Effect of MRSA Irradiation by 632, 532, and 405 nm (Red, Blue, and Green) Diode Lasers on Antibiotic Susceptibility Tests

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Abstract

Background: Since the discovery of penicillin and till now, antibiotics are considered the most important regime in treating bacterial infections, insipid the fast development of bacterial resistance to many antibiotics over decades. Methicillin resistance *Staphylococcus aureus* (MRSA) is one of these bacteria types that emerged resistance to all current antibiotic classes. Fortunately, many studies proved phototherapy as a promising supporting to antibiotics in clinical fields.

Objectives: The purpose of this study is to evaluate the effect of three lasers wavelengths (red, blue, and green) with 50 mW power density on the susceptibility of clinical MRSA isolates.

Materials and methods: 45 clinical identified MRSA isolates are exposed to 632, 532, and 405 nm (red, blue, and green) Diode lasers. The susceptibility tests with 12 antibiotics are determined by disk diffusion method and minimum inhibition concentration (MIC) tests.

Results: The results demonstrate an increasing in the clear zone of antibiotics for all the isolates, especially for the red wavelength which was more effective, rather than the blue and green lasers which come next respectively. The effective inhibition doses of the antibiotics are decreased for lasered MRSA. The red wavelength is again more effective than the two others lasers.

Conclusion: the visible laser wavelength (red, blue, and green) improved the *in vitro* action of the antibiotics against MRSA.

Keywords: Methicillin resistance *Staphylococcus aureus* (MRSA), Diode Laser, disk diffusion test, minimum inhibition concentration (MIC) tests.

Introduction:

Staphylococcus aureus is an important cause of serious infections in the hospitals as well as the community, mean while the increasing rates of methicillin resistance *S. aureus* (MRSA) strains among *S. aureus* species have been a cause for concern (1, 2). Unfortunately, this pathogen has been particularly efficient at developing resistance to antimicrobial agents, such as when it acquired *mecA* gene. This gene makes them resistant to all beta-lactam antibiotics (3, 4, 5). This is why many physicians are concerned that several bacterial infections may soon become untreatable (1, 2, 4). This growing problem has become a major clinical and public health case nowadays as this rapid development is faster than the production of new antibiotic generations. As a reflex, many researchers are prompted to find other alternative antimicrobial approaches to which it is hypothesized that bacteria will not be easily able to develop resistance, and one of these was the laser light. (6, 7, 8, 9)

The laser light is characterized by its monochromatisity, coherence, directionality which makes it efficient for many medical fields. The Diode laser device is one of these lasers that are widely used for many reasons, including lower cost, availability in a variety of wavelengths region of the spectrum, narrow emission band. Furthermore, the arrays can be constructed in various sizes to accommodate large areas without generating any heat, which may cause unwanted tissue damage. (8, 10)

Many publications have been performed to determine the action of low-intensity visible laser light on various cells, and this action depends on many factors, two of these important factors is the laser wavelength, and the presence of photoreceptor (a molecule within the cell) in the target cell, which are usually cytochromes (11, 12, 13). These components in the respiratory chain of the bacteria will absorb the energy of the specific wavelength of that laser leading to changes in their redox properties and causing an alteration the metabolic statues of the cell (6, 11, 12, 14). Many studies claim blue, red and near IR wavelength parts of the spectrum for their responsibility in killing various pathogens (6, 12, 15, 16). Kim *et. al* reported the inhibition effect of 625 nm laser on *Ps. aeruginosa* , *E. coli*, and *S. aureus* (6). Other researches combined laser treatment with antibiotic to enhance the susceptibility of *Ps. aeruginosa*, this phathogen became more sensitive to Gentamycin after 532 nm laser irradiation (8). In another experiment Bumah *et. al* studies the bactericidal effect of 470 nm light and hyperbaric oxygen (HBO) on MRSA, ending to a bacteria growth

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suppression even when the HBO does not act synergistically with 470-nm light (7).

This paper aims to study the effect of three different laser wavelengths (blue, green and red) on the susceptibility of MRSA isolates to 12 antibiotics, and evaluating the differences in the results among these three lasers combining antibiotics upon these bacteria, and could this photo-effect enhance the treatment of this pathogenic bacteria or not.

