**PROPOSED TITLES**

**Fourier-transformed infrared microscopy of blue light/UV irradiated and vancomycin treated methicillin-resistant *Staphylococcus aureus***

**The nature of cell death in blue light/UV and vancomycin treated methicillin- resistant *Staphylococcus aureus* by Fourier-transformed infrared spectroscopy**

**Characterizing treated methicillin-resistant *Staphylococcus aureus* using Infrared spectromicroscopy**

Violet V Bumah1\*, Ebrahim Aboualizadeh2\*, Daniela S Masson-Meyers1, Janis Eells1, Carol J Hirschmugl2ᵠ, Chukuka S Enwemeka3

1Department of Biomedical Sciences, College of Health Sciences, University of Wisconsin-Milwaukee, Milwaukee, United States of America

2Physics Department, University of Wisconsin-Milwaukee, Milwaukee, United States

3San Diego State University, San Diego, California USA

**\*Co-first author (V.V.B and E.A contributed equally to this work)**

**ᵠCorrespondence to:**

Carol J Hirschmugl, PhD

Physics Department

College of Letters and Science

University of Wisconsin-Milwaukee

3135 North Maryland Ave.

Milwaukee, WI 53211, USA

Phone: +1-414-229-5748

Email: [cjhirsch@uwm.edu](mailto:cjhirsch@uwm.edu)

**ABSTRACT**

**Background:** Treatment of antibiotic resistant bacteria is a serious problem worldwide. In the case of methicillin resistant Staphylococcus *aureus* (MRSA), it has been reported that death due to MRSA infection now exceed the death due to HIV/AIDS; a situation that calls for intensive measures to address the epidemic. MRSA is a bacterium that is resistant to many antibiotics and leads to fatal infections. Vancomycin is one of the current effective drugs for MRSA suppression *in vivo* and ultraviolet (UV) light for environmental sterilization. With the ongoing antibiotic resistance, our laboratory and those of others have shown that blue light inactivates MRSA, and other gram positive and gram negative bacteria; but the mechanism of bacterial cell suppression remains unclear. Whereas the mechanisms for vancomycin and UV light inactivation have been elucidated, the mechanism of blue light suppression is not clear, hence the thrust of this study which involves characterizing the nature of cell death using biomarkers of bacterial metabolism. **Objectives:** Fourier transform infrared spectromicroscopy was employed to detect signatures of living and dying cells to investigate the nature of cell inactivation in blue-light/UV irradiated (470 nm and UV) and vancomycin treated MRSA. **Methods:** MRSA was cultured in tryptic soy broth (TSB), spun down at 13000g for 5 minutes and 2x109 CFU/mL suspended in broth. Two µl of this suspension was lightly coated on diamond windows and irradiated with 121 (3x) and 220 J/cm2 (4x) of blue light; or placed under UV lamp for 12 hours; or mixed with a concentration of 2 and 40µg/ml of vancomycin. Control samples were neither irradiated nor vancomycin treated. One set of control sample (C1) was not incubated (left in ambient air), while the other set of controls (C2) and treated MRSA were incubated at 37ºC for 24 hours and FTIR assay performed. Aliquots of TSB and vancomycin were also placed on diamond windows to determine the contribution of these solutions to the FTIR spectrum. Bacteria mounted on diamond optical windows are assessed in transmission mode with a Bruker Vertex 70 IR spectrometer and a Bruker Hyperion 3000 IR Microscope equipped with a thermal blackbody source. The setup is equipped with a 64 x 64 pixel focal plane array (FPA) detector and enables us to generate hyperspectral cube of data. After FTIR, a swap of MRSA on the diamond windows from all groups was obtained and cultured on tryptic soy agar (TSA) at 37ºC for 24 hours. Bacterial growth was then determined. **Results:** blue-light/UV-radiated samples revealed biomakers associated with DNA, nucleic acids and protein while infrared peaks in carbohydrates region including glycogen and cellulose were found in vancomycin-treated MRSA. There was luxuriant MRSA growth observed in the control group that was incubated at 37ºC (C1) followed by that which was placed in ambient temperature (C2), while approximately 150, 3, 0 and 4 CFU’s were observed in 363 J/cm2, 880 J/cm2, UV and vancomycin treated samples respectively. **Conclusion**: These findings provide valuable insights to light therapy community and helps for better understanding of the MRSA signaling pathway toward treatments at cellular level.

Keywords: FTIR, Blue light, UV, vancomycin, PCA analysis, MRSA

**1. Introduction**

Methicillin resistant Staphylococcus aureus (MRSA) is a gram positive bacterium which infects the skin, soft tissues and generally starts as swollen painful red bumps but can quickly turn into deep, painful abscesses that require surgical draining. If infection accesses deep tissue it may cause potential life-threatening infections in bones, joints, surgical wounds, the bloodstream, heart valves and lungs. To combat this infection a myriad of antibiotics have been used. However, their effectiveness against Staphylococci infections has substantially decreased due to the development of resistant strains of bacteria (Howell-Jones et al., 2005; Liu 1999). The search for more efficacious remedies and alternative measures to address the problem has heightened. Therapies under investigation include a myriad of natural and synthetic products such as antibacterial clay (Otto and Haydel, 2013), combined honey and antibiotics (Müller et al., 2013), hyperbaric oxygen (Turhan et al., 2009), photodynamic therapy (Dai et al., 2010), blue light phototherapy (Bumah et al., 2013; Dai et al., 2013; Enwemeka et al., 2008; Enwemeka, 2013; Maclean et al., 2014, Bumah et al., 2015a,b; Masson-Meyers et al., 2015) and combined blue light and hyperbaric oxygen (Bumah et al, 2015c).

