Title: Rose bengal and riboflavin mediated photodynamic therapy to inhibit methicillin-resistant *Staphylococcus aureus* keratitis isolates

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Short Title: Photodynamic Therapy for Methicillin-resistant *S. aureus*

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is among the leading causes of bacterial keratitis.¹ *S. aureus* produces toxic exudates that activate natural host immune responses in the cornea. In an attempt to clear the infection, the host releases proinflammatory cytokines which results in scarring and functional damage to the cornea. In the most severe cases *S. aureus* keratitis can cause a secondary corneal melt, with a potential corneal perforation that can lead to endophthalmitis.²

Traditionally, clinical treatment of bacterial keratitis has been empirical with a recent shift towards culture-guided practice. A patient with presumed infectious keratitis will likely receive monotherapy with a fourth generation fluoroquinolone as a first-line treatment.³ Repeated and inappropriate utilization of antibiotics has led to the rise in drug resistant strains of bacteria such as *Methicillin-resistant Staphylococcus aureus* (MRSA).⁴ Increased resistance of MRSA to current fluoroquinolones and other common ocular antibiotics has led to limited and less than optimal treatment options.⁵

One promising alternative treatment is photodynamic therapy (PDT), which has been explored to treat a variety of diseases.⁶⁻⁸ Photodynamic therapy involves the activation of a photosensitizing agent with light at a specific wavelength band. When the photosensitizer is activated to an excited state, it interacts with ambient oxygen to produce either type I or type II reactions which can lead to cell death by activating apoptotic pathways along with many other interactions.⁹

In ophthalmology, PDT has been widely applied for the treatment of choroidal neovascularization in patients and has been tested for its effects on corneal neovascularization.^{8,10} Currently, the most common application of PDT in the cornea is for corneal collagen crosslinking (CXL) to treat keratoconus using riboflavin as a photosensitizing agent and ultraviolet-A (UV-A) irradiation first established under the Dresden protocol (0.1% riboflavin solution and UV-A irradiation for a total of 5.4 J/cm²).¹¹ Studies have shown the value of using riboflavin CXL following the Dresden protocol as an adjunct therapy for infectious keratitis.^{12,13}

The efficacy of rose bengal, a commonly used ocular stain, as an alternate photosensitizer has been investigated for inhibiting the growth of microbial isolates.¹⁴⁻¹⁶ One group found the minimum concentration of rose bengal needed to inhibit fungal isolates to be 280 μ M (0.03%), which was used as one of the concentrations of rose bengal in our study.¹⁶ It was hypothesized that PDT is effective in eradicating multidrug-resistant MRSA strains *in vitro*. To test this, two clinical MRSA strains were exposed to 0.03% and 0.1% rose bengal-mediated PDT and 0.1% riboflavin-mediated PDT and observed for growth inhibition.

MATERIALS AND METHODS

All work for this experimental study was performed at the Ophthalmic Biophysics Center and Ocular Microbiology Laboratory, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, Florida, USA.

Figure 1 provides a general overview of methods and workflow of the *in vitro* PDT experiments.

Bacterial Strain and Growth (Inoculum)

Inocula were prepared from two different multidrug-resistant MRSA strains that were isolated from corneas of patients with confirmed infectious keratitis.

The parent cultures of both strains came from patients with similar clinical scenarios. Both patients had chronic exposure keratitis that was not well-treated and required previous prolonged hospital admission where they were treated with a variety of ocular fortified and non-fortified drops. There was no or minimal response using fluoroquinolones. Both patients presented to Bascom Palmer Emergency Room with corneal perforation secondary to infectious keratitis (MRSA). Patients were managed with therapeutic graft surgery and fortified vancomycin.

MRSA Strain 1 (MRSA-1) was resistant to four classes of antibiotics (x4): fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin), macrolides (erythromycin), beta-lactams (penicillin, oxacillin), and lincosamides (clindamycin). MRSA Strain 2 (MRSA-2) was resistant to six classes of antibiotics (x6): fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin), aminoglycosides (gentamicin), macrolides (erythromycin), sulfonamides (trimethoprim sulfate), tetracyclines (tetracycline, doxycycline), and beta lactams (penicillin, oxacillin). *In vitro* susceptibilities were based on systemic MIC breakpoints.