Material and methods:

Bacterial isolates: 45 clinical identified MRSA isolates (urine, sputum, wounds, and nasal swabs) have been used in this experiment; all of them were previously preserved in nutrient broth with glycerol at -18 °C till the experiment date (17). The bacteria were inoculated in brain heart infusion agar (HIMEDIA, India) by quadrant streaking (17) to obtain single colonies preparing for laser treating.

Lasers parameters: Three different Diode lasers (Shenzhen Kelly Long Technology Co.) were used to irradiate MRSA isolates, these lasers have different wavelengths which were, 405 nm, 532 nm, and 632 nm. The output power was 50 mW for all these laser devices.

Photo- irradiation: In June 2015, the irradiation step for each laser was done by fixing the laser device perpendicularly on a stand; the laser's beam opening was directed above the surface of an opened cultivated plate. At each laser treatment run, the beam was pointed to a single 48 hr old colony for 10 min. The distance between the laser and colony on agar plate surface was 0.5 cm, the beam diameter was 0.4 cm (for the three lasers). The power density calculated by dividing laser power to the beam's exposed area (8, 16), and that will be 0.4 W/cm2.

Disk Diffusion Test: After laser irradiation each irradiated colony was transferred and spread on a Mueller-Hinton agar (HIMEDIA, India) plate. Then the antibiotic disks placed on the surface of the agar and incubated for 24 hr at 37 °C. The following are the 12 antibiotics that were used in this test: Vancomycin, Rifampin, Gentamycin, Fusidic acid, Clindamycin, Imipenem, Azithromycin, Tetracycline, Trimethoprim-sulfamethoxazole, Neomycin, Chloramphenicol, and Linezolid (Mast Group LTD. UK). The diameter of the clear zone was recorded. The susceptibility was determined according to the manufacturer's recommendations (18).Two additional plates for each isolate were prepared; the first plate is for non irradiated isolate as a negative control, while the second plat, was for irradiated isolate without antibiotic disks for the next MIC test (positive control).

The Minimum Inhibition Concentration Test (MIC): The antibiotics standard powders were obtained from Mast Group. The stock solutions and macrodilution ranges for each antibiotic prepared as described by Andrews (19). The inoculum of bacterial suspension was prepared by harvesting bacterial growth (from the positive control plate) with a sterile spreader to a glass test tube with 5 ml Mueller-Hinton broth (HIMEDIA, India). The tube was shaken by vortex before incubation for 30 min at 37 °C. Next, the growth suspension was adjusted to 0.5 McFarland standard solution (18) which is equal to 107 CFU/ml.

In a 96-well sterile microtiter plat the rows (12 well tray) labeled for the antibiotic dilution solutions, while the columns (8 well tray) labeled for the tested antibiotics. In the wells, 100 µL of each antibiotic dilution and 100 µL of bacterial inoculum solution were dispensed; a positive control (non irradiated bacteria) was also included in this test for each isolate. The microtiter plat was covered and incubated at 37 °C for 24 hr. The result determined as the lowest concentration of antibiotic at which there is no visible growth (19).

The three previous tests were done in duplicating run to ensure accurate results. Least significant difference –LSD test was used to significant compare between means in this study.

Results

The results of **Disk Diffusion test** are shown in Fig. (1). The effect can be clearly noticed after the illumination of the isolates with red, blue and green laser light against 12 antibiotics as compared with control. An increasing in the clear zone diameters of all antibiotics are mostly significant after lasers illumination, insipid the differences in the susceptibility of MRSA to those antibiotic. The 632 nm laser wavelength (red column) appears to be more effective than the other two lasers (the columns in Figure (1) represent the average values of clear zone's diameters of the 45 isolates for each laser wavelength). Mean while the blue laser (405 nm) comes next in its effectiveness to MRSA. The last order is for the effect of wavelength 532 nm laser in comparison with control (non irradiated samples).

The susceptibility average of MRSA isolates to the antibiotics Vancomycin, Rifampicin, Fusidic asid, Trimethoprim-sulfamethoxazole, and Chloramfenicol in this study appears to be sensitive in controls, after irradiation the sensitivity increases. Even though, the different rates for each wavelength are not equal for the tested antibiotics, or for the 3 lasers on the same antibiotic. On the other hand, the means of the control isolates are resistant to Gentamycin, Clindamycin, Imipenem, Azithromycin, Tetracycline. After lasers irradiation, they remain resistance even with the increasing in clear zone diameter. An exception is noticed in Neomycin and Linezolid (Fig 1).

