Bactericidal Efficacy of Dielectric Barrier Discharge Plasma on Methicillin-Resistant *Staphylococcus* aureus and *Escherichia coli* in Planktonic Phase and Colonies *In vitro*

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ABSTRACT: Increasing emergence of antibiotic resistance in bacteria during the past few decades has become a major public health concern and has reduced the efficacy of conventional therapies. The objective of the current study is to examine the bactericidal efficacy of atmospheric pressure nonthermal dielectric barrier discharge (DBD) plasma on methicillin-resistant *Staphylococcus aureus* (MRSA) as a common musculoskeletal pathogen and *Escherichia coli* (*E. coli*) as another cause of bone, joint, and soft tissue infections in planktonic phase and in colonies. In planktonic phase, results demonstrated complete decontamination of *E. coli* and MRSA cells on agar plates with a bacterial surface density of 1.27×10^5 colony-forming units (CFU)/cm^2 following 30 seconds and 60 seconds of plasma treatment, respectively. Reduction rates of 7 $\log_{10}$ steps in the number of viable CFUs in *E. coli* colonies was observed following 5 minutes of plasma treatment. The colony growth was halted, and no more growth in colony size was observed during a 24-hour monitoring period. For MRSA colonies, results demonstrated 2 $\log_{10}$ to 7 $\log_{10}$ steps reduction in the number of viable CFUs in each colony after 5 minutes of plasma treatment. Results indicated that plasma-treated colonies will have prolonged lag time during generation of the growth curves. Finally, diffusion of nitric oxide into the agarose gel was confirmed as a reactive agent that was effective in the decontamination process.

KEY WORDS: plasma, DBD, sterilization, decontamination, MRSA, *E. coli*, colony

I. INTRODUCTION

Emergence of antibiotic-resistant bacteria is an increasing concern in wound infections. These organisms colonize wounds, often necessitating aggressive wound management with a very limited choice of antibiotics. *Staphylococcus aureus* is one of the common musculoskeletal pathogens causing osteomyelitis. An infection in bone, cartilage, and surrounding tissues as a result of injury (open wounds) or surgery combined with antibiotic resistance to *S. aureus* is a serious problem.[1-4]

Several conventional techniques are used for the prevention and management of wound infection. It is nonetheless a gold standard to treat patients with deep wounds

with antibiotic prophylaxis to prevent bacteria from being released into the blood stream and cause infection complications. This procedure is combined with debridement and surgical processes. Washing wounds with antibiotic solutions and use of topical antibiotics are controversial.[5] In some cases, silver and other noble metals are potential allies in fighting pathogens in wounds.[6] Considering that antibiotics do not reach the source of the wound infection, only the area around the wound, and that bacteria are becoming resistant to antibiotics, these methods are not very efficient in the treatment of antibiotic-resistant species. Thus, there is considerable need for an efficient and nondestructive way to decontaminate wounds and prevent germs from spreading. This need fuels the search for other possible methods. Research currently underway is aimed at developing new techniques, including ozonation, and use of dry mist–generated hydrogen peroxide, plant extracts, and nonthermal plasma to combat antibiotic-resistant species.[7-11]

Atmospheric pressure nonthermal dielectric barrier discharge (DBD) plasma along with atmospheric pressure plasma jets (APPJ) with inert gases have been introduced as new, effective, and safe methods in the decontamination of living tissue.[12-14] Plasma, the fourth state of matter, is a cocktail of electrons, uncharged gas molecules, atoms, free radicals, ions, and UV photons.[15] Different forms of plasma can be generated using different energy sources, including lasers, heat, electrical current, and a strong electromagnetic field. In this study, DBD plasma is used, which is of particular interest in research on its possible biomedical use.[16] Several studies suggest that reactive species produced in plasma, such as nitric oxide and hydroxyl radicals, have antimicrobial effects and can be useful in the decontamination of human tissue.[17-20] The application of DBD plasma on human tissue is particularly promising because of its low temperature, the straightforward setup, and low electric current.[21] In addition, using air as the working gas in atmospheric pressure provides a huge advantage over other plasma systems in which gas delivery systems should be used. DBD plasma treatment is a contact-free and painless procedure, causing no harm to the tissue while killing many known pathogens and also promoting wound healing.[22]

The current study focuses on the efficacy of DBD plasma treatment on bacteria in planktonic phase and in colonies. Bacteria were grown *in vitro* as planktonic cultures and in colonies as a common form of bacterial cell aggregation to compare the efficacy of DBD plasma treatment on each phase.[23]

The aim of this work is to study the effect of plasma in the decontamination of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* in planktonic cultures and in colonies *in vitro*.

II. MATERIALS AND METHODS

A. Bacterial Culture and Sample Preparation

1. Planktonic Bacterial Culture

To study the effect of plasma treatment on planktonic culture, one lyophilized cylinder of *E. coli* (NCTC 10538, Microbiologics) was rehydrated in super broth (SB) (Fisher

Scientific, MA). Dehydrated pellets of MRSA strains ATCC BAA-1556, ATCC BAA-1717, and ATCC 33591 (ATCC,VA), were rehydrated in brain-heart infusion (BHI) broth (Sigma-Aldrich, MO), tryptic soy broth (TSB) (Fisher Scientific, MA), and nutrient broth (Sigma-Aldrich, MO), respectively. Each culture was streaked onto an agar plate using sterile swabs and incubated at 37°C for 24 hours. A single colony of each strain was then transferred and inoculated in liquid broth. It was then grown overnight on a shaker platform at 37°C. The culture was centrifuged and the supernatant was removed. The pellet was resuspended in 20 mL of liquid broth with 15% glycerol and transferred to microcentrifuge tubes, maintained at -80°C freezer until further use.