Parent cultures of each strain were plated on nutrient agar (W31, Hardy Diagnostics, Santa Maria, California, USA) and placed in an incubator (Precision Scientific, Scientific Products, Miami, FL, USA) at 30°C for 18-24 hours. On the day of experimentation, MRSA strains were transferred to tryptic soy broth (R064890; Remel Products, Lenexa, KS) and concentrations standardized to 1.5×10^8 colony forming units (cfu/mL) using a spectrophotometer (Novaspec Plus 80-2117-50; Biochrom LTD, Cambridge, England) and serially diluted to the final working concentration of 1.5×10^5 cfu/mL.

Photosensitizer Preparation

Solutions were prepared on the day of experimentation and kept in light protected test tubes to prevent photobleaching of the photosensitizers. The 0.1% and 0.03% rose bengal solutions were prepared by dissolving 100 mg and 30 mg of rose bengal (198250; Sigma-Aldrich, St Louis, MO, USA), respectively, in 100 mL of high purity water (NERL 9805; Fisher Diagnostics, Middletown, VA, USA). The 0.1% riboflavin solution was made by dissolving 100 mg of riboflavin-5-phosphate (R7774; Sigma-Aldrich, St. Louis, MO, USA) in 100 mL of high purity water.

The MRSA-1 and MRSA-2 suspensions were mixed with the appropriate photosensitizer solution or high purity water for a final bacterial concentration of 1.5×10^4 cfu/mL. Under minimal light conditions, corresponding to switching off the room light with residual 2 lux or $0.20 \mu\text{W}/\text{cm}^2$ across the visible light spectrum, 1 mL aliquots of the MRSA suspension were pipetted onto nutrient agar plates (15 x 100 mm Nutrient Agar plate W31; Hardy Diagnostics, Santa Maria, CA, USA), for a total preparation time of approximately 10 minutes.

Experimental groups were conducted in triplicate and the plates were divided into four groups for each strain of bacteria: (1) MRSA only control, (2) MRSA with 0.1% rose bengal, (3) MRSA with 0.03% rose bengal, and (4) MRSA with 0.1% riboflavin. Each group was exposed to either (1) dark or (2) ambient light or (3) irradiation of either green LED for rose bengal or UV-A for riboflavin.

The ambient light group was exposed for 30 minutes, the green LED irradiation was performed for 34 minutes and the UV-A irradiation was performed for 30 minutes. After irradiation, all plates were immediately placed inside a lightproof container to prevent further light activation and stored in an incubator at 30°C.

Irradiation Sources

Two irradiation sources were built in-house using an array of light emitting diodes (LEDs) (Figure 2). The green LED irradiation source, used to activate rose bengal, contains LEDs in an array of diameter of 47 mm. The green irradiation source had a peak wavelength of 518 nm ($I_{40\%}$: 500–541 nm) (L1-0-G5TH45-1; LEDSupply, Randolph, VT, USA) and produced an average irradiance of $2.8 \text{ mW}/\text{cm}^2$. The UV-A irradiation source, used to activate riboflavin, contains LEDs in an array of 37 mm. The UV-A irradiation source had a peak wavelength of 375 nm ($I_{40\%}$: 370–383 nm) (L7-0-U5TH15-1; LEDSupply, Randolph, VT, USA) and produced an average irradiance of $3.0 \text{ mW}/\text{cm}^2$.

The ambient light condition was produced by the laboratory fluorescent illumination of 300 lux and produced an irradiance of $107 \mu\text{W}/\text{cm}^2$ across the visible light spectrum. Irradiances were measured at the surface of the agar plates using a photo power meter (Model 1916C; Newport, Irvine, California, USA). Spectra for each of the four light sources were recorded using a spectrometer (SM442; Spectral Products, Putnam, Connecticut, USA).

Percent Inhibition Measurements

Agar plates were placed on a custom-built retroilluminator¹⁷ and photographed every 24 hours with a digital camera (16.2 MP, Nikon D7000; Nikon Inc, USA) with resolution of 31.7 px/mm. Percent growth was measured with a custom-made software written in LabVIEW 6 (National Instruments, Austin, TX, USA). For every plate, a central zone was selected for measurement corresponding to the irradiation source diameter (47 mm for green irradiator and 37 mm for UV-A irradiator).

