Photodynamic Inactivation of Klebsiella Pneumonia Isolated from Burn Wounds

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Abstract

Background and Objectives: As a result of considerable consume of antibiotics, the increase of drug- resistance bacteria is one of the greater troublesome the arts to popular health- one approach that may be used to reduce potential shortcomings perhaps the inactivation of these bacteria by photodynamic therapy (PHDT).
Aim: study the antimicrobial effect of methylene blue (MB) –mediated photodynamic therapy (PDT) on Klebsiella pneumonia isolated from burn wounds.
Methods: We selected 20 strains of bacteria for this project. Antimicrobial susceptibility was performed using Kirby-Bauer method; the most resistant Klebsiella pneumoniae isolate to 12 antibiotics was selected and irradiated by laser light. The antimicrobial effect of different MB concentrations and laser doses were evaluated for determining the optimal PHDT parameters. Four experimental groups were included: group1: photosensitizer alone, group2: laser diode irradiation alone, group3: combination of MB and laser irradiation. Influence of laser light doses (4.8, 9.6, 14, and 19J/cm2) in association with different MB concentrations (30, 50, 70, and 90µg/ml. CFU/ml were performed to estimate the viability of bacteria. The antimicrobial effect of PHDT was assessed with percent of reduction in viable count.
Results: The antimicrobial effect of PHDT on K. pneumoniae was dose dependent to laser light. In group1, without laser irradiation did not show any antimicrobial effect on K. pneumoniea, in group2, laser irradiation alone with 20mw/cm2 and different doses (4.8, 9.6, 14, and 19J/cm2) did not modify bacterial growth. Group3, significant difference in viability of bacteria after treatment by PHDT. The adequate PHDT parameters were light irradiance 20 mw/cm2, and MB concentration 50µg/ml and exposure time 12min, dose 14J/cm2. Complete killing of K.pneumoniea at a dose 19J/cm2 and MB concentration 90µg/ml.
Conclusion: Methylene blue mediated PHDT had an antibacterial efficacy in vitro on Klebsiella pneumonia isolated from burn wounds. Complete killing of K. pneumoniae at a dose 19 J/cm2 and MB concentration 90 µg/ml..

Key Words: Burn Wounds, Photodynamic Therapy (PHDT), Mediated Photodynamic Therapy (PDT).
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Introduction

Infection is the largest familiar problems in burn patients enter to the hospitals generating high morbidity and mortality [1].

Patients with burn wounds greater sensitive to nosocomial infections, this is associated to immune compromised state of patients with burn wounds due to longer stay in hospital, altered physiology, invasive intervention and disrupted skin barrier. Treatment with suggest antibiotic can be lifesaving in burn patients. The major problems is the increasing resistance that prevent achieve this goal [2].

K- Pneumonia is one of the commonly bacteria isolated from burn wound [3] it is almost frequently apart of family Enterobacteriaceae, produce plasmid mediated extended spectrum beta lactamases. [4].

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The increase of bacterial strain with multi drug resistant is an essential hazard to national health and rise in mortality rates, cost of health care. [5], [6]. K. pneumonia appear as a main health – associated infections (HSIS). HSIS produce K. pneumoniae strains progressively acquired great level of antimicrobial drug resistance. K. pneumoniae is a fermentative gram negative bacillus, member of family of Enterobacteriaceae which is most commonly associated with producing infection of UTI and pneumonia [7]

K. pneumoniea infections may happen in inherent organs like lung tissue, wound tissue and bladder, accordingly light activation must pass through tissue with adequate power to generate efficient PHDT, therefore, the interpretation best photosensitizer, and light dose. So the principle case of bacterial resistance is misuse and over use of antibiotics. [5], [6], therefore, there is a need for alternative method for killing K. pneumonia. Photodynamic therapy (PHTD) one possible approach which based using low intensity light in presence of a photosensitizer agent to killing an organism. In this treatment method, excitation photosensitizer by absorbed light irradiation at appropriate wavelength translates the sensitizer to triplet state to produce oxygen free radicals. The reactive oxygen specie motivation of oxidation of proteins in cytoplasmic, lipids, and nucleic acid membrane, so the risk produce the eradication of micro-organism. 8] Because free radicals form thoroughly non- specific and deactivate different locations in the cell, the bacteria produce no resistance against this method. [9] PHTDT has been established to be sufficient for eradication of microorganisms. It can inhibit or kill a wide range of microbial pathogens. [10] especially Methicilline-resistance , Vancomicine resistance, Enterococcus species and multi-drug resistance, Pseudomonas aeruginosa species and other gram positive and gram negative bacteria., [11] [12], [13].

Methylene blue (MB) which belongs to phenothiazinium compounds, is a chemical dye, and is used in clinical antimicrobial treatment. It is considered as an essential photosensitizer for its high capability to generate reactive oxygen species and on human cells has less toxicity. [14] and. [15].

