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The Bactericidal Effect of CO_2 Laser on *Pseudomonas aeruginosa* Isolated from Wound and Burn Infections, In-Vitro

Eman N. Naji* ***Aqeela A. Ali*****
Baydaa F. Hamzah****

* Biology Department/ College of Science/ University of AL-Mustansiriyah
**Medical City/Teaching Laboratories Department
*** Periodontics Department /College of Dentistry/ University of AL-Mustansiriyah.

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Abstract:

A total of 60 cotton swabs are collected from patients suffering from burn wound and surgical site infections admitted to Baghdad Teaching Hospital and Burn Specialist Hospital in Baghdad city during 9/2013 to 11/2013. All cotton swabs are cultured initially on blood agar and MacConkey agar and subjected for standard bacteriological procedures for bacteriological diagnosis. Twenty samples out of sixty are identified as *Pseudomonas aeruginosa* by conventional methods. The results of antibiotic susceptibility test illustrate that the antibiotics resistance rate of *Pseudomonas aeruginosa* isolates is as follows:100% (20\20) for ceftriaxone, cefepime and carbencillin, 70% (14/20) for amikacin, 65%(13/20) for tobramycin, ceftazidim and gentamycin, 55% (11/20) for ciprofloxacin and norfloxacin, 50% (10/20) for piperacillin and impeneme, 30% (6/20) for aztreonam. All *Pseudomonas aeruginosa* isolates are investigated for detection of some virulence factors (haemolysin, protease, lipase enzymes, and extracellular pigments) and biofilm formation. The results of virulence factors reveal that all the isolates are haemolysin, protease, lipase enzymes and extracellular pigments producer, while 95% of the isolates are biofilm producer. Six isolates are selected to irradiation by using CO_2 laser according to the results of antibiotic susceptibility and virulence factors at power densities (2000, 2500, and 3000) W/cm^2 with exposure time (60 and 90) second. The results of CO_2 laser irradiation illustrate that CO_2 laser irradiation lead to a reduction in the mean value of the viable number CFU/ml of *Pseudomonas aeruginosa* isolates with the increase of the power density and exposure time. The results of the statistical analysis by using analysis of variance (ANOVA) one way and least significant differences-LSD show that there are statistical significant differences in the mean of the viable number CFU/ml between different power densities and different exposure times. After irradiation, antibiotic susceptibility and virulence factors tests of the irradiated strains are performed.

The current study concludes that CO_2 laser has bactericidal effect on *P. aeruginosa* isolates without any effect on its antibiotics susceptibility and virulence factors.

Key words: *Pseudomonas aeruginosa*, Antibiotic Susceptibility Test, Virulence Factors, Carbon Dioxide (CO_2) Laser.

Introduction:

The use of laser for therapy has become very common in the medical field, it is considered the standard of

care particularly in ophthalmology, otolaryngology and dermatology [1]. The bactericidal effect of different lasers on gram positive and gram negative bacteria is demonstrated by distinct authors' worldwide [2]. Carbon dioxide (CO_2) laser has been extensively used in the next two decades as an incision tool in increasingly wide areas, such as neurosurgery, dermatology and plastic surgery, otorhinolaryngology, ophthalmology, gynecology, and general surgery [3]. It is able to treat safely epidermis and dermis because the wound can be restored quickly and easily from the surrounding normal skin though injured at epidermis and dermis by laser [4].

Wound infection is one of the health problems that are caused by the invasion of pathogenic organisms in different parts of the body. In the developing countries, large number of people die daily of preventable and curable diseases such as wound infections [5]. Burns are among the main causes of death of humans in the world. The World Health Organization reports that over 90% of burns occur in developing or underdeveloped nations, where the mortality for large burns (over 40% total body surface area) approaches 100%[6]. Infections are considered to be one of the most important and potentially serious complications in people with burns[7]. Burn wounds are a suitable site for multiplication of bacteria within 48 hrs and are more persistent richer sources of infection than surgical wounds, mainly because of the larger moist exposed area involved and longer duration of patient stay in the hospital [8]. The infection of burn wounds with multiple organisms, with super added problem of drug resistance, illustrates the need for a drug policy by the hospitals for burn and wound patients. The isolated bacteria exhibits multiple resistant to antibiotics [9].

