The Influence of Helium- Neon Laser on Methicillin – Resistant *Staphylococcus aureus* using Photosensitizer(TBO)

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Abstract

The main objective of this study is to determine whether the use of He-Ne laser 632.8 nm, 7.5mW in combination with toluidine blue O (TBO) is an effective tool to eradicate MRSA, and the effect of laser light on the antibiotics sensitivity in vitro.

Ten isolates were methicillin-resistant ***Staphylococcus aureus***, after testing for their sensitivity to oxacillin 6µg/ml screening test and cefoxitin 30µg antibiotic disk.

Following exposure to laser light with a wavelength of 632.8 nm in the presence of TBO at a concentration of 50µg/ml at various exposure times (5 min , 10 min , 15 min), highly significant statistical reduction in the viable count was achieved at the three exposure times , but 100% killing of cells was observed at exposure time of 15 min.

While in the absence of the photo sensitizer there is no significant effect of the laser irradiation neither on the viable count at different exposure times observed, nor on changing their sensitivity to antimicrobial agents.

Introduction

Many lasers have been used succesfully for treating many cases of infection that caused by bacteria such as ***E.coli, Staphylococcus aureus, Pseudomonas aeruginosa.***

Throughout recorded history, humans have suffered infections caused by ***Staphylococcus aureus.*** In the fact ***S. aureus*** is currently one of the most common cause of infections in hospitalized patients.(1)

Colonization, occurs when the ***Staphylococcus*** bacteria are present on the body without causing illness. Approximately 25% to 30% of population is colonized in the nose with ***S.aureus*** or methicillin resistant ***Staphylococcus aureus*** (MRSA) bacteria at a given time (2).

The field of antimicrobial chemotherapy is one of the most constant challenge, particularly in view of the rapid evolutionary changes and wide variety of

pathogens encountered. [3] In the past decades great advances have been made against microbes with the advent of agents such as B-lactam antibiotics , but since ***S. aureus*** had acquired resistance to penicillin , semi synthetic penicillin derivative and a wide variety of antimicrobials , there has been a continuing battle to manufacture new antibiotics that are successful in inhibiting or killing MRSA , because of both the diversity and the severity of the infections caused by these organisms.[4]

Since methicillin resistance is now widespread in hospitals all over the world , therapy has become cumbersome .

Anew and even more threatening development is the emergence of strains with reduced susceptibility to glycopeptides. [5] It has been shown by several Iraqi studies the prevalence of MRSA in the Iraqi hospitals and it is the source of infection in hospitalized patients. [6] [7] [8] [9]

What is worrying is that MRSA appears to be just as the tip of the iceberg [1] So , the emergence of MRSA strains of **S. aureus** with resistance to multiple antibiotics requires the rapid and more efficient methods for screening patients , colonized or infected by MRSA (since routine oxacillin tests failed to detect all MRSA populations) There is a pressing need for alternative method for detecting MRSA.

Also it requires the development of novel therapeutic strategies for the elimination of ***S. aureus*** from infected wounds and carriage sites. [10]

One possible approach is to use light activated antimicrobial agents to achieve lethal photosensitization of the organism this involves treating the microbe with alight activated chemicals , termed photo sensitizer .

Killing of bacteria in this way has been demonstrated with a wide variety of Gram – positive and Gram – negative organisms , e.g. ***S.aureus*** including MRSA [5] [11]***, Porphyromonas gingivitis*** [12] [13] [14] ***, Streptococcal species*** [15] [16] [17] and ***E.coli***. [18]

MRSA strains of ***Staphylococcus aureus*** are one of the major causes of hospital acquired infection of surgical wounds , and infections associated with indwelling medical devices , throughout the world , causing significant infections and morbidity in many patients. [5] [11] [19]

This bacteria causes a variety of superficial infections occur most frequently and are characterized by intense suppuration local tissue necrosis , and the formation of pus – filled local a abscess. Such as pyoderma (impetigo) , folliculitis , furuncles (boils) , abscesses and carbuncles. [20]

MRSA has emerged as a nosocomial pathogen of major importance in pediatric patients . Infection occurs most often in hospitalized individuals with underlying predisposing medical conditions. [21] Half of all Staph. that circulated in hospitals is resistance methicillin , the standard drug for therapy at that time.[22]

The present work is an attempt to study and quantify the effect of He- Ne (632 .8 nm) laser with the combination of toludin blue O (TBO) at different experimental parameters on the viability &susceptibility to antimicrobial agents MRSA .