Prior to plasma treatment, *E. coli* and MRSA samples were removed from the -80°C freezer, thawed, and cultured in liquid broth overnight on a shaker platform at 37°C. The cultures were diluted to obtain a cell concentration of 10^8 CFU/mL. The concentration was confirmed by a microplate reader (SpectraMax Plus 384, Molecular Devices, CA) and verified by a colony counting assay. Serial dilutions of 10^7, 10^6, 10^5, 10^4, and 10^3 CFU/mL were prepared. 100 µL of each dilution (for *E. coli* and all MRSA strains) were spread over agar plates using sterile spreading beads. The plates were treated with plasma for various times to study the antibacterial effect of the plasma treatment.

2. Bacterial Colonies

To evaluate the effect of plasma treatment on bacterial colonies, *E. coli* and MRSA ATCC BAA-1556 were used. This strain of MRSA is the most resistant to plasma treatment in the planktonic phase. Samples were taken out of the -80°C freezer, thawed, and cultured overnight in SB and BHI, respectively, on a shaker platform at 37°C. Cultures were diluted to reach a cell concentration of 10^8 CFU/mL. These concentrations were verified with a microplate reader. Serial dilutions of 10^3 and 10^4 CFU/mL of each culture of *E. coli* and MRSA were prepared. 100 µL of each dilution was spread over sterile SB and BHI agar plates, respectively, using sterile spreading beads. Then the agar plates were incubated at 37°C for 12 hours until the colonies had grown and were visible on the plates. The plates were then treated with plasma to study the bactericidal efficacy of the treatment.

B. Dielectric Barrier Discharge Plasma Treatment

DBD plasma was generated using a copper core electrode insulated by a Teflon hollow cylinder and a quartz dielectric barrier as the high voltage electrode. A schematic of the experimental setup is shown in Figure 1. The copper rod with 25-mm diameter was insulated with a 10-mm layer of Teflon, and a 1-mm thick quartz plate was used as the dielectric barrier. The samples were placed on a grounded plate, and the air gap between the high voltage electrode and sample surface was adjusted to 1 mm. The direct plasma treatment was applied on a circle in the center of the sample plates that created an area with a 25-mm diameter (core electrode diameter). This area was referred to as the plasma treated area. The applied maximum high voltage output was 10 kV at 500 Hz and had a 3-µsec pulse width, with a power of 0.12 W/cm^2.

Agar plates inoculated with planktonic bacteria were placed on the grounded electrode, and the high voltage electrode was placed 1 mm from the sample surface, as shown in Figure 1. Samples were treated with plasma for 15 seconds, 30 seconds and 60 seconds, and untreated samples were used as the control. After plasma treatment, plates were incubated for 24 hours at 37°C for further characterization of the effect of the DBD plasma treatment. All of the experiments were performed in triplicate.

For bacterial colonies, inoculated agar plates were incubated for 12 hours to grow bacterial colonies. The agar plates were placed on the grounded electrode, and the high voltage electrode was positioned at 1 mm from the sample surface. Plates were treated with DBD plasma for 1, 2, 3, 4, and 5 minutes, with untreated (0 minutes) samples used as the control. Microbiological analysis and quantification of the surviving populations of bacteria were performed.

C. Microbiological Analysis

1. Planktonic Bacterial Culture

To investigate the effect of plasma treatment on planktonic cultures, plasma-treated plates were incubated and monitored daily for 72 hours to check for further bacterial growth on the plasma treated area. To investigate whether the effect of plasma treatment was bacteriostatic or bactericidal, the clear plasma-treated area of each plate was swabbed after 24 hours of incubation, using a sterile cotton swab, and streaked on another sterile plate. The cloudy, covered area was also swabbed as a control and streaked on a sterile plate. Plates were incubated for another 24 hours to analyze the effects of plasma treatment.

2. Bacterial Colonies

To analyze the effects of the plasma treatment on colonies, the untreated control colonies and plasma-treated colonies were monitored. After plasma treatment, plates were
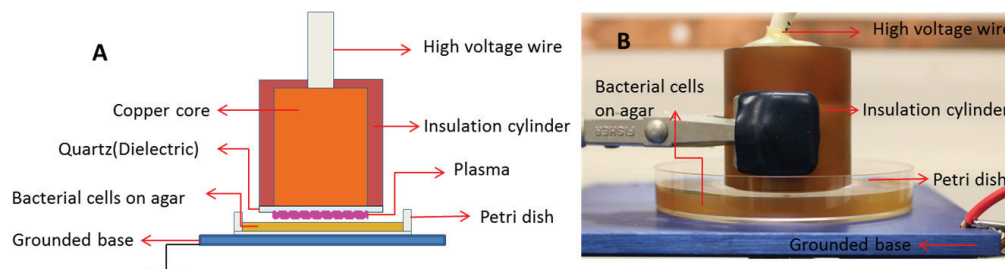

