Received: 16 February 2021 | Revised: 16 September 2021 | Accepted: 6 November 2021

DOI: 10.1111/jam.15366

Journal of Applied Microbiology

ORIGINAL ARTICLE

# Bactericidal effects of high-energy visible light on common otitis media pathogens

**Shae D. Morgan**[1] | **Deborah Yoder-Himes**[2] | **Douglas Jackson**[3] | **John Naber**[3] | **Rachel Berry**[1] | **Elizabeth Cash**[1] | **Swapna Chandran**[1] | **Thomas Roussel**[4]

[1]Department of Otolaryngology – Head and Neck Surgery & Communicative Disorders, University of Louisville, Louisville, Kentucky, USA

[2]Department of Biology, University of Louisville, Louisville, Kentucky, USA

[3]Department of Electrical and Computer Engineering, University of Louisville, Louisville, Kentucky, USA

[4]Department of Bioengineering, University of Louisville, Louisville, Kentucky, USA

**Correspondence**
Shae D. Morgan, Program in Audiology, 500 South Preston Street, Suite 414, Louisville, KY 40202, USA.
Email: shae.morgan@louisville.edu

**Funding information**
University of Louisville School of Medicine

## Abstract

**Aims:** This study assessed the use of high-energy, visible light on the survival rates of three bacteria commonly found in middle ear infections (i.e. otitis media; *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae*).

**Method and Results:** Bacteria were cultured and then subjected to a single, 4-h treatment of 405 nm wavelength light at two different intensities. All three bacteria species were susceptible to the light at clinically significant rates (>99.9% reduction). Bacteria were susceptible to the high-energy visible (HEV) light in a dose-dependent manner (lower survival rates with increased intensity and duration of exposure).

**Conclusions:** The results suggest that HEV light may provide a non-surgical, non-pharmaceutical approach to the therapeutic treatment of otitis media.

**Significance an Impact of the Study:** Given the growing concerns surrounding antibiotic resistance, this study demonstrates a rapid, alternative method for effective inactivation of bacterial pathogens partly responsible for instances of otitis media.

## INTRODUCTION

The American Academy of Pediatrics stated that in the United States about 5,000,000 cases of acute otitis media (AOM) occur every year (Kaur et al., 2017), most commonly between the ages of 6 and 11 months (Casselbrant & Mandel, 2015). Otitis media arises from an inflammation of mucous membranes in the middle ear space (Ilechukwu et al., 2014). When inflamed, the Eustachian tube (which connects the middle ear space to the throat) ceases to regulate pressure in and drain fluid from the middle ear space. When the Eustachian tube fails to function properly, middle ear pressure drops below ambient levels as oxygen is absorbed, which can result in increased secretion of fluid into that space. This negative pressure results in pain and discomfort for the individual. The Eustachian tube also allows a conduit through which bacteria from the upper respiratory tract enter the middle ear space and colonize. Children who experience AOM within the first 12 months of life have a 70% chance of experiencing a subsequent AOM infection (Kaur et al., 2017). With such high prevalence and recurrence rates, effective treatment and precautionary measures are warranted to improve the otologic health of these patients.

Current treatment options for otitis media include watchful waiting, antibiotic use and surgery. During a watchful waiting period, the patient is observed without any form of applied management, in the hopes that the infection will resolve on its own with time (Casselbrant & Mandel, 2015). Antibiotics are routinely prescribed to treat AOM; however, given the high recurrence of infection, there is some concern that the bacteria will quickly develop antibiotic resistance. Indeed, recent studies have reported that resistance to clinically relevant antibiotics is high (>50%) in clinical AOM isolates of *Streptococcus pneumoniae*, *Moraxella catarrhalis*

  

and *Haemophilus influenzae* (e.g. Jacobs et al., 1998; Sillanpaa et al., 2016) and is continuing to rise. Lastly, surgical procedures to treat OM include a simple myringotomy (puncturing the eardrum) or a myringotomy followed by the insertion of a pressure-equalizing tympanostomy tube. Myringotomy can help relieve the pressure and pain associated with AOM, and tympanostomy tube placement is implemented when there are frequent episodes of AOM to further avoid repeated use of oral antibiotics (Casselbrant & Mandel, 2015). However, both are surgical interventions that require children to undergo general anaesthesia.

High-energy visible (HEV) light (between 380 and 450 nm wavelengths) has been shown to kill a wide array of bacterial pathogens, including one species commonly present in episodes of otitis media, *S. pneumoniae* (Amin et al., 2016; Barneck et al., 2016; Wong et al., 2017). HEV has been long used in dentistry for resin curing (Yoshino & Yoshida, 2018), and more recently has been suggested as a diagnostic tool for oral cancers (Tatehara & Satomura, 2020) and as an anti-biofilm antibacterial agent (Zhang et al., 2020). It is also frequently used in dermatology as an antimicrobial agent, a treatment for chronic skin conditions and wound healing, and for diagnostic purposes (Amin et al., 2016; Ansaripour et al., 2017; Dai & Hamblin, 2017; Falcone et al., 2018; Maytin et al., 2018). The mechanism underlying this inhibition may be through the excitation of surface porphyrins leading to the production of reactive oxygen species in the cytoplasm of the cells, which can damage many macromolecules in the cell (Wang et al., 2017). Previous studies have analysed the effect of HEV light on other Gram-positive bacteria, such as *Staphylococcus aureus*, and many species of Gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii* (e.g. Barneck et al., 2016). In almost all cases, there were significant reductions in bacterial survival following dose-dependent exposure to HEV light. However, these studies were limited by the number of isolates tested, the type of clinical isolates used or the physiological relevance of the conditions used in the studies. So far, there has been no systematic study performed on multiple isolates of common otitis media pathogens, including *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*. We hypothesized that HEV light exposure would be effective against these pathogens since *M. catarrhalis* and *H. influenzae* are both *Gammaproteobacteria* and are thus relatively closely related to Gram-negative bacteria such as *E. coli* and *P. aeruginosa* for which HEV light was particularly effective at reducing bacterial survival. Additionally, although *S. pneumoniae* has only been tested in a limited fashion, a single isolate from sputum sample was previously shown to lose viability after HEV light treatment (Barneck et al., 2016). Thus, we suspected that otitis media clinical isolates of *S. pneumoniae* would be susceptible to HEV light as well.