Materials and methods

35 samples were taken from the surface of burn, wound, and nasal vestibulum (of medical and non medical staff as a carrier) using a sterile cotton swab. All samples were screened for infection and nasal colonization with S. aureus at Al-Yarmouk teaching hospital units in Baghdad .

The samples were directly inoculated onto sheep blood agar and mannitol – salt agar , incubated at 37C^{o} for 24hr .

Bergys manual of systemic bacteriology (1986) was considered for the final identification of ***S.aureus*** by using Microscope examination, Culture characteristics and Biochemical tests[23].

Identification of MRSA Strains

1)Oxacillin Agar Screening Method:

The inoculum was prepared by making a direct broth or saline suspension of isolated colonies selected from an 18-24h plate (anon- selective medium such as blood agar). The suspension is adjusted to match the 0.5 McFarland turbidity standard. The test was performed by inoculation a ***S.aureus*** isolate on Muller – Hinton agar with Nacl (4% w/v) and that contains 6µg oxacillin / ml. The agar is inoculated using a cotton swab that was dipped into a direct colony suspension equivalent to 0.5 McFarland standard , the plate is incubated no higher than 35C^{o} for 24 hours , and examined carefully with transmitted light for evidence of small colonies (1 colony) , indicating oxacillin resistance. [24]

2)Cefoxitin Disk Diffusion Test

Three to five colonies of the same morphology are selected from an agar plate culture . The growth is transferred into a tube containing 4 to 5 ml of tryptic soya broth , then the broth culture is incubated at 35C^{o} until it achieves or exceeds the turbidity of 0.5 McFarland standard (usually 2-6 hours).

A sterile cotton swab dipped into a suspension of the organism , and pressed against the side of the tube to remove any excess fluid , is streaked on the surface of Muller – Hinton agar for several times in a different directions to ensure complete cover of the plate.

After allowing the plates to dry for approximately 5 minutes , but no more than 15 minutes ,cefoxitins 30 µg disk was applied onto the surface and pressed gently to ensure complete contact with the agar surface.

Finally , the plates were incubated invertely at 35-37C^{o} for 16-18hr.[24]

Susceptibility to antimicrobial agents was determined by the agar disk diffusion method , by procedures outlined in the guidelines of the national comitte for clinical laboratory standards. [24]

Antibiotics used in this study following the agar disk diffusion method were vancomycin , rifampicin and gentamaicin.

A spectral study for the photo sensitizer was done to ensure that the photosensitizer used in this study toludin blue O (TBO) has absorption maximum matches the wavelength of the He-Ne laser (632.8 nm).

This was done by dissolving TBO in a distilled water at the same concentration used in the experiments , and by the use of spectrophotometer , the absorbency of the dye was measured for the visible spectrum range 400-700 nm while distilled water was used as control .

The laser used in this study was a CW Helium – Neon gas laser (Griffin and George , Britain) , with a measured output power of 7.5mW , which emits light in a collimated beam with diameter 4mm and wavelength 632.8nm.

Irradiation of Bacterial Sample for Sensitivity Test

Aliquots (20 µl) of a suspension of the organism , prepared as described above, were transferred to a sterile ependroff tube . Samples were , then , exposed to a measured amount of laser light and for different exposure times (5,10,20 minutes) . Control ependroff tubes were not exposed to the light source.

Following exposure to light, the suspension from each ependroff was plated onto Muller-Hinton agar by a sterile cotton swab through streaking it over the entire sterile agar surface. After applying the drug disks, the plates were placed invertely in an incubator at 35-37 C^o for 18h.

Irradiation of Bacterial Samples in the Presence of TBO

Preparation of TBO

A stock was prepared by dissolving 5 mg of TBO powder in 100 ml distilled water to get the concentration of 50 µg /ml.

The solution was sterilized through a 0.22 Millipore filter paper, and then the solution kept in the dark till use.

Laser treatment

The organism was grown aerobically in tryptic soya broth at 37C^o for 16h, harvested by centrifugation and resuspended in a sterile saline solution 0.85% , then serial dilutions were done.

Aliquots (15 µl) of a suspension of the organism from the desired dilution ($1*10^9$ cfu/ml) were transferred to a sterile ependroff tube and an equal volume of a filtered – sterilized solution of TBO in a saline was added to each ependroff to give final concentration of 50 µg /ml , and mixed together . Samples were then exposed to a measured amount of laser light for different exposure times (5,10,15,20) minutes.

Control ependroffs exposed to laser light in the absence of the photo sensitizer . Further ependroff tubes were considered as a control neither sensitized nor exposed to the light source.