P. aeruginosa is an opportunistic pathogen that causes various infections [10]. It is a major cause of nosocomial infection [11]. *P. aeruginosa* plays a prominent role as an etiological agent of serious infections in patients with burn wounds [7]. *P. aeruginosa* has been selected due to their high prevalence in burn wound and surgical site infections. These species of bacteria have caused higher rate of morbidity in patients due to the high antibiotic resistance pattern towards the traditional use of antibiotics. Therefore, the use of CO_2 laser which is independent towards the antibiotic resistance pattern of bacteria could prove beneficial to treat the burn and wound infection. The current study focuses on in-vitro effectiveness of CO_2 laser to kill this bacterial species.

Materials and Methods:

Specimen Collection and Bacterial Identification

The cotton swabs are collected from burned wound and surgical site infections cultured on blood agar and MacConkey agar and subjected for standard bacteriological procedures including morphological, biochemical and API 20E diagnosis [12].

Antibiotic Susceptibility Test:

Antibiotic susceptibility test is performed by Kirby-Bauer's disk diffusion technique as follows: A sterile cotton swab is dipped into bacterial suspension matching to the McFarland solution (1.5×10^8 CFU/ml) for each isolates and streaked it in three directions on the surface of Muller-Hinton agar plates then left (5-10) min on room temperature. By using a sterile forceps, the selected antibiotics (12 antibiotics 6 in each plate) are put on the surface of plate and left for five minutes, incubated at 37°C for (18-24) hrs then the zones of

inhibition are measured in millimeter by using ruler [13].

Detection of Some Virulence Factors:

Detection of Haemolysin Production

Blood agar plate is streaking with a single colony of an overnight growth from brain heart infusion agar and incubated at 37 °C for (18-24) hrs. The appearance of cleared zone indicates a positive test, as described by [14].

Detection of Protease Enzyme:

A single colony of an overnight growth from brain heart infusion agar is cultured on skimmed milk agar by picking of the colony, incubated at 37°C for (24-48) hrs. The appearance of cleared hydrolysis zone indicates a positive test, as described by [14].

Detection of Lipase Enzyme:

Egg yolk agar is inoculated with a single colony of an overnight growth from brain heart infusion agar and incubated at 37°C for 48 hrs [14].

Detection of Extracellular Pigments Production

Pseudomonas agar medium is used to detect the bacterial isolates ability to produce the extracellular pigments pyocyanin and pyoveridin. This medium is inoculated with a single colony of an overnight growth from brain heart infusion agar and incubated at 37°C for (18-24) hrs.

Biofilm Formation Assay:

The biofilm formation assay is achieved according to the method described by [15]. The results are interpreted depending on the classification of bacterial adherence and biofilm formation by the Tissue Culture Plate (TCP) method.

CO_2 Laser System

The CW CO_2 laser system (DS-40 U, Daeshin Enterprise co., Ltd., Korea) emits laser light at 10600 nm, IR light. The power densities are (2000, 2500 and 3000) W/cm² and the exposure time is (60 and 90) second.

Selection of Bacterial Isolates for CO_2 Laser Irradiation

Six isolates of *P. aeruginosa* are chosen for CO_2 laser irradiation according to the results of antibiotic susceptibility test and virulence factors, where three isolates were resistant to antibiotics and the other three isolates are sensitive to antibiotics in order to compare the effect of CO_2 laser irradiation in all the chosen bacterial isolates.

Irradiation procedure

Bacterial colonies are picked up from the brain heart infusion agar to a test tube containing 9 ml of normal saline then mixed by vortex to get homogenous suspension compared with the McFarland solution (1.5×10^8 CFU/ml). Standard suspension of bacterial growth with dilution of (10^{-6} viable cell/ml) is chosen from the other serial dilutions for *P. aeruginosa* irradiation, 400 µl of this suspension is placed in a sterile Eppndroff tube. The irradiation experiments are done in sterilized hood and the hand piece of CO_2 laser is fixed perpendicularly on the opening of the Eppndroff tube. The bacterial suspension is subjected to laser irradiation experiment at different power densities (2000, 2500, and 3000 W/cm^2) and the exposure time is (60 and 90) second. After irradiation, 100 µl of the irradiated suspension is spread on the surface of brain heart infusion agar plates for each isolate and incubated aerobically at 37 ºC for (18-24) hrs, three replicates are used for each bacterial isolate. After incubation, the viable cells count CFU/ml is determined by using of the digital colony counter [16].