Among these therapies the antibiotic, vancomycin, a glycopeptide (glycosylated peptide) obtained from *Streptomyces orientalis* with bactericidal activity against most organisms including MRSA and bacteriostatic effect on enterococci has been proven to be very effective. The mechanism of action is associated to its binding to D-alanyl-D-alanine portion of bacterial cell wall precursors (N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG)), preventing transpeptidase from cross-linking the peptidoglycan layer thereby interfering with cell wall synthesis. The peptidoglycan layer gets less rigid and more permeable, causing cellular contents of the bacteria to leak out, and bacterial autolysins activated to destroy the cell wall by lysis. (Walter et al., 1974; Clinical Pharmacology, Wikipedia). Vancomycin may also alter the permeability of bacterial cytoplasmic membranes and may selectively inhibit RNA synthesis. Even with these mechanisms elucidated, there are several other biomarkers of bacterial cell death that could be investigated to further provide information that could be useful determinants for efficacy and potential development of resistance. FTIR is a technique that provides information on quantitative profile of the overall biochemical composition (DNA, RNA, proteins, membrane and cell wall components) of intact cells, and could be used to investigate changes of these markers in dying cells. Hence, exploring chemical changes using an efficacious antimicrobial such as vancomycin in comparison with other methods, which have not clearly described cell death mechanism is of interest here.

The involvement of one of such technologies, monochromatic radiations as a viable inactivation protocol for MRSA *in vitro*, is gaining interests. Previously, our group proposed a paradigm shift in therapy, after our experiments in which we successfully eradicated two strains of MRSA with 405 nm and 470 nm light in vitro; as opposed to the efforts by others to achieve the same goal with photodynamic therapy (PDT) which is well known for its toxicity to adjoining tissues and cells (Howell-Jones et al., 2005; Malik et al., 1990). Since then, our research team and others have shown that 405 nm, 415 nm and 470 nm blue light inactivates cultures of *Staphylococcus aureus* (both MRSA and methicillin susceptible *Staphylococcus aureus* (MSSA)), *Escherichia coli* and other bacterial pathogens(Enwemeka et al., 2008; Enwemeka et al., 2009; Maclean et al., 2009; Lipovsky et al., 2010; Wasson et el., 2012) however, the mechanisms involved remain unclear.

Monochromatic irradiations involving UV (100 to 400nm) light which is in close proximity to blue light in the electromagnetic spectrum, has also been utilized to sterilize environments that harbor bacteria, including MRSA with the mechanism through which death is brought about well defined. Notably, DNA damage resulting in the formation of a very stable cyclobutane pyrimidine dimer (CPD) and the (6-4) photoproduct (6-4PP) (Moné et al., 2001) whose repair, usually by excising or removing the two bases and filling in new nucleotides is fairly efficient. These photoproducts are potent inhibitors of transcription by RNA polymerase II (RNA pol II) (Moné et al., 2001). The longer the exposure to UV light, the more thymine dimers are formed in the DNA and the greater the risk of an incorrect repair or a "missed" dimer (Rammelsberg, 1998). If cellular processes are disrupted because of an incorrect repair or remaining damage, the cell cannot carry out its normal functions. If the damage is not too extensive, cancerous or precancerous cells are created from healthy cells, but if it is widespread, the cell is directed to apoptosis, or programmed cell death (Baxter, 1998). Since UV causes damage to biological material through DNA damage, it will be interesting to see if blue light exhibits similar changes/trends in biomarkers investigated through FTIR.

Studies shedding light on the antimicrobial mechanisms of visible light in general, have implicated the production of reactive oxygen species (ROS) in living cells following absorption by photoacceptor molecules including cytochromes (cytochrome c oxidase), porphyrins, flavins and NADH(Lipovsky et al., 2009; Lipovsky et al., 2010). These endogenous photosensitizers exhibit absorption spectra with peaks in the blue region of the spectrum. Some bacteria also possess the same endogenous photosensitizing molecules(Lipovsky et al., 2009; Lipovsky et al., 2010). and many studies have shown that low fluence irradiation at wavelengths between 450-550 nm exerts phototoxic actions on gram negative and gram positive bacteria(Wilson and Yianni, 1995; Maclean et al., 2009; Wasson et al., 2012). These reports suggest that ROS production is a viable mechanism of phototoxicity.

To gain insight into the potential mechanisms involved in blue light therapy, our research have examined blue light irradiated MRSA colonies at the molecular level by observing the effect of blue light on femA and mecA genes implicated in antibiotic resistance; analyzing the protein profile of whole cell lysates; and conducted genomic fingerprinting. The results in these studies indicated that no observable differences in genomic fingerprinting were seen using pulsed field gel electrophoresis (Sma I digestion) and DiversiLab repetitive PCR. Studies by our lab have revealed that blue light disrupts the transmembrane potential of MRSA which is one of the potential leads under investigation at the moment. Also ongoing studies exploring the use of rapid high-throughput whole genome sequencing technology to deduce any potential differences in MRSA isolates are being conducted. Some preliminary data on irradiated MRSA sequences has been obtained by our team (Daum et al., 2015) and the implications of these findings are under investigation. Nevertheless, investigating other biomarkers of cell death is important to provide some insight into mechanisms involved in blue light inactivation, hence the use of FTIR in this study.