Following exposure to light , the samples were spread over the surfaces of nutrient agar plates , and grown overnight at 37C^o.

Laser Parameter Measurement

The laser parameter for each experiment was calculated as follows:-

$$Power\ density = \frac{Power\ (P)}{Area\ (A)} = W / cm^2$$

Where:-

P=The output power of the laser light. (watt)

A=The exposed area to the laser beam. (cm^2)

Evaluation Criteria

The irradiation samples for sensitivity test , were taken out from the incubator after overnight growing at $37C^o$, the inhibition zones around each antibiotic disk is to be measured and compared with the zone diameter interpretive standard published by NCCLS. [25] from the measures, the susceptibility of the organism for the used antibiotics whether it is sensitive, intermediate or resistant and the effect of laser irradiation on the organism susceptibility will be known.

While the irradiated samples that contain the photo sensitizer , both of the ependroff tubes that used as a control were cultured immediately on nutrient agar and incubated at $37C^o$ overnight , and the total viable count for the samples were calculated.

Results and discussion

35 isolates were identified according to the following results of cultural characteristics, microscopic and biochemical examination .

Cultural Characteristics

Thirty-five isolates (eleven from burn samples, twelve from wound samples , and twelve from carriers) of the suspected ***S.aureus*** species were golden-yellow colonies on nutrient agar and change the color of phenol red from red to yellow in mannitol-salt agar. Colonies on blood agar produced a surrounding transparent zone of beta hemolysis.

Microscopic Examination

Under light microscopic examination after staining with gram stain , they were gram-positive , spherical cells (0.5-1.5 µm) in diameter.

Cells occurring singly or in pairs and in irregular clusters non-spore forming.

Biochemical Test

The results of the biochemical tests for ***S.aureus*** isolates are shown in Table (1)

Table (1) Biochemical tests for ***S.aureus*** isolates.

Biochemical test	Result
Catalase	+
Coagulase	+
Growth on Mannitol Salt agar	+
Acid Production from Sugar Media:	
Sucrose	+
D-trehalose	+
D-ribose	+
Mannitol	+
(+) : Positive Reaction	

Identification of MRSA

All ***S.aureus*** isolates were tested against oxacillin (6 µg/ml), (which considered as a screening test & the routine method for detecting MRSA

Eight of the isolates were MRSA after over night incubation at temperature 30-35 C^o, while on using cefoxitin disk diffusion test (the new & alternative method) the inhibition zone diameters for methicillin – resistant ***Staphylococcus aureus*** (MRSA) and methicillin–susceptible ***Staphylococcus aureus*** (MSSA) isolates were very distinct when using 30 µg cefoxitin disks. Ten MRSA isolates showed cefoxitin inhibition zone diameters of < 27mm, and all MSSA isolates showed larger diameters after incubation for 16-18hr at 37C^o.

According to our results, cefoxitin 30 µg disk diffusion method performed beter than oxacillin screening agar for identifying MRSA. Oxacillin screening agar failed to detect all MRSA isolates correctly . ***S.aureus*** isolate that gave a cefoxitin diameter of < 27mm can be identified as MRSA.

This result is in agood agreement agreed with the result obtained by Felten *eta*l[26] and confirming the result of Skov *etal*[27] who showed in his study that cefoxitin 30 µg disk performed on Iso-Sensitest Agar (ISA) using standard conditions , with a sensitivity 100%, and an interpretive zone diameter of S>29 and R<29. While the difference in the zone diameter ranges in the studies is related to the difference in agar type.

This implies that the cefoxitin disk test is an available alternative to oxacillin screening test at 37C^o.

Susceptibility to Antimicrobial Agents

Ten MRSA isolates were tested for their sensitivity to antimicrobial agents by disk diffusion method.

It has been shown that 70% of the isolates were resistant to Rifampicin 80% were resistant to Gentamicin and the isolates were cefoxitin resistant and oxacillin (6 µg/ml) resistant . Rendering these antibiotics ineffective as a therapeutic agents against MRSA.

Results of our study indicated that isolates which were resistant to all antibiotics, showed sensitivity to vancomycin. Resistant to vancomycin was not observed but, 50% of the isolates were vancomycin intermediate, this made this bacteria a great threat to the public health and hospital environment.