## MATERIALS AND METHODS

### Chamber design and light source set-up

The test fixture design included three isolated sample wells and was designed in SolidWorks (v.2019; Dassault Systèmes and exported as a stereolithography file. This file was imported into Cura (v.4.4.1; Ultimaker), which is an open-source 3D printer slicing application that was used to program the print parameters (layer height = 0.2 mm, wall thickness = 2 mm, infill density = 90%, and print speed = 60 mm $s^{-1}$). A *g-code* file required to 3D print the part was created in Cura, saved to a USB drive, and uploaded to a fused deposition modelling 3D printer (Ender 3; Shenzhen Creality 3D Technology Co., Ltd.) using Natural Clear (no colour additive) poly-lactic acid (PLA) filament (3D Solutech, 1.75 mm, Lot No. 1906010). PLA was chosen due to its reported short-term biocompatibility (Athanasiou et al., 1996). Due to observed degradation from autoclaving for sterilization, these test fixtures were single use after initial sterilization. The final print parameters were used to ensure the sample medium would not leak from the chamber wells, which was accomplished by building up multiple individual layers of the filament. The properties of the tympanic membrane (TM) are variable (e.g. thickness; Van der Jeught et al., 2013), but some work suggests that the TM offers little reflectance of light at the target frequencies (Doladov et al., 2001), so a surrogate TM was not involved in this stage of testing.

### Light source

The intensities were measured using a G&R Labs Model 325 UV light meter with a 420-nm probe that has sufficient bandwidth for measuring the 405 nm LEDs. Spectral content (emitted wavelength) of the light sources was not expected to vary from manufacturer specifications. The measurements were made in a 37°C chamber, and the temperature rise from light exposure was measured.

### Bacteria isolates

Clinical isolates were collected with permission from a local hospital and typed in the clinical microbiology lab using mass spectrometry. Bacteria were collected from a variety of locations in the body, and the conditions of their collection and growth are specified in Table 1. All

**TABLE 1** Clinical isolates tested for each of the three bacteria. Isolate origins, preferred growth medium and growth conditions are listed

| Isolate ID | Bacteria species | Isolate | Growth media | Growth conditions | Isolate origin |
|---|---|---|---|---|---|
| 19-016 | *Streptococcus pneumoniae* | 1 | THB | 5% $CO_2$, not shaken | Ear isolate (OM) |
| 19-030 | *S. pneumoniae* | 3 | THB | 5% $CO_2$, not shaken | Blood isolate |
| 19-051 | *S. pneumoniae* | 2 | THB | 5% $CO_2$, not shaken | Unknown |
| 19-018 | *Moraxella catarrhalis* | 1 | TSB/BHI | Shaken | Ear isolate (OM) |
| 19-024 | *M. catarrhalis* | 2 | TSB/BHI | Shaken | Sputum |
| 19-027 | *M. catarrhalis* | 3 | TSB/BHI | Shaken | Sputum |
| 19-033 | *Haemophilus influenzae* | 2 | sBHI | Shaken | Ear isolate (OM) |
| 19-045 | *H. influenzae* | 3 | sBHI | Shaken | Ear isolate (OM) |
| 19-050 | *H. influenzae* | 1 | sBHI | Shaken | Unknown |

*Note:* At least one isolate from each species was obtained from an acute case of otitis media (OM).

Abbreviations: BHI, brain–heart infusion broth; sBHI, supplemented brain–heart infusion broth; THB, Todd–Hewitt broth; TSB, tryptic soy broth.

bacteriological experiments were done in accordance with guidelines set forth by the University of Louisville Institutional Biosafety Committee (Protocol: 15–090).

## Bacterial growth analyses and maintenance

Each of the clinical isolates of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* were grown in triplicate for 24 h. For hourly intervals, samples were analysed by optical density at 600 nm ($O.D._{600}$). Measurements were taken until they reached an $O.D._{600}$ between 0.8 and 1.2, which indicated mid-log phase, and viable cell count analyses were performed. After this, bacterial strains were routinely grown in their respective mediums and incubated at 37°C incubator with shaking (for *M. catarrhalis* and *H. influenzae*) or in 5% $CO_2$ with no shaking (for *S. pneumoniae*).

## Survival assays

Cultures were grown overnight in their respective media (see Table 2), diluted (1:10 to 1:50, depending on overnight growth) and grown to mid-log phase. One ml of culture was centrifuged at 21,000× g at room temperature for 1 min. The supernatant was discarded, and the bacterial pellet was resuspended in 1 ml of sterile 1×phosphate-buffered solution (PBS). 500 µl of this solution was deposited into each of two sterile wells on the 3D printed chambers (described above). The chambers were placed in a 37°C incubator room and one of the two samples was exposed to the HEV light source while the other was not. The chambers ±light rigs were placed under opaque containers to limit light exposure of objects outside the container, as well as to limit any broad-spectrum light exposure from the incubation room.