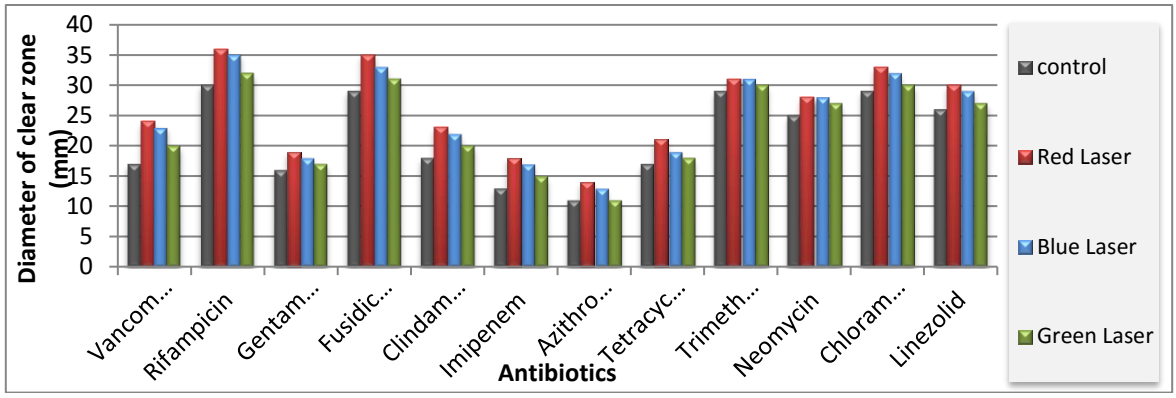

Figure 1. Effect of 632 nm (red), 405 nm (blue), 532 nm (green) lasers on disk diffusion test of irradiated MRAS (each column is the average value of the antibiotic's clear zone diameter).

The percentages of MRSA sensitivity test to the 12 antibiotics (Table-1). All isolates are sensitive 100% to Vancomycin and Linezolid before and after red, blue, and green lasers treatment. A slight increasing in all other antibiotics are noticed after illuminations and according to these results its appeared that the visible laser beams (red, blue and green) enhances the susceptibility of MRSA to antibiotics. (Table-1).

Table 1. Antibiotic Sensitivity Percentage of Illuminated MRSA Isolates by Red, Blue, and Green Lasers.

Laser / Antibiotic	Control (non irradiation) %	632 nm (red) %	405 nm (blue) %	532 (green) %
Vancomycin	100	100	100	100
Rifampicin	85	94	92	88
Gentamycin	9	15	13	10
Fusidic acid	90	97	95	92
Clindamycin	16	23	21	18
Imipenem	15	28	23	18
Azithromycin	6	16	13	9
Tetracycline	10	19	18	14
Trimethoprim-sulfamethoxazole	88	80	83	84
Neomycin	39	55	53	47
Chloramphenicol	70	80	78	73
Linezolid	100	100	100	100

The results of **MIC test** came synchronized with that in the first test (disk diffusion test), the red wavelength decreased (all most significantly) all antibiotics concentrations that needed to inhibit MRSA growth (the columns in fig. 2 represented the average values of antibiotic concentration of the 45 MRSA isolates). The MIC values of the 632 nm laser were lower than the two other lasers except in Vancomycin, Rifampicin, Fusidic acid, and Clindamycin. The green laser remained the less effective wavelength on bacteria cell. On the other hand, the blue laser had an intermediate effect between red and green wavelength. Again, like the result in the first test, visible laser wavelengths decreased the effective dose concentration of the antibiotics against MRSA bacteria cells. (Fig. 2)