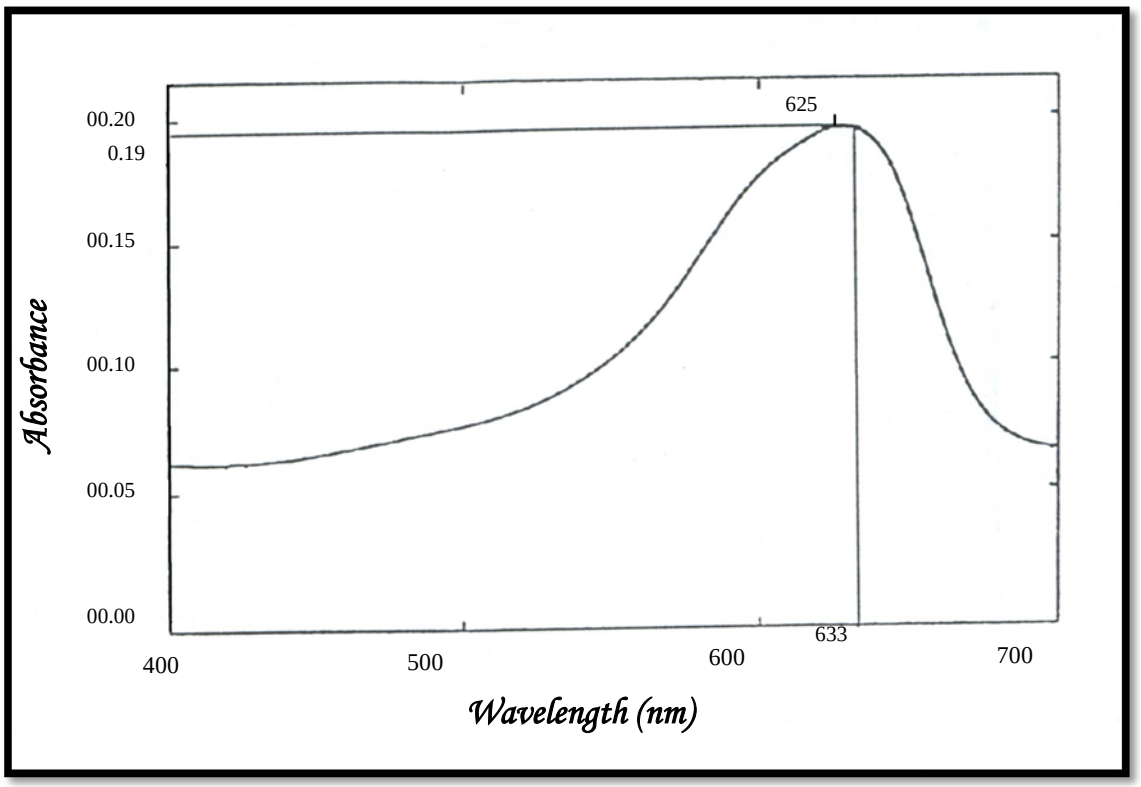
These results agreed with results of Al-Nasiri; 2004[8] and Al-Shkhli :2003[7].

Spectral Study of the Photosensitizer

Spectral study of the photosensitizer was done to ensure that TBo which was used as a photosensitizer in this study, has absorption maximum corresponding to the helium-neon laser. In which this would produce high yields of singlet oxygen when exposed to red light 632.8nm.

The results of this study showed that the peak absorption of this dye was 625nm.

This made toludine blue O is an efficient photosensitizer for photoeradication of methicillin-resistant strains of ***Staphylococcus aureus***. Figure(1) illustrate the absorption spectrum of TBO ranging from 400 to 700 nm and its corresponding absorbancy at wavelength 632.8nm.



Figure(1): The absorption spectrum of TBO

The Effect of Laser Light

After irradiation of bacterial suspension with the He-Ne laser for different exposure times (5,10,20) minutes. The isolates with intermediate resistance to vancomycin before laser irradiation, become sensitive to it , while remaining resistant to the other ones.

On the other hand, the isolates that were sensitive only to vancomycin remain within the sensitivity range even after laser irradiation , and there was no significant change for there susceptibility to the other antibiotics

For the isolates that were sensitive to all antibiotics excluding cefoxitin before exposure to the light source, remain sensitive to them.

Photosensitization Sensitivity Tests:

When 30µl of a suspension of MRSA containing $1*10^9$ cfu was exposed to He-Ne laser light for 5min and 10min in the presence of TBO at a concentration of 50µg/ml, a reduction in the viable count was achieved when compared with a control sample that was neither sensitized nor irradiated [noted as L.(laser light), S.(sensitizer)].

