Violet 405 nm Light: A Novel Therapeutic Agent Against β-Lactam-Resistant Escherichia coli

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Background and Objective: Approximately 1.7 million patients are affected by hospital-acquired infections every year in the United States. The increasing prevalence of multidrug-resistant bacteria associated with these infections prompts the investigation of alternative sterilization and antibacterial therapies. One method currently under investigation is the antibacterial properties of visible light. This study examines the effect of a visible light therapy (VLT) on β-lactam-resistant Escherichia coli, a common non-skin flora pathogen responsible for a large percentage of indwelling medical device-associated clinical infection.

Materials and Methods: 405 nm light-emitting diodes were used to treat varying concentrations of a common laboratory E. coli K-12 strain transformed with the pCIG mammalian expression vector. This conferred ampicillin resistance via expression of the β-lactamase gene. Bacteria were grown on sterile polystyrene Petri dishes plated with Luria-Bertani broth. Images of bacterial growth colonies on plates were processed and analyzed using ImageJ. Irradiance levels between 2.89 ± 0.19 and 9.45 ± 0.63 mW cm⁻² and radiant exposure levels between 5.60 ± 0.39 and 136.91 ± 4.06 J cm⁻² were tested.

Results: VLT with variable irradiance and constant treatment time (120 minutes) demonstrated significant reduction (P < 0.001) in E. coli between an irradiance of 2.89 mW cm⁻² (81.70%) and 9.37 mW cm⁻² (100.00%). Similar results were found with variable treatment time with constant irradiance. Log₁₀ reduction analysis produced between 1.98 ± 0.53 (60 minute treatment) and 6.27 ± 0.54 (250 minute treatment) log₁₀ reduction in bacterial concentration (P < 0.001).

Conclusions: We have successfully demonstrated a significant bacterial reduction using high intensity 405 nm light. Illustrating the efficacy of this technology against a β-lactam-resistant E. coli is especially relevant to the need for novel methods of sterilization in healthcare settings. These results suggest that VLT using 405 nm light could be a suitable clinical option for eradication of β-lactam-resistant E. coli. Lasers Surg. Med. © 2015 Wiley Periodicals, Inc.

• Greater than 6 log₁₀ reduction in β-lactam-resistant E. coli when treated with visible light therapy.

Key words: antibiotic resistance; sterilization; ultraviolet (UV) light; visible light therapy; visible spectrum light

INTRODUCTION

Hospital-acquired infections (HAIs) occur in approximately 1 in 25 patients, resulting in over 1.7 million patients affected annually in the United States [1,2]. It is estimated that 25.6% of these infections are device-associated [1]. Over 150 million intravascular catheters are placed in the Unites States each year with 2.65 billion dollars spent to treat the resulting device-associated infections [3]. The current standard of care for the treatment of device-associated infections involves removal of the infected hardware and treatment with first-line broad-spectrum antibiotics until culture and sensitivity data allow for a more tailored antibiotic regimen. The increasing prevalence of multidrug-resistant bacteria often necessitates a use of second-line antibiotics with a greater side-effect profile and may lead to loss of efficacy of these critical therapeutics [4]. Drug resistance is considered a natural evolutionary process and over 90% of Staphylococcus aureus strains are resistant to penicillin and other related antibiotics [5]. The National Healthcare Safety Network reported as much as 83% of blood stream infections and 20–40% of all HAIs were caused by drug resistant species [4]. It is clear that standard drug

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treatments are not always appropriate to stop HAIs and more effective methods of sterilization should be implemented. Medical device manufacturers have attempted to solve the issue of hardware-associated infections through several novel approaches including coating at-risk surfaces with bactericidal and bacteriostatic agents such as chlorhexidine or silver compounds. These coatings are intended to inhibit bacterial and fungal colonization of the device.

