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Inhibition of *Klebseilla pneumoniae* by Visible and Near-IR Radiation

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Abstract: We have studied the effect of blue light on *Mycobacterium smegmatis*, and the effects of combined blue and infrared on *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* and found that inhibition can be obtained. This research sought to determine the potential for visible and near-IR radiation to inhibit *Klebseilla pneumoniae*. Additionally, the study sought to determine whether wavelength, dose or a combination of wavelength and dose could produce a significant inhibitory effect. *K. pneumoniae* was tested due to its addition to the "urgent threat" list compiled by the Centers for Disease Control. The organism was treated in vitro with 464nm, 850nm and a combined 464 & 850nm light emitted from a supraluminous diode (SLD) array. Doses of 3, 10, 30, 45, 60 Joules per square centimeter (J/cm2) were used. Colony counts were compared to untreated controls using a Repeated Measures ANOVA. One-way ANOVA with Tukey HSD was used for post hoc analysis. The results revealed statistically significant inhibition of *K. pneumoniae* for wavelength, dose and interaction of wavelength and dose (Main Effect $F_{1,9} = 3971.89$, $p = 0.00$; Interactive Effect $F_{1,9} = 645.68$, $p = 0.00$). *Post hoc* analysis revealed that the combined 464 & 850nm wavelength at 45 and 60 J/cm^2 were significantly effective and different from the other treatment conditions. A maximum kill rate of 96.19% was achieved with the combination blue/IR wavelength at 60 J/cm^2. We concluded that a combined visible and near-IR radiation at 45 and 60 J/cm^2 is an effective inhibitor of *K. pneumoniae*.

Keywords: Visible Light, Near-IR, Bactericidal Effect, Photobiomodulation

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1. Introduction

Antibiotics-resistant microbes represent an urgent health risk. The Centers for Disease Control (CDC) report that each year more than two million Americans are infected with antibiotic resistant bacteria.[1] These difficult to treat infections lead to approximately 23,000 deaths yearly. Unquestionably, the standard pharmaceutical approach to managing bacterial infections is becoming increasingly less effective.[2,3] Carbapenem-resistant Enterobacteriaceae (CRE) such as *Escherichia coli* and *Klebseilla pneumoniae* are highly resistant to many classes of antibiotics.[4] These highly resistant organisms carry the New Delhi Metallo-Beta-Lactamase -1 (NDM-1) gene on the plasmid, making transference of antibiotic resistance possible to same and similar organisms.[4] This situation has led the CDC to rank the threat associated with microbes such as *K. pneumoniae* as urgent.[1]

In response to the urgency posed by mounting antibiotic resistance, Bush et al have called for the investigation of non-antibiotic approaches for the prevention of and protection against infectious diseases.[5] Light energy has been demonstrated to have potential benefit in terms of microbial inhibition. Various wavelengths from ultraviolet (UV), through the visible range, and into the infrared (IR) spectrum have been shown to produce from moderate to significant inhibition. Unfortunately, energies in the UV spectrum can produce unwanted changes in the host tissues.[6]

Visible light, to our knowledge, has not been shown to include risks to the host tissue. Blue light (400 – 495 nm) inhibits *Pseudomonas aeruginosa*,[7-9] *Staphylococcus aureus* [8-10] and *Mycobacterium smegmatis* [11]. The effectiveness of blue light as a bactericidal agent appears to be enhanced when combined with IR wavelengths.[8-10] Combined red (624 nm) and IR (850 nm) wavelengths are effective against the growth of *Candida albicans*.[12-13] The source of the antimicrobial effect associated with visible and IR energies is a photobiomodulation where mitochondrial chromophores in the treated organism absorb light photons and produce singlet oxygen or free radicals that are lethal to the microbe.[14]

K. pneumoniae, as stated above, has become a rising threat to human health. Its resistance to normal pharmacological treatment is foreboding. *K. pneumonia* accounts for 8% of all nosocomial infections in the western world and is the second only to *E. coli* as the leading cause of urinary tract infections. [15] This organism possesses a polysaccharide capsule which contributes to its virulence and is important in the prevention of phagocytosis and survival within the host. [16] *K. pneumoniae*, like other gram negative bacteria, is inherently resistant to a number of antibiotics which affect gram positive organisms such as *Staphylococcus aureus*. The lipid rich nature of its outer membrane prevents absorption and penetration of these antibiotics, limiting their effectiveness.[17] CRE such as *K. pneumoniae* have an added advantage because they also produce an enzyme (plasmid mediated) that is capable of destroying the activity of carbapenems, which are often the last resort for antibiotic-resistant organisms.[4]

The purpose of this experiment is to determine whether visible and/or IR wavelengths are effective inhibitors of *K. pneumoniae.* The organism was chosen due to its established antibiotic resistance and it demonstrated ability to share that resistance to same and similar microbes.