FIG. 1: A) Schematic view of the DBD plasma system. The DBD plasma system was used for the treatment of *E. coli* and MRSA cells in planktonic phase and in colonies. **B)** Image of DBD plasma system

incubated for another 12 hours to observe the effect of the treatment, after which the respective sizes of treated and untreated colonies were measured and compared. For the purpose of this comparison, the diameters of the colonies were measured before plasma treatment and immediately after plasma treatment, and after 12 hours of post-treatment incubation. The measurements were done by analyzing the pictures of the samples using public domain software ImageJ (National Institutes of Health [NIH], USA). The diameter of at least 10 colonies from each group was measured, and the values are shown in the results (Figure 6) as the mean ± standard deviation.

a. Quantification of the Viable CFUs within Plasma-Treated Colonies

To determine the number of viable CFUs in samples treated with plasma for 1, 2, 3, 4, or 5 minutes and the untreated control samples, colonies were scrapped off from the agar plates using a sharp sterile needle tip and transferred to 1 mL of liquid media. The suspension was homogenized by pipetting up and down and then vortexing. Serial dilution was performed for the suspension, and 100 µL of each dilution was inoculated on an agar plate and incubated at 37°C for 24 hours. After 24 hours the number of bacterial colonies was counted, and the number of viable CFUs in each colony was calculated.

b. Assessment of Regrowth of Plasma-Treated Colonies in Fresh Media

To study the regrowth of viable bacterial cells, plates were seeded with ~2×10^3 CFU/mL of bacteria and incubated for 12 hours. Following incubation, plates were treated with DBD plasma for 1, 2, 3, 4, or 5 minutes; untreated colonies were used as controls. Immediately after the plasma treatment, single colonies were scraped off of the plasma-treated area using a sharp sterile needle tip and transferred to 4 mL of liquid media. The suspension was homogenized by vortexing and incubated on a shaker platform at 37°C. The regrowth of bacterial cells in fresh media was monitored by reading the absorbance at 600 nm (OD600) every 20 minutes for 140 minutes for MRSA and for 200 minutes for *E. coli.* The dynamics of the bacterial growth were measured by plotting the cell growth (absorbance) against the incubation time. Using this OD_{600} value and the standard curve of each bacterial species, a standardized regrowth curve of each treated colony was plotted.

D. Penetration of DBD Plasma-Generated Nitric Oxide in Agarose Gel

The plasma treatment was conducted at ambient environmental conditions. Studies showed that nitric oxide is likely the most well-known agent in decontamination when plasma is ignited in air.[24,25] Nitric oxide introduced to the medium rapidly reacts with oxygen and produces stable metabolites of nitrate (NO_3) and nitrite (NO_2), which can be detected by Griess reaction.[26,27] To study the penetration of the nitric oxide in agar after plasma treatment, a series of agar plates was prepared and seeded with ~2×10^3 CFU/mL of bacteria and incubated for 12 hours, after which the plates were treated with

DBD plasma for 1, 2, 3, 4, or 5 minutes. A Griess Reagent Kit (Molecular Probes, Life Technologies, CA) was used to study the penetration of nitric oxide into agarose gel after treatment. 100 µL of each reagent, R1 (sulfanilic acid) and R2 (*N*-[1-naphthyl] ethylenediamine), was pipetted onto the plasma-treated plate, then spread and incubated for 15 minutes to allow the reagents to diffuse into the agar and for the reaction and color change to happen.

E. Statistical analysis

A minimum of three samples ($n = 3$) were used for experimental and control groups. All statistical differences were determined using the *t*-test in Microsoft Office Excel data analysis add-in with a 95% confidence interval ($p < 0.05$). A *P* value less than 0.05 was considered to be statistically significant. All values are reported as the mean ± the standard deviation of the mean.

III. RESULTS

A. The Efficacy of DBD Plasma Treatment on Planktonic Bacterial Culture

The decontamination effect of the plasma treatment on planktonic bacteria is represented in Figure 2. The results show complete decontamination of the plasma-treated area with a surface density of 1.27×10^5 CFU/cm^2 for MRSA strains ATCC BAA-1556, ATCC BAA-1717, and ATCC 33591 after 60 seconds of treatment, and for *E. coli* NCTC 10538 after 30 seconds of treatment. The plasma-treated area is defined as a circle with a 25-mm diameter on which the plasma is applied directly. After the center of each plate (seeded with 10^8 CFU/mL) was treated with plasma for 60 seconds, complete decontamination on the plasma treated area was observed, and after 24 hours of incubation at 37°C, no bacteria growth took place on the plasma-treated area.

To study whether the effect of plasma treatment is bactericidal or bacteriostatic, the clear plasma treated area was swabbed and streaked on a sterile agar plate. Plates were monitored for 72 hours, and after this period of incubation, no bacteria regrowth was observed. As a control, the untreated area was also swabbed and streaked on a new agar plate; after 24 hours of incubation bacterial growth could be seen (Fig. 3, *B*).