Using MRSA isolates (1,2,3,4) substantial reduction in the viable count was achieved , after exposure to laser light for 5min and 10min, as shown obviously in Fig(2) indicate the reduction of the viable count in different exposure times after laser irradiation

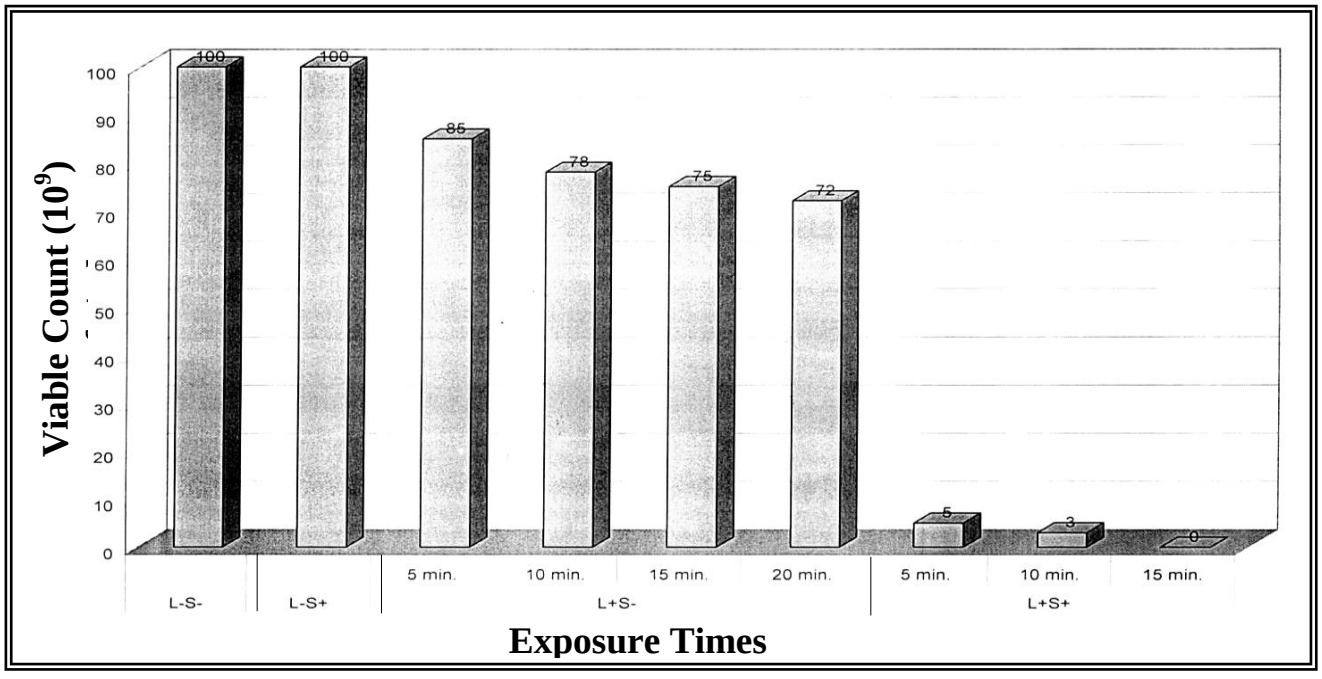


Figure (2):Viable count of suspension of MRSA before and after various periods of exposure to light from a helium / neon laser at power density 59.7 W/cm^2 in the presence and absence of 50µg/ml of toluidine blue O. L-S-; suspensions exposed to neither sensitizer nor laser light; L-S+, exposed to sensitizer in the absence of laser light ; L+S-, exposed to laser light in the absence of sensitizer ;L+S+, exposed to laser light in the presence of sensitizer

The laser light alone has no significant effect on the viability of the organism. Further more without laser light, the dye alone did not cause significant reduction in the viable count.

While for all of the isolates when 30µl of a suspension containing $1*10^9$cfu/ml of the organism was exposed to the red light 632.8nm for 15 min, 100% of the cells being killed.

As a final result, the effect of laser light in combination of a photo sensitizer there was highly significant reduction in the viable count .

The result of this in vitro investigation have demonstrated the efficiency of TBO to photo eradicate methicillin-resistant ***Staphylococcus aureus*** at a concentration of 50µg/ml and exposure times of 5min , 10min and 15min.

In the absence of the photo sensitizer the laser alone has no significant reduction effect in the viable count of the organism , neither the dye alone had a significant effect.