2. Methods and Materials

The organism used for this study was *K. pneumoniae* (ATCC 13883), a microorganism that is part of the normal microbiota of the gastrointestinal tract. This encapsulated, gram negative rod is well known to cause upper respiratory tract and wound infections. Like other facultative anaerobes, this organism is able to grow well anaerobically but grows much better in the presence of oxygen (ambient air). *K. pneumoniae* has no special growth requirements and grows well on any nutritive-type media. TSA or Tryptic soy agar (Difco, Detroit, USA), a non-selective medium, was chosen to cultivate the organism and for colony counts. TSA has a high protein content (casein and soy bean digests) and is used to grow a wide variety of organisms both fastidious and non-fastidious. The organism was incubated at 37° C for a period of 20 hours. Use of a 20-hour-old culture is standard microbiological practice and serves to minimize the lag time for new growth.

Using a sterile cotton-tipped swab, material was removed from the 20-hour old culture and added to sterile deionized water to form a suspension equivalent to a 0.5 McFarland Standard (1.5×10^8 CFU/ml). The suspension was then diluted 1/1000 using 100 microliter automatic pipettes for purposes of accuracy and reproducibility. All dilutions were made immediately before the treatment with light.

The bacterial suspension (10 ml) was poured into a sterile 60 X 15 mm, polystyrene petri dish with the cover removed. The light source was placed directly over the suspension in the petri dish and after light exposure; a 10 microliter automatic pipette was used to remove an aliquot of the treated bacterial suspension. The irradiated suspension was then inoculated onto 60 X 15 mm polystyrene petri dishes containing TSA. The diluted bacterial suspension was applied to the surface of the TSA plates in a star-streak pattern to enable colony counts to be performed after 18 to 20 hours of incubation at 37° C.

For this experiment, we chose to illuminate the cultures using a pair of SLD light pads that emitted a band of light focused around the primary wavelengths of 464nm and

850nm. The pads consisted of a 353 cm^2 illuminating surface area comprised of 176 SLDs with a maximum power output of 5160 mW. Dose was calculated in J/cm^2. Since output for the pad was held constant, adjustment in time of irradiation provided the dose (3, 10, 30 45 and 60 J/cm^2). The Dynatron® 705^{Plus} Solaris™ (light source used in this experiment) automatically calculates time of irradiation when desired dosage is selected. The rate of the delivery of light energy ranged from 16.7 to 19.88 mW/cm^2 (See Table 1). After 20 – 24 hours of incubation, colony forming units (CFUs) were counted and compared to controls.

Table 1: Light energy details

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Dose (Jcm^{-2})	Wavelength (nm)	Power Output (mW)	Rate of Delivery ($mWcm^{-2}$)	Time (sec)
3	464	2090	16.7	180
10	464	2090	16.7	600
30	464	2090	16.7	1,800
45	464	2090	16.7	2,700
60	464	2090	16.7	3,600
3	850	5160	16.7	180
10	850	5160	16.7	600
30	850	5160	16.7	1,800
45	850	5160	16.7	2,700
60	850	5160	16.7	3,600
3	464 & 850	4092	19.88	150
10	464 & 850	4092	19.88	503
30	464 & 850	4092	19.88	1,509
45	464 & 850	4092	19.88	2,263
60	464 & 850	4092	19.88	3,018

Jcm^{-2} = Joules per centimeter squared
nm = nanometers
mW = Milliwatts
$mWcm^{-2}$ = Milliwatts per centimeter squared
sec = Seconds

2.1 *Statistical Analysis*

Data were descriptively analyzed by producing a kill rate value. Kill rate was determined by subtracting the mean treated trials CFUs from the mean CFUs in controls. The result was divided by the control CFU mean and multiplied by 100 to produce a percentage value ([Control – Treated]/Control X 100). A two-way repeated measures ANOVA was performed to determine the main and interactive effects of wavelength and dose in terms of kill rate observed. Since significant effects were observed, a one-way ANOVA and post hoc Tukey's Honest Significant Difference were employed to identify the homogenous subsets. SPSS 20 was the software package employed for the data analysis.

3. Results

Table 2 displays the outcome of the data collection in terms of kill rate at each dose level by wavelength. The most effective combination of dose and wavelength was 60 J/cm^2 using a combination of 464 and 850nm (96.19% kill rate).

Table 2: Kill rate by dose and wavelength K.Pneumoniae

Table 2. Kill rate by dose and wavelength. *K. pneumoniae*

Wavelength (nm)	N	Dose (Jcm^{-2})	Kill Rate (%) +/- SD
464	10	3	16.93 +/- 3.74
464	10	10	10.64 +/- 2.96
464	10	30	18.74 +/- 3.21
464	10	45	-15.78 +/- 23.65
464	10	60	1.42 +/- 8.45
850	10	3	11.97 +/- 4.28
850	10	10	3.14 +/- 2.84
850	10	30	9.07 +/- 2.30
850	10	45	54.17 +/- 5.25
850	10	60	60.12 +/- 6.87
464 & 850	10	3	41.23 +/- 6.88
464 & 850	10	10	42.93 +/- 7.49
464 & 850	10	30	45.34 +/- 5.17
464 & 850	10	45	84.40 +/- 1.27
464 & 850	10	60	96.19 +/- 1.40