The images were converted to grayscale and background illumination was normalized, image contrast was increased and images were segmented by applying a series of binary threshold filters.

The resulting black and white image had white pixels for bacterial growth and black pixels for no growth. The area of growth was calculated by summing the total number of white pixels. Percent growth was determined by dividing total growth by the total pixel area of the irradiation zone. Percent growth values were validated by comparing to manual segmentation methods and were found to be within the same range of precision.

$$\text{Percent Growth} = (\# \text{ white pixels} / \text{total \# of pixels in selected zone})$$

The control group was the dark condition inoculated with MRSA and corresponded to 100% growth. All other experimental groups were compared to this value. To calculate percent inhibition, percent growth was subtracted from 100%, as the control group has 0% inhibition.

$$\text{Percent Inhibition} = 100\% - (\text{experimental group percent growth} / \text{control dark group percent growth})$$

Statistical tests were performed to assess and compare the percent inhibition between each experimental group. Statistical significance was set at $p < 0.05$.

RESULTS

Seventy-two hours after irradiation, images were taken and percent inhibition was calculated. Results are displayed in Figure 3.

0.1% and 0.03% rose bengal showed 100% inhibition of both MRSA strains in both ambient light and green LED irradiation groups. In dark conditions, 0.1% rose bengal demonstrated 100% inhibition in both strains, while 0.03% rose bengal showed 94% inhibition in MRSA-1 and 100% inhibition in MRSA-2. Photographs taken 72 hours after MRSA growth with rose bengal and exposure to either dark, ambient or green LED irradiation are displayed in Figure 4.

Riboflavin with UV-A irradiation showed inhibition in both MRSA strains, 98% in MRSA-1 and 100% in MRSA-2. Riboflavin with ambient light showed 13% inhibition in MRSA-1 and 15% inhibition in MRSA-2. Riboflavin in dark conditions showed 11% inhibition in MRSA-1 and 3% inhibition in MRSA-2. Photographs taken 72 hours after MRSA growth with riboflavin and exposure to either dark, ambient or UV-A irradiation are displayed in Figure 5.

In groups without photosensitizers, under green LED irradiation, inhibition was 50% in MRSA-1 and 28% in MRSA-2. Under UV-A irradiation, inhibition was 79% in MRSA-1

and 81% in MRSA-2. In the ambient light group, MRSA-1 had more growth than in the dark group, corresponding to -7% inhibition.

A one-way ANOVA test was conducted to determine the significance of variance among groups. It was observed that there was a significant effect of photosensitizer and irradiation at $p < 0.05$ on percent growth of both strains of MRSA. MRSA-1 [$F(2,12) = 190.67$, $p < 0.001$] and MRSA-2 [$F(2,12) = 861.46$, $p < 0.001$]

Furthermore, a Tukey's HSD test was conducted post-hoc to compare the significance between individual groups. It was observed that the experimental groups could be divided into three tiers of inhibition with no significant overlap. The presence of rose bengal (dark, ambient, green LED irradiation) or riboflavin with UV-A irradiation showed the most percent growth inhibition (>98%). A second tier of inhibition involving use of an irradiation source without photosensitizer (green LED and UV-A irradiation) resulted in moderate growth inhibition (28-81%). A third tier of inhibition consisted of groups with no photosensitizer and groups with riboflavin that did not receive UV-A irradiation. These groups, whose error of standard deviation overlapped with maximum growth, were not considered significantly different from 0% inhibition (0-15%).

DISCUSSION

The results of the study support the hypothesis that PDT can inhibit growth of MRSA isolates. In this study, 0.1% rose bengal was the most effective agent for growth inhibition of two different multidrug-resistant clinical MRSA strains. The 0.03% rose bengal solution was also effective in inhibiting growth in ambient and green LED irradiated groups, but showed slightly less efficacy in MRSA-1 under dark conditions. 0.1% riboflavin with UV-A irradiation inhibited MRSA growth within the irradiation zone, comparable to the results achieved with rose bengal for MRSA-2; however, 0.1% riboflavin with UV-A irradiation was not as effective as either 0.1% or 0.03% rose bengal in MRSA-1. Furthermore, riboflavin under ambient light and dark conditions demonstrated minimal inhibition of both MRSA strains.