Results and Discussion

The results of *P. aeruginosa* isolation from burn and surgical site infections in 2013 reveal that *P. aeruginosa* is the most common bacteria isolated from burn and wound infections at

percentage 55% and 45% respectively, as shown in (Figure1). This result is in accordance with those gathered from other studies in Iraq as a study by [17] who finds that these percentages are 51.9 % and 40.4 % respectively, and [18] who finds that *P. aeruginosa* is the most common pathogen isolated from burn and wound infections with a percentage of 70% and 60% respectively. [19] Claims that *P. aeruginosa* represents 52.2% of all pathogens isolated from contaminated wounds and burns infections. [20] Reports that *P. aeruginosa* is the most frequently isolated pathogen in burn wound infections. [21] Reveals that *P. aeruginosa* is a common isolate representing 66.7% of isolates cultures of infected burns. The result of this study reveals that the most common bacteria isolated from burn and wound infections is *P. aeruginosa*, this could be as a result of the fact that healthcare workers carry this microorganism in their wears and stand the chance of transmitting them to immune compromised burn wound patients. Also, this organism is opportunistic and can only cause infection in patients with breached immunity; this can also be seen in wounds caused by trauma or open wounds [22].

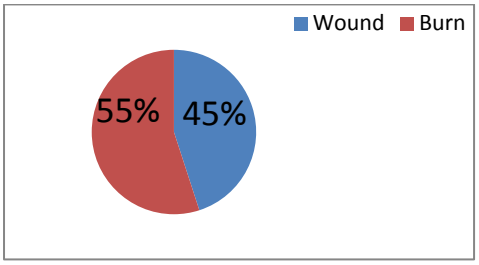

Fig. 1: The Percentage of *Pseudomonas aeruginosa* Isolation among Wounds and Burns Infections.

The results of the antibiotics resistance percentage of *P. aeruginosa* isolates in (Table 1, Figure 2), show that the percentage of multidrug resistance of *P. aeruginosa* isolates which mean resistance to three or more than three antibiotics is 100%. This rate is in agreement with the study of [23] and [24] who noted that 95.7% and 96% of *P. aeruginosa* isolates are multi-drug resistance. Multidrug resistance is frequent, and clinical isolates resistant to virtually all anti-pseudomonal agents are increasingly being reported. It has a natural resistance mechanism to many antibiotics because of a resistance transfer plasmid, extra genetic material carried in the cells with genes that code for proteins that destroy antibiotic substances. However, acquired antibiotic resistance of *P. aeruginosa* during treatment is a common phenomenon [25].

Table 1: The Antibiotics Resistance Percentage of *P. aeruginosa* Isolates against 12 Antibiotics

Antibiotic	Code	Resistant rate% (No. of the isolates)
Carbencillin	PY	100 (20/20)
Cefepime	FEP	100 (20/20)
Ceftriaxon	CRO	100 (20/20)
Amikacin	AK	70 (14/20)
Ceftazidime	CAZ	65 (13/20)
Gentamycin	CN	65 (13/20)
Tobramycin	TOB	65 (13/20)
Ciprofloxacin	CIP	55 (11/20)
Norfloxacin	NOR	55 (11/20)
Impenem	IPM	50 (10/20)
Piperacillin	PRL	50 (10/20)
Aztreonam	ATM	30 (6/20)