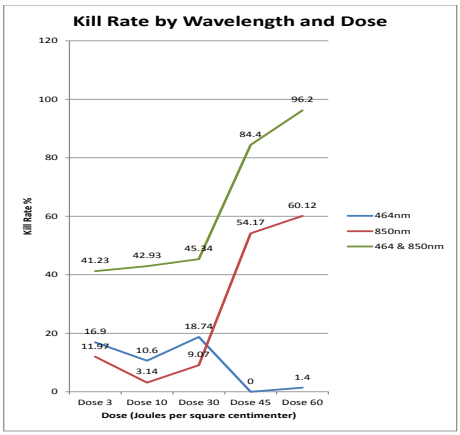
nm = Nanometer

Jcm^{-2} = Joules per square centimeter

SD = Standard Deviation

A two-way repeated measures ANOVA (wavelength X dose) demonstrated a significant main and interactive effect (Main Effect $F_{1,9}$ = 3971.89, p = 0.00; Interactive Effect $F_{1,9}$ = 645.68, p = 0.00). A one-way ANOVA with Tukey's Honest Significant Difference was used for post hoc analysis. The post hoc analysis demonstrated that the combination of 464 and 850nm wavelength was the most effective condition in terms of wavelength and the doses of 45 and 60 J/cm^2 were most effective doses. These combinations represented statistically significant and homogenous subsets. Figure 1 displays these data in a graphical fashion.

Figure 1. Kill Rate by Wavelength and Dose. *K. pneumoniae*



nm = Nanometer

Figure 1: Kill rate by wavelength and dose K.Pneumoniae

4. Discussion

Alternatives to pharmaceuticals to achieve bacterial inhibition must be developed if the goal of limiting the spread of infection is to be achieved. *K. pneumoniae* is a perfect example of why alternatives are needed. The application of light energies is promising because it can be easily applied, the dose and delivery are controllable and predictable, and the potential effectiveness is clearly demonstrated in this research. Kill rates that exceed 90% are equivalent or superior to most drug therapies.

This experiment was performed in an *in vitro* manner. It should be pointed out that generalization to the *in vivo* situation is not necessarily supported by these data. However, Dai, et al [7] demonstrated that light, administered in a similar manner, could significantly inhibit *Pseudomonas aeruginosa* growth in burn wounds on live mice. It does appear there is potential for light application to inhibit bacterial growth while leaving the host unharmed.

Our decision to include an IR wavelength (850 nm) should be discussed. In some of our earliest work we found that the addition of IR energy could improve outcomes.[9] We chose to include IR in these earlier experiments because of the work done by Karu.[18] We recently note that Lee, et al have also demonstrated the photomodulation derived inhibition potential associated with IR.[14] As is demonstrated in our data (See Table 2), the addition of the combination of blue and IR wavelengths is superior, in terms of inhibition, to either wavelength delivered alone. Our work was not designed to particularly identify the mechanism(s) that produce the observed significant inhibition of *K. pneumoniae*. Rather, we sought to determine whether the inhibition was possible. Having demonstrated the effectiveness of these wavelengths, we would refer to Karu [18] to offer possible explanation. Visible and near-IR radiation may produce,

1. Changes in redox properties of the respiratory components of the cell,
2. Generation of singlet oxygen,
3. Localized transient heating of the absorbing chromophores, and
4. Increased superoxide anion production with subsequent increase in concentration of H_2O_2.

Any, all or none of these factors may be the reason we saw improvements in outcome when IR was added. Our work does not directly answer this question, but future research is certainly indicated.

Another factor may be at work in this experiment. *K. pneumoniae*, like other gram-negative bacteria, has an envelope that acts as a regulatory structure. A primary reason many antibiotics are less effective inhibitors of *K. pneumoniae* is that this envelope restricts the entry of the antibiotic into the cell. Additionally, *K. pneumoniae* secretes a capsule that inhibits the ability of white blood cells to perform phagocytosis and diminishes the effectiveness of antibodies, leaving the organism free to invade host tissues. It is possible that the IR wavelength alters the properties of these structures, allowing a greater potential for the light energy to be absorbed and, therefore, those events outlined by Karu [18] to take place. We are currently examining this possibility as part of a related investigation.

The data collected in this research strongly support the effectiveness of a combined 464 and 850nm radiation as an effective inhibitor of *K. pneumoniae*. The doses that proved effective have not, to our knowledge, been shown to cause any detrimental effects to human cells.[19] There is evidence to support the connection between *in vitro* experiments related to bacterial inhibition and potentially effective methods for *in vivo* application.[7]

5. Conclusions

Based on the data collected in this experiment, we have drawn the following conclusions.

1. *K. pneumoniae* can be effectively inhibited with a combination of visible and near-IR radiation.

2. There is a significant interaction between wavelength and dose in terms of inhibition effectiveness.

3. A combination 464 and 850nm wavelength applied at doses of 45 and 60 J/cm^2 yields a significant inhibition (up to 96%) of *K. pneumoniae in vitro*.

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