Infrared microspectroscopy has been proven to identify microbial species and subspecies(Helm et al., 1991; Mariey et al., 2001; Amiali et al., 2011). This nondestructive method provides a quantitative profile of the overall biochemical composition (DNA, RNA, proteins, membrane and cell wall components) of intact cells and therefore major organisms can be identified by an infrared absorption spectrum. Closely related strains can be differentiated by analyzing spectra using a variety of chemometric techniques(Mariey et al., 2001; Lamprell et al., 2006). Cell death leads to chemical modifications in infrared absorption spectrum due to the changes in biochemical composition during apoptosis. Thus, with the ability to detect the infrared features, chemical alterations can be monitored on a cell-by-cell basis. The measurement can be completed in less than 10 minutes for an area of 64×64 µm2, so one can monitor too many cells during apoptosis within field of view.

Key biomarkers of bacterial metabolism and cell death are attributed to DNA composition, nucleic acids and protein profile. Also, the intent to use vancomycin and UV treated MRSA samples alongside blue light irradiated MRSA was considered based on the established efficacies of these treatments in combating the bacteria. Therefore, investigation of the biomarkers of bacterial metabolism and cell death using FTIR, characterizes irradiated and vancomycin treated MRSA, with the goal of obtaining an insight into mechanisms involved in bacterial inactivation when biomarkers are altered.

**2. Materials and Methods**

**2.1 Experimental Design**

Five different experimental groups of samples were evaluated using FTIR. Group I: Control 1, comprised of untreated MRSA incubated at 37ºC for 24 hours; Group II: Control 2, comprised of untreated MRSA incubated in ambient air for 24 hours; Group III: Irradiation with 880 J/cm2 of blue 470 nm light, incubated at 37ºC for 24 hours; Group IV: UV-treated MRSA; Group V: Vancomycin-treated MRSA.

**2.2 Bacterial culture**

MRSA USA300 strain (ATCC® BAA-1680) was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The strain was identified by standard procedures; including PCR. For the experiments, a colony of MRSA was grown in TSB at 37ºC for 24 hours. The culture was then centrifuged at 13000 rpm for 5 minutes, supernatant discarded and pellet re-suspended in broth. Aliquots of 2µl of 2x109 CFU/mL were placed on diamond optical windows (size 5.5 mm diameter-600 μm thick) for either irradiation or vancomycin treatment and then incubated at 37ºC for 24 hours before FTIR assay.

**2.3 Photoirradiation of MRSA**

**2.3.1 Blue light irradiation**

A Dynatron Solaris 708 (Dynatronics Corp., Salt Lake City, UT) fitted with a 470 nm light probe was used to irradiate the cells. Briefly, the irradiation protocol involved the use of a 5.0 cm2 applicator, which was clamped at a distance of 1–2mm perpendicularly above each diamond window containing 2 µl of 2x109 CFU/mL MRSA suspended in sterile saline. The applicator has a cluster of 32 light-emitting diodes (LEDs), emits 470 nm blue light with a spectral width of 455–485 nm, and has a rating of 150 mW average power and 30 mW/cm2 irradiance. The applicator is cooled with a built-in fan, thus eliminating thermal radiation. In preliminary studies, we ascertained that the device did not generate any measurable temperature rise within the range of fluencies used in this study [Enwemeka et al., 2009]. Fluences of 121 J/cm2 irradiated thrice (cumulative dose, 363 J/cm2, total irradiation time 60 mins) at 30minutes interval and 220 J/cm2 irradiated four times (cumulative dose, 880 J/cm2, total irradiation time, 144 mins) at 30 minutes interval were applied. After irradiation, the diamond windows were incubated at 37ºC for 24 hours and FTIR assay performed.

**2.3.2 UV irradiation**

The diamond windows containing 2x109 CFU/mL MRSA suspended in sterile saline were placed inside a biosafety hood and the UV light turned on for 12 hours. Samples were incubated at 37ºC for 24 hours and analyzed using FTIR.

**2.3.3 Vancomycin treated MRSA**

Bacterial culture of 2x109 CFU/mL had 2 and 40µg/ml of vancomycin added and incubated at 37ºC for 24 hours. FTIR assay was then performed.

**2.4 FTIR Assay:** ***In situ*, real-time monitoring of cell death**

This experiment was conducted with MRSA cells in situ. Briefly, Bacterial cells were cultured in tryptic soy broth (TSB), and 2x109 CFU/mL suspended in broth. Aliquots of 2µl were lightly coated on diamond windows. Fourier transform infrared widefield microscopy used to detect chemical properties of biomacromolecules. Bacterial cells mounted on diamond optical windows are assessed in transmission mode with a Bruker Vertex 70 IR spectrometer and a Bruker Hyperion 3000 IR Microscope equipped with a thermal blackbody source. The setup is equipped with a 64 x 64 pixel focal plane array (FPA) detector and enables us to generate hyperspectral cube of data (x, y, Abs(λ)). The data are collected with 512 co-added scans and 4 cm-1 spectral resolution with a pixel projection of 1.1×1.1μm2 on the sample plane using a 36× Cassegrain microscope objective (numerical aperture 0.5) and a 15× aperture objective (N.A 0.4).

Background was measured from a clean area on the diamond window and the ratio of sample measurement to this background is evaluated as an absorption spectrum. Although bacterial cells are diluted in broth medium, the contribution of the broth in an absorption spectrum is negligible (5%) based upon measuring pure broth mounted on diamond window. Bacterial cells that are mounted on the window are left to dry for overnight and there is no cell fixation prior to FTIR measurements. 4096 individual spectra (64×64 pixels within a field of view and every single pixel generates a full spectrum) were collected per single measurement and preprocessed (i.e. baselined and vector normalized) for further analysis.