There are many studies exploring the PHDT effect of different dose and different photosensitizers. [16], [14], [17], [18], [19]

The purpose of this study was to determine best photosensitizer concentration and study the effect of different exposure time (doses) of laser on killing K. pneumoniae in vitro.

Methods

Twenty swab samples were collected from skin areas of burn wounds using sterile swabs in transport media. These swaps were taken from in patents in AL- Yarmook teaching hospital in Baghdad, during period from April 2018 to June 2018.

The samples were cultured on blood agar, MacConky agar and incubated aerobically at 37c for 24 hours. In health center laboratory (Baghdad) under aseptic condition the collected specimens were streaked directly on blood agar incubated for24hours at 37c. Specimen isolates were identified depending on microscopic examination, biochemical test and APi-20E (bio-merieux/france).

Kirby- Bauer method was followed as described by (WHO2003) [20] to carry out the antibiotic susceptibility test for 10 antibiotics and according to laboratory standards institutes [20] 10 isolates of Klebsiella were elected for this study. Antimicrobial susceptibility test was done for each isolate.

Antimicrobial susceptibility test by the disk diffusion method was performed to determine the resistance patterns of the isolates to the following antibiotics : cefotaxime (CTX.30µg), ceftriaxone (CT 30µg), ceftazidime (CAZ 30µg), cefepime (cefep 30µg), gentamicine (GEN 10µg, amicacine (AK30µg), piperacilline / tazbactam (PIP/TAZ100/ 10µg), ampicillin (AMP, 10µg), amoxicilline/ clavulanic (Ax/CLA 30µg) , imipenem (IMI,10 µg) [oxide, England, disc contents according to clinical and laboratory standards institute (CLSI) guidelines]. [21]

All antimicrobial testing was performed on Muller-Hinting agar technique and data interpreted according to CLSI guidelines [21] one isolate of K. pneumoniae was selected from ten isolates that resistant to all antibiotic used. Activation of selected isolate for 18 hours using brain heart infusion broth (HIMEDIA/India). The results of susceptibility testing were categorized into susceptible resistance, intermediate.

Laser used in this project was 100mw diode laser (UK scientific Ltd) emitting radiation with radiation with wavelength of 660nm. Intensity (irradiance) 20mw/cm^2 obtained when the distance between laser and the surface of microtiter plate was adjusted, irradiated area at the surface of microtiter plate 5cm^2. Exposure times were 4,8,12, and 16

minutes corresponding to doses 4,8 J/cm^2, 9.6 J/cm^2, 14 J/cm^2, and 19J/ cm^2 respectively.

MB (Merk Company) was selected and prepared freshly for each experiment in distilled water. PH= 7.4, kept in dark and filtered- sterilized by using 0.22µm Millipore filter paper. MB is considered as photosensitizer and has maximum absorption at 668and 609 nm which corresponding to the wavelength of 650 nm of laser used in this project. [22]

Photosensitizer concentration was prepared using 10mg/ml then diluted by distilled water four concentrations were prepared 30, 50, 70, and 90 µg/ml).

More resistance isolate of K.pneumonia was selected for 10 isolates according to the results of 12 antibiotics sensitivity test. The selected isolate was then cultured on MacConky agar at 37c for 16-18 hours. The optical density was measured, for bacterial suspension using spectrometer at 530nm wavelength gave the absorbance of 0.5 after that , serial dilution from10^{-1} – 10^{-8} was performed by adding 1ml of the suspension of bacterial culture to 9ml of physical saline solution (0.85%) in sterilized test tubes.

Aliquot of 100µl of each dilution was spread on a plate containing nutrient agar, using L-shape spreader. Six plates were prepared for each assay for each assay and incubated aerobically at 37c for 18- 24hours. After incubation, the number of colonies counted in each plate using colony counter.

A bacterial suspension with dilution of 10^{-5} was chosen from the other serial dilutions according to the viability count .The colony forming units per millilitre (CFU/ml) was then calculated using following formula [23].

$$\mathbf{CFU/ml} = \frac{\mathbf{number\ of\ colonies}}{\mathbf{volume\ plated\ (ml)} \times \mathbf{dilution\ used}}$$

$$\frac{\mathbf{CFU}}{\mathbf{ml}} = \mathbf{(No.\ of\ colonies)} \times \mathbf{dilution\ factor} \times \frac{1}{\mathbf{volume\ plated}}$$

$$\mathbf{CFU/ml} = \mathbf{(No.\ of\ colonies)} \times \mathbf{dilution\ factor} \times \mathbf{10}$$

CFU/ml: colony forming unit per ml

Volume plated =100µl=0.1 ml

Irradiation procedure accomplished by using 96 well- Microtiter plate.