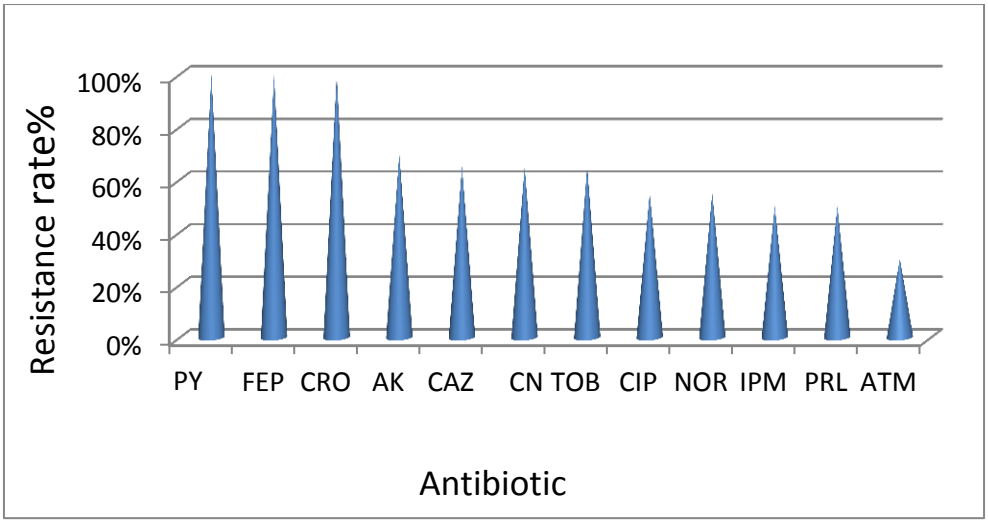

Fig. 2: Resistance Rates of *P. aeruginosa* to 12 Antibiotics

PY: Carbencillin ,FEP: Cefepime, CRO: Ceftriaxone, AK: Amikacin ,CAZ: Ceftazidime , CN: Gentamycin, TOB: Tobramycin, CIP: Ciprofloxacin, NOR: Norfloxacin , IPM: Impenem, PRL :Piperacillin, ATM: Aztreonam.

The results of detection of some virulence factors of *P. aeruginosa* display that it has many virulence factors associated with its pathogenicity. The results of virulence factors of the *P. aeruginosa* isolates reveal that all of the isolates are positive for haemolysin, protease, lipase enzymes and extracellular pigments, as shown in Figure 3, while 95% of the isolates are biofilm producer. In relation to protease production, the results outline that all of isolates have protease enzyme and this result like those of [26] who find that 85% of *P. aeruginosa* isolates have protease enzyme and [27] states that the vast majority of *P. aeruginosa* strains are shown to possess protease enzyme. Regarding the results of lipase production, the results reveal that all *P. aeruginosa* isolates are positive for lipase enzyme, this result like those of [20] who finds that all tested isolates of *P. aeruginosa* have lipase enzyme. The results of the current study show that all *P. aeruginosa* isolates are pigments producer. A similar finding is reported by [28] study where the rate of pigments production is 80%. The result of biofilm formation test reveals that 95% of the bacterial isolates are biofilm producer, this result is close to [29] who reports that 87.5% of *P. aeruginosa* isolates have the ability to form alginate biofilm while this rate decreases to 68.7% and 66% in [20] and [26] studies. Six isolates are chosen for CO_2 laser irradiation according to the results of antimicrobial susceptibility and virulence factors.