**TABLE 2** Light intensity measures (in mW $cm^{-2}$) for five different liquid media (PBS, phosphate-buffered saline; TH, Todd–Hewitt broth; TSB, tryptic soy broth and sBHI, supplemented brain–heart infusion broth) at different depths of measurement

| | Liquid medium | | | | |
|---|---|---|---|---|---|
| Depth (mm) | $H_2O$ | PBS | TH | TSB | sBHI |
| 0 | 24.0 | 23.5 | 23.0 | 24.3 | 24.5 |
| 3 | 24.2 | 25.3 | 20.9 | 14.4 | 12.1 |
| 6 | 25.3 | 27.9 | 17.2 | 13.2 | 5.4 |
| 9 | 27.6 | 28.7 | 14.1 | 8.2 | 2.8 |
| 12 | 27.8 | 28.3 | 11.1 | 7.9 | 1.3 |
| 15 | 27.5 | 28.0 | 9.2 | 5.5 | 0.7 |

Bacteria were exposed to the HEV light at two different intensities (10, 20 mW $cm^{-2}$), and samples were measured from the wells at different durations of exposure (0–4 h) for a repeated measures experimental design. At each time point (0, 1, 2, 3 and 4 h), 20 µl of the solution was withdrawn from each well (test and control), serially diluted (up to a maximum of a $1:10^7$ dilution) and plated onto chocolate agar plates to ascertain bacterial concentrations.

## Statistical analysis

Biological replicates were analysed using linear mixed-effects models via the *lme4* (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017) packages in *R* (version 3.5.1; R Development Core Team, 2018). We aimed to collect 540 measures (3 replicates × 3 isolates × 3 species × 2 light

intensities × 2 conditions × 5 time points = 540 total). Some isolates (19-033, 19-045, 19-050, 19-051) from two species (*H. influenzae*, *S. pneumoniae*) were tested twice due to piloting that occurred using final test conditions, and we decided to include data from both attempts in the analysis (adding 120 measurements to the analyses). Due to experimenter error, isolate 19-027 (*M. catarrhalis*) was not tested in the 20 mW $cm^{-2}$ condition, and data from the final time point were not measured for all *H. influenzae* trials in the 20 mW $cm^{-2}$ conditions. Matched data points (trial and control) were excluded if the control well demonstrated a 3-log (99.9%) or greater reduction over time ($n = 52$), suggesting some alternative mechanism may have resulted in the death of the control and test cells. In addition, there were 10 data points excluded due to having a zero-point value at the initial measurement (time 0). As the metric for analysis was reliant on a comparison to the initial time point measure, subsequent time points were discarded. After these additions and exclusions, the total number of observations included in the statistical model was 550 data points, grouped by nine clinical isolates (540 anticipated + 120 repeated from pilot data − 48 from experimenter error − 62 from exclusion criteria = 550 trials).

The raw data showed logarithmic reductions in growth, which made them unsuitable for linear regression analysis. Furthermore, despite our best attempts to control the quantity of bacteria in a solution at the start of the experiment (by growing each colony to a similar $O.D._{600}$ reading), variations in the amount of the bacteria present at the start of each trial were present in the data. We used two transformations of the data in an attempt to account for these limitations that would affect the statistical comparison. The final dependent variable was calculated by (1) scaling the data relative to the bacterial growth at time point zero (0) to account for differences in the initial growth of each sample and species and (2) performing a $\log_{10}$ transformation of the scaled data. These adjustments yielded a modified survival fraction (MSF) that represented the log reduction of the bacteria relative to the growth of the bacteria at the initial time point, which was used as the dependent variable in the model. Negative MSF values (MSF−) can be easily converted into the percentage of bacteria that were killed in response to the treatment using Equation (1). Positive MSF values (MSF+) can be converted into the percentage of bacteria growth using Equation (2).

$$\text{Percent bacteria reduction} = (1 - 10^{\text{MSF}-}) * 100, \quad (1)$$

$$\text{Percent bacteria growth} = 10^{\text{MSF}+} * 100. \quad (2)$$

Visual inspection of the residuals in the model (using the *hnp* package in *R*; Moral et al., 2017) confirmed assumptions were met after these transformations. To aid interpretation, it is worth clarifying that positive MSF values indicate growth from the baseline, whereas negative values indicate a decline from the baseline. In cases where there was no growth observed at all after treatment ($n = 60$), we imputed the value of a 7-log reduction (−7 MSF) for the statistical analyses, which represented the limit of detection in these experiments and allowed the log transformation to be completed on non-zero, finite values.

Time (0, 1, 2, 3 and 4 h), light intensity (10 and 20 mW $cm^{-2}$) and condition (test and control) were included as fixed effects in the model, with each specific isolate included as a random intercept term. The test well position (left, middle, right; see Figure 1) was included as a fixed factor in a preliminary model but did not significantly contribute ($p = .35$) and was, therefore, excluded. Main effects were assessed using the *anova* function in *lmerTest*, which implements the Satterthwaite approximation for degrees of freedom to calculate *p*-values (Satterthwaite, 1941). In contrast to using likelihood ratio tests (another popular method for assessing significance of variables in linear mixed-effects models), this approach has been shown to yield more conservative estimates that are less prone to Type I error (Luke, 2017).