B. The Efficacy of DBD Plasma Treatment on Bacterial Colonies

The decontamination effect of the plasma treatment on bacterial colonies is represented in Figures 4 and 5. The black circles on the plates show the plasma-treated area. The small marks on the plasma-treated area are where colonies were scraped off for further characterization. As shown, *E. coli* colonies stopped growing after 5 minutes of plasma treatment. For MRSA strain ATCC BAA-1556, colony size is approximately unchanged after 24 hours of incubation at 37°C. Colonies from test samples with shorter treatment times did grow after 24 hours of post-treatment incubation at 37°C. However, growth
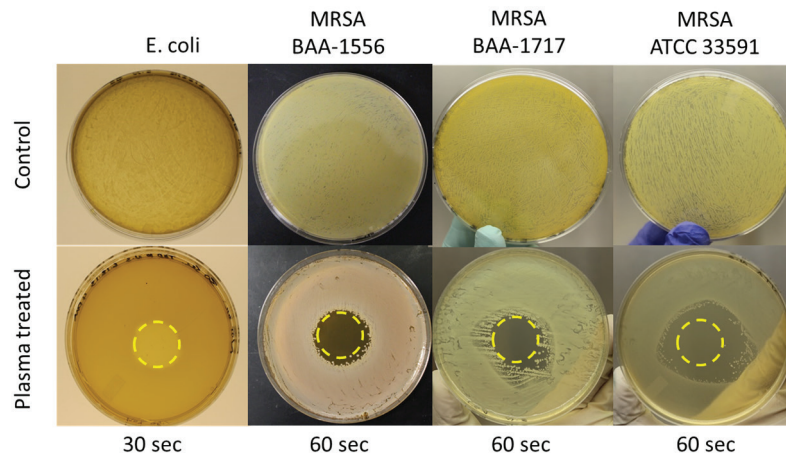
FIG. 2: Plates seeded with planktonic bacterial culture with the concentrations of ~10^8 CFU/mL creating a bacterial surface density of ~ 10^5 CFU/cm^2. Complete sterilization on the plasma-treated area (dashed circles) is achieved for *E. coli* after 30 seconds and for MRSA ATCC BAA-1556, MRSA ATCC BAA-1717, and MRSA ATCC 33591 after 60 seconds of plasma treatment
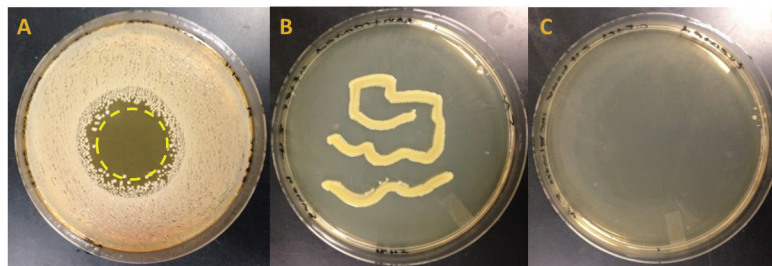

FIG. 3: A) MRSA (ATCC BAA-1556) culture on agar after treatment and 24 hours of post-treatment incubation. The plasma-treated area that (dashed circle) is completely clear. **B)** Untreated area was swabbed and streaked on a sterile agar plate. After 24 hours of post-treatment incubation, bacterial growth is present. **C)** Plasma treated area is swabbed and streaked on a sterile agar plate. No bacterial regrowth was observed after 3 days of post-treatment incubation.

in the treated colonies was less substantial than in the untreated colonies after 12 hours of incubation, and as treatment time increased, the level of growth in the colonies decreased, when measured after 24 hours of post-treatment incubation at 37ºC. Figure 6 shows the final diameter of the *E. coli* colonies incubated for 12 hours following various lengths of plasma treatment.
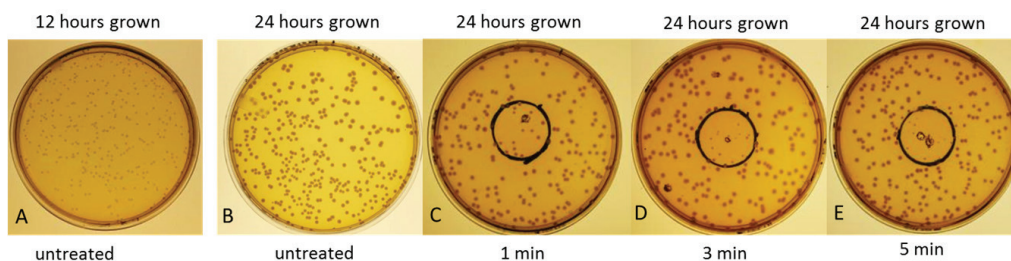
FIG. 4: A) *E. coli* colonies incubated for 12 hours. After colonies are grown for 12 hours, they are plasma treated for 1 to 5 minutes to observe the effect on the colony growth process. **B, C, D,E)** *E. coli* colonies after 1, 3, and 5 minutes of plasma treatment and an additional 12 hours of post-treatment incubation. The black circles show the plasma treatment area. Results demonstrated that the colonies in the plasma-treated area are smaller than untreated colonies that had gone through the normal growth process.
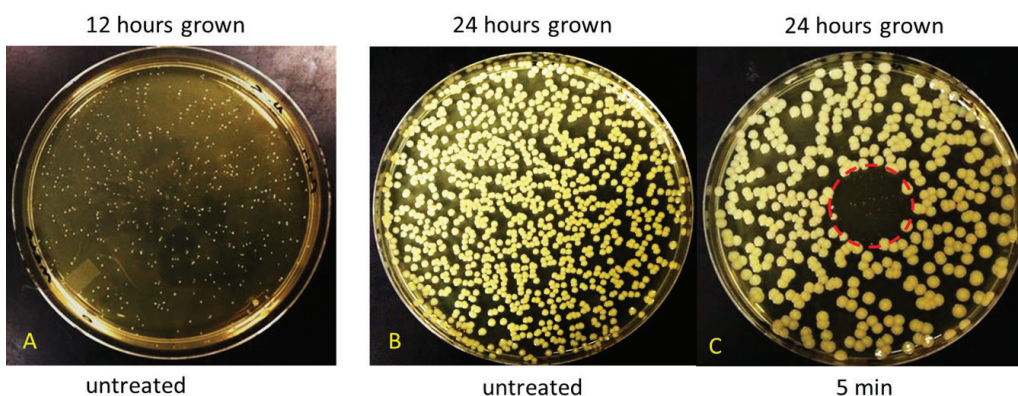