Unfortunately, these solutions have had minimal efficacy in the clinical setting in reducing device-associated infections [6,7]. Given the magnitude of the problem of indwelling medical device-associated infections, more efficacious solutions must be found. One novel approach under investigation is harnessing the potential of visible light therapy (VLT). VLT has shown substantial antimicrobial efficacy against pathogenic bacteria and has received clearance for clinical application for acne treatment by the United States Food and Drug Administration (FDA) [8]. In addition, bacterial resistance to VLT has not been observed. This is thought to be due to a different antimicrobial mechanism, compared to traditional pharmacological antibiotics, which avoids the method in which bacteria become drug-resistant [9,10]. The most commonly used classes of antibacterial drugs target cell wall biosynthesis, protein synthesis, or DNA replication and repair. However, once an antibiotic is introduced clinically, it will only have a period of months to years until a statistically significant efficacy reduction appears [11]. The most common methods of antibiotic resistance seen in bacteria are: chemical or enzymatic modification of the drug, transmembrane pumps which reduce intracellular drug concentrations below therapeutic levels, and bacterial target structure modification resulting in a reduced affinity for the drug [10].

Although others have demonstrated the bactericidal efficacy of 405 nm light on the drug resistant Gram-positive bacterium methicillin-resistant Staphylococcus aureus (MRSA) and non-resistant Gram-negative bacteria, few studies have investigated its efficacy against common drug-resistant Gram-negative organisms [12–19]. In particular, Escherichia coli are Gram-negative bacilli commonly present in the gastrointestinal tract of humans. According to the United States Center for Disease Control, E.coli is one of the most common pathogens that cause nosocomial infections in addition to S. aureus and Pseudomonas aeruginosa [20]. Wavelength specific VLT against the spectrum of pathogens responsible for indwelling device-associate clinical infections may offer a novel, cost-effective, and sustainable agentagainst these life-threatening infections. In this study we explore the efficacy of 405 nm VLT against E. coli, one of the most common isolates of HAIs and indwelling medical device-associated infections [21,22].

MATERIALS AND METHODS

Bacteria Preparation

The common laboratory E. coli K-12 strain (fhuA2 lac(del) U169 phoA glvV44 Φ80 laczZ(del)/M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) transformed with the pCIG mammalian expression vector was used for all experiments. The pCIG plasmid confers ampicillin resistance to the E. coli strain via expression of the β-lactamase gene. The pCIG plasmid also conferred expression of green fluorescent protein, which was used to verify strain selection. Sterile polystyrene 100 × 15 mm² Petri dishes were plated with Luria-Bertani (LB) broth (Lennox L3022). LB selective media contained 0.1 mg/ml ampicillin in all experiments. For consistency in counting colony forming units (CFUs) between experiments, dilution series were made from cultures inoculated with 3–4 well-separated bacterial colonies and grown to saturation. Serial dilutions were prepared by taking 0.5 ml of the saturated bacterial solution and adding it to 4.5 ml of the LB broth stock solution. Time and power trial plates were diluted in this fashion by a factor of 10² for each control and treated plates. Log₅₀ reduction plates were diluted by a factor of 10³ for control and between 10¹ and 10² for treated. Each plate was seeded with 200 µl aliquots and the liquid was spread until uniformly distributed. Optimal plating densities were determined in pilot experiments in order to yield 10–15 CFUs cm⁻².

Experimental Setup

The 405 nm light-emitting diodes (LEDs) were selected (Bivar #: UV5TZ-405-15) and wavelength was verified using a visible light spectrometer (Ocean Optics USB2000). LEDs had a peak wavelength at 404.63 nm ± 1.74 and had a spectrum of 16.57 nm ± 0.89 at 50% relative peak intensity. Nine LEDs were wired in a parallel circuit and arranged in a 3 × 3 grid pattern. This setup was placed onto a tray customized for an incubator. Custom partitions were created to ensure no light transferred between treatment areas (Supplementary Fig. S1).