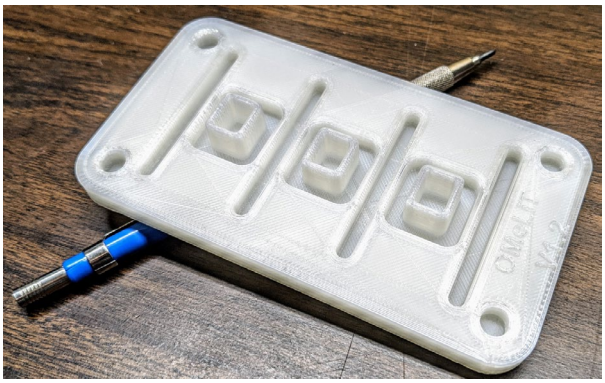

**FIGURE 1** 3D printed polylactic acid test fixture with three light-isolated wells for performing triplicate experiments

## RESULTS

### Chamber design

A physical model that was moderately representative of the middle ear space was first constructed to simulate the primary area of the middle ear space (visible through the TM) using a box model of the middle ear that was approximately 8 mm wide × 10 mm high and 5 mm deep. To account for additional fluid-filled space, and to address concerns of the light penetrating to the depth of the

middle ear space, the model was approximately 8 mm deep, allowing for an approximate volume of 0.65 mL (Figure 1). A disposable test fixture was constructed based on these specifications to provide consistent exposure to the test light so that experimental process variations were not introduced and would not become a factor in outcomes. Light-emitting diodes (LEDs) were chosen as our light source, with LED intensity primarily as a function of forward current. A constant intensity was obtained by driving each LED independently with an adjustable constant current source. The current source was fabricated on a custom printed circuit board that includes the LEDs on the bottom side (Figure 2). The board was placed on standoffs that placed the LEDs at a fixed height and position above the sample wells. Light blocking panels extended downward to block light from adjacent LEDs.

## Testing the light rig

The desired intensity values used in the present study were based on previous work using light to treat bacteria (Barneck et al., 2016) that showed effective dosing at 10 mW $cm^{-2}$ on a surface plating of the bacteria. Given that we were attempting to treat a volume of inoculated medium rather than a thin surface layer, we tested at 10 and 20 mW $cm^{-2}$. The light devices were calibrated such that the target intensity was achieved at the bottom of the well. Actual measured intensities varied from 8.2 to 10 mW $cm^{-2}$ for the 10 mW $cm^{-2}$ condition and from 16 to 20 mW $cm^{-2}$ for our 20 mW $cm^{-2}$ condition. The distance from the eardrum to the medial wall of the middle ear space is approximately 5 mm. Table 2 shows the intensity of the HEV light source (measured in mW $cm^{-2}$) at different depths of different media relevant to this experiment (water, various growth media and PBS). The growth media absorbed much of the light as the depth increased, which varied by medium, and both water and PBS had an amplification effect on the light with increasing depth. Based on these results, we determined that PBS would be the most suitable medium in which to perform these tests, which also provided a normalized medium in which to test the light conditions, given that each bacteria grew best in different growth media.

All analyses were performed in an incubator room at 37°C to simulate body temperature. Thus, we measured the increase in heat at the bottom of the test wells due to the use of the light source at the different intensities in an incubation chamber for the time duration of the experiment. The results from those tests indicated that a typical increase in temperature associated with the light treatment was approximately 3.3 and 4.5°C for the 10 and 20 mW $cm^{-2}$ test conditions, respectively. Although the LED light sources undoubtedly contributed to this increase in temperature, heat generated by the circuit board itself (powering three LEDs per test tray) may have also been a contributing factor. Temperatures were held below levels reported to cause damage (Stoll, 1977).

**FIGURE 2** LED source over 3D printed (PLA) test fixture with three isolated sample wells

## Testing the overall effect of HEV on bacterial pathogens

We first assessed whether HEV affected survival of species over time. Data for the three isolates of each species were collected and analysed in aggregate. Descriptive information on the starting concentrations for each species is provided in Table 3. Data averaged for each species and test condition are presented in Figure 3. The initial statistical model allowed us to check for a dosage effect (time and intensity) irrespective of bacterial species, thus pooling the data across all three bacteria to increase the statistical power of the model. In agreement with our statistical hypotheses, we found a significant effect of condition ($F_{(1,538)} = 146.5$, $p < .001$), intensity ($F_{(1,546)} = 14.5$, $p < .001$) and time ($F_{(1,538)} = 105.1$, $p < .001$).

The overall effect of treatment (when accounting for exposure duration and light intensity) was a −1.83 survival fraction, which is a 99% reduction (see Equation 1) for treated wells compared with control wells. We further hypothesized that the higher light intensity would yield

**TABLE 3** Initial bacteria concentrations at time point 0 averaged across isolate and replicate for each species. Values are $\log_{10}$ transformed for ease of comparison

| Bacteria species | $\log_{10}$ (mean) | $\log_{10}$ (min) | $\log_{10}$ (max) |
|---|---|---|---|
| *Streptococcus pneumoniae* | 8.4637 | 6.5051 | 8.9542 |
| *Moraxella catarrhalis* | 8.3917 | 7.2553 | 9.3820 |
| *Haemophilus influenzae* | 8.5483 | 7.9031 | 9.0792 |