It is important to note that all of the plates were prepared in the laboratory with the lights off, and the exposure was measured to be 2 lux for a total time of five minutes. Although the light level was very low, this means that even the dark groups had some measurable exposure to possible photoactivation of the photosensitizers, not that the rose bengal was inherently toxic.

In groups without photosensitizers that underwent UV-A irradiation, inhibition was 79% in MRSA-1 and 81% in MRSA-2. While it was observed in our study that UV-A light alone has the ability to significantly inhibit MRSA growth, the inhibitory effects of UV-A light on DNA replication in certain strains of bacteria is known and this mechanism can be repaired by many cells, with certain microorganisms being more susceptible to UV-A irradiation than others.¹⁸ Moreover, it has been known that UV-A exposure in humans induces the process of cataractogenesis and is dose dependent.¹⁹ The introduction of a photosensitizer such as riboflavin, in addition to increasing bacterial inhibition efficacy,

also provides protection by absorbing UV-A irradiation, preventing higher energy dosages from transmitting into deeper layers and inducing cataracts.¹¹

Previous work has explored the effect of PDT on *S. aureus* using other photosensitizers including porphyrins, xanthines, and toluidine; however it should be noted that these are not approved for clinical use.²⁰⁻²³ The two photosensitizers used in our study, rose bengal and riboflavin, offer the benefit of being routinely used in clinical practice.

The inhibitory effect of riboflavin and UV-A irradiation on different microbes has been previously investigated *in vitro*.^{13,24} Martins et al. tested several corneal pathogens, including Methicillin susceptible and Methicillin resistant *S. aureus* strains, with the riboflavin/UV-A irradiation combination *in vitro* and demonstrated this effectively inhibited growth.¹³ Makdoui et al. investigated the effect of varying riboflavin concentrations and irradiation time on *S. epidermis*, *S. aureus*, and *P. aeruginosa*. They found no clear correlation when changing the riboflavin concentration, but increasing the irradiation time of UV-A light from 30 minutes (5.4 J/cm²) to 60 minutes (10.8 J/cm²) increased the inhibitory effect.²⁴ The studies of Martins et al. and Makdoui et al. are consistent with our findings showing that riboflavin with UV-A irradiation causes maximum growth inhibition.^{13,24}

In vivo studies to evaluate the efficacy of CXL utilizing the Dresden protocol have been performed for patients with infectious keratitis.²⁵⁻²⁷ Iseli et al. pioneered CXL for treating patients with corneal melting associated with severe infectious keratitis as a last resort before emergency keratoplasty. In four of the five cases, melting was halted and keratoplasty was avoided. In addition, corneal infiltrate, both bacterial and fungal, were reduced and cultures returned negative for microbes. The only case that was not successful was related to *Fusarium* keratitis.²⁶ A larger prospective *in vivo* study by Said et. al reported that the use of the Dresden protocol, as an adjunct to traditional antimicrobial therapy, showed no complication of perforation or infection recurrence in 21 eyes. This was markedly different from the control group who received only antimicrobial therapy and had a complication rate of 21%.²⁸

Rose bengal PDT in non-ophthalmic settings has been investigated by Schafer et al. who studied its effects on *E. coli*, *D. radiodurans*, and *B. subtilis* bacteria for water purification purposes. Their work demonstrated that rose bengal PDT irradiated by visible light (300-600 nm) was able to cause cell death in gram positive and gram negative bacteria.¹⁴ Our results are consistent with their work. Banks et al. have previously tested rose bengal's ability to cause cytotoxic and photodynamic cell death of *S. aureus* in solid media and liquid suspensions in relation to food sterilization. Using 15 W fluorescent irradiation (170 and 315 μ E/m/s for 0-120 min), it was determined that oxygen is required to increase the cytotoxic effect of rose bengal and this was reduced in the presence of quenchers crocetin and L-histidine.²⁹ Though Banks et al.'s results are consistent with the results of this study, it should be noted that in their experimental setup, the rose bengal was immobilized into the agar and then the bacterium was inoculated on top. The immobilized rose bengal agar will slowly release the photosensitizer over time and have a prolonged killing effect, whereas in our

experimental setup, the rose bengal solution was mixed with *S. aureus* and then placed onto a petri dish. This more closely simulates a one-time topical application of rose bengal and its interaction with the bacteria on the ocular surface.