**2.5 Determination of CFU after FTIR assay**

After FTIR, a swap was obtained from the diamond windows of all groups and cultured on tryptic soy agar (TSA) at 37ºC for 24 hours. Bacterial growth was then determined.

**2.6 Statistical analysis**

Principal Component Analysis (PCA) is a well-known statistical method to decompose a dataset into bilinear latent variables called principal components (PCs) and reduces the dimensionality of the data while retaining most of the variations in the data set. In principal component analysis the first component is accredited to an average intensity observed at every spectral data point and the second component represents the variations from the first component and subtle differences between groups of samples can be identified with standard statistics. PCA was performed on the single pixel spectra generated from measurements on both untreated and treated bacterial cells. The principal component loadings are used to infer the components and strength of components that correspond to extermination mechanism of bacteria by different treatments. In addition scatter plot from PC scores reveals clear clusters for control I, control II and treated samples with different methods. Every single point in the scatterplot is assignable to an individual spectrum and entails the chemical composition of a related pixel. The first three PCs were used to create 2D scatter plots due to their highest variability. To have better understanding of chemical changes attributed to cell wall components, DNA and protein denaturation, the mid-infrared spectral region 1800-900cm-1 is chosen for the PCA analysis. PCA analysis is performed using “R” package version 3.1.2.

Results from colony counts are expressed as CFU’s per diamond window/plate. All data are shown as mean±SEM and were analyzed using One-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test (GraphPad Prism 5.01 software, GraphPad Software Inc., USA). The level of statistical difference was set at p<0.05.

**3. Results and discussion**

Bacteria were treated exclusively to comprehend the cell signaling during exposure to stimulus. Infrared absorption average spectra concentrating on mid-infrared fingerprint region and the corresponding second derivative spectra, variation trends generated from PCA analysis and clear clusters in the scatterplots associated with distinctive group of samples (five groups termed in previous section) will be discussed. Additional results showing the growth of MRSA per treatment after FTIR measurements is shown.

3.1 **Spectral features:**

Figure 1 shows infrared absorption average spectra for MRSA samples maintained at distinctive environmental circumstances. Alive samples (red spectrum), Vancomycin-treated samples (tortoise spectrum), control samples (green spectrum) and blue-light/UV-light induced samples (blue/purple spectrum) zoomed in spectral region 1800-900cm-1 is shown in Figure 1. Infrared absorption bands assignable to important functional groups of carbohydrates, protein and backbone of DNA are identified. Distinctive features per treatment in the spectral region (900–1300 cm−1) contain primarily the features due to phosphate and carbohydrate functional groups were observed.

Infrared bands that are overlapping for all groups of samples attributed to nucleic acids region shown on Figure 1 are listed below:

Peaks at 995 cm-1 stems from C-C, C-OH, C-H ring and side group vibrations, C-O stretching of cellulose at 1060 (±2) cm-1, C-O-C stretching at 1118(±4) cm-1, symmetric and asymmetric stretching of phosphate group at 1087 and 1238 cm−1, and C=O stretching of ester functional groups in lipids at 1745 cm-1 are identified.

In addition, several bands at 1027,1155 and 1176 cm-1 that are due to stretching vibrations of C-O and C-O-C groups in carbohydrates are indicative of MRSA response to the treatment with vancomycin. The vibrations of pure vancomycin dominate average spectrum recorded from vancomycin-treated MRSA and it is due to the fact that high concentration (0.04 mg) of vancomycin was applied to MRSA to make sure that bacterial cells are deteriorated. Spectral reproducibility was obtained by measuring four replicates per sample and eight measurements per replicate and the peak positions and relative intensity of peaks were highly consistent.

The spectral region 1800–1500 cm−1 is primarily associated with absorptions due to the secondary structure of proteins. In the second derivative spectra, three amide I peaks at 1681, 1654 and 1637cm-1 and a peak at 1544cm-1 attributed to Amide II are observed. For all groups of samples, the strongest peak in protein region is 1654cm-1 and the position of this peak remains unchanged except for vancomycin-treated MRSA in which the peak shifts to 1652 cm-1 that could be related to the transformation of secondary structure of protein from β-sheet structure to α-helix or random coil. Another remarkable difference in this region is the reduced intensity seen in the Amide II peak at 1544cm-1 for the control samples that were deprived of nutrients.

3. 2 **Differentiation of MRSA based on treatment:**

Principal component analysis was employed to differentiate between groups of samples and in particular discriminate between untreated (control I, control II) and either blue-light radiated, UV-light radiated and vancomycin-treated MRSA. The motivation behind classification of aforementioned three groups was identifying the pathway toward breakage of bacteria due to the fact that any of these treatments has a known mechanism in killing cells. The first three PCs account for 99% of the total variability of data and therefore used to create scatter plots. In this work, the biological response of MRSA to every treatment is compared with respect to control I and control II groups. The scatter plot from scores exhibits clear clusters for control I, control II and blue-light radiated samples (Figure 2-A), UV-light radiated samples (Figure 2-B) and vancomycin-treated samples (Figure 2-C) and they are separated into three distinguishable clusters and dispersed with above 95% specificity.