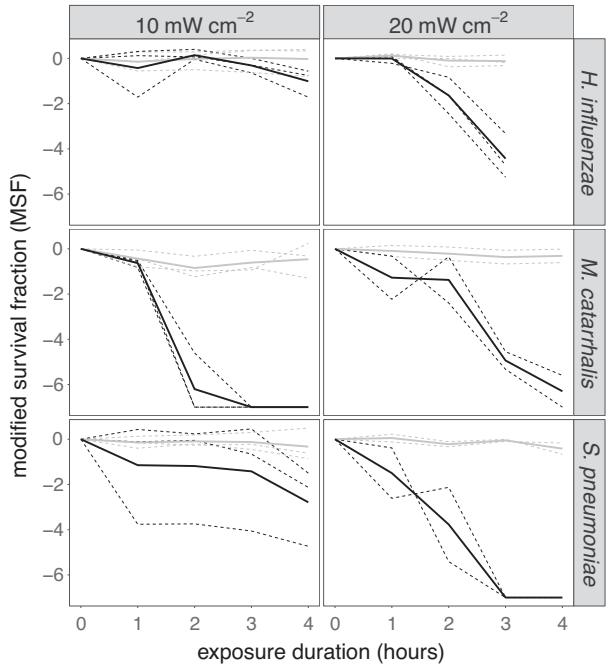


**FIGURE 3** Average and individual test and control bacterial survival rates for each isolate (averaged across replicates) of the three bacterial species tested at two intensities for up to 4 h of duration. Note that the 4-h time point was not measured for *Haemophilus influenzae* for the 20 mW $cm^{-2}$ condition due to a premature termination of the experiment. Gray lines: Control ($n = 275$). Black lines: Test ($n = 275$). Solid lines: Bacteria averaged across isolates. Dashed lines: Isolate averaged across replicates

lower survival rates than the lower light intensity. The main effect of intensity was less pronounced than it was for the treatment wells alone, with a mean difference of MSF of −0.47 (−0.916 MSF for 10 mW $cm^{-2}$; −1.39 MSF for 20 mW $cm^{-2}$). This significant effect was driven by the treatment wells and tempered by the lack of change in the control wells over time.

Next, we hypothesized that longer exposure durations would yield lower survival rates than shorter durations. Control wells did not show significant reductions in bacterial concentration over time, whereas treatment wells demonstrated a significant reduction in bacterial concentrations over time. Table 4 provides the MSF value means for control and test conditions across the five time points tested, along with a converted percentage reduction value (see Equation 1). The relative decrease of bacteria over the different time points was 0.00, −0.50, −1.13, −1.81 and −2.11 MSF at timepoints 0, 1, 2, 3 and 4 h, respectively. In other words, approximately every two hours, the number of viable bacterial colonies decreased 10-fold. This effect was also driven by the reduction of bacteria in the treatment condition compared to the control condition. The interaction effect, which would illustrate the actual treatment effect across time for different doses of HEV light, was statistically significant ($F_{(1,537)} = 98.2, p < .001$), which supported our dose-dependent treatment hypothesis.

## Species-dependence of HEV efficacy

Although the previous analysis examined the data aggregated across species, we further analysed the effect of light treatment on each individual species. A linear mixed-effects model was used for each species to test the main effects of treatment condition, light intensity and time, with individual isolates included in the model as random intercept terms. A summary of these models can be found in Table 5. Each species demonstrated a significant main effect of treatment, time and light intensity, as well as the anticipated time x treatment condition interaction observed in the aggregate data (all $p < .01$). These findings suggest that each species was separately influenced by the light treatment, and that the general effects of these independent variables were not influenced by a single bacterial species. Although we lacked the statistical power to examine effects at the level of each clinical isolate, visual inspection of the bacterial reduction for each isolate (see Figure 3) indicates similar susceptibility to the light by all clinical isolates from each of the species examined in this study.

# DISCUSSION

## Main effects of condition, intensity and duration of exposure

The primary goal of treating the infected middle ear space in otitis media is reducing the bacterial burden on the body as it heals. Thus, the aim when using antibiotics or another alternative treatment strategy should be to balance the amount of resources the body uses in fighting the infection of bacteria versus the source of inflammation of the Eustachian tube. In this study, HEV light was able to effectively reduce the number of living of bacteria present for three of the pathogens most commonly associated with otitis media. All treatment wells showed significant reductions in the number of colony forming units after exposure to the light, compared with control wells. This effect was dose dependent; in general, the intensity of the light and duration of the exposure significantly affected the magnitude of the reduction. As the light irradiance (or intensity) and duration of the exposure increased, number of surviving colonies decreased. At the higher intensity and with longer durations, we were able to achieve a reduction of bacteria that was greater than 99.99% for all bacteria after 3 h of treatment, with a reduction greater

**TABLE 4** Average modified survival fractions (MSFs) and percent reductions for control and test conditions across all time points, averaged across all bacteria species and isolates

| | | Time (h) | | | | |
|---|---|---|---|---|---|---|
| **Condition** | **Measure** | **0** | **1** | **2** | **3** | **4** |
| Control | MSF | 0 | −0.133 | −0.215 | −0.184 | −0.284 |
| | percent reduction | 0% | 26.38% | 39.05% | 34.54% | 48.00% |
| Test | MSF | 0 | −0.866 | −2.04 | −3.44 | −3.93 |
| | percent reduction | 0% | 86.39% | 99.09% | 99.96% | 99.99% |

**TABLE 5** Summary of model statistics used to assess the main effects and interactions for each bacterial species

| **Species** | **Effect** | ***F*** | ***df*** | ***p*** |
|---|---|---|---|---|
| *Haemophilus influenzae* | Condition | 15.6 | (1, 219.88) | <.001 |
| | Light intensity | 16.2 | (1, 221.92) | <.001 |
| | Time | 17.1 | (1, 220.09) | <.001 |
| | Time × condition | 12.5 | (1, 218.89) | <.001 |
| *Moraxella catarrhalis* | Condition | 111.4 | (1, 140) | <.001 |
| | Light intensity | 7.9 | (1, 140) | <.01 |
| | Time | 91.2 | (1, 140) | <.001 |
| | Time × condition | 139.6 | (1, 139) | <.001 |
| *Streptococcus pneumoniae* | Condition | 68.3 | (1, 173.96) | <.001 |
| | Light intensity | 26.7 | (1, 174.29) | <.001 |
| | Time | 36.8 | (1, 173.96) | <.001 |
| | Time × condition | 30.9 | (1, 172.96) | <.001 |

than 99.9999% for *S. pneumoniae* and *M. catarrhalis* after 4 h of treatment (*H. influenzae* was not measured at 4 h of treatment, further discussed below).