FIG. 5: A) MRSA colonies incubated for 12 hours. **B)** MRSA colonies after 5 minutes of plasma treatment and an additional 12 hours of pos-treatment incubation. The colonies in the plasma-treated area (dashed circles) are smaller than the colonies outside of the plasma-treated area.

1. Quantification of the Viable CFUs in Plasma-Treated Colonies

Colonies were treated on the plates for 1 to 5 minutes; the control was untreated. A portion of each sample was scraped off and transferred to 1 mL of fresh media. Serial dilution was performed and dilutions were plated. Colonies were counted after 24 hours, and the number of viable CFUs was calculated for each colony. Samples were prepared in triplicate; at least three colonies from each plate were removed and the number of CFUs in each was calculated. The results for *E. coli* and MRSA colonies are shown in Figures 7 and 8 respectively. The values are mean ± standard deviation of at least
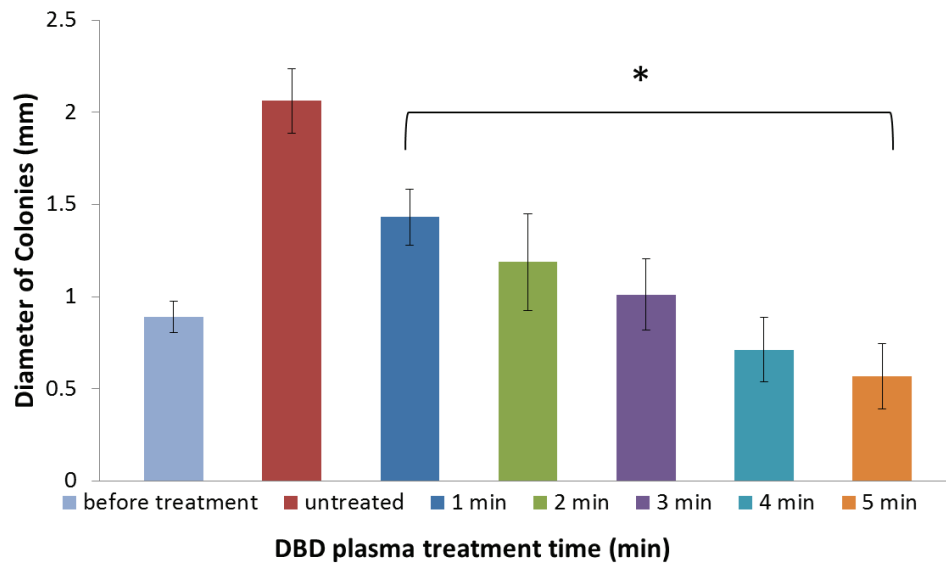
FIG. 6: The first bar represents the diameter of the colonies before plasma treatment. The rest of the bars show the final diameter of *E. coli* colonies after additional 12 Hours of post-treatment incubation. The results are expressed as mean ± standard deviation of at least 10 samples. Asterisks (*) denote statistical differences from the untreated group. ($P < 0.0001$ [*t*-test])
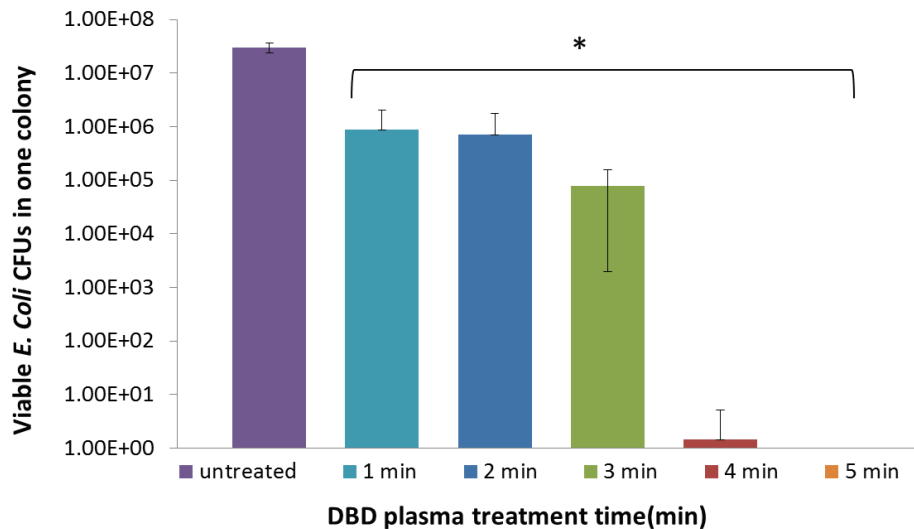