Light intensity from the LEDs were measured to determine overall output power using a digital power meter (PM100USB, Thorlabs, Newton, NJ) and sensor (S140C, Thorlabs, Newton, NJ). Diode intensity was measured pre- and post-treatment to determine irradiance. Untreated and treated (exposure to 405 nm light) areas within the same plate were measured for CFUs in order to control for potential plate-to-plate differences in bacterial plating densities. A flexible opaque polyethylene sleeve was placed to cover half of the plate. The other half of the plate was left unobstructed and light sources were placed approximately 3.75 cm above the surface of the plate. Circular areas on both the control and treated sides with a radius of 1.02 cm were marked on the plate and used to focus the light in a treated area. All measurements of CFUs were done with respect to bacteria inside this 1.02 cm radius circle. Radiant exposure levels are defined as the intensity of light in an area for a certain amount of time giving it units of mW/cm² · sec or W/m²·sec. Varying power levels of light intensity as well as irradiation time led to several different radiant exposure levels tested.

Bacterial Growth Analysis

After 24 hours of growth, high-resolution images were taken of each plate using a digital 18-megapixel camera (Canon T2i DLSR). The camera was mounted on a stand...
with the lens 61 cm from the plate surface. Images were processed and analyzed using ImageJ (National Institute of Health). Supplementary Figure S2 shows various stages of image processing including a whole Petri dish photograph, a binary image, and individual colony count outlines. Individual colonies were selected based on a size and circularity protocol. Size was determined by pixel count. A range of 200–4000 pixel was determined based on the average isolated colony size [2]. A circularity value range of 0.2–1.0 was selected. A value of 1.0 indicates a perfect circle, whereas a value approaching 0.0 indicates an increasingly elongated polygon.

The resulting CFUs counted at different radiant exposures were plotted as survival fraction versus radiant exposure. The survival fraction values were generated based on a log10 normalization of the resulting CFUs.

Statistical Analysis

Continuous data of individual survival fractions were defined as the quantity of colony forming units normalized by taking log10 of said value in treated and untreated control groups. Differences in continuous data were assessed using a Wilcoxon signed-rank test using JMP software (SAS, Cary, NC). Data was binned based on variable component in each experimental setup. For example, in time variable testing, data groups were binned by time, in log10 reduction testing, groups were binned based on concentration and time. Differences in binned data were assessed using a paired t-test using Microsoft Excel 2013. A P-value of less than 0.05 was set as the a priori level of significance.

RESULTS

Variable Irradiance With Constant Exposure Time Analysis

The bactericidal effect of the 405 nm light on ampicillin-resistant bacteria was first determined using light of varying irradiance. Irradiance was modulated by controlling the voltage and current applied to each diode determined as a percentage based on the manufacturer’s suggested forward voltage and current (3.4 volts, 30 mA). The values were 25%, 50%, 75%, and 100% of this voltage, which produced 9.43 ± 0.72, 16.05 ± 0.79, 26.15 ± 2.30, and 30.30 ± 1.25 volts across each diode, respectively. Figure 1 shows the results for the exposure of ampicillin-resistant E. coli to 405 nm light delivered at increasing light irradiance and with time being held constant at 120 minutes. There was a significant reduction in E. coli starting at an irradiance of 2.89 mW cm−2 resulting in an 81.70% reduction in bacterial growth (P < 0.001). Each subsequent increase in irradiance yielded a statistically significant bacterial reduction (P < 0.001). Maximum bacterial eradication (100.00%) was observed after an irradiance of 9.37 mW cm−2 for 120 minutes (65.90 J cm−2) was applied (P < 0.001). Table 1 lists the bacterial counts observed at each treatment intensity.