Figure 3 illustrates a survival reduction for each bacterial species and each isolate within each species, which supports the viability of the HEV light as a potential therapeutic agent that would be locally effective against multiple harmful pathogens present in otitis media. These results add to previous research in *S. pneumoniae* in which a 120 min exposure to HEV at ~5 to ~10 mW/cm$^{-2}$ was found to be effective at reducing a single strain of *S. pneumoniae* by ~85% (Barneck et al., 2016). Interestingly, *M. catarrhalis* appeared to be equally, if not more, susceptible to the light at 10 mW cm$^{-2}$ than at the 20 mW cm$^{-2}$ condition. This slightly contrasts with a previous study on *M. catarrhalis* which found HEV to effectively kill though only a single, much higher intensity (60 mW cm$^{-2}$) was used (Liu et al., 2020). Perhaps this was due to the different growth media used to grow the bacteria at the two intensities, or due to unidentified sources of error. In addition, of note is that no data were collected at the fourth hour of analysis using a 20 mW cm$^{-2}$ intensity for *H. influenzae* due to experimenter error; however, the general trends remain consistent with the other two bacteria types. We note that we were unable to find any previously published studies using HEV on *H. influenzae*, suggesting this is a unique aspect to this study. Future research is needed to determine the relative effectiveness of this approach compared with or in tandem with antibiotics.

Our measurement of the reduction of bacteria with treatment was limited by our imputed values for conditions that showed no bacterial growth after treatment (e.g. 20 mW cm$^{-2}$ intensity, treatment condition, 4 h of exposure). It is possible that true reduction values were 100% in these conditions or an MSF value that approaches negative infinity. Since including negative infinity for some values of a dependent variable prohibited statistical analysis, we imputed values when a 100% reduction was observed. The maximum reduction possible based on our test methods (if not 100%) was an MSF of −7 due to our methods of serial dilution and drip plating; and conditions measured at −7 were modified from a true value of negative infinity. Thus, the reported bacteria reduction means may be conservative estimates of the true reductions observed. We also acknowledge some instances of a reduction of the bacteria survival in the control condition. Recall that once grown to a mid-log phase, bacteria were removed from their growth media and suspended in PBS. We believe this placement in a less supportive environment (e.g. no shaking, nutrients or $CO_2$ supplement) may have resulted in

the observed modest reductions in bacteria survival in the control condition.

This work was intended to be a preliminary investigation into the use of HEV light as a potential therapeutic treatment option for otitis media. There are many limitations and factors yet to be addressed by future inquiry. For example, the mechanisms of cell death are still unclear. Some work suggests that the primary mechanism of cell death is the overproduction of reactive oxygen species by photosensitive chromophores, which disrupts the cellular processes and results in apoptosis (Wang et al., 2017). Therein lies a primary concern for using this type of therapy as a treatment alternative for otitis media. Photosensitizing porphyrins are not exclusive to bacteria cells, as they are also present in healthy tissue cells. It is possible that an exposure to the treatment light at similar intensities would have a negative effect on healthy tissue cells (ear canal, TM, ossicles, etc.). The method of presenting the light to the infected ear space would be by placing the light source into the ear canal (in a device similar to a modern hearing aid) and shining the light to the middle ear space through the TM. Thus, damage or injury to the TM and other healthy tissues must be minimized for this approach to be viable.

Another concern is the increased temperature caused by the light exposure. Raising the temperature of one ear independently of the other results in a vestibular system response that induces vertigo in the patient (Coats et al., 1976). The temperature differences observed in this study may result in such an effect, although no studies have measured the effects of vertigo using light as a stimulator, nor at temperature differences so near body temperature (within 1–4°C). Additional research would be required to quantify the vestibular side effects of light treatment. If significant effects are observed at treatment doses, temperature effects could be easily mitigated by using a pulsed light source or with sensor technology that shuts off the light if the measured heat exceeds some problematic level.

Yet another concern is whether HEV light can penetrate through biofilms. There is some evidence that bacterial pathogens form biofilms in the middle ear during AOM in animal models and in humans (Ehrlich et al., 2002; Hall-Stoodley et al., 2006). Previously published literature in the microbiology field has begun to explore the survival rates of bacterial and fungi in biofilms exposed to HEV and found it to be mostly effective (Bumah et al., 2020; Ferrer-Espada et al., 2019; Ferrer-Espada et al., 2020; Halstead et al., 2016; Tsutsumi-Arai et al., 2019; Wang et al., 2016). However, most of these studies explored these effects on in vitro biofilms, used different wavelengths, intensities, and duration of treatments.