A bacterial suspension with dilution of 10^{-5} was chosen from other serial dilution. Which give 30-300 CFU/ml, five millilitres of diluted bacterial suspension with dilution 10-5 was put in sterilized test tube and not exposed to laser light (control).

Plates for each assay and incubated at 37c for 18-24 hours. After incubation, the number of colonies was counted in each plate, and the CFU/ ml was calculated. Two ml of bacterial suspension was distributed in the wells of microtiter. Beam spot size 5 cm^2.

Then after irradiation (100µl of bacterial suspension exposed to laser spread on a plate containing nutrient agar, using L-shape spreader. six plates were prepared for each assay incubated aerobically at 37c for 18-24 hours (irradiation groups). Bacterial suspension were mixed and incubated with MB at different concentrations (30, 50, 70, 90 µl/ml) for 15 min in dark and at room temperature to study the effect of irradiation on sensitized K. pneumonia. Samples were irradiated for four different periods. The photosensitized loaded cells were washed twice with sterile distal water and centrifuged (7000rpm for 10 min). Aliquot of 100 µl of treated cells placed in 96 well microtiter plate and irradiated with red light 660 nm.

In order to maintain the sterility of culture during irradiation the microtiter plates were kept covered in this study. A novel two steps in this method to reduce the effect of data variability attributed to environmental or procedural minor differences were:

1. Freshly prepared bacterial suspension for each experiment.
2. Each experiment has its control plates.

100µl from control bacterial suspension spread on nutrient agar by L- shape spreader. Suspension with dilution 10^{-5} not exposed to laser light (control plates).

To determine the inhibition effect, the total number of viable count was used and per cent of reduction given in following formula:

Percent of reduction = A-B/A×100% [24]

A= CFU/ml before irradiation. B= CFU/ml after irradiation.

Statistical Analysis

Statistical package for social science –version 25 (Statistical Packages for Social Sciences- version 25) was carried out for analysing data.

Data were presented in simple measures of mean, standard deviation, and range (maximum, minimum values).

The significance of difference of different means (quantitative data) was tested using Students-t-test

for difference between two independent means or Paired-t-test for difference of paired observations (or two dependent means), Data were reported as means ± standard deviation and (95% confidence interval) for normally distributed variables. The analysis of variance (ANOVA) were used to compare continuous parametric variables between more than two groups.

Results No significant effect on viability of K. pneumoniae was observed when the bacteria are irradiated with laser light. The results showed that in absence of photosensitizer, the laser alone had no significant difference when compared with control groups. Table (1)

No significant effect on viability of K-pneumoniae when bacterial suspension was mixed with MB Table (2).

No antimicrobial toxicity of MB concentrations when incubated in the dark for 15 min. (Table).

Table 1. The effect of laser irradiation at different exposure time on viability K. pneumoniae (CFU/ml).