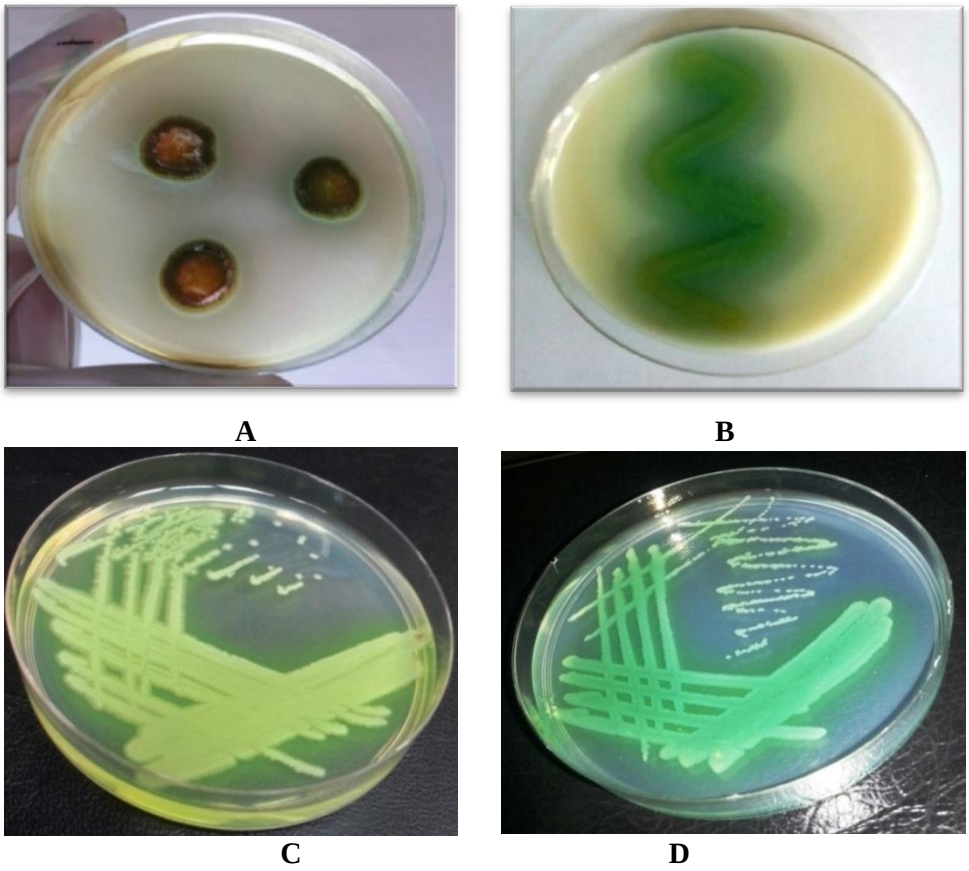

Fig. 3: Positive Result for: A, Lipase Enzyme Production on Egg-yolk Agar. B, Protease Enzyme Production on Skim Milk Agar. C and D, Pigments Production on Pseudomonas Agar (C, Pyoveridin Pigment, D, Pyocyanin Pigment)

After CO_2 laser irradiation, the results show a reduction in CFU/ml of *P. aeruginosa* isolates as compared with control group, as shown in (Table 2 and3, Figure 4 and 5). According to the results of the statistical analysis by using analysis variance of (ANOVA) one way and least significant differences-LSD test, it is found that there are statistical significant differences in the bacterial number between power densities (2000, 2500 and 3000)W/cm²and exposure times (60 and 90) second. The highest statistically significant differences ($P<0.001$) are detected between exposure times (60 and 90) second compared with each other and with control as well as the power densities. Generally, reduction in the viable number of *P. aeruginosa* isolates is observed with the increase of the power densities and exposure time. These results agree with the results of a study by [30] who finds that CO_2 laser has a bactericidal effect on pathogenic bacteria, and also agree with the study of [31] who reports that CO_2 laser kills 100% of *Staphylococcus aureus* at power 6W and exposure time 10 ms while 97% of the bacteria are killed at power 4W and exposure time 10 ms. In a study by [32] they reveal the bactericidal effect of CO_2 laser by using power 2W, Continuous Wavelength (CW) for decontaminating *P. gingivalis* bacteria and the results show a significant reduction. [33] Demonstrates the bactericidal effect of CO_2 laser on *S .sanguinis* and *P. gingivalis* at power 2W, exposure time 10 sec and power 4W, exposure time 60 sec.

Table 2: Results the Effect of CO_2 Laser Irradiation on the Mean Value of Viability of *P. aeruginosa* Resistant Strains (P12, P16, P18) and Killing Percentage % at Exposure Time (60 and 90) Second.

Power density W/cm²	P12					P16					P18				
	Control CFU/ml	60sec Mean	%	90sec Mean	%	Control CFU/ml	60sec Mean	%	90sec Mean	%	Control CFU/ml	60sec Mean	%	90sec Mean	%
2000	87	27	68.96	14	83.91	270	168	37.78	9	96.67	68	28	58.82	5	92.65
2500	87	8	90.81	0	100	270	15	94.44	0	100	68	0	100	0	100
3000	87	0	100	0	100	270	8	97.04	0	100	68	0	100	0	100