Our results show that rose bengal is very effective for *in vitro* inhibition of two different multidrug-resistant strains of MRSA ocular isolates. The isolates that were tested came from patients in situations where traditional antimicrobials proved ineffective and eventual surgical intervention and therapeutic graft was required. Hundreds of methicillin susceptible and resistant *S. aureus* strains exist and remain to be tested. However, given their unique ocular origin and multidrug-resistant characteristics, these two strains were used as proof of principle that PDT could be effective against MRSA strains in ocular emergencies.

This study was limited by the fact that it was conducted on agar plates, which only mimic the ocular surface; however moderate to severe cases of infectious keratitis have deep corneal stromal involvement. One study used freshly enucleated and de-epithelialized rabbit eyes with rose bengal and green light irradiation and found that the rose bengal penetrated to a depth of 200 μm .³⁰ Deeper penetration of rose bengal which, like riboflavin, is electronegative, can be achieved using iontophoresis.^{31,32} Future studies will need to investigate the penetration of these photosensitizers into the deeper levels of the stroma, as well as evaluate any potential toxicity to the cornea.

In regard to photosensitizer choice, our results demonstrate that using rose bengal mediated PDT is more effective than riboflavin in the inhibition of MRSA isolates. Currently, riboflavin offers the advantage of being routinely used in CXL to treat keratoconus outside of the United States. Rose bengal is a commonly used and FDA approved dye for ocular surface staining; however, the use of rose bengal in PDT is not.

Further studies on rose bengal's safety in PDT applications are needed before clinical use can be considered. Cherfan et al. demonstrated an increase in Young's modulus of the cornea after rose bengal CXL with no adverse effects on keratocyte viability.³⁰ In ophthalmology clinics, 1% rose bengal strips are used to detect conjunctival defects.³³ Transitioning rose bengal from a clinically accepted ocular surface stain to an antimicrobial agent could be straightforward. The results of this experiment offer evidence to support the idea that a concentration ten-fold less than the rose bengal strips is or may be sufficient to inhibit MRSA growth under dark and ambient lighting conditions. As most patients spend their days in ambient lighting or in natural sunlight, rose bengal remaining on the ocular surface could be further activated throughout the day and a topical application might help fight cases of superficial keratitis.

Compared to topical antibiotics, the direct topical administration of photosensitizers for PDT could prove advantageous in providing a localized treatment for bacterial keratitis. PDT may offer an alternate adjunct therapy for MRSA, potentially combating infection and decreasing bacterial load without introducing possible antibiotic resistance. Future studies on *in vivo* models will be needed to move this modality forward for clinical practice.

ACKNOWLEDGEMENTS:

a. Funding/Support:

This work was supported in part by The Florida Lions Eye Bank (Miami, FL), the Edward D. and Janet K. Robson Foundation (Tulsa, OK), Drs. KR Olsen and ME Hildebrandt, NIH center grant P30EY14801, an unrestricted grant from Research to Prevent Blindness (New York, NY) to the Department of Ophthalmology, and the Henri and Flore Lesieur Foundation (Chicago, IL).

b. Financial Disclosures:

The following financial disclosures were reported:

Jean-Marie Parel: Grants from NIH, DOD, InnFocus LLC (Miami, FL), EyeGatePharma (Waltham, MA), Australian Government Cooperative Research Centre scheme (Vision CRC, Sydney). All patents are assigned to the University of Miami.

The following authors have no financial disclosures: Francisco Halili, Alejandro Arboleda, Heather Durkee, Mukesh Taneja, Darlene Miller, Karam A. Alawa, Mariela C. Aguilar, Guillermo Amescua, and Harry W. Flynn Jr.

c. Other Acknowledgments such as Statisticians, Medical Writers, Expert contributions.

