ABSTRACT

Heterogeneous Response to 470 nm Blue Light Amongst 25 *Staphylococcus aureus* Isolates

Meredith Rosenthal

Director: Tamarah Adair, Ph.D.

Staphylococcus aureus infections are an increasing concern for the United States as it is currently the most prevalent cause of hospital acquired infections. The growing antibiotic resistance amongst these bacteria has called for new treatments, including photodynamic therapy, which uses light to kill microorganisms. We tested the variance of the response to blue light of 25 different isolates of *S. aureus*. Using 470 nm blue LEDs with an approximate forward power of 80 mW and an average luminance of 4.22 klux, we exposed 1 ml *S. aureus* cultures for 30 minutes and measured the optical density at 600 nm 18 hours after treatment. We compared the blue light treatment to a control group and found that there was a wide degree of variance with inhibition values ranging from 97% to an increased growth rate of 8%. Using flow cytometry, we measured cell counts at 4 hours after exposure to blue light for 3 strains identified as highly sensitive and 3 strains identified as highly resistant. There was a range of 80-99.9% inhibition when examining live cell counts. However, the discrepancy between the more and less sensitive strains was still apparent when examining live to dead cell ratios. A variation in susceptibility to blue light indicates a genetic trait may be responsible for this phenotype. Future studies will explore the genetic differences in these strains.

APPROVED BY DIRECTOR OF HONORS THESIS:

_____

Dr. Tamarah Adair, Department of Biology

APPROVED BY THE HONORS PROGRAM:

_____

Dr, Andrew Wisely, Director

DATE: _____

HETEROGENEOUS RESPONSE TO 470 NM BLUE LIGHT AMONGST 25 *STAPHYLOCOCCUS AUREUS* ISOLATES

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

Meredith Rosenthal

Waco, Texas

May 2014

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to acknowledge the College of Arts and Sciences and URSA for a grant and invaluable resources that allowed me to complete my research. I would like to thank the Department of Biology specifically for the opportunity to participate in the Summer Undergraduate Research Program (SURP) 2013 during which I gathered much of my data. I would like to thank Jerry Milner for his construction of the updated light box used in our lab as well as helping with emergency repairs. I would most of all like to thank my advisor, Dr. Tamarah Adair, who went above and beyond the call of mentorship to provide invaluable knowledge and to help me through the frustrating times of research. Lastly, I would like to thank my lab mates past and present who have assisted me and have collaborated ideas including Gregg Fox, Kayla Fricke, Carlo Manzana, Suk Namkung III, Bayless Drum, Jeanne Samake, Will Jones, and Sarah Yuen.

How far that little candle throws his beams! So shines a good deed in a weary world.
— William Shakespeare, *The Merchant of Venice*

CHAPTER ONE

Review of Literature

Staphylococcus aureus is a Gram-positive, nonmotile, facultatively anaerobic cocci. Its name is derived from the Latin meaning of *aureus,* golden, due to the carotenoid pigments produced during growth. It can colonize mucosal surfaces, particularly those of the anterior nares, and is part of the normal flora on human skin. The organism is not inherently pathogenic but can cause an array of diseases. Shedding of the bacteria is common due to their often external presence on the body and is responsible for many hospital–acquired infections. *Staphylococci* are susceptible to high temperatures and disinfectants, but can survive on dry surfaces for long periods. Therefore, proper hand-washing techniques of medical personnel and proper sanitation of fomites are necessary for limiting the spread of the organism.[1]

S. aureus causes disease through either the production of toxins or the direct invasion and destruction of tissue. It can cause a variety of ailments from impetigo, boils, and food poisoning to pneumonia, bacteremia and even death. Additional concerns include increased rates of infections associated with implants and foreign objects placed in the body such as catheters, shunts, and breathing tubes. In a survey done by Morgan et al. in 2004, *S. aureus* was the most commonly (76%) identified cause of skin and soft-tissue infections in patients presenting to emergency departments in 11 US cities.[2] Even more alarming is the increase in the total number of infectious cases involving *S. aureus*. From 1999-2005, there was an estimated 62% increase (294,570 vs. 477,927) in the number of hospitalizations involving *S. aureus*-related infections.[3]

More concerning is the growing resistance of *S. aureus* to the antibiotics used as the primary treatment for these infections. Only two years after the introduction of penicillin for medical use, a penicillin-resistant *S. aureus* isolate was observed in a hospital. Penicillin-resistant isolates have since been found in the community and currently an approximate 80% of all *S. aureus* strains are resistant to this antibiotic. The trend continued with methicillin with the discovery of a resistant isolate in 1961, only two years after the introduction of this β-lactam drug.[4] Methicillin resistance is due to the acquisition of the *mecA* gene. It is suggested that methicillin-resistant *S. aureus* (MRSA) can acquire its *mecA* gene through horizontal transfer from coagulase-negative *Staphylococci* species, such as *S. epidermidis* or *S. haemolyticus*.[5, 6] The ability to acquire DNA from the environment allows for rapid genetic evolution of bacteria, and quickens the widespread resistance to antibiotics. This, combined with the overuse of antibiotics, has caused antibiotic-resistant bacteria to overwhelm society's healthcare concerns requiring the search for new treatments to combat these infections.

As early as 1993, it was evident that MRSA was no longer limited to patients at risk for nocosomial infection with predisposing factors such as prolonged hospitalization, invasive or surgical procedures, indwelling medical devices, or exposure to antibiotics.[7, 8] A distinction was made in the scientific community for infections acquired inside the hospital (HA-MRSA), and never before observed infections acquired from the community (CA-MRSA). CA-MRSA infections are on the rise and have even begun to outnumber HA-MRSA infections. HA-MRSA has been divided into the subcategories of community-onset HA-MRSA, where health risk factors are present but a *S. aureus* infection is observed within 48 hours of hospital admission, and hospital-onset HA-

MRSA. CA-MRSA and HA-MRSA have differences in genotype and phenotype. Besides being classified by location of onset, MRSA can also be classified using multilocus sequence typing (MLST) and PCR of *mec* cassettes and CCR genes. CA-MRSA is most commonly associated with USA300, SCC*mec* IV, and the PVL toxin; HA-MRSA usually correlates with the strain USA100 and SCC*mec* II.[9, 10] A survey of 12 participating facilities in Minnesota found the following trends with CA-MRSA and HA-MRSA: CA-MRSA had a younger median age of 23 years vs. 68 years in HA-MRSA, the CA-MRSA isolates were more sensitive to other antibiotics, and HA-MRSA was associated with more invasive infections such as respiratory, urinary, or blood infections whereas CA-MRSA primarily presented as skin and soft tissue infections.[9]

Overall, MRSA has become an increasing burden over the years in the United States. A review by Appelbaum compared the percentage of *S. aureus* isolates that were resistant to methicillin to be <1% in northern Europe vs. >40% in southern and western Europe with the greatest percentage (>50%) in the U.S.[11] Review of the National Hospital Discharge Survey records from 1995-2005 indicate that *S. aureus* infections resistant to methicillin increased 119%, or about 14% per year. In 2005, there were 11,406 *S. aureus*-related deaths of which 6,639 were MRSA. However, there was an overall decrease of 3.7% to 2.4% in the percentage of *S. aureus*-related hospitalizations that resulted in death during this time.[3] Using data from July 2004 to December 2005, the national estimated adjusted incidence for invasive MRSA was 37.54 per 100,000 persons with a breakdown of 2.46 per 100,000 for community-onset HA-MRSA (HACO), 9.91per 100,000 for hospital onset HA-MRSA (HO), and 5.59 per 100,000 for CA-MRSA (CA).[10]

Infection incidence rates were reevaluated more recently in 2011 by Dantes et al. who found that there was a 27.7% decrease of HACO infections, 54.2% decrease in HO infections, but only a 5% decrease in CA infections. This corresponds to an estimated 30,800 (31%) fewer invasive MRSA infections in the USA in 2011 compared to 2005.[12] The large decrease in HO infections (54%) may be attributable to an increased awareness of the burden of MRSA infections in the United States which resulted in greater local and national infection prevention measures in health care settings. However, the increase in the percentage of MRSA infections acquired outside of a hospital setting is concerning because there are currently no clear guidelines on how to control transmission in the community.

Additional concerns posed by *S. aureus* for the United States include the percentage of the healthy population that are carriers for this organism, the increased morbidity and mortality of MRSA, and the incredibly high financial cost of MRSA. It is estimated that 20% of the population are persistent carriers of *S. aureus* in the anterior nares, 60% are intermittent carriers, and 20% are persistent non-carriers. There is concern for individuals becoming infected by the strain carried in their nasal passages or infecting others, especially for those in healthcare settings with associated risks. Individuals with a MRSA infection have a greater risk for more severe complications, morbidity, and even mortality than if they had a methicillin-sensitive *S. aureus* (MSSA) infection.[13] In addition, MRSA infections cost much more financially than MSSA infections, with a per patient-day cost difference of $9,744 for MRSA vs. only $4,442 for MSSA infections. This suggests that MRSA cost the healthcare system, both patients and

hospitals, an additional $830 million- $9.7 billion in 2005 before considering the indirect costs related to patient pain, illness, and time spent in the hospital.[3, 14, 15]

The threat of *S. aureus* infections does not stop with MRSA. When methicillin is ineffective against a particular strain, glycopeptides such as vancomycin are used. Vancomycin was introduced into the US healthcare setting in 1958, and vancomycin intermediate *S. aureus* (VISA) strains were first seen in 1996. The first vancomycin resistant *S. aureus* (VRSA) was later discovered in 2002 and seven total strains have been reported during the period 2002-2006.[16, 17] It is believed that VRSA strains acquired the *vanA* operon through conjugal transfer of plasmids from vancomycin-resistant *Enterococcus faecilis.*[18] All VRSA strains identified have been proven sensitive to other antibiotics, but the emergence of MRSA and VRSA show that alternative treatments to antibiotics are in immediate demand.

Suggested alternative treatments include natural antibacterial compounds, cationic antimicrobial peptides, bacteriophages, and staphylococcal vaccines.[19] However, there are many complications with the efficacy and consistency of these treatments that currently prevent them from being viable options. For example, Lu et al. discusses the problems with bacteriophage therapy which include the host range of the bacteriophage, the evolution of bacterial resistance *in vitro*, manufacturing, systemic side effects, and the delivery of the bacteriophage to the site of infection and to the cells themselves due to difficulty in penetrating the biofilm of *S. aureus.*[20] A *S. aureus* vaccine that would mechanistically employ antibodies to target the bacteria for destruction, has yet to been successfully produced due to the extensive survival mechanisms *S. aureus* has against humans. These include proteases that degrade antibodies, interference strategies for

blocking complement in opsonization, and many others. As of yet, trials of *S. aureus* vaccines in mice models have not shown positive results, and at best personalized vaccine cocktails may be required.[16]

Another potential treatment option is light-based anti-infectives. These include ultraviolet C (UVC) irradiation therapy, photodynamic therapy (PDT), and blue light therapy. The proposed advantages of these treatments are the ability to eradicate microbes regardless of antibiotic resistance and the improbability of the development of resistance to these light-based therapies due to the non-specific nature of their targets.[21] UVC irradiation has the best potential ability to inactivate microorganisms using a wavelength of 250-270 nm which is absorbed by the nucleic acids of microbial cells causing DNA damage. However, UVC can also cause damage to human DNA with prolonged exposure being carcinogenic, eliminating it from being an ideal treatment for MRSA infections, although it is currently successfully used to sterilize equipment and rooms.