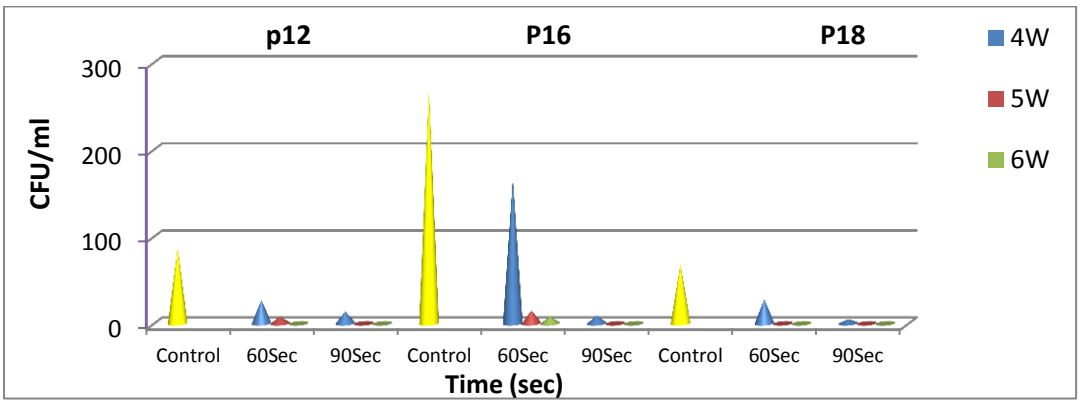

Fig. 4: The Reduction in CFUs/ml of *P. aeruginosa* Resistant Strains (P12, P16, P18) after CO_2 Laser Irradiation Using Output Power from (4-6)W with Exposure Times (60 and 90) Sec Corresponding to Power Densities (2000, 2500 and 3000) W/cm^2 .

Table 3: Results the Effect of CO_2 Laser Irradiation on the Mean Value of Viability of *P. aeruginosa* Sensitive Strains (P7, P9, P10) and Killing Percentage % at Exposure Time (60 and 90) second.

Power density W/cm²	P7					P9					P10				
	Control CFU/ml	60sec Mean	%	90sec Mean	%	Control CFU/ml	60sec Mean	%	90sec Mean	%	Control CFU/ml	60sec Mean	%	90sec Mean	%
2000	112	27	75.89	0	100	280	189	32.5	21	92.5	157	75	52.23	8	94.90
2500	112	0	100	0	100	280	45	83.93	6	97.86	157	3	98.09	1	99.36
3000	112	0	100	0	100	280	18	93.57	0	100	157	8	94.90	1	99.36

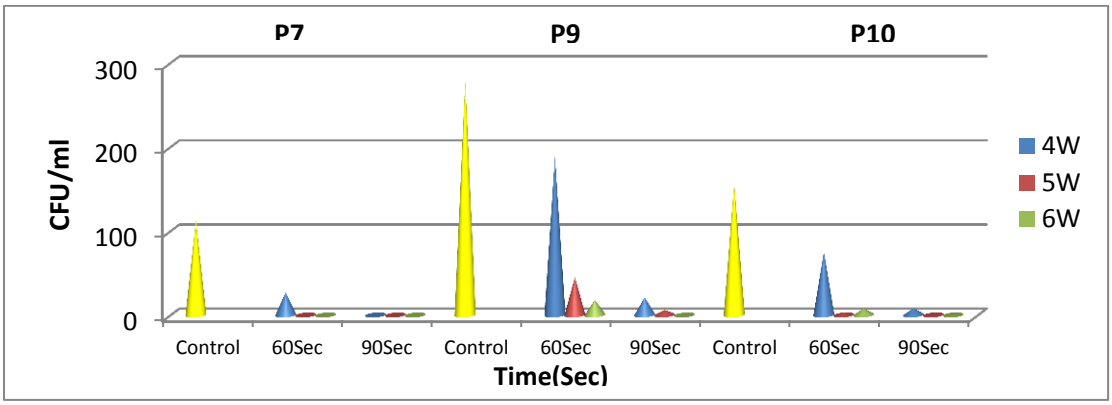

Fig. 5: The Reduction in CFUs/ml of *P. aeruginosa* Sensitive Strains (P7, P9, P10) after CO_2 Laser Irradiation Using Output Power from (4-6)W with Exposure Times (60 and 90) Sec Corresponding to Power Densities (2000, 2500 and 3000) W/cm^2 .

Generally, reduction in the viable number of all the bacterial isolates is observed with the increase of the power densities and exposure time this may be due to the thermal effect of CO_2 laser. When the energy of the CO_2 photons is absorbed by water which is considered the main light-absorbing molecules (in the bacterial cell water the main component is affected by CO_2 laser) it will be converted into heat energy then the heat will diffuse causing a rise in the temperature in the surrounding tissue.which can cause a range of thermal effects from tissue coagulation to vaporization [34]. As the temperature is raised, the large, specially configured molecules necessary for life are shaken open. Most proteins, DNA, RNA, membranes and their integral structures start to unwind or melt at temperatures ranging from (40-100) °C, the result is denaturation or loss of function [35]. The results of antibiotics sensitivity test after CO_2 laser irradiation show no changes in diameter of inhibition zone between the irradiated isolates of *P. aeruginosa.* It has been found that CO_2 laser does not have any effect on the ability of the tested bacterial isolates to produce haemolysin, protease, lipase, coagulase enzymes , extracellular pigments and biofilm formation this may be due to the fact that the production of these enzymes is correlated with the bacterial chromosome and not easily effected by the short exposure time of laser irradiation, where the laser does not cause any mutation in the bacterial chromosome