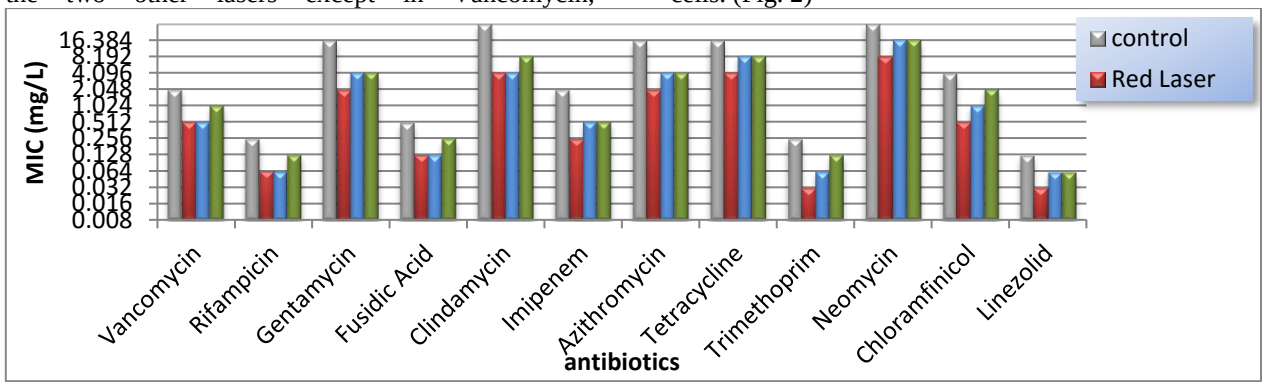

Figure 2. Effect of 632 nm (red), 405 nm (blue), 532 nm (green) lasers, on the MIC of antibiotics of irradiated MRAS (each column is the average value of the antibiotic's dilution).

Discussion:

Phototherapy has been a field of advanced research in the recent years emerging as a new approach for treating numerous diseases. In the study, some included antibiotics are the most commonly recommended antibiotics for MRSA (used individually or in a combination manner) (4, 20, 22, 23, 24, 25), and the others are chosen to include more different groups of antibiotics (according to their mechanisms) in this study (5, 21, 26, 27).

Bactericidal effects of Diode lasers irradiation have been reported, with results differing by wavelength (6, 8). The wavelength factor appears to be a very important factor as many studies report (6, 14, 28, 29, 30), however the power density (50 mW) is the same for all the three laser wavelengths, meanwhile the results come different (Fig 1, 2 and Table 1). The effect is due to the energy absorption of specific wavelength by cytochromes. This action causes an alteration in cell metabolism (11, 12, 31). *S. aureus* bacteria have three cytochromes which are: cytochrome *a* (with peak absorption at 600) (32, 33), cytochrome *b* (with peak absorption at 554 and 558) (32, 33), and cytochrome *bd* (34, 35) (a compound of cytochromes *b*558, *b*595 and *d* (12, 34, 35). The peak absorption of the reduced form of cytochrome *d* is around 630 nm (31,34, 35). It is believed to be the photoacceptor molecule for red wavelength (11). As cytochromes are electron transfer proteins in the respiratory system, so the excessive absorbed energy will lead to an unusual biological effect in the cell, like effecting on the influence of the redox state of the cytochromes and the rate of electron flow. Cause a shifting of cytochrome to the reduced form (which naturally promotes electron transport). The result of these events is an increase of proton motive force (pmf, $\Delta\mu H+$, ATP pool, increase the acidification of the cytoplasm, and the generation of toxic free radicals) (11, 12). These photochemical and photophysical changes are believed to occur in the cell membrane (where the respiratory chain components take place (31, 36)). On the other hand, the inactivation of blue wavelength (405 nm) to MRSA is the result of photo-excitation of intracellular porphyrins (also a component in respiratory chain). The subsequent of this excitation will generate a cytotoxic ROS such as singlet oxygen, which is a well-recognized trigger of cell death (11, 28, 29, 37, 38)