Photodynamic therapy (PDT) is different from UVC irradiation in that it utilizes visible light to activate a pre-applied photosensitizer (PS) drug to its excited state, producing reactive oxygen species (ROS) that chemically attack and alter a wide range of biomolecules in the cell.[21] This method has greater bactericidal results in Gram-positive bacteria due to the porosity of the outer wall through which the PS can enter the cell. PDT has potential for treatment of fungi, viruses, acquaculture, waste water, and with food disinfection. PDT using blue light with a wavelength between 400 and 500 nm has shown to be effective against a variety of organisms. Blue light has occasionally been reported to be phototoxic to mammalian cells at certain spectrum ranges in a wavelength-

dependent manner. Thus, an ideal use of blue light in therapy should choose an optimal wavelength that selectively excites the PS with minimized effect on the chromophores in mammalian cells.[22] Contrarily, a different study found that 470 nm blue light actually enhanced wound healing in excision sites in a rat model. It is postulated that blue light affects the nitric oxide (NO) pathway in the mammalian tissues of the rats by recovering mitochondria inhibited by NO gas release and enhancing the amount of growth factors released which are important mediators in wound healing.[23]

The results of PDT vary greatly between different pathogens. Nakonechny et al. used white light and the PS methylene blue (MB) or toluidine blue (TBO) on both *S. aureus* and *E. coli*. In the presence of MB, *S. aureus* resulted in a 6.5 log reduction in colony forming units (CFU) when using a power of 12.1 mW cm^{-2} for 1 hour, whereas only a 2.2 log reduction was seen in *E. coli*. This research indicates that there is a dose-dependent effect on the viability of these organisms: at a moderate power of 1.6 mW per cm^2, the *S. aureus* with PS was reduced only 10-fold. Interestingly, the control cells (with no PS) were reduced 6-fold with the higher power (12.1 mW cm^{-2}) of white light.[24] PDT has also shown to have positive results in the treatment of *L. major* (Leishmaniasis), *Acne vulgaris,* herpes simplex virus, papillomavirus, *H. pylori*, *T. rubum* (onychomycosis, a toenail infection), and *A. actinomycetemcomitans* (dental infection). However, there is no current PDT treatment that would be safe for humans, effective against all pathogens, and whose effectiveness is greater than current treatment options, including that of antibiotics.[21]

A similar pattern was seen with PDT using blue light on *Propionibacterium acnes*, a Gram-positive organism that normally inhabits human sebaceous glands and is

the major cause of acne. Ashekanzi et al. found that bacterium pre-treated with δ-aminolevunic acid (ALA) in clostridial broth had enhanced production of porphyrins. The increase in porphyrin levels paralleled the appearance of low density areas in the middle of the cells suggesting leakage of intracellular components. Additionally, asymmetrical septation and undivided elongated cells were also observed which lead to a lytic process that caused bacterial cell death. Bacterial cell counts were diminished after PDT with one illumination of 75 J cm^{-2} decreasing the cell-count by two orders and 100 J cm^{-2} causing a three order decrease. However, there was a reduction in viability even without the addition of ALA, decreasing one to two-fold with one dose, two-fold with two consecutive doses, four-fold with two doses timed 24 hours apart, and five-fold with three consecutive illuminations.[25] Thus, although the viability was decreased to a greater degree with the addition of the ALA, it was not necessary to be bactericidal.

There is much concern over the safety and side effects of photosensitizers, the vessels that allow transfer and translation of light energy into ROS via a type II chemical reaction. There are three families of PS: porphyrins, chlorophylls, and dyes, each with their own setbacks. A complication with porphyrins is the prolonged photosensitivity after administration. This could be minimized by photobleaching in which just enough PS is delivered to kill the unwanted cells, with no PDT occurring in non-targeted tissues. However, no PS can currently accomplish this preciseness clinically. Some PS of the chlorine family have shown to cause vascular damage and the commonly used ALA can cause pain in PDT therapy, especially because it is not very active and requires high energy levels and long treatments. Many dyes are naturally hydrophobic and require special liposomal preparation for efficient delivery.[26] Additionally, the long-term effects

of PS have not been studied extensively and there is concern of harmful side-products that can be produced after being metabolized. Thus, PS and their effects require much research and development before these products can be safely used in medicine.

There has also been some evidence of variation amongst different strains in response to PDT using 624 nm red light. This could prove to be problematic in establishing a basic light therapy protocol for *S. aureus* infections should this trend also exist with the use of blue light. Grinholc et al. tested 81 *S. aureus* strains: the ATCC 25904 strain, 40 MRSA, and 40 MSSA. Four MRSA strains were considered resistant (0-0.03 log reduction), and 20 MRSA and 15 MSSA strains were considered intermediately resistant (0.2-0.9 log reduction). For the MSSA strains tested, 62.5% were considered sensitive to PDT vs. only 40% of the MRSA strains. When the antibiograms of individual strains were compared to PDT results, no correlation could be noted. They reported that the non-biofilm producing strains were more sensitive to PDT.[27] It must be kept in mind that this study added a PS to the medium. Red light illumination without a PS does not inactivate *S. aureus,* and the mechanism behind photodynamic inactivation may be different for red and blue light.

The ability to form a biofilm poses additional obstacles in persistent *S. aureus* infections. Biofilms provide an additional layer of protection for *S. aureus* and can decrease the effectiveness of compounds intended to be taken up or interact with the cell, such as antibiotics and PS. Using the photosensitizer TMP, Di Poto et al. studied the disruption and dispersal of the biofilms of three *S. aureus* strains with PDT. Addition of 10 μM of TMP (non-toxic dose) with the highest light dose used (150-200 J cm^{-2}) resulted in a 30-70 fold decrease in viability compared with untreated controls and those

with TMP but kept in the dark. Notably, confocal laser scanning microscopy (CLSM) images of the biofilms subjected to PDT showed increased permeability to propidium iodide, indicating the cell membrane could be an important site of photodamage. Also, cell detachment and consequent disruption to the biofilm architecture was observed with the PDT-treated samples.[28] Thus, PDT used in conjunction with an antibiotic or other treatment may have synergistic effects. However, increasing maturity and corresponding thickness may result in a decreased killing effect.

More interest has arisen about the possibility of bactericidal effects of light without the addition of a photosensitizer. Hamblin et al. found that only 30 J cm^{-2} of broadband visible light could inactivate *H. pylori*, but not *E. coli.* This suggests that *H. pylori* is at least 100,000 times more sensitive than *E. coli.* Additionally, *H. pylori* was much more sensitive than *P. acnes*, requiring two to three times less light to produce 100 to 1,000 times more killing. Multiple strains of *H. pylori* were tested, including laboratory and clinical strains, one of which was doubly antibiotic resistant, and all were photoinactivated. There was a positive correlation over several orders of magnitude between porphyrin fluorescence and cytotoxicity, suggesting that an accumulation of a metal-free porphyrin produced ROS upon illumination. The determined fluorescence peak corresponded with that for the porphyrins CP and PPIX. The most effective wavelength ranges to inactivate *H.* pylori were determined to be 375-435 nm followed by 435-475 nm. Overall, there was a 99.9% reduction in viability of all strains tested with 20 J cm^{-2} of 405 nm light.[29]

Inactivation of *S. aureus* by broadband visible light was shown through the research done by Lipovsky et al. With the maximum fluency used of 180 J cm^{-2}, two

strains showed 55.5% and 99.8% reductions in colony counts. However, both strains had increased proliferation at a lower fluency of 18 J cm^{-2}. The more sensitive strain produced 10 times more secreted and intracellular porphyrins and greater levels of ROS whereas the more resistant strain had two times more carotenoids. Carotenoids are known to be an antioxidant and can potentially protect the cell from the damaging effects of ROS. Conversely, the increased amounts of porphyrins could increase the amount of energy transferred from the light to cellular machinery in the presence of oxygen to make ROS.[30]

Lipovsky et al. later sought to determine the most effective wavelength, either 415 or 455 nm, in the visible range to inactivate *E. coli* and *S. aureus* while measuring the amount of ROS produced (via spin-trap paramagnetic resonance spectroscopy (TEMPO) measurements). The more effective wavelength was 415 nm for both species. There was an increase in cell count for *S. aureus* when illuminated with 30 J cm^{-2}(5 minutes) of 415 nm light, a 50% decrease with 60 J cm^{-2}, and a maximum 90% reduction with 120 J cm^{-2}. The wavelength 455 nm resulted in only a 50% reduction with 120 J cm^{-2}. *E. coli* was nearly 100% inactivated at 415 nm with the smallest dose of only 30 J cm^{-2} and showed a dose-dependent reduction in viability with 455 nm. Unlike *S. aureus*, neither 415 nm nor 455 nm enhanced proliferation of *E. coli* at any fluency.[31] The increased proliferation of *S. aureus* at low fluencies could be problematic in terms of wound healing, so more detailed data is needed on the consistency of these results and the minimum dosage required to inactivate all strains of *S. aureus*.

Maclean et al. also sought to determine the most effective wavelength to inactivate *S. aureus*. Absolute doses of 23.5 J cm^{-2} were delivered to the samples and

significant log reductions were observed for wavelengths in the 400-420 nm range. The greatest log reduction of 2.4 occurred at 405 nm. A variety of different bacteria were tested for dose/$\log_{10}$ reduction response to 405 nm light with a MSSA strain of *S. aureus* having the greatest reduction of 7.2 (units of $\log_{10}$ (N/N_0 per J cm^{-2})), followed by MRSA with a value of 9, and *Staphylococcus epidermidis* with 9.1. Notably, *Pseudomonas aeruginosa* had a value of 42.9 and *E. coli* 58.1.[32] Thus, it is evident that different bacteria respond drastically differently to 405 nm light, and infections with multiple bacteria species would either require great amounts of blue light or combination treatments using blue light and antibiotics. Also notable in Maclean's work is the finding that regardless of the initial population density, similar amounts of blue light energy was required to achieve a similar $\log_{10}$ reduction. For example, a 3-$\log_{10}$ reduction in CFU count required 36 J cm^{-2} for both 10^3 and 10^7 CFU/ml and 41 J cm^{-2} for 10^9 CFU/ml.[32]

Enwemeka et al. explored the inactivation of *S. aureus* with blue light without the addition of a photosensitizer. Their initial research focused upon the effects of blue light on MRSA *in vitro* by streaking cultures onto Tryptic Soy Agar (TSA) plates, exposing them to 405 nm light for different periods of time (corresponding to 0-60 J cm^{-2}), and comparing the CFU after 24 hours of incubation with controls. This was the first report that blue light can photo-destroy a HA-MRSA and CA-MRSA strain of *S. aureus*. The data displayed a non-linear dose-dependent curve: increases in energy fluencies between 1.0 and 15 J cm^{-2} resulted in greater bacteria death than similar increases between 15 and 60 J cm^{-2}. This suggests that consecutive low doses of blue light is more effective than a single higher dose. Both *S. aureus* and *P. aeruginosa* were tested and inactivation rates reached 90% and 95.1% respectively. *P. acnes* showed no response to the blue light in

this experiment. In addition, 470 nm light was tested, and it was found that 405 nm had greater killing rates for both bacterial species.[33]

Enwemeka et al. tested 470 nm blue light because of its ubiquity, ease of use, and low cost. The 405 nm superluminous diodes (SLDs) were invalidated due to the need to filter the UV light produced with this diode. The 470 nm light does not contain UV light in its spectra and is therefore safer to humans. There was a statistically-significant ($p < 0.05$) dose-dependent non-linear trend, similar to that with 405 nm. Both the CA-MRSA and HA-MRSA had over 90% inactivation with a dose of 55 J cm^{-2}. More than 40% inactivation was seen with 7 J cm^{-2} and over 80% with only 35 J cm^{-2}.[34] This indicates that low doses of 470 nm blue light may be possible as a less expensive and potentially safer treatment for MRSA infections. Enwemeka is now attempting to increase the inactivation percentages with greater penetration in order to kill the remaining few colonies that survive irradiation.[35]

Gaupp et al. wrote an inclusive review of the response to oxidative stress of different *Staphylococcus* species. Endogenous stress can occur which leads to monoatomically-reduced oxygen that interacts with flavoproteins to generate further reactive oxygen species (ROS). ROS can also be produced with Fenton chemistry in which iron reacts with H_2O_2 to generate $HO\cdot$. The close proximity of Fe^{2+} to DNA suggests that ROS generated by Fenton chemistry can interact with DNA, as well as amino acids and proteins. *Staphylococcus* have many means to resist oxidative stress, such as via the membrane-bound staphyloxanthin, the main pigment of *S. aureus,* which is a potent antioxidant because its numerous conjugated bonds quench toxic singlet oxygen. *Staphylococci* also have detoxifying enzymes, mechanisms to maintain metal

homeostasis, DNA protection and repair mechanisms, and protein damage repair pathways. Staphylococcal response to oxidative stress has been shown to depend on the chemical nature of the oxidant. Changes in the bacterial metabolic status are "sensed," creating signals to alter the activity of the redox-responsive and metabolite-responsive regulators. When damage to the cell becomes so great that the DNA is damaged, *Staphylococci* can activate the SOS system where a highly conserved global DNA damage repair system is activated, which involves the LexA regulon. This response may activate virulence genes and mobile genetic elements. [19]

Maisch et al. demonstrated that ROS play a specific role in photodynamic inactivation of *S. aureus*. After the photosensitizer absorbs light, energy is transferred to the triplet state through which either charge or energy is transferred to a substrate or molecular oxygen to generate ROS. Viability was decreased 20-fold with a low concentration of *S. aureus*, and 8-fold for a high concentration, suggesting increased oxygen levels are needed for greater cell densities. During illumination with the laser light for 6 minutes (54 J/cm^{-2}), the low cell concentration showed only a slight reduction in oxygen saturation from 98% to 93% whereas the high cell concentrations had a decreased oxygen saturation from 90% to 60%. During a second experiment where the light source was not switched off after illumination, the oxygen concentration decreased to 82% saturation for the low cell density samples and decreased to 0% saturation for the high cell concentration.[36] Thus, inactivation of *S. aureus* requires oxygen, lending to the hypothesis that ROS are being produced and are the bactericidal agent.