Conclusion:

The current study concludes that CO_2 laser has a bactericidal effect on *P. aeruginosa* without any effect on its antibiotics susceptibility and virulence factors. Reduction in the viable number of *P. aeruginosa* isolates is observed with the increase of the power densities and exposure time.

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ألتأثير القاتل لليزر ثاني اوكسيد الكاربون على بكتريا الزوائف الزنجارية المعزولة من اصابات الجروح والحروق- خارج جسم الكائن الحي

ايمان ناطق ناجي* **عقيلة عبد الزهرة علي ****

بيداء فليح حمزة***

*الجامعة المستنصرية/كليةالعلوم- قسم علوم الحياة
**دائرة مدينة الطب/قسم المختبرات التعليمية
***الجامعة المستنصرية/كلية طب الاسنان- قسم امراض و جراحة ماحول الاسنان

الخلاصة:

تضمنت الدراسة جمع 60 مسحة من مرضى مصابين بأخماج جروح العمليات و جروح الحروق من مستشفى بغداد التعليمي ومستشفى الحروق التخصصية في مدينة بغداد للفترة من شهرايلول ولغاية شهرتشرين الثاني لسنة2013. زرعت المسحات على وسطي اكار الدم واكارالماكونكي واخضعت للتشخيص البكتريولوجي بأستخدام المعايير البكتريولوجية القياسية. تم تشخيص 20 عينة من مجموع 60 عينة على انها تعود للنوع البكتيري *Pseudomonas aeruginosa* اعتمادا على الطرق التقليدية.اظهرت نتائج اختبار فحص الحساسية للمضادات الحياتية ان نسب مقاومة عزلات بكتريا *Pseudomonas aeruginosa* للمضادات الحياتية كانت كالاتي: 100% (20/20) لمضادات, Carbencillin Cefepime وCeftriaxone و70% (14/20) لمضاد Amikacin و 65 % (13/20) لمضادات ,Tobramycin Cftazidime وGentamycin و55% (11/20) لمضادي Ciprofloxacin و Norfloxacin و50% (10/20) لمضادي Piperacillin وImpenim و 30% (6/20) لمضاد Aztreonam . تم اختبار بعض عوامل ضراوة العزلات البكتيرية(انزيمات الهيمولايسين والبروتيز واللاييز والصبغات خارج الخلوية) والقابلية على تكوين الاغشية الحيوية واظهرت النتائج ان جميع العزلات البكتيرية كانت منتجة لانزيمات الهيمولايسين والبروتيز واللاييز والصبغات خارج الخلوية بينما 95% من العزلات اظهرت القدرة على تكوين الاغشية الحيوية. اختيرت ست عزلات للتشعيع بCO_2 ليزر اعتمادا على نتائج اختبار فحص الحساسية للمضادات الحياتية وعوامل الضراوة عند كثافة الطاقة (2000, 2500 و 3000) واط/سنتمترمربع وزمن تعرض (60 و90) ثانية. اظهرت نتائج التشعيع ان CO_2 ليزرادى الى تناقص في قيمة CFU/ml لبكتريا *P. aeruginosa* وبزيادة كثافة الطاقة وزمن التعرض واوضحت نتائج التحليل الاحصائي بأستخدام تحليل التباين بأتجاه واحد واقل فرق معنوي ان هناك فروقات معنوية في قيمة CFU/ml بين مختلف كثافة الطاقة وزمن التعرض. بعد التشعيع اجري فحص الحساسية للمضادات الحياتية وعوامل الضراوة للعزلات المشععة. استنتج من الدراسة ان CO_2 ليزر له تأثير قاتل على بكتريا *Pseudomonas aeruginosa* دون التأثير على حساسيتها للمضادات الحياتية و عوامل ضراوتها.

الكلمات المفتاحية: بكتريا الزوائف الزنجارية، اختبار فحص الحساسية للمضادات الحياتية، عوامل الضراوة، ليزر ثاني اوكسيد الكربون.