Moreover, by means of PC loadings, infrared absorption bands ascribed to DNA are representative of the variations observed as a consequence of radiation with blue light and UV light. In particular, C-C stretching of backbone at 968(±1) cm-1, symmetric and asymmetric stretching of the PO2− ion in Phosphates at 1085 and 1238 (±2) cm-1 and stretching of base pair carbonyl at 1714 (±1) cm-1 are highlighted in Figure 3-A for blue and purple spectra. The peak at 1238 cm-1, which is originating from PO2- functional group in phosphates, and known as one of the A-DNA markers, is more pronounced in blue-light radiated samples. Comparatively speaking, UV-irradiated MRSA exhibits the signals toward 1228 cm-1 peak in phosphate region, which is a B-DNA marker (Figure 3-A). There is also not considerable variation in the relative intensity of A-DNA to B-DNA bands between control I and control II groups of MRSA in phosphate region and most of the variations between these groups were observed in protein region. It is worth noting that the peak at 1230cm-1 seen in average absorption spectrum from vancomycin-treated MRSA does not experience any shift in either PC1 or PC2 loadings, however in comparison with untreated MRSA, this band looks narrower and is found at 1230cm-1.

Figure 3-B shows additional infrared absorption bands in carbohydrate spectral region (1200-900 cm-1) including 995, 1014 1060 and 1155 cm-1 for UV/blue-light-radiated and vancomycin-treated MRSA. The third component of variation was used for the samples treated with vancomycin because the second component was highly dominated by the absorptions from vancomycin. PC loadings in protein region (1800-1500 cm-1) depict two primary infrared bands associated with Amide II at 1544cm-1 and Amide I at 1654cm-1 and two additional shoulders at 1556cm-1 and 1637 cm-1 for all treatments while the last two shoulders are more prominent in vancomycin-treated samples.

3.3 **MRSA signaling pathway in response to treatments**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium that is notorious to be resistant to many antibiotics and treatments and leads to lethal bloodstream contagions and surgical site infections. Therefore, it is critical to have in depth understanding of the response of MRSA when it is exposed to a treatment at cellular and molecular level. One of the major goals of this study was determining the impacts of the blue-light radiation on the MRSA and assess if irradiating via blue-light could potentially be an alternative treatment to the existing techniques. Couple of eminent methods that are known for rupturing the bacterial cells [ref] was examined to assist us in evaluating what is happening with blue-light radiation. Hitherto, It is postulated that DNA is the main target for the UV-irradiation damages [ref] or protein denaturation is reported as a consequence of autoclaving on bacterial endospores [ref] and lastly exposing the bacterial cells to antimicrobial, which is one of the foremost clinical methods with regards to MRSA infections [ref].

Findings from principal component analysis suggest that strong and discernible peaks associable to DNA, nucleic acids and proteins, are the major components that play role in the damages by UV/blue-light radiation. Finding a correlation between clusters of points in the scores 2D scatterplot and the variations seen in PC loadings is critical. One can infer that all positive bands that are highlighted in PC loadings [figure2-A, blue and purple spectra] are related to the cluster of points that are mostly positive in the scatterplot and troughs in the loadings are associated with negative scores [figure 3-A]. The largest peak or trough in the loading plot causes the largest variance in the score plot. One of the major contributors to the variance observed in the score plot is due to the DNA marker of antisymmetric phosphate stretching vibration. Examination of the loading plot for UV-radiated MRSA reveals this peak at 1230 (±2) cm-1 (B-DNA marker band) while it is seen at 1240cm-1 (A-DNA marker band) for blue-light radiated MRSA. It tells us that although DNA damage is predominant when MRSA is exposed to irradiation, distinctive infrared absorption bands are signaling toward the pathway of death in individual treatments. PCA plots clearly suggest that the sections of DNA in MRSA that are in A-like conformation state are biomarkers of blue-light radiation while the sections of DNA with B-like form are associated with UV-light damage. We hypothesize that blue-light radiation can detect stretching modes of DNA known to be interacting with proteins or ions which induce the A conformation [ref-B.W]. It has also been shown in some spores that transition to A-like conformation could happen through saturation with DNA protecting proteins [ref 11,12]. This feature is unique and differs from the observed effect of the UV-light radiation in DNA breakage [reference to known uv-damage]. Furthermore, evaluating the average absorption and second derivative spectra from vancomycin-treated MRSA, PC-1 and PC-2 loadings depicts the νasym (PO2- ) peak at 1230cm-1 as a result of treatment through vancomycin. These findings imply that MRSA is undergone distinguishing conduit to DNA damage when exposed to different stimuli.

In addition two peaks associated with C-O stretches observed in the light treated samples at the same peak positions and relative intensities. The presence of a peak at 1060(±2) due to cellulose as one of the major cell wall components [ref] and another shoulder at 1027 cm-1 are another alterations occurred at nucleic acids region (1300-900 cm-1) during UV/blue-light irradiation. Infrared absorption spectra and PC loadings in the spectral region 1800-1500cm-1 associated with the secondary structure of proteins are dominated by Amide II and Amide I normal vibrations where the former peak is located at 1544cm-1 and the latter appears approximately at 1654cm-1. Additional Amide I shoulder at 1637cm-1, which has been reported as a β-sheet conformation in secondary structure of proteins [ref] is another MRSA marker for UV-light and blue-light radiated samples.