The significance of oxygen depletion in *S. aureus* after exposure to 400 nm light without the addition of a photosensitizer was studied by Maclean et al. Three oxygen

scavengers (ascorbic acid, catalase, and dimethylthiourea [DMTU]) were used to illustrate the effect of oxygen depletion in photo-irradiation of *S. aureus*. A 30-minute exposure resulted in a 1.0 $\log_{10}$ reduction for all three scavengers. Thirty minutes of light with no scavenger resulted in close to total destruction of the initial population of 2.2 x 10^5 CFU/ml. When comparing oxygen-enhanced and non oxygen-enhanced samples after 30 minutes of exposure, both achieved a 5 $\log_{10}$ reduction although the oxygen-enhanced samples achieved this more quickly with a 4.4 $\log_{10}$ reduction after 25 minutes (450 J cm^{-2}) versus a 1.2 $\log_{10}$ reduction. Thus, oxygen is needed to achieve high levels of inactivation of *S. aureus* in an efficient time-frame. Non-light-exposed samples, both oxygen-enhanced and not oxygen-enhanced, showed no inactivation. Additionally, when the irradiance levels (75 and 30 mW cm^{-2}) were adjusted for time of exposure, the same total dose of 450 J cm^{-2} was delivered. It was found that the level of inactivation is a function of the dose, independent of the irradiance used. This paper theorized that the mechanism of visible-light inactivation of *S. aureus* is the photo-excitation of naturally occurring intracellular porphyrins that function as endogenous photosensitizers.[37]

In summary, there is promising evidence that a range of wavelengths of blue light (400-470 nm) can kill *S. aureus* with the addition of a known photosensitizer using PDT. Additional studies demonstrate that the addition of a photosensitizer is not required, making the clinical applications of blue light for *S. aureus* infections even more valuable. Although there is evidence of variation in the effectiveness of PDT for different *S. aureus* isolates when using red light, there has not been studies published evaluating the effects of blue light on numerous *S. aureus* strains. Thus, there was a need to determine if a heterogeneous response to different isolates to blue light exists. This information will

have clinical implications of how this therapy can be applied to patients and to help determine the mechanism of blue light inactivation of *S. aureus*.

CHAPTER TWO

Introduction

Staphylococcus aureus is a Gram-positive cocci that forms grape-like clusters and is aerobic or facultatively anaerobic.[1] It is often found as a commensal organism of the human nose: 20% of the population persistently carries *S. aureus*, 30% carry it intermittently, and 50% are considered non-carriers.[38] However, should *S. aureus* breach the skin barrier, it can cause a range of ailments from superficial wound infections to invasive, life-threatening disorders including pneumonia, bone and joint infections, bloodstream infections, toxic shock syndrome, and endocarditis.[39] *S. aureus* has become a recurrent cause of infection in both hospital and community settings, and is the most frequent presentation of skin and soft tissue infections in emergency departments in the U.S. as well as the most common cause of hospital-acquired infections.[40]

Along with the rise in *S. aureus* infections includes those resistant to antibiotics. Presently, less than 5% of *S. aureus* strains are susceptible to penicillin.[33] It is estimated that 50% of *S. aureus* strains are classified as methicillin resistant *S. aureus* (MRSA) which has become common since its recognition in 1961, and costs the United States $4-7 billion annually.[15] MRSA was contained in hospital settings (hospital-acquired (HA) MRSA) for approximately 40 years, but the more recent community-acquired MRSA (CA-MRSA) infections are becoming more common. Even more alarming, is the increased virulence of CA-MRSA, such as with the appearance of Panton-Valentine leukocidin gene (PV-luk) which is associated with a particularly lethal form of hemolytic pneumonia in children.[11] This rapid increase in virulence and antibiotic resistance is due

to the readily adaptable nature of *S. aureus.* Variable regions constitute 22% of the *S. aureus* genome and horizontal transfer events are common.[40, 41] This has become an ever-increasing concern as there is no consistent standard within U.S. health-care settings for controlling the transmission of MRSA.[42] This has contributed to further antibiotic resistance such as vancomycin-intermediate (VISA) and resistant (VRSA) *S. aureus* strains, which further limit the treatment options of *S. aureus* infections.[18]

As fewer antibiotics effective against *S. aureus* infections become available and the incidence of *S. aureus* infections increases, the discovery of new, alternative treatments has become a necessity. Examples of alternative treatments include antibacterial natural compounds, cationic antimicrobial peptides, bacteriophages, and staphylococcal vaccines.[43] However, these treatments are either weak in their bactericidal effects on *S. aureus* or are not bactericidal for all strains. A more promising treatment option is photodynamic therapy (PDT) which involves the addition of chemical photosensitizers to the bacteria and a subsequent exposure of light. Specifically, the use of blue light against *S. aureus* has been explored with and without the addition of a photosensitizer.[33]

One proposed mechanism for the bactericidal effects of blue light on *S. aureus* is the formation of reactive oxygen species (ROS). ROS, such as hydroxyl and hypochlorite, may be formed when blue light interacts with components in the cell or its environment.[19] Although *S. aureus* has many detoxifying enzymes, metal homeostasis pathways, and ways to protect and repair DNA and proteins, ROS can overwhelm the cell by damaging biomolecules, resulting in cell death.

Blue light has been shown to have varying effects across species and has been tested on *P. acnes, H. pylori, and E. coli.*[22] Blue light of wavelengths 405 nm and the safer 470 nm have both been shown to inhibit the growth of MRSA.[33, 34] One study, using 624 nm red light and photoporphyrin diarganate as a photosensitizer, found evidence of variation to the response of blue light among 81 strains of *S. aureus*.[27] No mechanism was proposed in this experiment but this variation is significant because it indicates that this is due to a genetic component. As with other types of treatment such as antibiotics, if some strains are more resistant due to a genetic component, there is a potential for acquired resistance to red light, which would decrease the viability of PDT in a clinical setting. Variation in the response to PDT has also been reported using broadband visible light with samples tested in nutrient broth. This experiment tested only two strains of *S. aureus*, one of which was considered more "resistant" to visible light (99.8% vs. 55.5% inhibition). Illumination took place in nutrient broth, but the conclusions drawn indicated that the variation was due to endogenous photosensitizers.[30] Measurements of free radicals, porphyrins and carotenoids were compared for the two strains and the authors concluded that the variation in pigment molecules produced varying amounts of ROS, causing the differences in inhibition.

A review of the literature concerning the photoinactivation of *S. aureus* reveals a variety of experimental designs, light sources, photosensitizers, and proposed mechanisms for inactivation. To address this confusion, we have developed a simple light box that is amenable to standard culturing techniques and allows for many replicates and controls. Using both MRSA and MSSA nasal isolates with known genetic variation, we explored the response to 30 minutes of exposure to 470 nm blue light, at both 4 hours

after illumination and 18 hours for 25 total strains. Our results suggest that the effect of blue light among *S. aureus* cells in liquid culture varies over 10-fold.

CHAPTER THREE

Materials and Methods

Bacterial isolates

Twenty-five *S. aureus* strains (15 MSSA and 10 MRSA) isolated from the nasal passages of healthy individuals collected as part of a five-year longitudinal study at Baylor University were supplied by T. Adair. The isolates were characterized through fermentation on mannitol salt, Gram-staining, and positive results to catalase and coagulase tests. To include strains with a variety of genetic backgrounds, strains were selected based on variations in the response to 12 antibiotics and to phage K infection. Resistance patterns to antibiotics were identified using a Kirby-Bauer disc-diffusion method. PCR was performed on the MRSA strains to classify the SCC*mec* type I-V.[44] Isolate stocks were kept frozen on cryobeads at -20ºC or glycerol stocks at -80°C. Cryobeads for the selected 25 strains were placed in 5 ml of BHI and grown overnight at 35°C. For the first experiment, streaks from the overnight cultures were made on tryptic soy agar (TSA) master plates, which were used for up to two weeks for subsequent experiments. For the flow cytometry experiments, a 1:1 ratio of this overnight culture and glycerol was made and frozen at -80ºC. This frozen stock was then used for all subsequent experiments.

Preparation of cultures

A portion of the fresh master plates or the frozen stock was placed in 5 mL BHI and grown overnight at 35°C. A 1:100 dilution of this culture was prepared in BHI and

grown for two hours at 35ºC to achieve an exponential growth phase. These cells were diluted to 1:10^4 to achieve an approximate cell concentration of 2000 CFU/ml determined by colony counts. One mL each of this dilution was placed in four wells of a 24-well microtiter plate for each isolate with up to 6 isolates tested per trial. This was repeated for a 'no light' control plate. The strain BU (Baylor University) SA2288 was prepared using the method listed above and was included in each of the 16 trials to measure variation between trials.

Blue light exposure and OD measurement after 18-hour recovery

To measure the variation in response to blue light exposure of 25 different isolates, 24-well microtiter plates were prepared as described with one placed in an opaque box and one under the blue light lid apparatus. The electrical diagram for the blue light box may be found in Appendix C. Each 1 cm^2 well of the blue light treatment plate was exposed to 30 minutes of 470 nm blue light (Kingbright) with a forward electrical current of 20 mA per diode.[45] The diodes have a voltage of 3.3 V when operated at 20 mA. This correlates to a fluency of approximately 120 J cm^{-2}. This power produces an average of 4 klux emitted per diode as measured with a luminometer. The control plate was placed in the same incubator (35ºC) and at the same time as the treatment group. No significant temperature change was observed while cultures were being exposed to the blue light. After the treatment, both plates were incubated for 18 ± 1.5 hours at 35°C.

After incubation, the cultures were resuspended by pipetting. The optical density, using a 600 nm filter (OD_{600}), was then recorded using the ELx800 BioTek plate reader and the BioTek Gen5 software. Each strain was measured in quadruplicate per treatment

(blue light or no exposure) per strain and each strain was repeated two to four times, with BUSA2288 included in all 16 independent trials. To account for possible variation and bias, strains were placed in different columns of the microtiter plate for subsequent trials. Average OD_{600} measurements were calculated for each strain and the percent inhibition was calculated for each strain using the equation

$$\frac{avg.\ no\ light\ \text{OD600} - avg.\ blue\ light\ \text{OD600}}{avg.\ no\ light\ \text{OD600}} x100\%.$$

Statistical testing performed included a Student's T-test pairing blue light and no light controls per strain for each individual trial and for the overall group. Chi-square testing was performed to determine if there was a correlation between methicillin resistance and sensitivity to blue light.

Blue light exposure and flow cytometry readings at 4 hours recovery

A second experiment was performed to test the effect of blue light on cells at the earlier time point of four hours post treatment. Flow cytometry was used to quantify the live:dead cell ratio and samples were prepared as stated above. Six strains were chosen based on the results from the 18 hour recovery experiment: three strains labeled 'sensitive' and 3 strains labeled 'resistant.' Strain BUSA2288 was also included to measure variation between trials. The blue light exposure procedure was the same (30 minutes) and cultures were incubated at 35ºC for four hours after exposure. Cultures were prepared for flow cytometry using the LIVE/DEAD Bac*Light*™ *Bacterial Viability and Counting Kit (L34856)* with the following modifications: the 1 mL samples were centrifuged at 10,000 x g for 6 minutes to pellet the cells. The supernatant was then removed and the samples were resuspended in 100 µL of filter-sterilized 0.85% NaCl. Twenty microliters of this prepared sample were aliquoted into 972 µL of sheath fluid

(BD Cat. No. 342003). Five microliters of the microsphere suspension and 1.5 µL each of green-fluorescent SYTO®9 and red-fluorescent propidium iodide were added to the sample to constitute a total volume of 1 mL.

Samples were analyzed according to the kit, using a BD FACS Calibur flow cytometer and the BD Cell Quest Pro software. We determined the optimal forward scatter (cell size measurement) setting for our *S. aureus* samples and used this as a standard to ensure only *S. aureus* cells were being measured. The settings used were: side scatter (SSC) of 366, forward scatter (FSC) of 398, FL1 set at 489, and FL3 set at 767. FL1 had a filter of 530/30 BP and FL3 had a filter of 670 LP. These settings allowed for the live cell population to be distinguished from the populations of the dead cells and microspheres (beads). Using the software, the *S. aureus* cells were gated and tallied. The density of cells in the bacterial culture was calculated with the following equation: $\frac{((\#\text{ of events in bacterial region})\text{x}(\text{dilution factor}))}{((\#\text{ events in bead region}) * 5{*}10^{-7})}$. Each strain was tested in triplicate and repeated three times for a total of 9 data points per strain (n = 9).