Variable Exposure Time With Constant Irradiance Analysis

The antimicrobial effect of 405 nm light was also determined by varying irradiation time. Voltage and current levels of each diode were held at the manufacturer’s suggested values (3.4 volts and 30 mA, respectively). The average irradiation during the time protocols was 9.24 ± 0.44 mW cm−2. Figure 2 depicts the results for the exposure of E. coli to 405 nm light delivered at increasing duration of time with irradiance being held constant. There was a significant reduction (P = 0.030, P < 0.001) in bacterial growth seen at each time point. It is interesting to note that statistically significant changes were shown in a duration of treatment as short as 10 minutes (5.59 J cm−2), in which a mean of 26% bacterial reduction was seen. Table 2 lists the bacterial counts observed for each treatment duration.

Log10 Reduction Analysis

Using the ASTM E2315-03 protocol (Standard Guide for Assessment of Antimicrobial Activity), E. coli was plated in
TABLE 1. Variable Irradiance With Constant Treatment Time

<table>
<thead>
<tr>
<th>Irradiance (mW cm$^{-2}$)</th>
<th>Time (minutes)</th>
<th>Radiant exposure (J cm$^{-2}$)</th>
<th>Treated (CFU)</th>
<th>Control (CFU)</th>
<th>Mean % reduction</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.89 ± 0.19</td>
<td>120</td>
<td>20.80 ± 1.39</td>
<td>3.67 ± 2.92</td>
<td>19.67 ± 2.33</td>
<td>81.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4.91 ± 0.23</td>
<td>120</td>
<td>35.37 ± 1.71</td>
<td>2.11 ± 1.83</td>
<td>20.00 ± 3.80</td>
<td>89.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>8.07 ± 0.06</td>
<td>120</td>
<td>58.09 ± 4.78</td>
<td>0.44 ± 0.72</td>
<td>17.30 ± 4.13</td>
<td>96.46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>9.37 ± 0.03</td>
<td>120</td>
<td>67.49 ± 2.21</td>
<td>0.00 ± 0.00</td>
<td>19.68 ± 5.35</td>
<td>100.00</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Mean percent E. coli CFU reduction following exposure to 405 nm light with treatment time held constant.

DISCUSSION

This study has demonstrated that 405 nm light has a significant bactericidal effect on β-lactam-resistant E. coli. This study set out with three aims: [1] to test the relative susceptibility and potential resistance of a medically common β-lactam-resistant microbe to 405 nm light [2] to determine the potential difference in efficacy between a brief high-intensity and a long low-intensity treatment of equivalent irradiant dose, and [3] to assess the potential log$_{10}$ reduction capability of 405 nm light on β-lactam-resistant E. coli.

Bactericidal Effect of 405 nm Light

The bactericidal effects of visible light, specifically violet spectrum 405 nm light, have been investigated by several groups. The most clinically established use of the bactericidal properties of this light is for the treatment of acne vulgaris. Ashkenzai et al., was able to demonstrate a 5 log$_{10}$ reduction of Propionibacterium acnes after illumination with 407–420 nm light at a radiant exposure of 225 J cm$^{-2}$ in an in vitro study [23]. The effectiveness of this light therapy for acne vulgaris has also been shown in multiple studies with clinical improvements reported from 64.7% to over 90% [24–28].

VLT has also been recently shown to be effective in the treatment of gastritis. Elimination of Helicobacter pylori has been demonstrated in vitro to have a 99.9% reduction in bacterial proliferation after exposure to 20.0 J cm$^{-2}$ of 405 nm light [19]. Ganz et al., demonstrated between 91% and 99% reduction of H. pylori colonies after a single treatment of 40.5 J cm$^{-2}$ with a 405 nm diode laser [15]. These results were replicated by Lembo et al., showing an 86% to over 97% reduction in H. pylori after 15–60 minutes of 408 nm VLT [29].

The efficacy of the broad-spectrum action of violet light against both Gram-positive and Gram-negative bacterial species has been well-documented [12,16–18,30,31]. A study by Maclean et al. compared the relative efficacy of violet light on several medically relevant bacterial species with an LED array delivering an irradiance of 10 mW cm$^{-2}$ to a bacterial suspension [13]. It was found that Gram-positive species were generally more susceptible (2.6–5.0 log$_{10}$ reduction) than Gram-negative species (3.1–4.7 log$_{10}$ reduction).