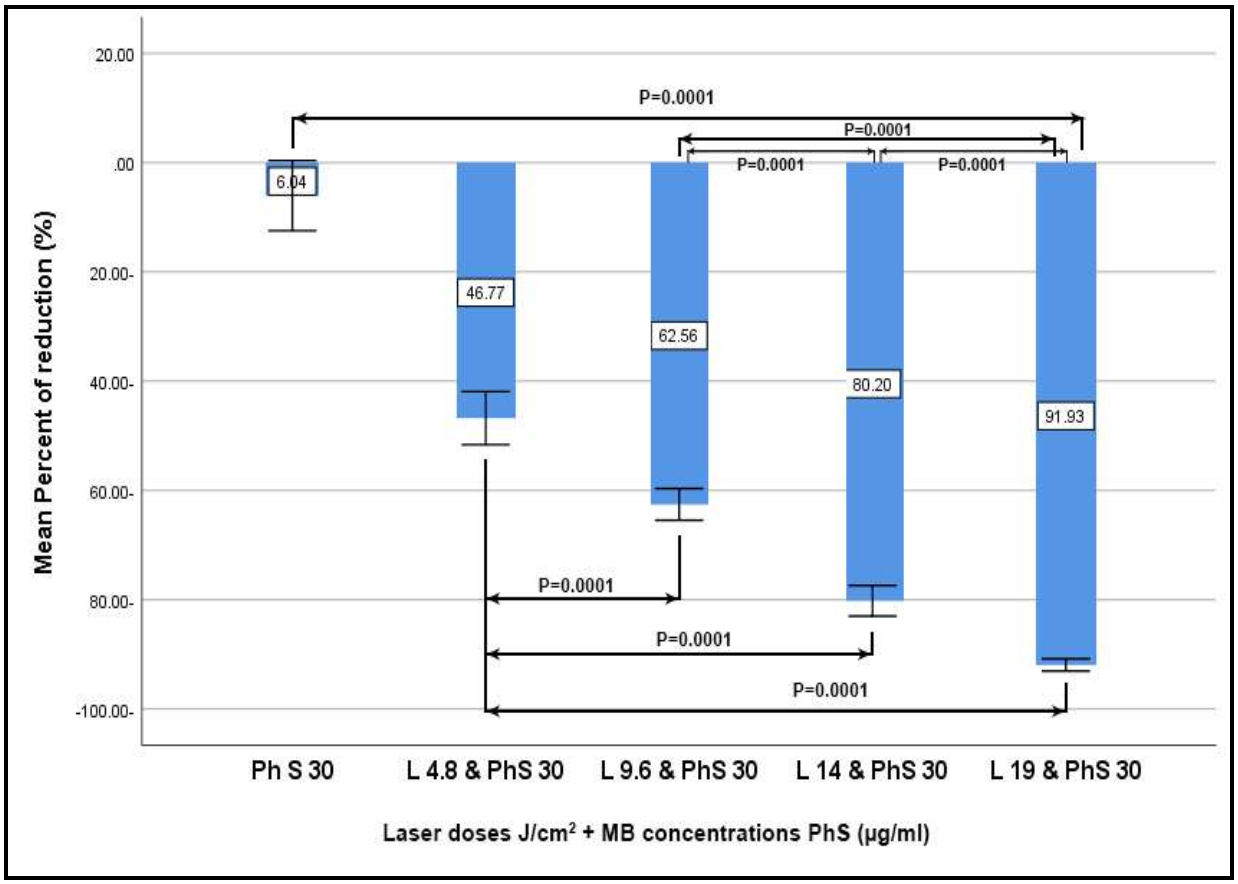
Dose J/cm²		CFU/ml = Number of colonies×10⁷ Laser treatment	Control	p-value t-test
4.8	m ± SD	108.23±54.69	111.23±54.63	0.311
	Range	(62.2-201.5)	(73.3-204.8)	
9.6	m ± SD	128.43±30.90	130.80±29.87	0.099
	Range	(79.8-162.5)	(82.2-161.3)	
14	m ± SD	125.73±34.82	126.63±33.54	0.596
	Range	(72.2-155)	(76-158.3)	
19	m ± SD	148.13±29.23	148.74±31.10	0.553
	Range	(109.8-184.7)	(107-186.7)	

Table 2. The viability of K. pneumoniae in presence of photosensitizers (MB) compared to control groups.

Photosensitizer concentration		CFU/ml = Number of colonies×10⁷ With PHS	Control	p-value t-test
30 µg/ml	m ± SD	142.30±27.13	169.60±68.17	0.306
	Range	(112.5-172.8)	(116-282.7)	
50 µg/ml	m ± SD	147.44±45.97	152.83±52.54	0.120
	Range	(104.3-237.3)	(108-256.8)	
70 µg/ml	m ± SD	158.17±15.03	159.10±15.73	0.297
	Range	(140.3-180.3)	(141.5-181.7)	
90 µg/ml	m ± SD	207.42±20.37	208.75±20.27	0.105
	Range	(191.7-237.3)	(192-238.2)	

Table 3. Mean values of CFU/ml and mean percentage of redaction of K.pneumoniea irradiated by diode laser in presence of MB in relation to control group

No. of colony of viable cells × 10⁷ CFU/ml								
Laser doses + photosensitizer concentrations					Control without laser and sensitizer		P-value	Percent of reduction %
Exposure time (sec)	Dose J/cm²	Concentrations µg/ml	Laser doses + photosensitizer concentrations		Control			
4	4.8	30	Range	86.7-112.3	Range	168.7-221.7	0.0001	46.77±4.86
			m ± SD	103.03±10.15	m ± SD	195.73±22.81		
	4.8	50	Range	68.8-85.5	Range	181.8-226.7	0.0001	62.44±1.11
			m ± SD	78.37±8.33	m ± SD	208.07±20.54		
	4.8	70	Range	43.2-69.7	Range	161.3-203.7	0.0001	69.66±3.57
			m ± SD	56.53±11.20	m ± SD	184.70±17.79		
	4.8	90	Range	9.3-12.3	Range	119.5-169.2	0.0001	92.51±0.72
			m ± SD	11.20±1.26	m ± SD	148.60±20.37		
8	9.6	30	Range	56.3-81	Range	154.8-198.5	0.0001	62.56±2.90
			m ± SD	67.97±9.41	m ± SD	179.90±22.25		
	9.6	50	Range	52.2-73	Range	198.2-259.8	0.0001	71.82±1.01
			m ± SD	63.80±8.85	m ± SD	226.80±28.55		
	9.6	70	Range	19.8-25.8	Range	144.7-194.2	0.0001	86.49±0.44
			m ± SD	23.77±2.33	m ± SD	177.43±19.94		
	9.6	90	Range	1.7-10.8	Range	131.7-155.7	0.0001	96.64±2.43
			m ± SD	4.60±3.78	m ± SD	142.07±10.26		
12	14	30	Range	31.5-42.2	Range	169.2-190.8	0.0001	80.20±2.81
			m ± SD	36.00±4.49	m ± SD	182.47±8.68		
	14	50	Range	11.3-17.7	Range	157.7-224.5	0.0001	92.19±1.07
			m ± SD	15.27±3.09	m ± SD	192.27±24.85		
	14	70	Range	6.5-8	Range	146.7-174.8	0.0001	95.33±0.29
			m ± SD	7.20±0.68	m ± SD	155.57±11.57		
	14	90	Range	2-6.8	Range	130.7-157	0.0001	97.39±1.80
			m ± SD	3.33±1.99	m ± SD	143.70±12.37		
16	19	30	Range	13.5-21.3	Range	172.7-225.3	0.0001	91.93±1.12
			m ± SD	16.13±3.30	m ± SD	200.57±25.09		
	19	50	Range	7.2-8.8	Range	176.8-211.5	0.0001	95.60±0.60
			m ± SD	8.43±0.72	m ± SD	193.97±12.65		
	19	70	Range	2.8-4.2	Range	121.8-180	0.0001	97.45±0.25
			m ± SD	3.57±0.54	m ± SD	143.87±27.29		
	19	90	Range	0.7-2.5	Range	157.5-196	0.0001	99.12±0.42
			m ± SD	1.57±0.67	m ± SD	173.13±15.95		