Statistical testing performed included a Student's T-test pairing blue light and no light controls per strain for each individual trial and for the overall group for both categories of live cell densities and live:dead ratios.

CHAPTER FOUR

Results

Blue light exposure and OD measurements after 18 hour recovery

Thirty minutes of 470 nm blue light exposure at 20mA per well (approximately 120 J cm^{-2}), was applied to 25 strains of *S. aureus***,** 10 MRSA and 15 MSSA, followed by a recovery time of 18 hours. Between 8 and 16 data points were gathered for each strain. As seen in Figure 1, when each strain was analyzed separately, the responses to blue light for the 25 strains were not homogenous and ranged from 88.74% reduction to a seemingly enhanced proliferation of -8.04%. Of the 25 strains, 12 had a statistically significant (Student's t-test $p < 0.05$) difference between blue light and the no light control which was interpreted as sensitive to blue light. The 13 remaining strains that were resistant to blue light ($p > 0.05$) varied between -9% to 27% inhibition.

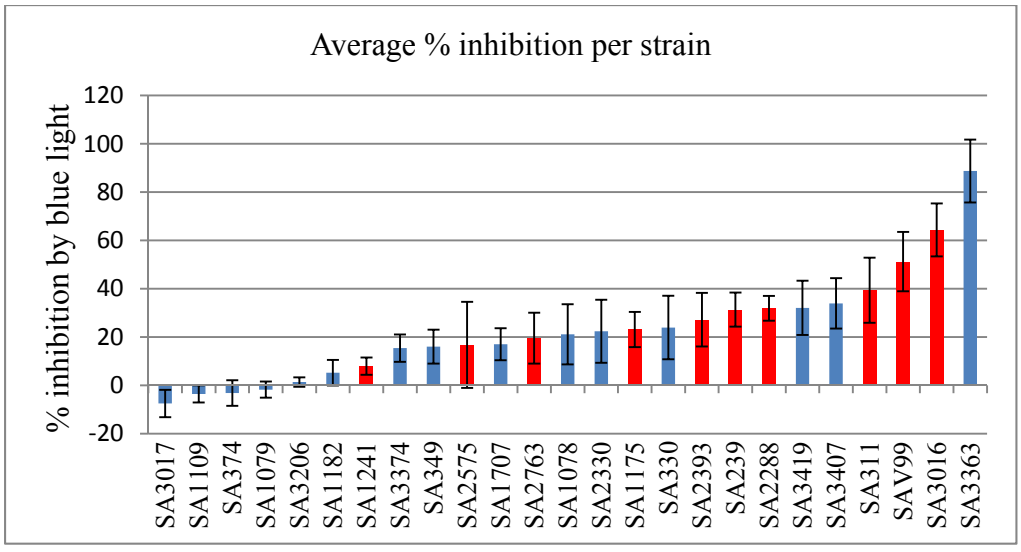


Figure 1. Average percent inhibitions by blue light per strain. Red bars reflect MRSA strains and the blue bars reflect MSSA strains. Bars show standard error.

There was no observable correlation between the sensitivity of a strain to blue light and its antibiotic resistance to methicillin. Of the 15 strains sensitive to methicillin, 5 (33%) were sensitive to blue light ($p < 0.05$). Of the 10 MRSA strains, 6 (60%) were sensitive to blue light ($p < 0.05$). Although a larger percentage of the MRSA strains had statistically significant inhibition by blue light, the comparison of methicillin susceptibility and sensitivity to blue light was not significant as can be seen with the Chi-Square table in Table 1 yielding a value of $c^2 (1, N = 25) = 2.57$, $p = 0.46$.

	$P < 0.05$ Sensitive to BL	$P > 0.05$ Resistant to BL	Total
MRSA	6	4	10
MSSA	5	10	15
Total	11	14	25
Chi-square results	$c^2 (1, N = 25) = 2.57$, $p = 0.46$		

Table 1. Chi-Square table evaluating the correlation between methicillin susceptibility and blue light inhibition.

Variation was observed within the strain BUSA2288 that was tested in every trial, ranging from average percent inhibitions of -36% to 85% (Figure 2). We interpret this variation as problematic for comparing strains after 18 hours of recovery using the plate reader, due to variations in the exact time of taking the measurement (18 ± 1.5 hours) and the observation that the 'no light' control had reached saturation. However, there were statistical differences in strains found consistently on either end of the spectrum (those that were very sensitive vs. highly resistant). This led to the investigation of three sensitive and three resistant strains, as classified by OD_{600} after 18 hours recovery, with a

more precise method, flow cytometry, while tightly controlling for suspected sources of variability such as initial age of culture, temperature of the incubator, exact time of recovery and growth stage of control cells.

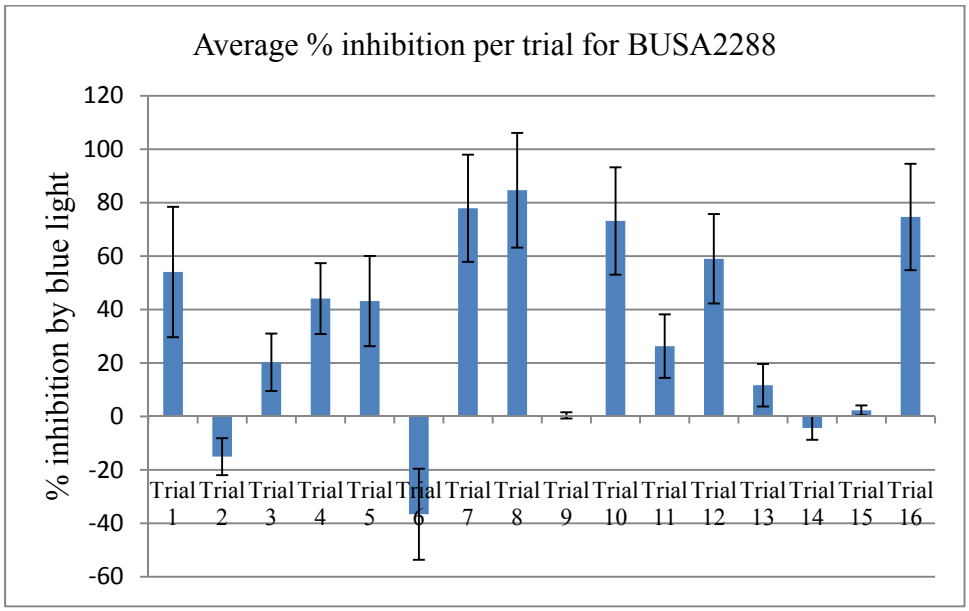


Figure 2. BUSA2288 was tested in each trial for (16 total) the first experiment evaluating the effect of blue light on *S. aureus* at 18 hours recovery post treatment. Variation ranged from -35% to 86% inhibition by blue light. Bars show standard error.

Blue light exposure and flow cytometry measurements at 4 hours recovery

To verify the results that some strains were more sensitive to blue light than others as determined by the optical density readings in the first experiment, a different method, flow cytometry, and a shorter recovery period of four hours post blue light exposure was used. Of the 25 strains tested, three MRSA strains classified as sensitive to blue light in the first experiment (BUSA3016, BUSAV99, and BUSA311, $p < 0.05$), and three MSSA strains (BUSA3017, BUSA1079, and BUSA1109, $P > 0.05$) classified as resistant. These 6 strains were re-evaluated using flow cytometry. The same dose of blue light (30 minutes) was applied to samples which were then incubated at 35ºC for

only four hours. Additionally, the strain BUSA2288, which was used as a control across trials for the optical density study, was evaluated with flow cytometry. Previous findings exploring the growth curve of BUSA2288 post blue light exposure suggest that the bactericidal effect of blue light is greatest after four hours of recovery.[46]

Each isolate was tested in triplicate per trial for a total of three trials. The ratios of live to dead cell densities were measured and calculated based off of individually calculated live and dead cell densities. The live:dead ratios for the blue light samples varied between 0.07 and 0.14 for the sensitive strains and 0.40 and 0.45 for the resistant strains (Figure 3). The density of live cells was also taken into consideration as these are the cells that can recover and cause infection. The percent difference in live cell densities, when comparing blue light and control, varied from 89%-99%. Thus, there was a decrease in viability for all strains tested at this time point. Student's T test $p < 0.05$ was used to determine the probability that these cells were significantly inhibited by blue light irradiation. The strains considered sensitive to blue light with the OD_{600} reading at 18 hours recovery showed a similar trend of being more sensitive to blue light at four hours than the three resistant strains. For all categories of evaluation, the resistant strains had a less significant difference between the blue light exposed and control samples than the sensitive strains (Figure 2). Thus, the propensity for a strain to either be sensitive or resistant to 30 minutes of 470 nm blue light can be seen as early as four hours post-illumination.

Of the 7 strains tested, 5 were statistically significant for inactivation by blue light when comparing the live cell densities (Table 2). The two strains that did not show statistically significant inhibition of live cell densities were BUSA1079 and BUSA1109,

two of the three strains classified as resistant according to the OD_{600} measurements after 18 hours recovery data as noted above. When comparing L:D ratios, only BUSA1109 did not show a statistically significant difference of L:D ratios between blue light and control groups. However, blue light did show some degree of inactivation when comparing live:dead cell ratios of blue light and controls for all strains, correlating to a range of 29% to 88% difference, and all strains showed at least 89% inactivation when looking solely at the density of live cells (Table 2).

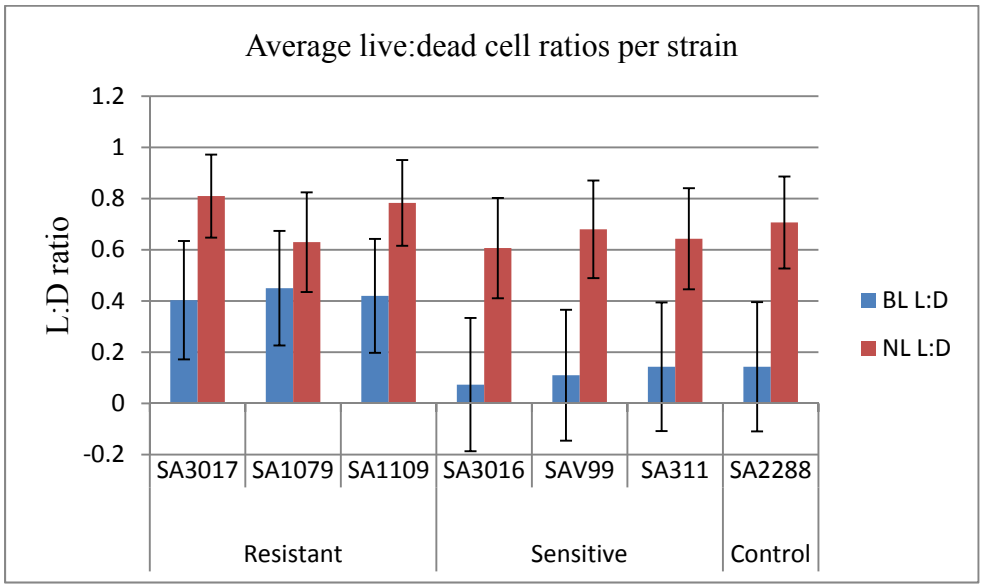


Figure 3. Three statistically significant ($p < 0.05$) strains (BUSA3016, BUSAV99, BUSA311), three resistant strains ($P > 0.05$, BUSA3017, BUSA1079, BUSA1109), and our control strain across trials (BUSA2288) were evaluated by flow cytometry after 4 hours post-treatment. BL = blue light. NL = no light. Bars show standard error.