Vancomycin is known as one of the noticeable members of glycopeptide antibiotics and clinically has been used for treating infection caused by MRSA due to the recognized ability in treating Gram-positive bacteria. Cell wall in the Gram-positive bacteria is comprised of peptidoglycan, which is a molecule made of sugars and polypeptides and the main goal of treatment via vancomycin is the known ability of antimicrobial in inhibiting the synthesis of peptidoglycans. Responses of MRSA to the treatment with vancomycin were distinctive compared to the radiated samples with either blue-light or UV-light. The infrared bands at 1014cm-1 generating from C-C and C-O-C stretching [ref], 1040 and 1155 cm-1 associated with glycogen in nucleic acids region and a peak at 1176 cm-1 assignable to asymmetric stretching of CO-O-C is observed. The variations in glycogen and carbohydrate spectral region could be interpreted as signatures of (13)(16)-β-D-glucans that are major component of cell walls [ref]. Another distinctive marker of MRSA in response to vancomycin treatment is the presence of a shoulder in protein region at 1556 (±2) cm-1, which has been reported as Amide I band (mainly C=O stretch)[ref].

Write a paragraph about growth experiment after FTIR .

**4. Conclusion**

Financial support was received from the SPARC Grant College of Health Sciences, University of Wisconsin-Milwaukee (Grant No. to C.S.E) , NSF grants CHE-1508240 and CHE -1112433 to C.J.H , Janis, please add grant number if it is needed. .

**References**

Howell-Jones RS, Wilson MJ, Hill KE, Howard AJ, Price PE, Thomas DW (2005) A review of the microbiology, antibiotic usage and resistance in chronic skin wounds. J Antimicrob Chemother, 55: 143–149.

Liu HH (1999) Antibiotic resistance in bacteria. A current and future problem. Adv Exp Med Biol, 455:387–396.

Graham J (2007) Deaths from drug-resistant bacteria top those from AIDS. Chicago Tribune, October 17.

Otto, C.C., Haydel, S.E., 2013. Exchangeable Ions Are Responsible for the In Vitro Antibacterial Properties of Natural Clay Mixtures. PLoS ONE 8, e64068.

Müller, P., Alber, D.G., Turnbull, L., Schlothauer, R.C., Carter, D.A., Whitchurch, C.B., Harry, E.J., 2013. Synergism between Medihoney and Rifampicin against Methicillin-Resistant Staphylococcus aureus (MRSA). PLoS ONE 8, e57679.

Turhan, V., Sacar, S., Uzun, G., Mustafa, S., Yildz, S., Ceran, N., Gorur, R., Oncul, O., 2009. Hyperbaric oxygen as adjunctive therapy in experimental mediastinitis. J. Surg. Res. 155, 111–115.

Dai, T., Tegos, G.P., Zhiyentayev, T., Mylonakis, E., Hamblin, M.R., 2010. Photodynamic therapy Methicillin-Resistant Staphylococcus aureus Infection in a Mouse Skin Abrasion Model. Lasers Surg. Med. 42, 38–44.

Bumah, V.V., Masson-Meyers, D.S., Cashin, S., Enwemeka, C.S., 2013. Wavelength and Bacterial Density Influence the Bactericidal Effect of Blue Light on Methicillin Resistant Staphylococcus aureus (MRSA). Photomed. Laser Surg. 31, 547–553.

Dai, T., Gupta, A., Huang, Y.Y., Sherwood, M.E., Murray, C.K., Vrahas, M.S., Kielian, T., Hamblin, M.R., 2013. Blue light eliminates community-acquired methicillin-resistant Staphylococcus aureus in infected mouse skin abrasions. Photomed. Laser Surg. 31, 531–538.

Enwemeka, C.S., Williams, D., Hollosi, S., Yens, D., Enwemeka, S.K., 2008. Visible 405nm SLD light photo-destroys methicillin-resistant Staphylococcus aureus (MRSA) in vitro. Lasers Surg. Med. 40, 734–737.

Enwemeka, C. S., Williams, D., Hollosi, S., Enwemeka, S.K., Hollosi, S., Yens D., 2009. Blue 470-nm light kills methicillin-resistant Staphylococcus aureus (MRSA) in vitro. Photomed. Laser Surg. 27, 221–226.

Enwemeka, C.S., 2013. Antimicrobial Blue Light: An Emerging Alternative to Antibiotics. Photomed. Laser Surg. 31, 1–3.

Maclean, M., McKenzie, K., Anderson, J.G., Gettinby, G., MacGregor, S.J., 2014. 405 nm light technology for the inactivation of pathogens and its potential role for environmental disinfection and infection control. J. Hosp. Infect. 88, 1–11.

Bumah VV, Masson-Meyers DS, Cashin SE, Enwemeka CS (2015a) Optimization of the antimicrobial effect of blue light on methicillin-resistant Staphylococcus aureus (MRSA) in vitro. Lasers Surg Med 47(3):266–272.

Bumah VV, Masson-Meyers DS, Enwemeka CS (2015b) Blue 470 nm light suppresses the growth of Salmonella enterica and Methicillin-resistant Staphylococcus (MRSA) in vitro. Lasers in Surgery and Medicine, 47: 595-601.

Bumah VV, Masson-Meyers DS, Quirk BJ, Buchmann E, Whelan HT, Enwemeka CS (2015c) The bactericidal effect of 470 nm light and hyperbaric oxygen on methicillin-resistant Staphylococcus aureus (MRSA). Lasers Med Sci 30(3):1153–1159.

Masson-Meyers D.S., Bumah V.V., Biener G., Raicu V. and Enwemeka C.S. (2015). The relative antimicrobial effect of 405nm LED and Blue 405nm laser on Methicillin resistant Staphylococcus aureus in vitro. Lasers in Medical Science DOI: 10.1007/s10103-015-1799-1.

Walter P. Hammes1 and Francis C. Neuhaus (1974). On the Mechanism of Action of Vancomycin: Inhibition of Peptidoglycan Synthesis in Gaffkya homari. Antimicrob Agents Chemother. 6(6): 722–728

Malik Z, Hanania J, Nitzan Y (1990) Bactericidal effects of photoactivated porphyrins - An alternative approach to antimicrobial drugs. J Photochem Photobiol B, 5:281-293.