FIG. 7: Number of viable CFUs of *E. coli* in each colony after various treatment times. The results are expressed as mean ± standard deviation of at least 7 samples. **Asterisks (*)** denote statistical differences from the untreated group. ($P < 0.0001$(*t*-test))
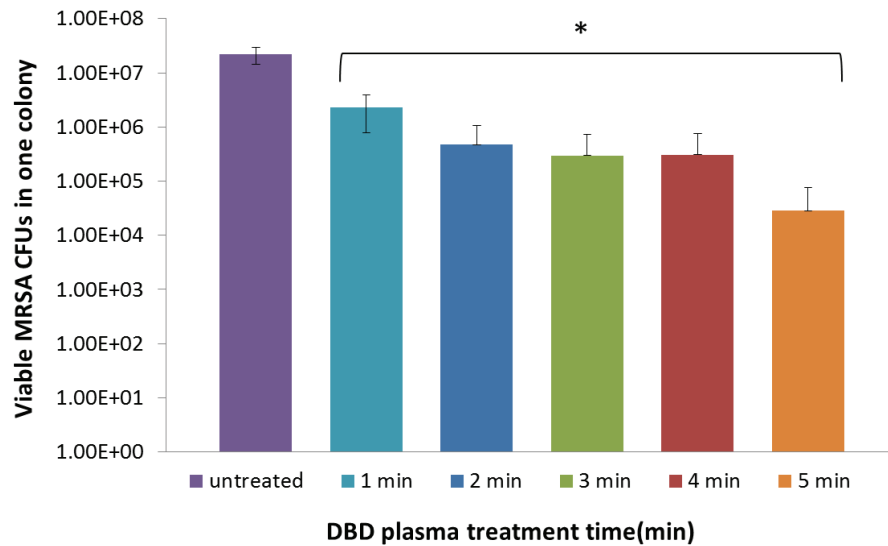
FIG. 8: Number of viable CFUs of MRSA in each colony after various treatment times. The results are expressed as mean ± standard deviation of at least 7 samples. Asterisks (*) denote statistical differences from the untreated group. (P < 0.0001 [*t*-test])

10 samples. Results show 7 $\log_{10}$ reductions for *E. coli* colonies after 5 minutes of plasma treatment. For MRSA colonies, 3 to 7 $\log_{10}$ reductions were observed after 5 minutes of plasma treatment. Longer treatment time is needed to achieve complete decontamination for MRSA colonies.

2. Assessment of Regrowth of Plasma Treated Colonies in Fresh Media

Colonies were cut and removed from the agar plate after treatment, transferred into 4 mL of liquid media, and incubated on a shaker platform at 37°C. The growth was monitored. The influence of the plasma treatment on the regrowth of treated *E. coli* and MRSA colonies is shown in Figures 9 and 10.