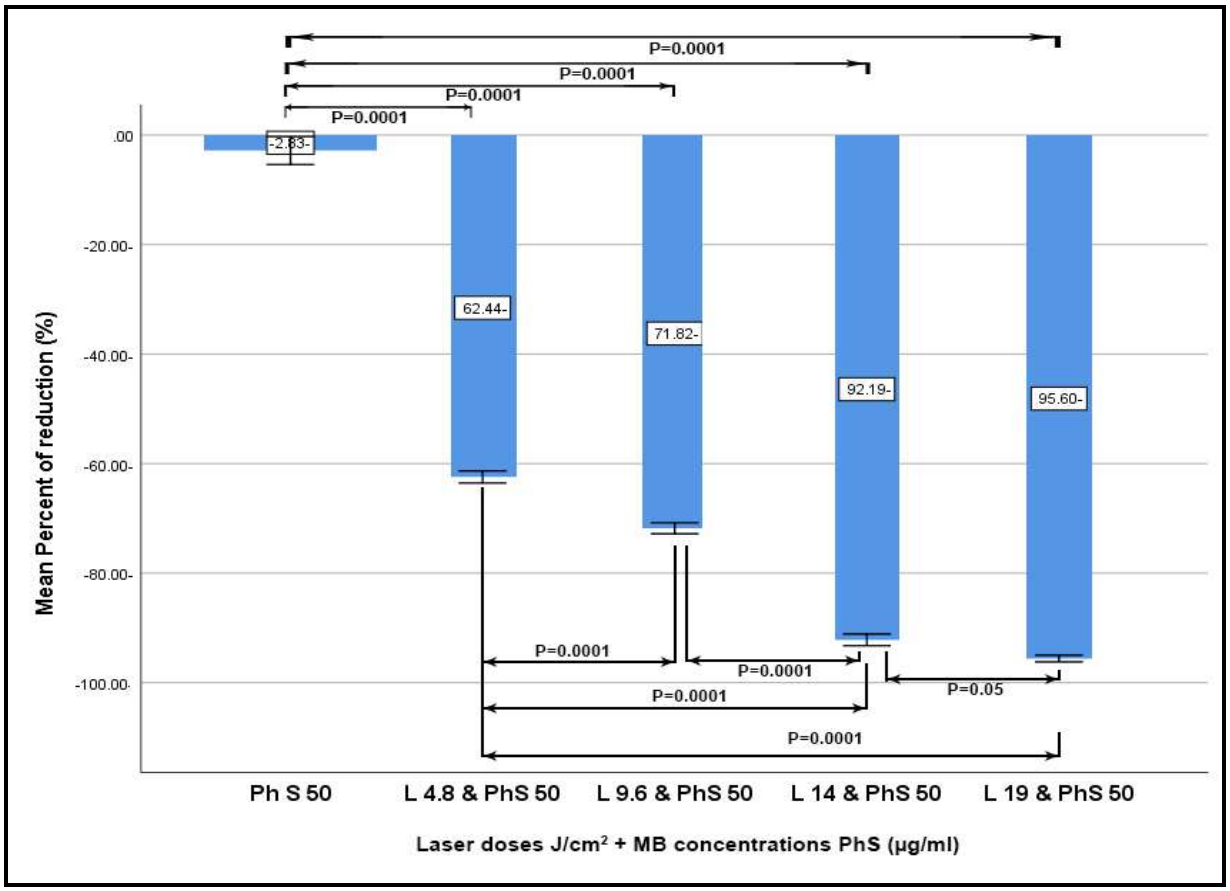


Figure 1. Dot diagram (with 95% confidence interval) showing the effect of varying laser doses with MB concentrations 30µg/ml and 50 µg/ml on mean percentage of reduction (%in viable count).