There has also been an interest in demonstrating the effectiveness of violet light against drug-resistant species.
An in vitro study by Maclean et al. demonstrated a 5 log_{10} reduction of MRSA in a liquid suspension after irradiating with 630–720 J cm^{-2} of >400 nm light [32]. A following study further showed that a 5 log_{10} reduction of MRSA can be achieved with a significantly lower radiant exposure of 45 J cm^{-2} when light is delivered specifically at the 405 nm wavelength [13]. Another research study produced similar results when irradiating both MRSA USA 300 and MRSA IS-853 at 55 J cm^{-2} with 405 nm light, achieving a reduction of 92.1% and 93.5%, respectively [16]. In Gram-negative bacteria, Hamblin et al. tested VLT on a strain of H. pylori that was resistant to multiple antibiotics [19].

Though studies have demonstrated the use of violet light to target MRSA and H. pylori, data is lacking on the efficacy of 405 nm light in the inactivation of β-lactam-resistant E. coli. β-lactam antibiotics act through inhibition of bacterial cell wall biosynthesis. MRSA and other β-lactam-resistant bacteria produce β-lactamases that provide resistance to commonly used β-lactam antibiotics like penicillin, cephamycin, and carbapenem. Hydrolysis of the β-lactam ring of the antibiotic will render it ineffective [33]. This study has established the efficacy of using 405 nm light to inactivate a medically common drug-resistant bacterium. We have constructed dose-response curves illustrating the irradiation necessary for the in vitro eradication of E. coli on plated growth media. This information may be useful for establishing the potential for clinical use. Due to the increasing numbers of HAIs and medically relevant drug-resistant microbes, this technology could provide another option for the clinical treatment of antibiotic-resistant bacterial infections.

**Mechanism of Action**

Although the antimicrobial mechanism of action of violet light has not been completely elucidated, research over the past decade has identified several critical areas of activity. It has been demonstrated that this process is oxygen dependent. Studies have shown that bacteria exposed to violet light under anaerobic conditions were able to resist the phototoxic effects and that decreasing the amount of reactive oxygen species (ROS) in an aerobic environment also reduced the phototoxic effect [32]. Conversely, other groups have shown that increasing the amount of oxygen significantly increased the rate of bacterial inactivation [32]. It has also been demonstrated that violet light illumination of endogenous porphyrins within bacteria results in their decreased proliferation [23]. Further, Hamblin et al. found that there is a strong positive correlation between amount of porphyrins contained

### Table 2. Variable Treatment Time With Constant Irradiance

<table>
<thead>
<tr>
<th>Irradiance (mW cm^{-2})</th>
<th>Time (minutes)</th>
<th>Radiant exposure (J cm^{-2})</th>
<th>Treated (CFU)</th>
<th>Control (CFU)</th>
<th>Mean % reduction</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.32 ± 0.51</td>
<td>10</td>
<td>05.59 ± 0.31</td>
<td>18.94 ± 7.71</td>
<td>27.42 ± 13.42</td>
<td>26.40</td>
<td>0.030</td>
</tr>
<tr>
<td>9.22 ± 0.55</td>
<td>50</td>
<td>27.67 ± 1.66</td>
<td>4.11 ± 3.59</td>
<td>27.54 ± 12.69</td>
<td>82.11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>9.37 ± 0.03</td>
<td>120</td>
<td>67.49 ± 2.21</td>
<td>0.00 ± 0.00</td>
<td>19.68 ± 5.35</td>
<td>100.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>9.13 ± 0.27</td>
<td>250</td>
<td>136.91 ± 4.06</td>
<td>0.00 ± 0.00</td>
<td>35.57 ± 23.38</td>
<td>100.00</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Mean percent E. coli CFU reduction following exposure to 405 nm light with variable treatment time and constant irradiance.