C. Penetration of DBD Plasma-Generated Nitric Oxide into Agarose Gel

The results of Griess assays on the plasma-treated area after different treatment times are shown in Figure 11. Color change indicates that nitric oxide diffused into the gel and that nitrite is present in the area. The nitric oxide diffusion area and color intensity are greater with increasing plasma treatment time, as shown in Figure 11 The nitric oxide diffusion area was measured using a threshold color plugin from the public domain software ImageJ (National Institutes of Health (NIH), USA). Images were converted to 8-bit grayscale, and their threshold was adjusted so that the colored area was distinct. The picture was then analyzed, and the diffusion change area was measured. In samples
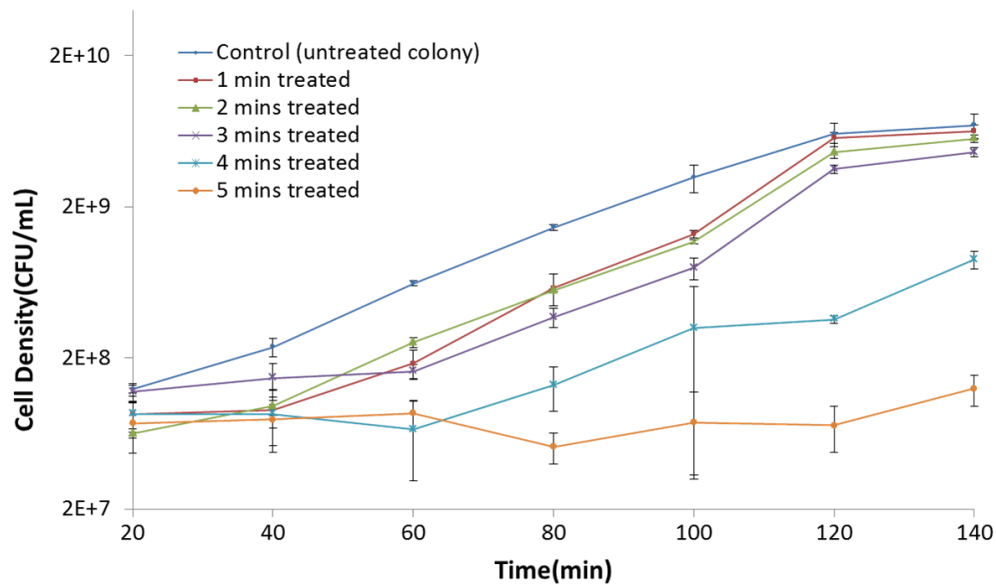
FIG. 9: Regrowth of *E. coli* Colonies in SB Media after Plasma Treatment. Treated colonies were transferred to fresh media, and the regrowth was monitored by reading the OD_{600} of the cultures every 20 minutes. Results are expressed as mean ± standard deviation of four samples.
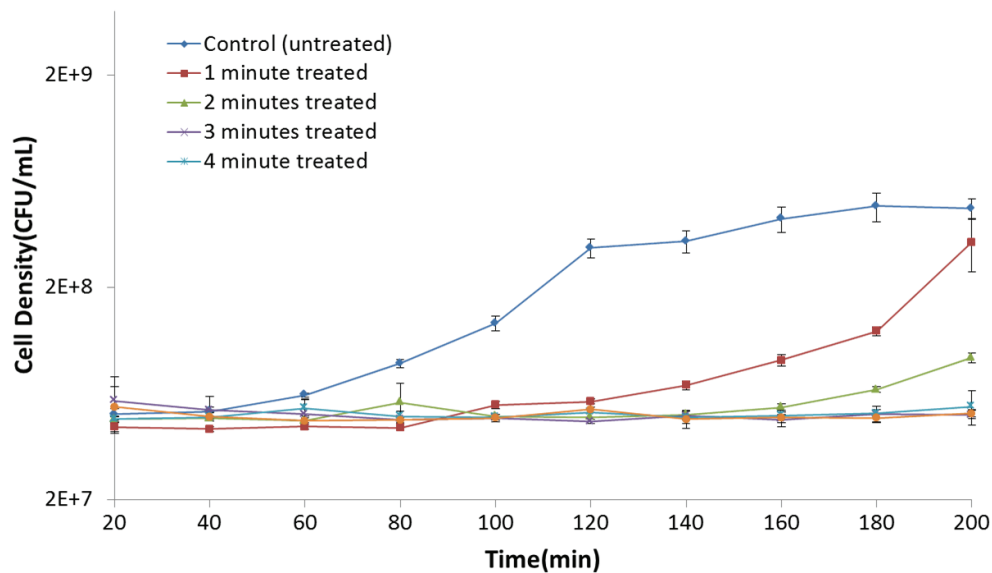

FIG. 10: Regrowth of MRSA Colonies in BHI Media after Plasma Treatment. Treated colonies were transferred to fresh media, and the regrowth was monitored by reading the OD_{600} of the cultures every 20 minutes. Results are expressed as mean ± standard deviation of four samples.
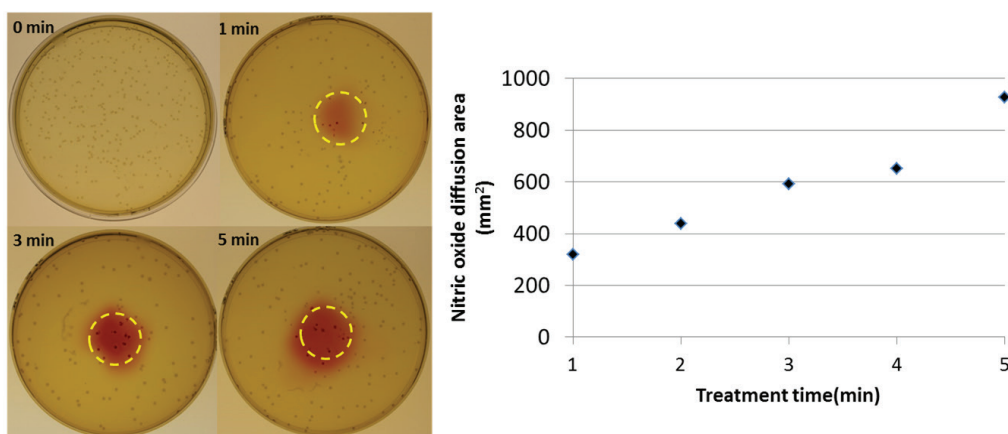
FIG. 11: Diffusion of nitric oxide in agarose gel is detected with the Griess assay after 1, 2, 3, 4, or 5 minutes of plasma treatment. The plasma-treated area is specified with the dashed circles. The diffusion area grows bigger with increasing the treatment time. The areas were measured using ImageJ software.

that were treated with plasma for 3 minutes, the diffusion area was larger than the area directly treated with plasma, which is equal to core electrode area.

IV. DISCUSSION

In this study, the decontamination efficacy of DBD plasma treatment on *E. coli* and MRSA was investigated in a their planktonic phase and in colonies. Results demonstrated that DBD plasma can kill antibiotic-resistant species.