As demonstrated by measuring the light in different growth media, the type of fluid the bacteria are in can dramatically influence the effective intensity of the light, as compounds in the medium may also absorb the light source energy. Fluid present in otitis media ranges from a clear runny liquid, to an opaque dense mucoid (Paparella et al., 1970). The HEV light should be tested on different viscosities of fluid (or on a variety of samples collected from patients via myringotomy), to determine penetration depth in different fluid types. Commonly, negative middle ear pressure is a precursor to otitis media as the Eustachian tube closes off, which results in fluid secretions in the middle ear that are not yet infected (i.e. serous otitis media; Buckingham & Ferrer, 1973). The fluid in this state is generally clear and less viscous, and so HEV light may be utilized in a preventative capacity when an uninfected middle ear with effusion is identified prior to acute infection. Lastly, in vitro studies are not always generalizable to in vivo applications, and this work will ultimately need to be extended to animal models and human trials once risk to healthy tissue is assessed in greater detail.

In conclusion, although potential barriers exist to the translation of this research to clinical populations with otitis media, this study shows promise that common pathogens are susceptible to HEV light at manageable exposure dosages. Future work could expand upon this foundation to explore alternative uses for the light, such as treating otitis externa (ear canal infection), bacterial biofilms that form on pressure-equalizing tubes and other applications where the infection is more accessible to the light source. We conclude that the three primary bacteria observed in cases of otitis media demonstrated significant susceptibility to the HEV light source for in vitro cell cultures which may be a viable therapeutic treatment or prevention option for individuals with acute or non-acute (serous) otitis media.

## ACKNOWLEDGEMENTS

This work was supported in part by a grant from the University of Louisville School of Medicine awarded to the first author. The authors would like to thank Scott Capece, Stacy Garrard, Meredith Del Castillo, Clarissa Story, Rhiannon Cecil and TJ Brandt for their assistance in training and preparing analyses and the many other tasks that make work in this area possible. We would also like to thank Dr. Alan Junkins at Norton Hospital for providing the clinical isolates used in this study.

## CONFLICT OF INTEREST

No conflict of interest has been declared.

## ORCID

*Shae D. Morgan* https://orcid.org/0000-0002-3263-7984

## REFERENCES

Amin, R.M., Bhayana, B., Hamblin, M.R. & Dai, T. (2016) Antimicrobial blue light inactivation of *Pseudomonas*

*aeruginosa* by photo-excitation of endogenous porphyrins: in vitro and in vivo studies. *Lasers in Surgery and Medicine*, 48, 562–568.

Ansaripour, A., Thio, H.B., Maessen, R. & Redekop, W.K. (2017) The cost–effectiveness of blue-light therapy in the treatment of mild-to-moderate psoriasis. *Journal of Comparative Effectiveness Research*, 6, 325–335.

Athanasiou, K.A., Niederauer, G.G. & Agrawal, C.M. (1996) Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. *Biomaterials*, 17, 93–102.

Barneck, M.D., Rhodes, N.L., De La Presa, M., Allen, J.P., Poursaid, A.E., Nourian, M.M. et al. (2016) Violet 405-nm light: a novel therapeutic agent against common pathogenic bacteria. *Journal of Surgical Research*, 206, 316–324.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67, 1–48. https://doi.org/10.18637/jss.v067.i01

Buckingham, R.A. & Ferrer, J.L. (1973) Middle ear pressures in eustachian tube malfunction: manometric studies. *The Laryngoscope*, 83, 1585–1593.

Bumah, V.V., Masson-Meyers, D.S. & Enwemeka, C.S. (2020) Pulsed 450 nm blue light suppresses MRSA and propionibacterium acnes in planktonic cultures and bacterial biofilms. *Journal of Photochemistry and Photobiology B: Biology*, 202, 111702.

Casselbrant, M.L. & Mandel, E.M. (2015) Acute otitis media and otitis media with effusion. In: Lesperance, M.M. & Flint, P.W. (Eds.) *Cummings pediatric otolaryngology*. , Philadelphia, PA: Elsevier Saunders, pp. 210–227.

Coats, A.C., Hebert, F. & Atwood, G.R. (1976) The air caloric test. A parametric study. *Archives of Otolaryngology*, 102, 343–354.

Dai, T. & Hamblin, M.R. (2017) *Visible blue light is capable of inactivating* Candida albicans *and other fungal species*. Mary Ann Liebert, Inc.

Doladov, M.Y., Kazakevich, V., Kalashnikov, I., Kupaeva, V. & Shcherbakov, V. (2001) Optical reflection spectrum of the human tympanic membrane. *Journal of Applied Spectroscopy*, 68, 708–710.

Ehrlich, G.D., Veeh, R., Wang, X., Costerton, J.W., Hayes, J.D., Hu, F.Z. et al. (2002) Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *The Journal of the American Medical Association*, 287, 1710–1715.

Falcone, D., Uzunbajakava, N.E., Van Abeelen, F., Oversluizen, G., Peppelman, M., Van Erp, P.E.J. et al. (2018) Effects of blue light on inflammation and skin barrier recovery following acute perturbation. Pilot study results in healthy human subjects. *Photodermatology, Photoimmunology and Photomedicine*, 34, 184–193.

Ferrer-Espada, R., Liu, X., Goh, X.S. & Dai, T. (2019) Antimicrobial blue light inactivation of polymicrobial biofilms. *Frontiers in Microbiology*, 10, 721.

Ferrer-Espada, R., Wang, Y., Goh, X.S. & Dai, T. (2020) Antimicrobial blue light inactivation of microbial isolates in biofilms. *Lasers in Surgery and Medicine*, 52, 472–478.

Hall-Stoodley, L., Hu, F.Z., Gieseke, A., Nistico, L., Nguyen, D., Hayes, J. et al. (2006) Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *The Journal of the American Medical Association*, 296, 202–211.