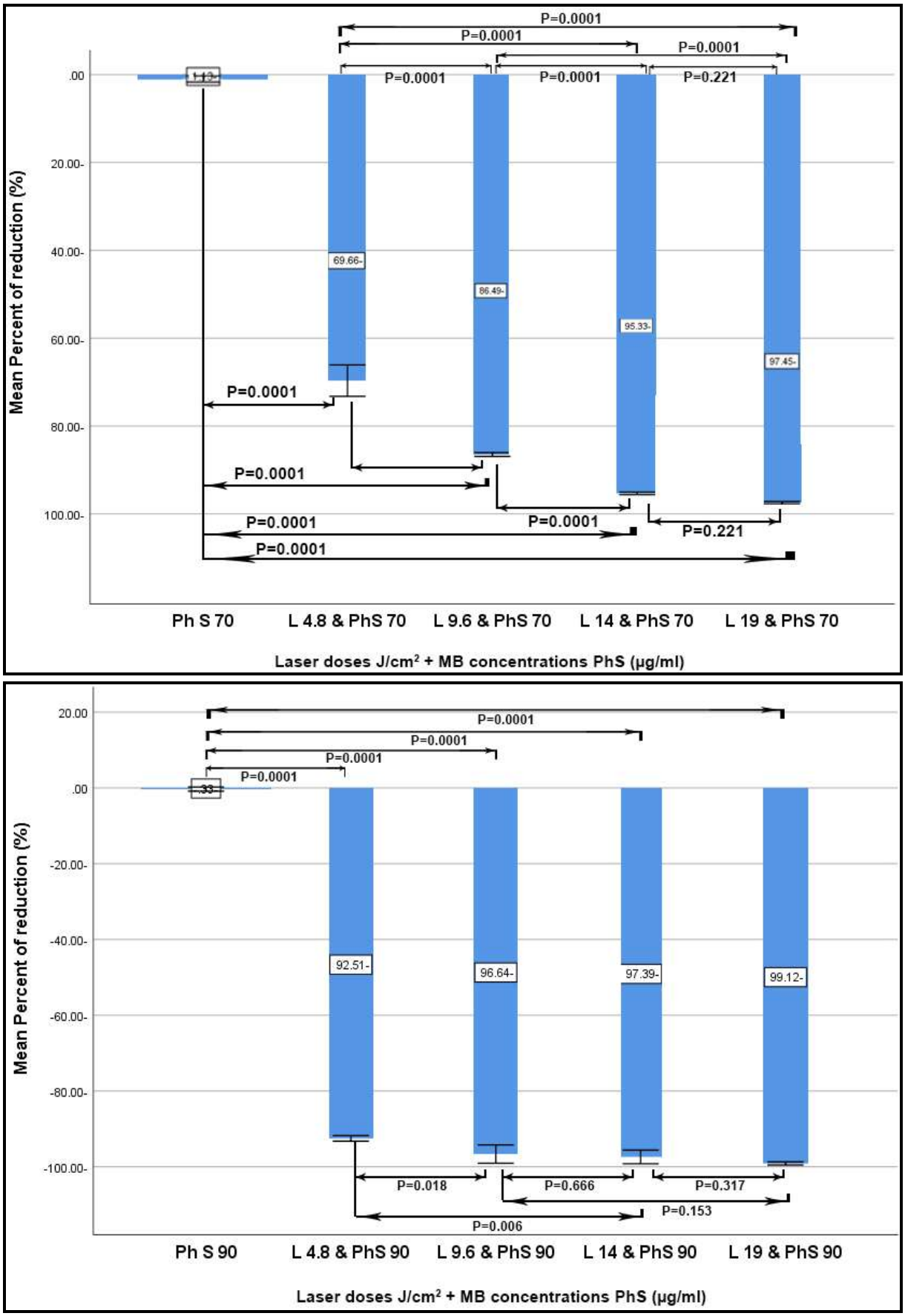


Figure 2. Dot diagram (with 95% confidence interval) showing the effect of varying laser doses with MB concentrations 70µg/ml and 90 µg/ml on mean percentage of reduction (%in viable count)