	OD_{600}, 18 hours recovery		Flow cytometry, 4 hours recovery			
Strain ID (BUSA)	% Difference OD (18 hr)	P value OD (18 hr)	% Difference live cells (4 hr)	P Value Live cells (4 hr)	% Difference L:D (4 hr)	P Value L:D (4 hr)
3016	63%	2.70×10^{-03}	96%	5.46×10^{-05}	88%	3.99×10^{-07}
V99	56%	2.78×10^{-04}	94%	4.81×10^{-05}	84%	1.76×10^{-06}
311	38%	2.66×10^{-02}	99%	1.18×10^{-03}	77%	3.03×10^{-05}
2288	36%	2.80×10^{-08}	99%	3.18×10^{-04}	79%	1.12×10^{-07}
1079	-1%	7.92×10^{-01}	90%	5.34×10^{-02}	29%	1.27×10^{-01}
1109	-3%	3.34×10^{-01}	94%	9.76×10^{-02}	46%	3.10×10^{-04}
3017	-9%	6.39×10^{-01}	89%	2.95×10^{-03}	50%	7.22×10^{-04}

Table 2. The side by side comparison of results from the first and second experiments utilizing OD600 and flow cytometry respectively, indicate there is a consistent pattern in the response to blue light among isolates across experiments Text highlighted in red designate no significant difference. BL = blue light and NL = no light

Thus, whether it is due to the time difference (4 hours vs. 18 hours recovery) or the experimental method (flow cytometry vs. optical density), the bactericidal effect of blue light is consistent for these 7 isolates in regards to its relative sensitivity to blue light when compared to other strains. Overall, when comparing the live:dead cell ratios, there was a statistically significant decrease in cell viability with blue light exposure (paired t-test : $t=10.50$, $p<0.0001$, 95% confidence interval: (0.2794,0.4099), mean=0.3447). The same was seen when comparing live cell densities between blue light and control samples (: $t=4.49$, $p<0.0001$, 95% confidence interval: (1.16×10^6 cells/mL, 3.00x106 cells/mL) mean=2.08×10^6 cells/mL).

CHAPTER FIVE

Discussion and Conclusions

Variation of 25 different S. aureus strains

The variation of the response to 470 nm blue light for multiple *S. aureus* isolates without the addition of a known photosensitizer has not been reported previously. These experiments specifically measured and compared the effects of 30 minutes of 470 nm blue light followed by 18 hours recovery on 25 individual isolates. The results show an overall inhibition of viability after blue light exposure. The variation seen within a specific strain (trial-to-trial variation) may be attributed to the saturation observed in the no light cultures and the variations in the time spent in recovery post treatment (18 ± 1.5 hours). In other words, the number of cells recovering to blue light exposure varied more than the number of cells not exposed to blue light which had reached saturation.

However, there was great variation amongst the strains in response to blue light exposure ranging from -8% to 89% inhibition. This variation was confirmed using flow cytometry by measuring the live:dead cell ratios on seven isolates four hours post blue light exposure. The isolates were chosen based on their response to 30 minutes of 470 nm blue light followed by an 18 hour recovery period: three sensitive to blue light ($P < 0.05$) and three resistant ($P > 0.05$). Flow cytometry confirmed that five of the seven strains showed inhibition by blue light at four hours post-treatment. Additionally, the three strains that were classified as sensitive to blue light in the first experiment (BUSA3016, BUVSA99, BUSA311) also proved to have greater inhibition when comparing live:dead ratios and live cell densities than the three resistant strains

(BUSA3017, BUSA1079, BUSA1109) at this time point. Thus, the relative sensitivity of a strain (as either sensitive or resistant to blue light) was consistent at both 18 and 4 hours after treatment, as can be seen in Table 2. This heterogeneous response suggests that there may be a genetic component attributable to these patterns.

Methicillin resistance was not found to correlate to the sensitivity of a strain to blue light. The percentage of strains that were statistically inhibited by blue light at 18 hours of recovery after treatment as measured with OD_{600} did not differ significantly between MRSA and MSSA as determined by Chi-Square testing (Table 1).

Mechanism of blue light killing of S. aureus

Evidence indicates that blue light inhibition of *S. aureus* may occur through the accumulation of reactive oxygen species produced through a light-sensitive chain reaction, as this has been observed in cases when *S. aureus* is exposed to visible light.[31] There is evidence suggesting that the free radicals produced in *S. aureus* subsequent to exposure to blue light damage the membrane, causing it to become compromised and resulting in death.[28] An important variable to consider in this experiment is the type of medium that the cells are grown in during blue light illumination. Our experiments were performed in BHI, an infusion from the brains and hearts of cows. The exact composition of BHI is not known, but when *S. aureus* is grown in BHI that has been illuminated with blue light prior to inoculation, inhibition of growth is still evident.[47] This indicates that photoreactive molecules are present in BHI. This photosensitizer could be an inexpensive tool to be used in clinical settings for inhibiting the growth of *S. aureus*. Future work is needed to discover and quantify this photosensitizer in addition to the definitive mechanism of blue light inhibition.

Regardless of the mechanism of blue light inactivation of *S. aureus*, this study does elucidate that the effect of blue light varies for different isolates of *S. aureus.* This indicates that a genetic component is responsible for this heterogeneous response. Potential biochemical pathways that could affect the response of *S. aureus* to blue light include membrane lipids, transmembrane proteins and regulatory proteins related to gene expression of enzymes that deactivate free radicals accumulated in the cell.

Clinical considerations

Although the use of blue light to treat *S. aureus* infections would be a relatively cost-effective and non-invasive treatment, there are several concerns with the clinical use of blue light. First, the variability seen in different strains, as seen in this experiment, may stem from genetic differences in the ability to either produce or quench ROS, or even a combination of these characteristics. This potential variability in ROS clearance would have implications in the use of blue light as a clinical treatment in multiple ways. One concern is that of a developed resistance to blue light stemming from a genetic mutation or alteration to the genetic impetus behind the variation observed.

Additionally, the heterogeneity in response to blue light presents difficulties in treatment as certain isolates would be more sensitive to blue light treatment than others. This would require further research to establish a standard treatment protocol with a minimum amount of blue light needed to effectively inactivate all strains. Continuing research is being conducted to determine the minimum dosage required for 90% inhibition of BUSA1182 following 30 minutes of blue light exposure and 18 hours of recovery after blue light treatment. Preliminary data shows that this strain has an average inhibition of 20% (making it one of the more resistant strains tested) with 30 minutes of

blue light and reaching 95% inhibition with two hours of blue light (Sarah Yuen, personal communication). This strain is resistant with the lower exposure of 30 minutes but it is considered sensitive to blue light with 2 hours of blue light treatment, showing that there is potential for strains to be considered sensitive to blue light after a certain dosage of blue light is used.

The second concern is that of safety. There have been recent studies on the effects of blue light on mammalian tissue. Dai et al. found that blue light successfully reduces the viability of *Pseudomonas aeruginosa* infections on burns in mouse models with no significant or irreversible damage to the mouse skin.[48] Other studies have shown 415 and 455 nm blue light to stimulate the growth of certain bacteria at low fluencies, necessitating the discovery of a minimum dosage of blue light to be used to prevent unwanted proliferation from occurring.[31] However, there have been contrary reports that higher doses of blue light decrease the viability of human splenocytes *in vitro,* which is presumably due to excessive ROS generation and exposure.[49] These mixed findings illustrate a need for caution in using blue light for clinical pursuits involving longer treatments until more thorough conclusions are made regarding the safety of blue light.

Lastly, our research was performed on a relatively low concentration of planktonic *S. aureus.* However, *S. aureus* often grows attached to a surface, forming a biofilm with a high density of cells, or invades the deeper tissue. Although our results show that blue light is effective in liquid culture, there is a need to validate this *in vivo.* There is some precedent in the treatment of neonatal jaundice that blue light can penetrate skin. The use of blue light to lower bilirubin levels in infants demonstrates that blue light can sufficiently penetrate the skin to interact with the porphyrins in the blood, although

there is concern for some ill long-term effects such as melanocytic nevus (MN) and damage to the eye.[50, 51] Research has also shown that blue light can penetrate a burn wound with great enough efficiency to reduce the viability of *Pseudomonas aeruginosa*.[48] Delivery of blue light to internal surfaces may require the use of nanotechnology or fiber optics. Additional studies are needed to better understand the degree and conditions under which blue light can penetrate mammalian tissue to reach pathogens.

Blue light from an LED may be better for preventative purposes as a relatively high dosage is required to kill a dense population of *S. aureus.* This can be seen in the recent development of MRSAid™ produced by a Canadian company as a method that utilizes a photosensitizer and a nasal light illuminator to decolonize the nasal passageways of patients.

Experimental limitations

Several sources of experimental error are possible in these experiments. In order to measure the consistency of this method, four replicates for BUSA2288 were included in each of the 16 trials performed to serve as an internal control across trials. This revealed a large amount of variability (-36% to 85%) in the percent inhibition to blue light within a strain (Figure 2). However, there was little variation amongst replicates performed within a single trial. The variation seen in these data points indicate that small inconsistencies such as incubation time, time of treatment, incubation temperature, age of culture, or stage of growth captured during the recovery period for a trial may have contributed to the variation. Thus, flow cytometry and stricter regulation of these variables were used in subsequent experiments to achieve less-variable results.

Additionally, individual LEDs in the light box used for treatments had differences in intensity and some wells received scattered light from all sides, whereas the wells on the outer rows receive scattered light from only 2 or 3 sides. The LEDs used have a small angle of coverage (Θ 1/2 =16º) and random placement of the samples was included in the design to reduce this variation. Optical density readings may have resulted in erroneously high readings if the cells were not completely resuspended before a measurement was taken. The cell density was not as great of a concern with the samples examined via flow cytometry due to lower concentrations at 4 hours recovery time. Additionally, the fact that sensitivity trends were the same for individual isolates with both the 18 hour recovery OD_{600} experiments and 4 hour recovery flow cytometry experiments help validate that the 18 hour recovery measurements hold value regardless of the variation seen amongst trials.

Modifications and future pursuits

Future pursuits include a close examination of BHI, the media in which the planktonic *S. aureus* cultures are grown, for the photoreactive molecules that are enhancing blue light inactivation of these bacteria. Further evaluation of the genes that are being expressed at different levels when exposed to blue light using RNA-Seq may also help elucidate the mechanism behind blue light killing of *S. aureus*. So far, bacteria have failed to produce consistent and complete resistance to blue light, so ongoing exposure and continuous passaging experiments to select for this trait are needed.

Conclusion

This study confirms that there is a wide degree of variation, ranging from an average -8% to 89% inhibition in response to 30 minutes of blue light for 25 different *S. aureus* isolates. The same response for blue light (sensitive or resistant) can be seen at an earlier and later time point (4 hours and 18 hours recovery post exposure to blue light) for 7 individual strains. This suggests that a genetic trait is causing this variability. The mechanism underlying blue light inactivation of *S. aureus* is poorly understood. Genetic variability may point to new targets for treatment, but may also be a mechanism for resistance to blue light to develop. In addition to research the resistance of *S. aureus* to blue light, research is required on a larger sample size of different isolates of *S. aureus* in a variety of environmental conditions with a variety of photosensitizers to determine the optimum dosages for complete inhibition of *S. aureus*. Before blue light is used as a therapeutic treatment or as a preventative strategy, further research is needed to ensure the safety of the host tissues as well as the exact response of the pathogen.

APPENDICES

APPENDIX A

Statistical Tables for 18 hour recovery experiment

Average data per strain per trial (total of 25) for 30 minutes of blue light measured with OD_{600} following 18 hour recovery.