In disk diffusion and MIC tests, the red wavelength appears to be more effective than the blue wavelength. The reason may be due to the amount of quantum energy that is absorbed by cytochrome *bd* as compared with porphyrins. This could explain why the 632 nm is more effective and has an important role in cell resistance to antibiotics. The results indicate the less effect of 532 nm wavelength on bacteria susceptibility rather than the two previously discussed wavelengths. However the decreasing in antibiotic resistance is noticed as compared with control. The mechanism of action of the green wavelength is not clear as suggested by Reznick *et. al.*. They find the combination of 532 nm laser with antibiotic gives more effective result than their effects separately, as it improves the Gentamycin action against *Ps. aeruginosa* (8). Another study finds a bactericidal effect of green light (525 nm) against *S. aureus* (6).Recently, researches mention the specific dependent action of light wavelength on certain molecules within the cells, the same as it is with the specific action of antibiotics and their targets in bacteria cells. These facts explain the results in this experiment. The tested antibiotics are related to different groups depending on their target in bacteria cells (cell wall, protein synthesis, ribosomes, RNA synthesis, and folic acid synthesis) (26). All the 12 tested antibiotics give positive results after laser treatment. In other words, the MRSA bacteria become more sensitive to these antibiotics and could be inhibited by less antibiotic doses, even with slight differences in these results. (Fig 1, and 2) It was noticed from the red laser's result the effect of red wavelength on MRSA resistance is not in the same rate with the 12 antibiotics. Some antibiotics become more effective than others like Vancomycin and Imipenem on irradiated bacteria, while the mostly less effective antibiotic is Trimethoprim-sulfamethoxazole. This effect may be related to action site of Vancomycin and Imipenem. They inhibit cell wall synthesis by binding with the peptidoglycan units and reducing the mechanical strength of the cell synthesis, as in Vancomycin (4, 25, 26). This effect with the mechanism action of 632 nm laser on the MRSA cytocrome *bd* caused an inhibition and increasing in the clear zone of these antibiotics and decreases the effective dose of those two antibiotics. The previously suggested mechanism could be similar to blue wavelength (as the 405 nm target is also respiratory component: porphyrins) and green wavelength. figure 3. The aminoglycoside antibiotic, like Gentamycin and Neomycin, inhibits protein synthesis by promoting protein mistranslation through the incorporation of inappropriate amino acids into elongating peptide strands. This process depends on respiration. Therefore, any changes in the activity of membrane-associated cytochromes that maintain the electrochemical potential power through the quinone pool will allow aminoglycosides to access the cell (26, 27). As a result, the action of laser wavelengths absorbed by cytochromes would be synchronized with aminoglycoside effect on bacteria cell. Figure 3.

The green laser appears to have less effect on MRSA as shown in figure (3), while, Linezolid, Trimethoprim-sulfamethoxazole, and Azithromycin have the lowest place. These antibiotics inhibit protein

synthesis in bacteria except Trimethoprim-sulfamethoxazole which is DNA synthesis inhibitor (25, 26). The latter antibiotic is the last one among red and blue wavelength.

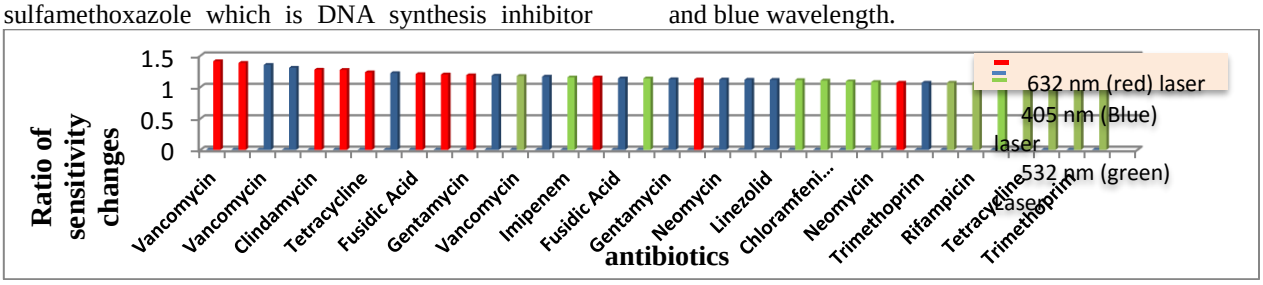

Figure (3). The change ratio of disk diffusion test after laser treatment

Conclusion:

The red laser wavelength (632 nm) was more effective on MRSA susceptibility to antibiotics than blue (532 nm) and green (405 nm) lasers respectively. From these data it was found that when the action of laser wavelength and antibiotic are on the same target or metabolic process within the bacteria cell, the effect or result will be best.

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