Moné MJ, Volker M, Nikaido O, et al. Local UV-induced DNA damage in cell nuclei results in local transcription inhibition. EMBO Reports. 2001;2(11):1013-1017. doi:10.1093/embo-reports/kve224.

Lipovsky A, Nitzan Y, Gedanken A, Lubart R. (2010) Visible light induced killing of bacteria as a function of wavelength: implication for wound healing. Laser Surg Med 42:467-472.

Lipovsky A, Nitzan Y, Friedman H, Lubart R (2009) Sensitivity of Staphylococcus aureus strains to broadband visible light. Photochem Photobiol, 85:255-260.

Kawada A, Aragane Y, Kameyama H, Sangen Y, Tezuka T (2002) Acne phototherapy with high-intensity, enhanced, narrowband blue light source: An open study in vitro investigation. J Dermatol Sci 30(2):129–135.

Baxter BK (1998). How does ultraviolet light kill cells? Scientific American, August 17, 1998. <http://www.scientificamerican.com/article/how-does-ultraviolet-ligh/>

Rammelsberg A (1998). How does ultraviolet light kill cells? Scientific American, August 17, 1998. <http://www.scientificamerican.com/article/how-does-ultraviolet-ligh/>

Wasson CJ, Zourelias JL, Aardsma NA, Eells JT, Ganger MT, Schober JM, Skwor TA (2012) Inhibitory effects of 405 nm irradiation on Chlamydia trachomatis growth and characterization of the ensuing inflammatory response in HeLa cells. BMC Microbiology, 12:176-186.

Wilson M, Yianni, C (1995) Killing of methicillin-resistant Staphylococcus aureus by low-power laser light. J Med Microbiol, 42:62-66.

Maclean M, MacGregor SJ, Anderson JG, Woolsey (2009) Inactivation of bacterial pathogens following exposure to light from a 405nm light-emitting diode array. Appl Environ Microbiol, 75:1932-1937.

Daum LT, Bumah VV, Masson-Meyers DS, Khubbar M, Rodriguez JD, Fischer GW, Enwemeka CS, Gradus S, Bhattacharyya S. (2015). Whole-genome sequence for methicillin-resistant Staphylococcus aureus strain ATCC BAA-1680. Genome Announc 3(2):e00011-15. doi:10.1128/genomeA.00011-15.

Helm D, Labischinski H, Schallehn G, Naumann D (1991) Classification and identification of bacteria by Fourier transform infrared spectroscopy. J Gen Microbiol, 137:69-79.

Mariey L, Signolle JP, Amiel C, Travert J. (2001) Discrimination, classification, identification of microorganisms using FTIR spectroscopy and chemometrics. Vibrational Spectrosc, 26:151-159.

Amiali MN, Goldingc GR, Sedmanb J, Simord AE, Ismailb AA (2011) Rapid identification of community-associated methicillin-resistant Staphylococcus aureus. Diagn Microbiol Infect Dis, 70:157-166.

Lamprell H, Mazerolles G, Kodjo A, Chamba JF, Noel Y, Beuvier E (2006) Discrimination of Staphylococcus aureus strains from different species of Staphylococcus using Fourier transform infrared (FTIR) spectroscopy. Int J Food Microbiol, 108:125-129.

Nasse M, Ratti S, Giordano M, Hirschmugl C (2009) Demountable Flow/Liquid Chamber for in vivo Infrared Microspectroscopy of Biological Specimen. Appl Spectrosc, 63:1181-1186.

Nasse MJ, Walsh MJ, Mattson EC, Reininger R, Kajdacsy-Balla A, Macias V, Bhargava R, Hirschmugl, CJ (2011) High resolution Fourier-transform infrared chemical imaging with multiple synchrotron beams. Nat Methods, 8:413-416.