Halstead, F.D., Thwaite, J.E., Burt, R., Laws, T.R., Raguse, M., Moeller, R. et al. (2016) Antibacterial activity of blue light against nosocomial wound pathogens growing planktonically and as mature biofilms. *Applied and Environmental Microbiology*, 82, 4006–4016.

Ilechukwu, G.C., Ilechukwu, C.G.A., Ubesie, A.C., Ojinnaka, C.N., Emechebe, G.O. & Iloh, K.K. (2014) Otitis media in children: review article. *Open Journal of Pediatrics*, 04, 47–53.

Jacobs, M.R., Dagan, R., Appelbaum, P.C. & Burch, D.J. (1998) Prevalence of antimicrobial-resistant pathogens in middle ear fluid: multinational study of 917 children with acute otitis media. *Antimicrobial Agents and Chemotherapy*, 42, 589–595.

Kaur, R., Morris, M. & Pichichero, M.E. (2017) Epidemiology of acute otitis media in the postpneumococcal conjugate vaccine era. *Pediatrics*, 140, 1–11. https://doi.org/10.1542/peds.2017-0181

Kuznetsova, A., Brockhoff, P.B. & Christensen, R.H.B. (2017) LmerTest package: tests in linear mixed effects models. *Journal of Statistical Software*, 82, 1–26.

Liu, X., Chang, Q., Ferrer-Espada, R., Leanse, L.G., Goh, X.S., Wang, X. et al. (2020) Photoinactivation of *Moraxella catarrhalis* using 405-nm blue light: implications for the treatment of otitis media. *Photochemistry and Photobiology*, 96, 611–617.

Luke, S.G. (2017) Evaluating significance in linear mixed-effects models in R. *Behavior Research Methods*, 49, 1494–1502.

Maytin, E.V., Kaw, U., Ilyas, M., Mack, J.A. & Hu, B. (2018) Blue light versus red light for photodynamic therapy of basal cell carcinoma in patients with Gorlin syndrome: a bilaterally controlled comparison study. *Photodiagnosis and Photodynamic Therapy*, 22, 7–13.

Moral, R.A., Hinde, J. & Demétrio, C.G. (2017) Half-normal plots and overdispersed models in R: the hnp package. *Journal of Statistical Software*, 81, 1–23.

Paparella, M.M., Hiraide, F., Juhn, S. & Kaneko, Y. (1970) Cellular events involved in middle ear fluid production. *Annals of Otology, Rhinology & Laryngology*, 79, 766–779.

R Development Core Team. (2018) *R: A language and environment for statistical computing*, 3.5.1 edition. Vienna, Austria: R Foundation for Statistical Computing.

Satterthwaite, F.E. (1941) Synthesis of variance. *Psychometrika*, 6, 309–316.

Sillanpaa, S., Sipila, M., Hyoty, H., Rautiainen, M. & Laranne, J. (2016) Antibiotic resistance in pathogens causing acute otitis media in Finnish children. *International Journal of Pediatric Otorhinolaryngology*, 85, 91–94.

Stoll, A.M. (1977) Thermal properties of human skin related to non-destructive measurement of epidermal thickness. *The Journal of Investigative Dermatology*, 69, 328–332.

Tatehara, S. & Satomura, K. (2020) Non-invasive diagnostic system based on light for detecting early-stage oral cancer and high-risk precancerous lesions—potential for dentistry. *Cancers*, 12, 3185.

Tsutsumi-Arai, C., Arai, Y., Terada-Ito, C., Takebe, Y., Ide, S., Umeki, H. et al. (2019) Effectiveness of 405-nm blue LED light for degradation of Candida biofilms formed on PMMA denture base resin. *Lasers in Medical Science*, 34, 1457–1464.

Van Der Jeught, S., Dirckx, J.J., Aerts, J.R., Bradu, A., Podoleanu, A.G. & Buytaert, J.A. (2013) Full-field thickness distribution of human tympanic membrane obtained with optical coherence tomography. *Journal of the Association for Research in Otolaryngology*, 14, 483–494.

Wang, Y., Wang, Y., Wang, Y., Murray, C.K., Hamblin, M.R., Hooper, D.C. et al. (2017) Antimicrobial blue light inactivation of pathogenic microbes: state of the art. *Drug Resistance Updates*, 33, 1–22.

Wang, Y., Wu, X., Chen, J., Amin, R., Lu, M., Bhayana, B. et al. (2016) Antimicrobial blue light inactivation of gram-negative pathogens in biofilms: in vitro and in vivo studies. *The Journal of Infectious Diseases*, 213, 1380–1387.

Wong, T.-W., Cheng, C.-W., Hsieh, Z.-J. & Liang, J.-Y. (2017) Effects of blue or violet light on the inactivation of *Staphylococcus aureus* by riboflavin-5′-phosphate photolysis. *Journal of Photochemistry and Photobiology B: Biology*, 173, 672–680.

Yoshino, F. & Yoshida, A. (2018) Effects of blue-light irradiation during dental treatment. *Japanese Dental Science Review*, 54, 160–168.

Zhang, L., Li, Y., Zhang, Q., Du, N., Li, X., Zhang, Q. et al. (2020) Antimicrobial activity of an implantable wireless blue light-emitting diode against root canal biofilm in vitro. *Photobiomodulation, Photomedicine, and Laser Surgery*, 38, 694–702.

Ho-w to cite this article: Morgan, S.D., Yoder-Himes, D., Jackson, D., Naber, J., Berry, R., Cash, E., et al. (2022) Bactericidal effects of high-energy visible light on common otitis media pathogens. *Journal of Applied Microbiology*, 132, 1856–1865. https://doi.org/10.1111/jam.15366