Recently, the rise of the use of light in conjugation with chemical photo sensitizers to treat antimicrobial infections is the subject of several studies.(4)

The present results is in a good agreement with the results obtained by Wilson *etal* (1995)(28), who showed that a great reduction in the viable count of MRSA was achieved using low power laser light in combination with a photo sensitizer.

There have been several reports of the ability of TBO to act as a photo sensitizer for bacteria.

Zanin *etal* (2002)(29) , had demonstrated the ability of killing oral microorganisms by low power laser light in the presence of a photo sensitizer.

(Al-Nuami: 2004)(18) found that three kinds of Gram negative bacteria (***Escherichia coli***, ***Pseudomonas aeruginosa*** and ***Salmonella typhi***) species could be sensitized by He-Ne laser light in the presence of the dye.

A study by (Al-Amirry :2003)(16) showed a great effect of low power laser light in combination of a photo sensitizer on ***Streptococcus mutans*** species.

The expected mechanism to make an effective action on the bacteria is photodynamic therapy where the criteria (Low power density, wavelength and exposure time are available).

When the photo sensitizer is illuminated with a light from low-power laser with an appropriate wavelength , it will be excited to a higher energy state, when falling back to the lower energy state , the emitted energy will react with cellular oxygen or/and other cellular components to produce reactive species such as singlet oxygen and free radicals. The site of action for the cytotoxic species produced during lethal photosensitization has been investigated in a number of studies, the three main sites are cell membrane, the nucleus and organelles.(14)

Increasing ion permeability and loss of fluidity is a result of the transfer of the triplet state photo sensitizer energy to molecular oxygen, forming the singlet oxygen which is the main bactericidal species, and cause lipid per oxidation,

which is a highly detrimental to cell membrane structure and function and cause cell death.[30] [31]

Since, singlet oxygen generated in the photosensitization process, has a very short life time and limited diffusion distance[11], this will ensure localization of its toxic effect to the microbes in the treated region only.

conclusions

1)Exposure of MRSA isolate to a laser light alone has no significant effect neither on the viability of bacteria, nor in changing its susceptibility to antimicrobial agents.

2) This investigation has demonstrated that TBO is an effective photo sensitizer for sensitization of MRSA.

3) Large numbers of the organism were killed with the use of TBO in a concentration of 50µg/ml, when low power density 59.7 W/cm^2 is used in (5 & 10) minutes exposure times.

4) The reduction amount of MRSA is 100% when using 50µg/ml TBO in combination of laser light for fifteen minutes.

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تأثير ليزر الهليوم – نيون في بكتريا المكورات العنقودية الذهبية المقاومة للميثسيلين باستعمال متحسس ضوئي(التوليدين الازرق)

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الخلاصة

هدف هذه الدراسة الخارج خلوية تحديد امكانية استعمال ليزر الهليوم– نيون (ذو الطول الموجي ٦٣٢,٨ نانوميتر، وبقدرة ٧,٥ ملي واط). وبوجود المتحسس الضوئي التوليدين الازرق (TBO) كأداة فعالة لقتل بكتريا المكورات العنقودية الذهبية المقاومة للمثسسيلين وفعاليتها في تغيير حساسيتها للمضادات الجرثومية.

أظهرت نتائج اختبارات الحساسية للمضادات الجرثومية الاوكساسلين والسيفوكستين ، عشر عزلات من بكتريا المكورات العنقودية الذهبية مقاومة للمثيسيلين.

أظهرت نتائج اختبارات الحساسية للاشعاع الليزري بطول موجي ٦٣٢,٨ نانوميتر بوجود المتحسس الضوئي التوليدين الازرق (TBO) وبتركيز ٥٠ مايكروغرام لكل مليلتر تناقص عال وملحوظ في اعداد الخلايا الحية للبكتريا وفي مختلف اوقات التعريض(١٥،١٠،٥) دقيقةً . وكانت نسبة القتل ١٠٠% عند زمن تعريض ١٥ دقيقةً.

بينت نتائج اختبار حساسية البكتريا للاشعاع الليزري بغياب المتحسس الضوئي التولدين الازرق (TBO) ، عدم وجود أي تغيير ملحوظ على عيوشية هذه البكتريا فضلا عن عدم تغيير حساسيتها للمضادات الحيوية في مختلف اوقات التعرض.

استنتج من هذه الدراسة انه استعمال ليزر الهليوم–نيون بوجود المتحسس الضوئي التوليدين الازرق (TBO) افضل طريقة منجزة لإزالة بكتريا المكورات العنقودية الذهبية المقاومة للميثسيلين.