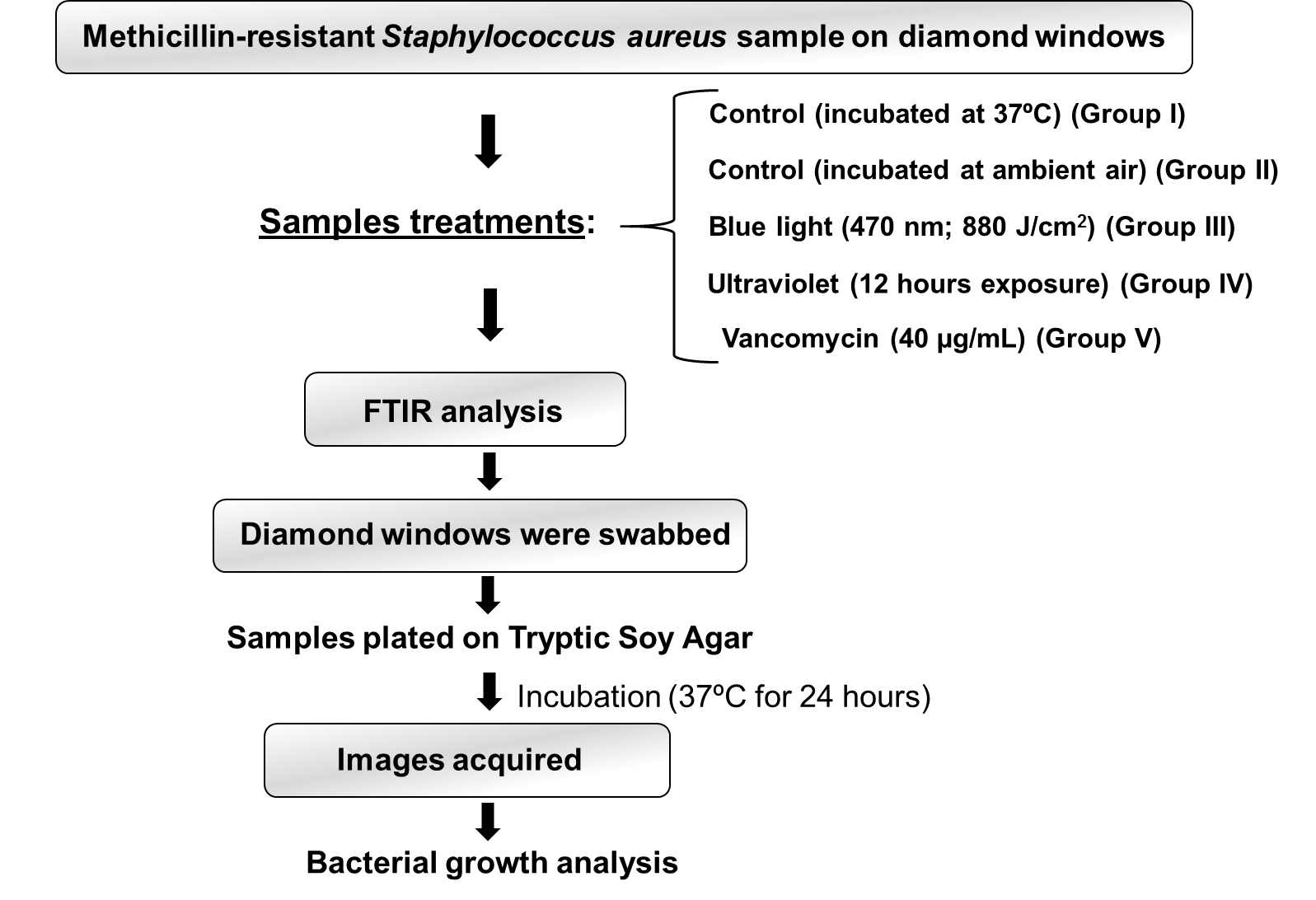
**Figure Legends:**

Figure2: The scatter plot from PC scores reveals clear clusters for control (samples deprived from nutrients) , alive and (A) blue light radiation-induced , (B) UV radiation-induced and (C) vancomycin-treated samples. They are following the same trends and in agreement with the direction that highlights the pathway toward death. Y-axis shows the 2nd component of variations (represents 7% of the original data) from 4000 vector normalized individual spectra and x-axis is the 1st component of the variations (represents 90% of the original data). The 3rd component of variation(PC-3) is used for vancomycin-treated samples.

**Fig 1.** Experimental design

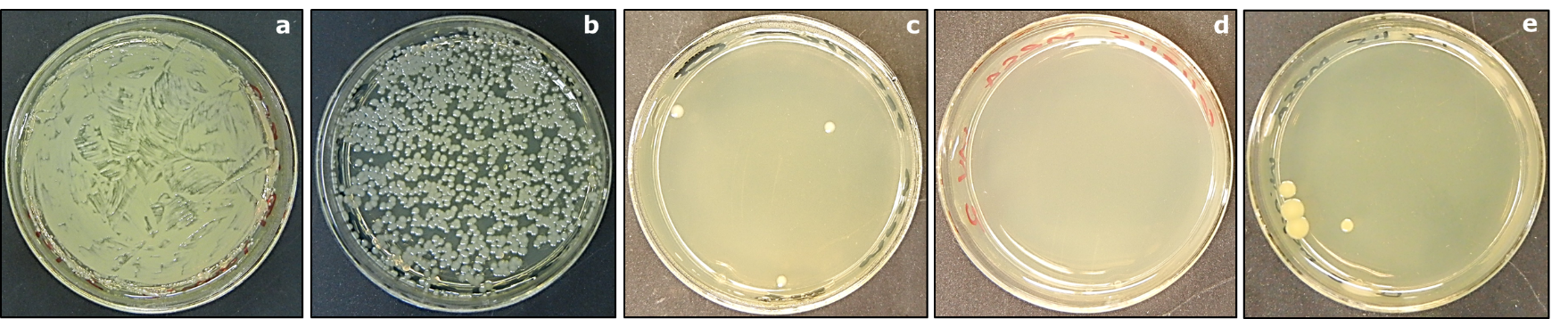
Fig.1: Infrared absorption average spectra for treated samples with different methods and alive sample for spectral region 1800-900 cm-1 attributed to carbohydrates and protein is shown. Functional groups including symmetric and asymmetric stretching of phosphate group, C=O stretching of ester functional groups in lipids and Amide groups associated with proteins are highlighted. Additional absorption bands from Vancomycin are highlighted in carbohydrate region in vancomycin-treated spectrum.

Figure 3: Second component of variations form averages (PC-2 loading) from different treatments are shown and infrared DNA bands are shown in (A) and additional bands, which are unique, and marker of any treatment is shown in (B). For samples treated with blue light, infrared signatures of A-DNA are revealed while for other treated samples (UV-treated, Autoclave, Vancomycin-treated) the signatures of B-DNA are found.









**Fig 5**. Post-FTIR bacterial growth analysis. a) Control (Group I), b) Control (Group II), c) Blue light- treated MRSA, d) UV treated MRSA, e) Vancomycin-treated MRSA