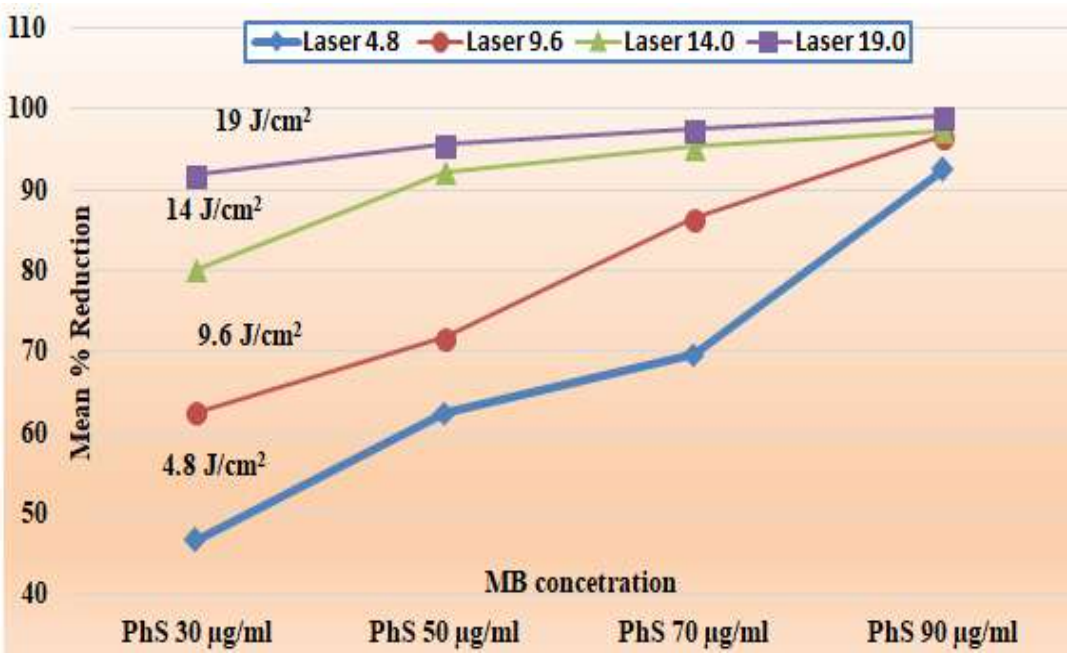


Figure 3. Lethal photosensitization of K. pneumonia with 30 ,50,70, and 90 µg/ml and irradiated with different doses from red

The antimicrobial effect of PHDT on K. pneumonia was dose dependent to laser irradiation and MB concentration. table (3)

When k. pneumoniae was exposed to red laser at a dose 4.8 J/cm² and treated with different concentrations of MB, CFU/ml decrease and significant (p<0.0001) reductions of 46.77%, 62.44%, 69.66%, 92.51% in viable count of K. pneumonia were achieved using concentrations 30, 50, 40, and 90 µg/ml respectively table (3) , fig (1)

Also, when K. pneumoniae exposed to dose 9.6 J/cm² of red laser and treated with different concentrations of MB, CFU/ml decrease and significant (p< 0.0001) reductions of 62.56%, 71.82%, 86.49% in viable count of K.pneumoniea were achieved using concentrations 30, 50, 70, 90 µg/ml respectively. Table (3) fig (1)

Exposure of K. Pneumoniae to lase light for (4, 8, 12, and 16) min, doses 4.8, 9.6. 14, and 19J/cm² in presence of different MB concentrations (30, 50,70, and 90) µg/ml each MB concentration treated with laser light at dose 4.8, 9.6,14, and 16 J/cm² the results indicate that there was significant difference in viability of bacteria CFU/ml between treated groups and control groups (p=0.0001, paired differences) also significant difference in percentage of reduction when compared with each other P=0.0001 using ANOVA –test at 0.05 level. fig (3)

As shown in fig (3): When K. pneumoniae exposed to 14J/cm ² of red light and MB concentration increased from 30, 50, 70, and 90µg/ml. significant difference in percentage of reduction between group exposed to (14J/cm2 + 30µg/ml) p= 0.0001 with 95% confidence interval, but no significant difference in per cent of reduction between group treated with (14J/cm2 +50µg/ml) and (14J/cm2 +70µg/mL) p=0.071 with 95% confidence interval, also no significant difference between group treated with (14J/cm² +70µg/ml) and group treated (14J/cm² +90µg/ml), p= 0.235 with 95% confidence interval.

When bacteria exposed to 19J/cm² of red laser and MB concentrations increased from 30, 50, 70, and 90µg/ml the reduction in viable count of 91.93%, 95.6 %, 97.45% and 99.12% also was stable from 50 to 90 µg/ml significant difference in percentage of reduction in viable count of bacteria between group exposed to (19J/cm2 +30µg/mL) and group treated with (19J/cm2 +50µg/mL) p=0.036. fig(3)

No significant difference in per cent of reduction between group treated with (19J/cm2 +50µg/mL) and (19J/cm2+70µg/mL), p=0.28 with 95% confidence interval, also no significant difference between group treated with(19J/cm2 +70µg/mL) and group treated (19J/cm2 +90µg/mL), p= 0.235 with 95% confidence interval , so the antimicrobial efficacy of PHDT was MB concentration – dependent up to 50µg/ml. Fig (3)

The effective PHDT parameters were intensity of laser used (irradiance (20mw/cm²) and MB concentration 50µg/ml and exposure time 12 min, dose 14J/cm2,complete killing at MB concentration 90µg/ml and exposure time 16min, dose 19J/cm².