### Fig. 3

E. coli Log_{10} reduction at variable radiant exposure: Inactivation of confluent concentrations of E. coli upon exposure to 405 nm light as a function time duration. Light was delivered at a constant power intensity (~9.1 mW cm^{-2}) with varying durations of time: 60, 120, and 250 minutes. (Top) A scatter plot showing the survival fraction of each plate in control and treated areas. Logarithmic trend lines are added to show the general relationship between control and treated plates as a function of radiant exposure. (Bottom) A bar graph showing binned survival fraction of grouped by concentration and treatment time of E. coli (10^{-1} at 250 minutes, 10^2 at 120 minutes, 10^3 at 60 minutes) Statistical significance in treated versus control. Statistical significance of P < 0.001 is noted with a double asterisk (**).
within bacteria and the cytotoxic effects of violet light [19]. These studies support an oxygen dependent mechanism which allows violet light to excite endogenous porphyrins to a high energy state. This energy is transferred to form ROS that cause intracellular damage. Bacterial and fungal proteins, lipids, and nucleic acids are particularly susceptible to these ROS [35].

The mechanism of action of 405 nm VLT is relevant when assessing the potential for microbe resistance. Although resistance to 405 nm light specifically has not been investigated, the potential development of microbial resistance to photodynamic inactivation has been studied. After repeated cycles of partial inactivation followed by regrowth, different bacterial species failed to develop resistance to the photodynamic process after 10 and 20 cycles [36,37].

It is possible that due to the multifactorial mechanism of action viable bacteria are less likely or unable to develop a resistance to VLT. Further studies are necessary to determine the extent to which bacteria are able to develop resistance. However, this study was able to illustrate that drug-resistant bacteria are susceptible to 405 nm VLT. The significant bacterial toxicity demonstrated makes VLT a potential alternative treatment for associated clinical infections.

Equivalent Radiant Exposure Dose

An in vitro study by Murdoch et al. showed significant differences in the bactericidal effect on Listeria monocytogenes at equivalent radiant exposure levels but varying irradiance and exposure time [18]. We performed similar preliminary testing on E. coli by reducing power levels and increased treatment time. The results supported the findings of Murdoch et al. in that we demonstrated a significant reduction of bacteria in treated plates even at 25% power and fourfold longer treatment times. However, due to the radiant exposure tested, both treatments in this study resulted in a 100% reduction in bacterial CFUs. Because of this, we cannot ascertain if one method is more efficacious than the other given equivalent radiant exposure. Additional testing will be performed in subsequent studies to determine efficacy trends at equivalent radiant exposure and varying irradiance and time levels.

Log10 Reduction

This study has also established the potential log10 reduction of 405 nm light on drug-resistant E. coli. The FDA requires that a minimum of a four-log10 reduction be achieved in order to be considered an antibacterial solution [38]. In order to be considered for approval, the FDA recommends following ASTM E2315-03 Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure or an equivalent method.

Using this ASTM protocol, we have demonstrated a greater than 6 log10 reduction. This is 100 times the reduction necessary to be defined as bactericidal by the FDA. The results from this study also illustrate the time and dose dependence of light technology. These results suggest that VLT using 405 nm light could be a suitable clinical option for sterilization in drug-resistant species of bacteria. The log10 reduction information provided may help direct future applications of VLT in a clinical setting.

CONCLUSIONS

We, along with other researcher groups, have successfully demonstrated a high efficacy at bacterial reduction using high intensity 405 nm light. Illustrating the efficacy of this technology against a β-lactam-resistant E. coli is especially relevant given the great need for novel methods of sterilization in healthcare settings. This information is also valuable in the context of the increasing occurrence of drug-resistant bacteria in HAIs and device-associated infections. The increasing incidence of these infections illustrate that current antimicrobial technologies are inadequate. Further research directives will focus on other common nosocomial infection causing agents, such as P. aeruginosa, S. aureus, and other drug resistant bacteria.

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