BUSA2288				
Trial	Average BL	Average NL	T-test	% Inhibition
6-Mar	0.40	0.88	9.07×10^{-2}	54.81
22-Mar	0.64	0.55	2.39×10^{-2}	-14.51
26-Mar	0.60	0.81	1.25×10^{-1}	25.21
27-Mar	0.39	0.70	4.80×10^{-3}	44.50
28-Mar	0.30	0.57	2.72×10^{-2}	46.10
10-Apr	0.70	0.52	8.52×10^{-2}	-34.62
18-Apr	0.12	0.54	0.00×10^{00}	77.78
24-Apr	0.09	0.59	0.00×10^{00}	84.75
7-May	0.54	0.55	9.28×10^{-1}	0.37
31-May	0.27	1.00	2.30×10^{-3}	73.10
5-Jun	0.48	0.60	1.78×10^{-1}	20.24
7-Jun	0.24	0.57	3.80×10^{-3}	58.13
13-Jun	0.35	0.41	1.75×10^{-1}	14.19
18-Jun	0.43	0.42	4.57×10^{-1}	-3.65
19-Jun	0.41	0.42	5.66×10^{-1}	2.52
25-Jun	0.10	0.39	3.00×10^{-4}	75.05
Average	0.38	0.59	2.80×10^{-8}	36.29

BUSA239				
Trial	Average BL	Average NL	T-test	% Inhibition
18-Apr	0.33	0.61	2.92×10^{-4}	45.58
18-Jun	0.50	0.61	5.93×10^{-3}	17.89
Average	0.42	0.61	1.61×10^{-4}	31.76

BUSA311				
Trial	Average BL	Average NL	T-test	% Inhibition
26-Mar	0.63	0.68	4.94×10^{-1}	8.59
5-Jun	0.18	0.61	6.41×10^{-4}	70.84
Average	0.40	0.65	2.66×10^{-2}	37.86

BUSA330				
Trial	Average BL	Average NL	T-test	% Inhibition
26-Mar	0.80	0.82	8.54×10^{-1}	2.58
5-Jun	0.43	0.87	4.58×10^{-2}	50.39
Average	0.62	0.85	7.32×10^{-2}	27.18

BUSA349				
Trial	Average BL	Average NL	T-test	% Inhibition
26-Mar	0.53	0.82	2.25×10^{-3}	35.56
5-Jun	0.50	0.49	4.06×10^{-1}	-2.72
Average	0.51	0.65	6.07×10^{-2}	21.24

BUSA374				
Trial	Average BL	Average NL	T-test	% Inhibition
26-Mar	0.56	0.61	3.45×10^{-1}	7.57
19-Jun	0.38	0.34	2.04×10^{-1}	-12.49
Average	0.47	0.47	9.77×10^{-1}	0.42

BUSA1078				
Trial	Average BL	Average NL	T-test	% Inhibition
27-Mar	0.65	0.63	8.08×10^{-1}	-2.24
7-Jun	0.31	0.57	4.43×10^{-2}	46.54
Average	0.48	0.60	1.65×10^{-1}	20.87

BUSA1079				
Trial	Average BL	Average NL	T-test	% Inhibition
27-Mar	0.64	0.62	1.84×10^{-1}	-3.98
13-Jun	0.47	0.52	5.30×10^{-2}	9.48
25-Jun	0.56	0.51	9.58×10^{-2}	-9.68
Average	0.55	0.54	7.92×10^{-1}	-1.36

BUSA1109				
Trial	Average BL	Average NL	T-test	% Inhibition
27-Mar	0.62	0.59	9.99×10^{-2}	-5.69
7-Jun	0.61	0.61	9.65×10^{-1}	-0.25
Average	0.61	0.60	3.34×10^{-1}	-2.93

BUSA1175				
Trial	Average BL	Average NL	T-test	% Inhibition
6-Mar	0.32	0.81	5.38×10^{-4}	60.53
22-Mar	0.60	0.63	3.15×10^{-2}	5.53
7-Jun	0.53	0.61	6.26×10^{-2}	12.79
Average	0.50	0.67	1.49×10^{-3}	25.98

BUSA1182				
Trial	Average BL	Average NL	T-test	% Inhibition
27-Mar	0.51	0.62	1.18×10^{-1}	17.18
13-Jun	0.42	0.42	9.19×10^{-1}	1.48
25-Jun	0.40	0.41	3.65×10^{-1}	3.36
Average	0.45	0.47	6.14×10^{-1}	4.52

BUSA1241				
Trial	Average BL	Average NL	T-test	% Inhibition
10-Apr	0.59	0.61	2.72×10^{-1}	3.42
18-Apr	0.52	0.65	2.83×10^{-2}	20.91
19-Jun	0.47	0.48	6.73×10^{-1}	1.26
Average	0.52	0.57	1.23×10^{-1}	9.34

BUSA1707				
Trial	Average BL	Average NL	T-test	% Inhibition
6-Mar	0.39	0.79	1.40×10^{-2}	50.59
22-Mar	0.58	0.61	2.65×10^{-1}	4.76
7-Jun	0.54	0.57	3.17×10^{-1}	4.40
Average	0.52	0.64	1.03×10^{-2}	19.99

BUSA1707				
Trial	Average BL	Average NL	T-test	% Inhibition
24-Apr	0.08	0.54	3.40×10^{-7}	84.59
7-May	0.62	0.52	6.81×10^{-2}	-19.56
19-Jun	0.37	0.38	4.65×10^{-1}	2.67
Average	0.38	0.47	2.25×10^{-1}	19.20

BUSA2393				
Trial	Average BL	Average NL	T-test	% Inhibition
24-Apr	0.12	0.61	7.01×10^{-6}	80.79
7-May	0.59	0.58	8.52×10^{-1}	-1.24
18-Jun	0.49	0.51	3.90×10^{-1}	3.41
Average	0.43	0.56	5.00×10^{-2}	23.18

BUSA2575				
Trial	Average BL	Average NL	T-test	% Inhibition
24-Apr	0.08	0.62	4.21×10^{-7}	86.67
7-May	0.52	0.63	4.71×10^{-1}	16.76
31-May	0.90	0.62	1.60×10^{-1}	-44.13
Average	0.53	0.62	4.84×10^{-1}	14.51

BUSA2763				
Trial	Average BL	Average NL	T-test	% Inhibition
24-Apr	0.21	0.63	1.99×10^{-3}	66.15
7-May	0.65	0.58	3.49×10^{-1}	-12.22
18-Jun	0.45	0.50	8.12×10^{-2}	10.28
Average	0.46	0.57	1.25×10^{-1}	18.32

BUSA3016				
Trial	Average BL	Average NL	T-test	% Inhibition
6-Mar	0.05	0.81	2.05×10^{-2}	93.43
22-Mar	0.55	0.56	6.23×10^{-1}	1.91
13-Jun	0.04	0.42	1.34×10^{-3}	90.39
25-Jun	0.03	0.21	1.00×10^{-2}	83.33
Average	0.18	0.48	2.70×10^{-3}	62.92

BUSA3017				
Trial	Average BL	Average NL	T-test	% Inhibition
6-Mar	1.14	0.89	4.96×10^{-3}	-28.12
22-Mar	0.61	0.66	4.12×10^{-1}	7.02
13-Jun	0.39	0.38	7.70×10^{-1}	-3.01
Average	0.68	0.62	6.39×10^{-1}	-8.97

BUSA3206				
Trial	Average BL	Average NL	T-test	% Inhibition
28-Mar	0.58	0.61	9.85×10^{-2}	5.33
19-Jun	0.46	0.45	6.39×10^{-1}	-2.40
Average	0.52	0.53	7.92×10^{-1}	2.07

BUSA3363				
Trial	Average BL	Average NL	T-test	% Inhibition
28-Mar	0.05	0.56	4.63×10^{-5}	91.64
5-Jun	0.07	0.52	6.26×10^{-7}	85.84
Average	0.06	0.54	3.42×10^{-11}	88.84

APPENDIX B

Statistical tables for flow cytometry tables.

Average data per strain (total of 7) per trial for 30 minutes of blue light measured with flow cytometry following 4 hour recovery.

BUSA2288						
Trial	Live BL	Live NL	% Inhibition Live	L:D BL	L:D NL	% Inhibition L:D
8-Jun	6.17×10^{3}	6.70×10^{6}	99.91	0.02	0.67	96.40
	2.05×10^{3}	6.10×10^{6}	99.97	0.01	0.83	98.84
	8.11×10^{3}	7.65×10^{6}	99.89	0.07	0.80	91.66
10-Jun	5.71×10^{4}	3.72×10^{6}	98.47	0.30	0.82	63.50
	4.69×10^{4}	3.68×10^{6}	98.73	0.23	0.78	70.48
	9.66×10^{4}	1.38×10^{6}	93.00	0.34	0.77	55.57
10-Sep	6.54×10^{4}	6.35×10^{5}	89.70	0.12	0.62	80.69
	7.99×10^{4}	6.48×10^{5}	87.66	0.15	0.62	75.16
	6.88×10^{4}	1.56×10^{5}	55.82	0.06	0.40	84.45
Average	4.79×10^{4}	3.41×10^{6}	98.59	0.15	0.70	79.27
T-test	2.94×10^{-3}			1.12×10^{-7}		

BUSA3016						
Trial	Live BL	Live NL	% Inhibition Live	L:D BL	L:D NL	% Inhibition L:D
8-Jun	1.88×10^{4}	1.36×10^{6}	98.62	0.05	0.84	94.14
	4.39×10^{3}	1.28×10^{6}	99.66	0.01	0.59	98.84
	2.07×10^{3}	1.84×10^{6}	99.89	0.01	0.94	99.22
10-Jun	2.44×10^{4}	1.08×10^{6}	97.75	0.03	0.61	94.78
	2.47×10^{4}	1.06×10^{6}	97.67	0.09	0.55	83.89
	1.65×10^{4}	8.74×10^{5}	98.11	0.07	0.53	87.84
10-Sep	6.80×10^{4}	4.86×10^{5}	86.02	0.11	0.40	72.91
	1.10×10^{5}	2.76×10^{5}	60.23	0.15	0.36	59.58
	9.99×10^{4}	4.14×10^{5}	75.87	0.16	0.63	74.36
Average	4.09×10^{4}	9.64×10^{5}	95.75	0.07	0.61	87.79
T-test	5.46×10^{-5}			3.99×10^{-7}		

BUSAV99						
Trial	Live BL	Live NL	% Inhibition Live	L:D BL	L:D NL	% Inhibition L:D
8-Jun	6.05×10^{3}	7.89×10^{5}	99.23	0.07	0.81	91.17
	4.08×10^{3}	1.21×10^{6}	99.66	0.02	0.90	97.60
	8.16×10^{3}	6.80×10^{5}	98.80	0.04	0.80	94.66
10-Jun	1.83×10^{4}	7.46×10^{5}	97.55	0.14	0.78	81.68
	1.42×10^{4}	6.73×10^{5}	97.89	0.09	0.82	88.66
	1.21×10^{4}	5.75×10^{5}	97.89	0.11	0.82	86.40
10-Sep	8.44×10^{4}	2.43×10^{5}	65.30	0.16	0.36	57.43
	7.59×10^{4}	4.10×10^{5}	81.48	0.14	0.49	71.28
	8.13×10^{4}	1.76×10^{5}	53.78	0.20	0.30	34.26
Average	3.38×10^{4}	6.11×10^{5}	94.46	0.11	0.68	83.95
T-test	4.81×10^{-5}			1.76×10^{-6}		

BUSA311						
Trial	Live BL	Live NL	% Inhibition Live	L:D BL	L:D NL	% Inhibition L:D
8-Jun	1.22×10^{4}	9.18×10^{6}	99.87	0.08	0.90	91.69
	1.01×10^{4}	6.80×10^{6}	99.85	0.10	0.90	88.63
	6.12×10^{3}	4.68×10^{6}	99.87	0.03	0.81	95.79
10-Jun	1.83×10^{4}	6.97×10^{6}	99.74	0.13	0.72	82.03
	1.82×10^{4}	7.81×10^{6}	99.77	0.14	0.72	80.06
	2.44×10^{4}	8.07×10^{6}	99.70	0.16	0.77	79.80
10-Sep	9.10×10^{4}	9.57×10^{4}	4.92	0.16	0.24	35.05
	5.93×10^{4}	2.13×10^{5}	72.21	0.27	0.33	18.42
	7.43×10^{4}	3.57×10^{5}	79.21	0.24	0.41	41.41
Average	3.49×10^{4}	4.91×10^{6}	99.29	0.15	0.64	77.47
T-test	1.18×10^{-3}			3.03×10^{-5}		

BUSA3017						
Trial	Live BL	Live NL	% Inhibition Live	L:D BL	L:D NL	% Inhibition L:D
8-Jun	2.65×10^{5}	6.59×10^{6}	95.98	0.74	0.81	8.13
	1.17×10^{6}	6.29×10^{6}	81.47	0.77	0.87	11.38
	1.53×10^{6}	5.71×10^{6}	73.25	0.76	0.75	-0.74
10-Jun	9.86×10^{4}	1.40×10^{6}	92.97	0.39	0.76	48.78
	5.71×10^{4}	2.46×10^{6}	97.68	0.30	0.72	57.54
	4.67×10^{4}	2.00×10^{6}	97.66	0.31	0.72	57.20
10-Sep	7.33×10^{4}	2.41×10^{6}	96.96	0.16	0.89	82.24
	9.14×10^{4}	3.03×10^{6}	96.98	0.10	0.89	88.35
	5.93×10^{4}	2.29×10^{6}	97.40	0.10	0.87	88.51
Average	3.76×10^{5}	3.57×10^{6}	89.48	0.40	0.81	50.11
T-test	3.18×10^{-4}			7.22×10^{-4}		

BUSA1079						
Trial	Live BL	Live NL	% Inhibition Live	L:D BL	L:D NL	% Inhibition L:D
8-Jun	1.61×10^{5}	1.03×10^{6}	84.47	0.42	0.69	38.81
	7.84×10^{4}	1.77×10^{7}	99.56	0.30	0.81	63.17
	2.43×10^{5}	3.69×10^{6}	93.40	0.53	0.66	20.40
10-Jun	9.12×10^{5}	2.80×10^{6}	67.48	0.79	0.76	-4.53
	9.99×10^{5}	6.32×10^{6}	84.20	0.70	0.81	12.78
	1.23×10^{6}	7.25×10^{6}	83.03	0.79	0.84	5.15
10-Sep	6.31×10^{4}	1.65×10^{5}	61.75	0.12	0.39	67.86
	9.82×10^{4}	2.09×10^{5}	53.01	0.22	0.32	29.87
	8.64×10^{4}	2.17×10^{5}	60.21	0.16	0.40	60.04
Average	4.30×10^{5}	4.37×10^{6}	90.17	0.45	0.63	28.68
T-test	5.34×10^{-2}			1.27 X 10-1		