Disscusion

In this study, we have examined whether K. pneumonia is sensitive to lethal photosensitization using different concentrations of MB in association with different doses of laser radiation (660nm). The results shown that K. pneumoniea can be inhibited by low intensity laser light after sensitization with MB. Table (3)

Irradiation of K.pneumoniea with light from semiconductor laser in absence of photosensitizer had no effect on viability of K. pneumoniae. (CFU/ml). Table (3)

The photosensitizer alone was found to have no bactericidal effect on K. pneumoniae without laser irradiation. The results obtained are in agreement with previous studies focused on MB as a photosensitizer and irradiation with visible red laser which had been shown that the gram negative bacteria can killed by low intensity laser in precence of suitable photosensitizers. [16], [25], [27], [26] .

Milson et al [28] have reported that H. pylori can be killed by low doses of laser light after sensitization with MB, irradiation of Pylori with light from either semiconductor or He-Ne laser had no effect on bacterial viability, in the absence of photosensitizes.

Hence AL-kashef et al [16] studied the influnce on clinical resistance strains and ATCC (American type culture collection) of Ecoli using photosensitizer of toluiden blue (TBO)and MB. Alaser light (adose 163.8J/cm^2 with MB (50µg/ml) able to reduceing 37.6 % in CFU/L ml of drug resistance E-coli and 53.1% of E-coli (ATCC 25922) Kashef .N [16],.

Kashef et al 2014 [25] examined the ability of PHDT on acinitobactor baumannii. The outcomee demonstrated the diminshing in vibility of live cell after PHTD with toludine blue and MB for five strains of acinetobactor Baumannii, moreover, PHTD diminished the minimum inhibitory concentrations of growth inhibitions in to gentmicin, ciproflaxin, Azithromicin, Impenem).

Another study performed PHDT by two photosensitizers at a concentration of 5µg/ml of porphine teratosylate (TMP)(N-Methyl-4-pyridyl) and MB on five strains of P.aeruginosa isolated from cystic fibrosis, and the PHDT used two photosensitizers of porphineteratosylate (TMP)(N-methyl-4- pyridyl) and MB at a concentration 5 µg/ml PHDT killed 99.9% of bacteria.[29]

The results obtained in this study have shown that lethal photosensitization based on exposure time to laser irradiation (dose). Table (3) fig (1,2). This findings confirm with other previous studies [28] [30], [31].

MB is commonly used as histological dye it's one of phenothiazinium group bind strongly to DNA, therefore the fundamental target for photosensitized action with MB may be DNA, which means that the resulting kill may include modification DNA with probability of mutagenicity. This must be focused on before undertaking any clinical studies [32] when a photon is absorbed by photosensitizer molecules it is elevated to a high energy state (triple stat) which then transfer its energy to an oxygen molecule producing single oxygen which(cytotoxic species). This processes can be used to kill bacteria is a mechanism termed PHDT or lethal photosensitization, which is a local treatment, non-cumulative and considered as appropriate antimicrobial strategy for the treatment of local infection.

The ability of MB to sensitize K. pneumoniae to kill low intensity laser light as demonstrated in this study may have clinical applications, if effective in vitro, in that could be used to kill these organisms in burn wounds prior to its repair. The irradiation of bacteria by red laser 660nm at different doses (4.8, 9.6, 14, and 19) J/cm^2 have no significant effect on viability of bacteria. Several factors like irradiance (watt/cm^2), exposure time (dose J/cm2) determine whether the results are due to thermal or photochemical effect [33]. The influence of low intensity laser on K.pneumonia is bio stimulation and not thermal, these results in accordance with previous literatures suggest that the irradiance is the most important parameters in bio stimulation effect of low intensity laser on bacterial culture. [34]. The use of extremely low irradiance to irradiate bacteria will give extremely low irradiance to irradiate bacteria will give no effect in all types of bacterial culture. [35].

The results of this study have shown that K.pneumoniea can be killed by low intensity laser light. Further work is needed to investigate if photosensitizer and laser light doses can be used to destroy the pathogenic bacteria in vivo without improper destruction to surrounding tissues, so if this can be happen, there is the principle technological challenge of transferring sufficient laser dose to all areas of infected burn wounds.

Conclusion

This study suggest that red laser (660nm) in combination with MB is hopeful for PHDT of burn infections caused by drug resistance K. pneumoniae

utilize this progress would may diminish the requirement for antibiotics in healing treatments.

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