxacillin	6(R)	10(R)
	Penicillin G	20(R)	24(R)
	Tetracyclin	9(R)	11(R)
	Trimethoprin-Sulfamethoxazole	17(S)	19(S)
	Vancomycin	13(R)	16(S)
	Gentamycin	10(R)	13(I)
	Refampin	12(R)	13(R)

Discussion

Increasing the bacterial resistance to penicillin was due to the production of B-lactamase enzyme that destroys the drug (Jawetz, 2007). For cephalosporins, resistance to this group of antibiotics can be attributed to poor permeation of bacteria by the drug. Lack of PBPs (the target site) for specific drug and by efflux system (Jawetz, 1991)).Gentamicin is bactericidal antibiotic inhibit protein synthesis; resistance is based on enzymatic destruction of the drug, frequently plasmid-mediated (Jawetz, 2007)) .Since 2002, several isolates of vancomycin – resistant *S.aureus* (VRSA) strains were isolated from patients world wide. The mechanism of resistance is the same as or similar to the transposon-mediated vancomycin resistance in enterococci (acquisition of Van Agene) (Jawetz, 1991,Campanile, 2009).In this article, the effect of near infrared high power Nd: YAG laser and green light from SHG Nd: YAG laser on *S.aureus* were monitored. Previous studies had shown the effectiveness of Q-switched Nd:YAG laser as bactericidal tool(Ward *et al.*,1996, Meral *et al.*,2003).Factors like energy density, exposure time ,light wavelength , beam diameter ,population and type of bacteria in the irradiation suspension affect minimum bactericidal energy level and indicate whether the resulting effect is due to photochemical or photo thermal. In this study the antimicrobial effect of near infrared laser is caused by photo thermal effect rather than photochemical. This fact is confirmed by (Andres *et al.,* 2000). In photo thermal effect; photons may be absorbed by any biomolecul, represented by water, protein, and DNA leading that molecule into an excited state. Collisions with other molecule increase their kinetic energy which leads to a temperature rise in (heat). Thermal effect on the bacterial cell leads to irreversible changes, such as conformational changes in protein due to the break of hydrogen bonds due to increasingly violent vibrations of the molecule as the temperature increases. Whenever protein change conformation ,it is no longer fulfilled function. The other changes which may occur inside bacterial cell due to thermal effect are reduction in enzyme activity, increasing membrane permeability, vaporization of water molecules ,increasing volume and gas bubbles are formed inducing thermal decomposition and mechanical rupture (Cox,2007,Niemz,2007).At a macromolecular level ,the helical structure of protein , DNA and RNA ,is excited to higher vibrational stages by infrared. At higher energy densities the resulting temperature rise leads to breakage of hydrogen bonds. (David *et al*,1999). The irradiation with 532nm green light from SHG Nd:YAG laser had the most inhibitory effect on the growth of *S.aureus*. The best results were obtained for both irradiated isolates at 0.955J/cm^2 for 420sec. The inhibitory of green laser light is due to photochemical effect. Irradiation of cells at certain wavelength ,stimulate some native components like porphyrins or flavoproteins, leading to specific biochemical reactions and the whole cellular metabolism can be altered. This leads to the release of singlet oxygen (Giese *et al.*,1980, Karu,1982). This suggestion which is in an agreement with (Aoki,2008) explained the absorption of Nd:YAG radiation by certain pigments contained in germs and bacteria ,which could make it ideal for killing bacteria.Coporphyrin is the predominant porphyrin in gram positive staphylococci and is responsible for the inactivation of cells (Nitzan *et al*,2004).This process is the change in redox

properties of the respiratory chain components following photo excitation of their electronic states, generation of singlet oxygen ,localized transient heating of absorbing chromophores ,and increasing super oxide anion production (Karu *et al*,1999).

In conclusion, the wide susceptibility of skin microbes to laser would indicate that this technique could be applied in vivo as a safe and effective method for treatment of burn ulcers diseases. Further studies on animal model are needed to demonstrate affectivity and safety of using laser in such treatment.

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الاستجابة الضوئية لبكتريا المكورات العنقودية الذهبية المقاومة للمثيسيلين المعزولة محليا" لليزر النيديميوم: ياك دراسة خارج خلوية

ياسمين زهير ابراهيم

معهد الليزر للدراسات العليا ، جامعة بغداد ، بغداد ، العراق

الخلاصة

هدفت الدراسة الحالية تقصي انتشار بكتريا المكورات العنقودية الذهبية المقاومة للمثيسيلين في ردهة الحروق في مستشفى الكندي ،حساسيتها للمضادات الحيوية والتأثير القاتل للأشعة تحت الحمراء المنبعثة من ليزر النيديميوم ياك عالي القدرة وذي طول موجي 1,064 نانوميتر ،والضوء الأخضر532 نانوميترالمنبعث من جيل التوافقية الثانية لليزر النيديميوم ياك بقدرات مختلفة على هذه البكتريا . شملت العينات أربعا" وعشرين عزلة لبكتريا المكورات العنقودية الذهبية من اربعة وستون مريضا" يعانون من جروح الحروق المتقرحة. مثلت بكتريا المكورات العنقودية الذهبية المقاومة للمثيسيلين 50%من الإصابات بالمكورات العنقودية الذهبية وامتازت بمقاومتها المتعددة للمضادات الحيوية المستخدمة في الاختبار. أدت المعالجه بالليزر الى زيادة قطر منطقةِ التثبيط لااغلب المضادات المستعملة فضلا أن بعض العزلات اصبحت حساسة اومتوسطة الحساسية ماعدا عقار البنسيلين،اظهرت النتائج بان الليزر لم يمتلك تاثيرملموس على المكورات العنقودية الذهبية المقاومة للمثيسيلين بعدوقت تعريض 180 ثانية،ماعدا عقاري الارثرومايسين والجنتمايسين والذي تغيرت من مقاومة الى متوسطة الحساسية، واصبحت حساسة للفانكومايسين . أظهرت نتائج التعرض للأشعة تحت الحمراء المنبعثة من ليزر النيديميوم ياك تأثيرا" مثبطا" عاليا" على عيوشية بكتريا المكورات العنقودية الذهبية المقاومة للمثيسيلين في قدرات مختلفة . وقد حصلت أفضل النتائج بعد التشعيع بواسطة الأشعة الخضراء 532 نانوميتر المنبعثة من جيل التوافقية الثانية لليزر النيديميوم ياك بقدرة 0,955 جول/سم^2 ،1260 نبضة، وبتردد تكرار للنبضة 3 هرتز.لم تظهر النتائج تأثيرا" حقيقيا" على حساسية البكتريا للمضادات الحيوية بعد التشعيع.النتائج المستحصلة أظهرت إمكانية تثبيط بكتريا المكورات العنقودية الذهبية المقاومة للمثيسيلين بنجاح بواسطة ليزر النيديميوم ذي التردد المضاعف.ان فعالية تأثير الضوء الأخضر532 نانوميتر المنبعث من ليزر النيديميوم ياك مختبريا"تجعله مرشحا" للاستخدام في علاج تقرحات الحروق الجلدية الملوثة بالمكورات العنقودية الذهبية المقاومة للمثيسيلين.