The authors would like to acknowledge the technical and scientific contributions of the following members of the Ophthalmic Biophysics Center (Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, Florida, USA) team: Cornelis Rowaan BS and William Lee for the fabrication of the UV-A irradiator; Cornelis Rowaan BS, Alex Gonzalez BA, and Juan Silgado for fabricating the green LED irradiator; Victor Hernandez MS and William Lee for their help fabricating the – retro illuminator; Carolina de Freitas MS, Mercury Sawatari, Keke Liu, and Kelly Mote for their assistance in the creation and verification of the custom LabVIEW program; Yu-cherng Chang BS for programming assistance in statistical analysis; and Carolina de Freitas MS for the design and display of experimental figures.

Martine Jager MD, PhD (Leiden University Medical Center, Department of Ophthalmology, Leiden, Netherlands), Florence Cabot MD and Nidhi Relhan MD (Ophthalmic Biophysics Center, Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, FL, United States) provided assistance preparing the manuscript.

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FIGURE CAPTIONS:

Figure 1. In vitro Photodynamic Therapy Experimental Overview to inhibit methicillin-resistant *Staphylococcus aureus* keratitis isolates. (Step1: Inoculum)

Clinically sampled Methicillin resistant *S. aureus* strains from patients with infectious keratitis were grown on nutrient agar for 24-hours, prepared in solution and adjusted to a standard concentration of 1.5×10^5 CFU/ml. **(Step 2: Photosensitizer)** Each photosensitizer solution (0.03%, 0.1% rose bengal or 0.1% riboflavin) was mixed with inoculum and plated onto nutrient agar. **(Step 3: Irradiation)** Plates were exposed to either dark, ambient light (30 minutes) or irradiation of either green light emitting diodes (34 minutes) or Ultraviolet-A (30 minutes) and grown in a light-protected box for 72 hours in an incubator. **(Step 4: Growth Inhibition)** At 72 hours, plates were photographed and analyzed with software to determine percent growth inhibition.

Figure 2. Irradiation Sources used in Photodynamic Therapy experimental protocol to inhibit methicillin-resistant *Staphylococcus aureus* keratitis isolates

(Left) green Light Emitting Diode irradiation source for rose bengal activation. (Right) Ultraviolet-A Light Emitting Diode irradiation source for riboflavin activation.

Figure 3. Percent growth inhibition of two strains of methicillin-resistant *Staphylococcus aureus* keratitis isolates 72 hours after Photodynamic Therapy with rose bengal or riboflavin.

Three photosensitizer solutions (0.03%, 0.1% rose bengal or 0.1% riboflavin) and control (water) were mixed with standardized inoculum and plated onto nutrient agar. Plates were exposed to either dark, ambient light (30 minutes) or irradiation of either green light emitting diodes (34 minutes) or Ultraviolet-A (30 minutes) and grown in a light-protected box placed in an incubator for 72 hours.

Figure 4. Agar plates photographed 72 hours after rose bengal- mediated photodynamic therapy of two strains of methicillin-resistant *Staphylococcus aureus* keratitis isolates

Inoculated agar plates photographed at 72 hours displaying control, 0.1%, and 0.03% rose bengal groups under dark, ambient light, and green light emitting diode irradiation conditions. White dotted line denotes 47 mm diameter corresponding to diameter of irradiation source.

Figure 5. Agar plates photographed 72 hours after riboflavin- mediated photodynamic therapy of two strains of methicillin-resistant *Staphylococcus aureus* keratitis isolates

Inoculated agar plates photographed at 72 hours displaying control and 0.1% riboflavin groups under dark, ambient light, and Ultraviolet-A irradiation conditions. Red dotted line denotes 37 mm diameter corresponding to diameter of irradiation source.

Figure 1. In vitro PDT Experimental Overview

Figure 2. Irradiation Sources

Figure 3. Percent growth in vitro PDT inhibition of MRSA

Figure 4. MRSA photographs post rose bengal mediated PDT

Figure 5. MRSA photographs post riboflavin mediated PDT