BUSA1109						
Trial	Live BL	Live NL	% Inhibition Live	L:D BL	L:D NL	% Inhibition Live
8-Jun	1.31×10^{6}	6.23×10^{5}	-110.44	0.75	0.67	-12.26
	3.95×10^{5}	2.85×10^{7}	98.61	0.60	0.97	38.47
	2.53×10^{5}	5.78×10^{5}	56.15	0.32	0.69	54.02
10-Jun	1.55×10^{5}	5.59×10^{6}	97.23	0.48	0.84	42.93
	2.53×10^{5}	6.79×10^{6}	96.28	0.58	0.85	31.36
	2.06×10^{5}	4.60×10^{6}	95.52	0.49	0.89	45.35
10-Sep	6.59×10^{4}	1.43×10^{6}	95.38	0.23	0.77	70.72
	6.44×10^{4}	1.15×10^{6}	94.39	0.16	0.73	77.58
	6.01×10^{4}	6.93×10^{5}	91.32	0.16	0.63	74.11
Average	3.07×10^{5}	5.55×10^{6}	94.47	0.42	0.78	46.50
T-test	9.76×10^{-2}			3.10×10^{-4}		

APPENDIX C

Detailed Protocol

Preparation of strains

1. Place 1-3 frozen cryobeads with stock *S. aureus* strains in 5mL BHI.
2. Incubate overnight at 35ºC.
3. Gently vortex. Streak onto TSA plate in attempts of isolating a single colony.
4. Incubate overnight at 35ºC.
5. Pick single colony with sterile tip.
6. Streak new TSA plate with single colony. Incubate it overnight at 35ºC. This is considered the new master plate.

Preparation of frozen glycerol stock

1. Inoculate 5mL BHI with streaked tip from master plate.
2. Incubate overnight at 35ºC.
3. Make a 1:1 ratio (1ml:1mL) of 40% glycerol and overnight culture in sterile tube.
4. Freeze at -80ºC.

Preparation of cultures for experiment

1. Scrape small amount of frozen glycerol stock (using sterile metal spatula) into 5mL BHI.
2. Incubate overnight at 35ºC.
3. Gently vortex.
4. Pipet 50 µL into 5mL BHI.
5. Incubate for two hours at 35ºC.
6. Gently vortex.
7. Pipet 50 µL into 5mL BHI.
8. Gently vortex.
9. Pipet 100 µL into 10mL BHI.

Preparing microtiter plate.

1. Label all appropriate wells (on the lid) and differentiate treatment and no treatment plates with a marking.
2. Aliquot 1mL into four wells of the treatment plate for each strain used.
3. Repeat for no treatment plate.
   Use sterile technique, such as working with a flame and changing tips.

Treatment

1. Clean blue light box with 10% bleach.
2. Place blue light board on top of treatment microtiter plate.
3. Place no treatment plate in an opaque box.
4. Place both plates in incubator set at 35ºC
5. Set forward current to 120mA for each row of blue lights.

6. Set timer for 30 minutes.
7. Remove both plates after 30 minutes of treatment with blue light.

Plating time 0 culture

To be done while plates are incubating and the treatment plate is being exposed to blue light.

1. Pipet 50µL of remaining 2mL of cultures (from preparing microtiter plate steps) onto TSA plate.
2. Dip glass wand into ethanol and place in flame.
3. After approximately 12 seconds, evenly spread culture on TSA plate.
4. Repeat four times for each strain.
5. Incubate plates overnight at 35ºC.
6. Count the number of colonies for each plate and calculate CFU/mL.

Measuring optical density

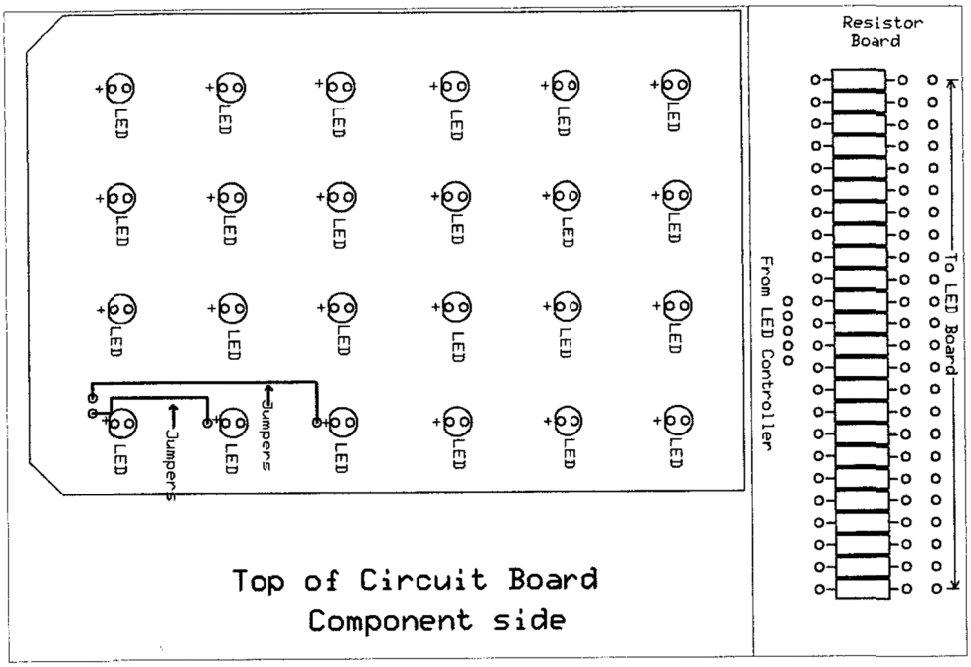
1. After 18 hour recovery growth in incubator at 35ºC, thoroughly resuspend samples by pipeting.
2. Take optical density measurement using a filter of 600nm on the ELx800 BioTek plate reader.
3. Transfer data into excel spreadsheet for later calculations.

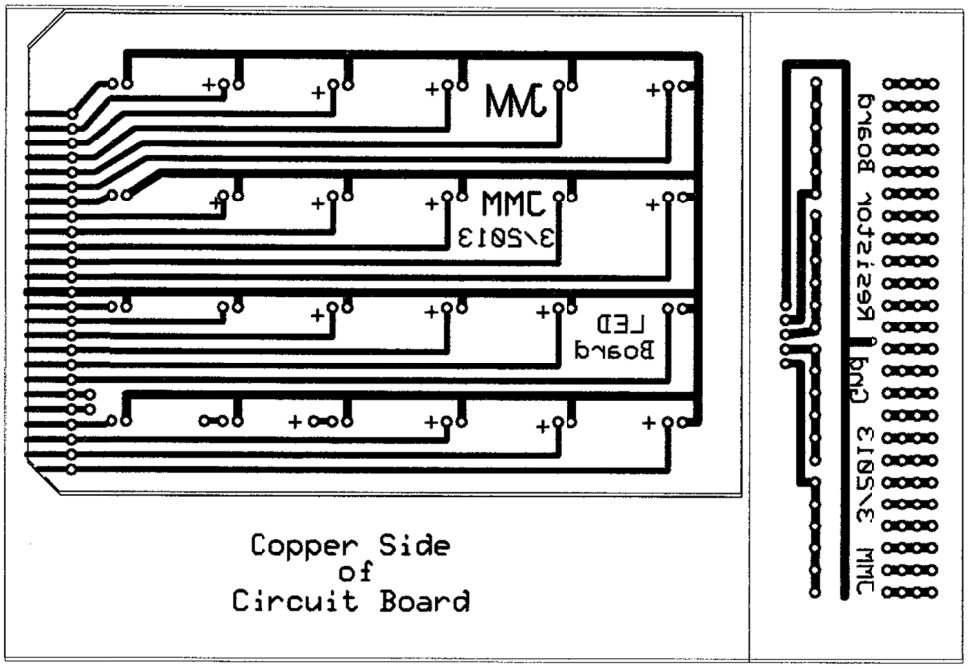
Preparation of samples for flow cytometry

1. After 30 minute incubation with blue light, turn off blue light. Allow the plates to incubate for intended number of hours.
2. Remove plates from incubator and thoroughly pipet each well to resuspend the sample.
3. Transfer all of the sample from a well to an appropriately labeled microcentrifuge tube.
4. Microcentrifuge the sample at 13,000xg for 6 minutes.
5. Drain away supernatant.
6. Resuspend pellet in 1000µL (if incubated 18 hours after treatment) or 100µL (if incubated 4 hours after treatment) filter-sterilized 0.85% NaCl.
7. At this point, samples can either be refrigerated overnight or can be directly prepared for analysis with flow cytometry.
8. Aliquot 20µL of sample into 972µL sheath fluid (in flow cytometry tube).
9. Add 1.5µL each propidium iodide and Syto 9 green dyes in a dark environment.
10. Incubate samples in the dark for 10 minutes.
11. While samples are incubating in the dark, sonicate microsphere standard for 10 minutes in a water bath.
12. Aliquot 5µL microsphere standard into sample tubes.
13. Run each sample until 5000 events are recorded on flow cytometer.
14. Analyze cell density via equation listed in materials and methods.

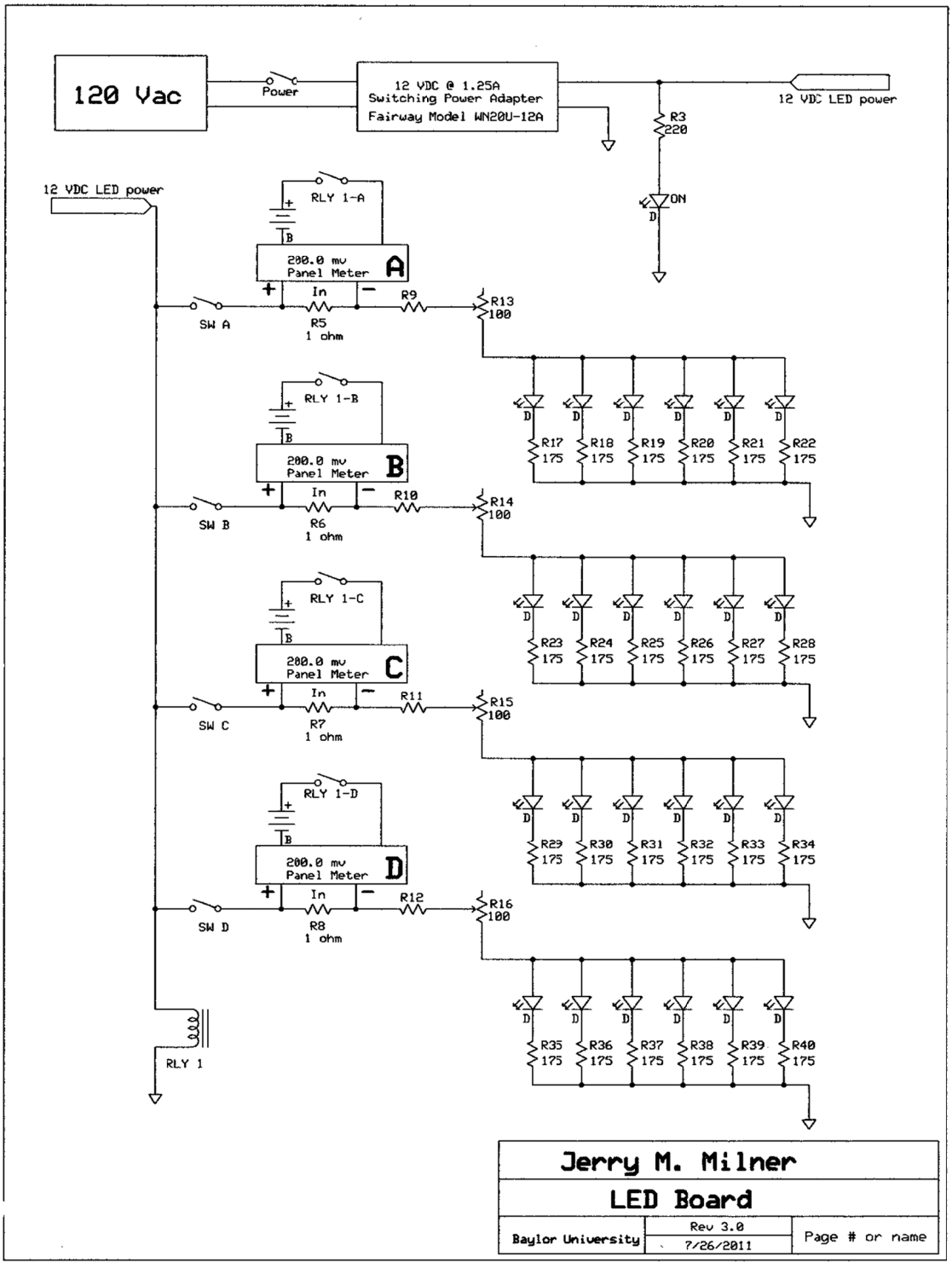
APPENDIX D

Schematic of the LED circuit board/light box used for blue light exposure to 24 well microtiter plate. Constructed by Jerry Milner.





Schematic of LED board used for blue light exposure. Constructed by Jerry Milner.



BIBLIOGRAPHY

1. Murray PR, Rosenthal KS, Pfaller MA, editors. Medical microbiology. 5th ed. Mosby; 2005. pp. 221-236 .

2. Moran GJ, Krishnadasan A, Gorwitz RJ, Fosheim GE, McDougal LK, Carey RB, Talan DA. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N Engl J Med 2006 08/17; 2014/01;355(7):666-74.

3. Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999-2005. Emerg Infect Dis 2007 Dec;13(12):1840-6.

4. Deurenberg RH, Stobberingh EE. The evolution of *Staphylococcus aureus*. Infection, Genetics and Evolution 2008 12;8(6):747-63.

5. Katayama Y, Ito T, Hiramatsu K. Genetic organization of the chromosome region surrounding *mecA* in clinical staphylococcal strains: Role of IS431-mediated *mec*I deletion in expression of resistance in *mecA*-carrying, low-level methicillin-resistant *Staphylococcus haemolyticus*. Antimicrob Agents Chemother 2001 Jul;45(7):1955-63.

6. Wielders CL, Vriens MR, Brisse S, de Graaf-Miltenburg LA, Troelstra A, Fleer A, Schmitz FJ, Verhoef J, Fluit AC. In-vivo transfer of *mecA* DNA to *Staphylococcus aureus* [corrected. Lancet 2001 May 26;357(9269):1674-5.

7. Herold BC, Immergluck LC, Maranan MC, Lauderdale DS, Gaskin RE, Boyle-Vavra S, Leitch CD, Daum RS. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. JAMA 1998 Feb 25;279(8):593-8.

8. Udo EE, Pearman JW, Grubb WB. Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. J Hosp Infect 1993 Oct;25(2):97-108.

9. Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Boxrud DJ, Etienne J, Johnson SK, Vandenesch F, Fridkin S, O'Boyle C, Danila RN, Lynfield R. Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. JAMA 2003 Dec 10;290(22):2976-84.

10. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA 2007;298:1763-71.

11. Appelbaum PC. MRSA--the tip of the iceberg. Clin Microbiol Infect 2006;12:3-10.

12. Dantes R, Mu Y, Belflower R, Aragon D, Dumyati G, Harrison LH, Lessa FC, Lynfield R, Nadle J, Petit S, Ray SM, Schaffner W, Townes J, Fridkin S, Emerging Infections Program-Active Bacterial Core Surveillance MRSA Surveillance Investigators. National burden of invasive methicillin-resistant *Staphylococcus aureus* infections, United States, 2011. JAMA Intern Med 2013 Nov 25;173(21):1970-8.

13. Blot SI, Vandewoude KH, Hoste EA, Colardyn FA. Outcome and attributable mortality in critically ill patients with bacteremia involving methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. Arch Intern Med 2002 Oct 28;162(19):2229-35.

14. McHugh CG, Riley LW. Risk factors and costs associated with methicillin-resistant *Staphylococcus aureus* bloodstream infections. Infect Control Hosp Epidemiol 2004 May;25(5):425-30.

15. Baddour MM. Chapter 1. the case for MRSA and treatment of methicilllin resistant staphylococcus aureus. In: Charles L. Koendi, editor. Methicillin-resistant *Staphylococcus aureus* (MRSA): Etiology, at-risk populations and treatment. New York: Nova Science Publishers, Inc.; 2009. p 1-1-54.

16. Holtfreter S, Kolata J, Bröker BM. Towards the immune proteome of *Staphylococcus aureus* – the anti-S. aureus antibody response. International Journal of Medical Microbiology 2010 2;300(2–3):176-92.

17. Sievert DM, Rudrik JT, Patel JB, McDonald LC, Wilkins MJ, Hageman JC. Vancomycin-resistant *Staphylococcus aureus* in the United States, 2002-2006. Clin Infect Dis 2008 Mar 1;46(5):668-74.

18. Schito GC. The importance of the development of antibiotic resistance in *Staphylococcus aureus*. Clinical Microbiology and Infection 2006;12:3-8.

19. Gaupp R, Ledala N, Somerville GA. Staphylococcal response to oxidative stress. Front Cell Infect Microbiol 2012;2:33.

20. Lu TK, Koeris MS. The next generation of bacteriophage therapy. Curr Opin Microbiol 2011 Oct;14(5):524-31.

21. Yin R, Dai T, Avci P, Jorge AE, de Melo WC, Vecchio D, Huang YY, Gupta A, Hamblin MR. Light based anti-infectives: Ultraviolet C irradiation, photodynamic therapy, blue light, and beyond. Curr Opin Pharmacol 2013 Oct;13(5):731-62.

22. Dai T, Gupta A, Murray CK, Vrahas MS, Tegos GP, Hamblin MR. Blue light for infectious diseases: *Propionibacterium acnes, Helicobacter pylori*, and beyond? Drug Resistance Updates 2012 8;15(4):223-36.

23. Adamskaya N, Dungel P, Mittermayr R, Hartinger J, Feichtinger G, Wassermann K, Redl H, van Griensven M. Light therapy by blue LED improves wound healing in an excision model in rats. Injury 2011 9;42(9):917-21.

24. Nakonechny F, Firer MA, Nitzan Y, Nisnevitch M. Intracellular antimicrobial photodynamic therapy: A novel technique for efficient eradication of pathogenic bacteria. Photochem Photobiol 2010;86(6):1350-5.

25. Ashkenazi H, Malik Z, Harth Y, Nitzan Y. Eradication of *Propionibacterium acnes* by its endogenic porphyrins after illumination with high intensity blue light. FEMS Immunol Med Microbiol 2003 Jan 21;35(1):17-24.

26. Allison RR, Downie GH, Cuenca R, Hu X, Childs CJ, Sibata CH. Photosensitizers in clinical PDT. Photodiagnosis and Photodynamic Therapy 2004;1(1):27,27-42.

27. Grinholc M, Szramka B, Olender K, Graczyk A. Bactericidal effect of photodynamic therapy against methicillin-resistant *Staphylococcus aureus* strain with the use of various porphyrin photosensitizers. Acta Biochim Pol 2007;54:665-70.

28. Di Poto A, Sbarra MS, Provenza G, Visai L, Speziale P. The effect of photodynamic treatment combined with antibiotic action or host defence mechanisms on *Staphylococcus aureus* biofilms. Biomaterials 2009 Jun;30(18):3158-66.

29. Hamblin MR, Viveiros J, Yang C, Ahmadi A, Ganz RA, Tolkoff MJ. *Helicobacter pylori* accumulates photoactive porphyrins and is killed by visible light. Antimicrob Agents Chemother 2005 Jul;49(7):2822-7.

30. Lipovsky A, Nitzan Y, Friedmann H, Lubart R. Sensitivity of *Staphylococcus aureus* strains to broadband visible light. Photochem Photobiol 2009;85(1):255-60.

31. Lipovsky A, Nitzan Y, Gedanken A, Lubart R. Visible light-induced killing of bacteria as a function of wavelength: Implication for wound healing. Lasers Surg Med 2010;42(6):467-72.

32. Maclean M, MacGregor SJ, Anderson JG, Woolsey G. Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. Appl Environ Microbiol 2009 Apr;75(7):1932-7.

33. Enwemeka CS, Williams D, Hollosi S, Yens D, Enwemeka SK. Visible 405 nm SLD light photo-destroys methicillin-resistant *Staphylococcus aureus* (MRSA) in vitro. Lasers Surg Med 2008 Dec;40(10):734-7.

34. Enwemeka CS, Williams D, Enwemeka SK, Hollosi S, Yens D. Blue 470-nm light kills methicillin-resistant *Staphylococcus aureus* (MRSA) in vitro. Photomed Laser Surg 2009 Apr;27(2):221-6.

35. University of Wisconsin-Milwaukee. Light as medicine? researchers explain how. ScienceDaily 2013 October 22, 2013.

36. Maisch T, Baier J, Franz B, Maier M, Landthaler M, Szeimies R, Bäumler W. The role of singlet oxygen and oxygen concentration in photodynamic inactivation of bacteria. Proceedings of the National Academy of Sciences 2007 April 24;104(17):7223-8.

37. Maclean M, MacGregor SJ, Anderson JG, Woolsey GA. The role of oxygen in the visible-light inactivation of *Staphylococcus aureus*. Journal of Photochemistry and Photobiology B: Biology 2008 9/18;92(3):180-4.

38. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of *Staphylococcus aureus*: Epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev 1997 Jul;10(3):505-20.

39. Hosgor-Limoncu M, Ermertcan S, Tasli H, Erac B, Yurtman AN. Chapter 7: Methicillin-resistant *Staphylococcus aureus*: Etiology, at-risk populations and treatment. In: Charles L. Kolendi, editor. Methicillin-resistant staphylococcus aureus (MRSA): Etiology, at-risk populations and treatment. New York: Nova Science Publishers, Inc.; 2009. p 329-329-364.

40. Lindsay JA, Holden MTG. *Staphylococcus aureus*: Superbug, super genome? Trends Microbiol 2004 8/1;12(8):378-85.

41. Ohno K. Chapter 12. risk factors for carrying methicillin-resistant *Staphylococcus aureus* at hospitalization in a pediatric surgical ward. In: Charles L. Kolendi, editor. Methicillin-resistant *Staphylococcus aureus* (MRSA): Etiology, at-risk populations and treatment. New York: Nova Science Publishers, Inc.; 2009. p 463-463-482.

42. Boyce JM, Cookson B, Christiansen K, Hori S, Vuopio-Varkila J, Kocagöz S, Öztop AY, Vandenbroucke-Grauls CM, Harbarth S, Pittet D. Meticillin-resistant *Staphylococcus aureus*. The Lancet Infectious Diseases 2005 10;5(10):653-63.

43. Kurlenda J, Grinholc M. Chapter 5. MRSA: The virulence, epidemiology and perspective diagnostics and therapy. In: Charles L. Koendi, editor. Methicillin-resistant *Staphylococcus aureus* (MRSA): Etiology, at-risk populations and treatment. New York: Nova Science Publishers, Inc.; 2009. p 75-75-141.

44. Zhang K, McClure JA, Elsayed S, Louie T, Conly JM. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 2005 Oct;43(10):5026-33.

45. Kingbright. 2013 March 29, 2013. Part number: WP7113QBC/D. <https://www.kingbrightusa.com/images/catalog/spec/WP7113QBC-D.pdf>. Accessed 2014 04/14.

46. Fox G. Investigating inhibitory synergy between blue light irradiation and antibiotic treatment of *Staphylococcus aureus*. BearDocs: Baylor University; 2013. Available from: http://hdl.handle.net/2104/8635.

47. Samake J. The effect of blue light irradiation of *S. aureus* in different media. 2013.

48. Dai T, Gupta A, Huang YY, Yin R, Murray CK, Vrahas MS, Sherwood ME, Tegos GP, Hamblin MR. Blue light rescues mice from potentially fatal *Pseudomonas aeruginosa* burn infection: Efficacy, safety, and mechanism of action. Antimicrob Agents Chemother 2013 Mar;57(3):1238-45.

49. Feuerstein O, Assad R, Koren E, Ginsburg I, Weiss EI, Houri-Haddad Y. Visible light promotes interleukin-10 secretion by sublethal fluences. Photomed Laser Surg 2011 Sep;29(9):627-33.

50. Oláh J, Tóth-Molnár E, Kemény L, Csoma Z. Long-term hazards of neonatal blue-light phototherapy. Br J Dermatol 2013;169(2):243-9.

51. Bhutani VK, Committee on Fetus and Newborn, American Academy of Pediatrics. Phototherapy to prevent severe neonatal hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. Pediatrics 2011 Oct;128(4):e1046-52.