Plasma is a cocktail of agents capable of decontamination. It contains a high concentration of reactive oxygen and nitrogen species, such as nitric oxide, hydrogen peroxide, and hydroxyl radicals.[28] In addition to the strong electric field between the closely spaced electrodes, two central mechanisms have been implicated in decontamination by plasma: the reactive species that are generated in the plasma can deactivate the bacterial cells either through direct physical bombardment of the membrane or by diffusing through the bacterial cell membrane and reacting with the membrane.[29,30]

The DBD plasma system was created and electrically characterized. Further the DBD plasma was used to treat *E. coli* and MRSA in the planktonic phase and in colonies for various treatment times. Results confirmed that DBD plasma treatment is decontaminating all the strains in planktonic phase and is damaging to the bacteria in colonies. Finally, we demonstrated that nitric oxide is diffusing more in the plasma-treated agar plates with increasing the treatment times.

The planktonic cultures of MRSA and *E. coli* were treated for various times by DBD plasma. After 60 seconds of plasma treatment of plates seeded with planktonic culture of all strains of MRSA (having the concentration of 10^8 CFU/mL) , complete decontamina-

tion on the plasma treated area was observed. The treated area had a bacterial surface density of 1.27×10^5 CFU/cm^2 (Figure 2). No bacterial growth occurred on the plasma-treated area after 24 hours of incubation. This happened for *E. coli* cells after 30 seconds of treatment (Figure 2). These results indicate that MRSA strains are more resistant to plasma treatment than *E. coli*. By decreasing the bacterial concentration and increasing the treatment time, the size of clear decontaminated area increases and will eventually expand beyond the size of the area that was directly treated with plasma, which is equal to the core electrode area. This suggests that the reactive species diffuse beyond the electrode area. Therefore, the effect of the reactive species in the decontamination process is chemical and not just related to the physical bombardment of the bacterial cells with ions or other charged particles. .

Bactericidal drugs kill bacteria directly, whereas bacteriostatic drugs simply inhibit bacterial growth. To test the bactericidal effect of the plasma treatment, plasma-treated bacterial cultures were transferred from the plasma-treated area that is decontaminated and clear, to a new agar plate. This would permit growth of any bacteria still present in the absence of antibacterial agents introduced to the agar plate following the plasma treatment. The agar plates were monitored for a few days, and no bacterial growth was observed (Figure 3). This suggests that the plasma treatment may have a bactericidal effect on the bacterial cells during the decontamination process.

The minimum time needed to completely inactivate MRSA in planktonic phase was 60 seconds, so this time was chosen as the minimum time to treat the colonies. Results demonstrated that longer treatment times are required to decontaminate the bacterial cells in colonies than in planktonic phase. This might occur because cell densities are higher in colonies. In addition, bacterial cells in colonies are encased in a polysaccharide matrix and as a result may be more resistant to the treatment. Colonies were treated for 1, 2, 3, 4, or 5 minutes. For all the treatment times, the plasma-treated *E. coli* colonies exhibited less growth at 24 hours after plasma treatment compared to untreated colonies (Figure 4). Also, the final diameter of the plasma-treated MRSA colonies at 24 hours post-treatment incubation are less than the diameter of untreated colonies (Figure 5).The final diameter of the *E. coli* colonies incubated for an additional 12 hours post–plasma treatment decreased as the treatment time increased (Figure 6). The diameter of the *E. coli* colonies before treatment was 0.88 ± 0.13 mm, which is slightly greater than the diameter of colonies after 5 minutes of treatment followed by 12-hour post-treatment incubation. This could represent some level of shrinkage in colonies due to bacterial cell damage after 5 minutes of plasma treatment.

Results demonstrated that plasma treatment significantly reduced the number of viable CFUs in colonies after 1 minute of plasma treatment (Figures 7 and 8), and monitoring the regrowth of treated colonies in liquid media justifies this finding. After quantification of the number of viable CFUs in plasma treated colonies, results indicate that for *E. coli* colonies 4 and 5 minutes of plasma treatment is resulting in 7 $\log_{10}$ reductions in all the colonies in the plasma treated area (Figure 7). For MRSA colonies more treatment time is needed for decontamination of all the colonies in the plasma treated

area as the colonies show more resistance to the plasma treatment compare to *E. coli* colonies. The results show a range of 2–7 $\log_{10}$ reduction in the number of viable CFUs from the areas treated with plasma for 5 minutes (Figure 8). This means that the effect of plasma treatment is not distributed uniformly on all the colonies, and longer treatments are needed to induce equal levels of decontamination in all the MRSA colonies in the plasma-treated area.

The length of the lag phase is dependent on a variety of factors, including change in physiology, nutrients, and time to recover from shock in the transfer, which we assume should be present for all the transferred colonies.[31] Time needed for recovery from damage and the size of the inoculum, which means the number of cells in each colony that are dividing normally, are two other important factors influencing the length of the lag phase.[32] Growth curves for colonies treated for various times are showing that colonies treated for longer times have a longer lag phase, which could mean that more damage is induced in the colonies with increasing treatment times. No regrowth of 4- and 5-minute treated *E. coli* colonies is observed in fresh media after 200 minutes incubation on a shaker platform at 37°C (Figure 9). However, monitoring the regrowth of MRSA colonies in fresh media for 140 minutes confirmed that extended treatment time is required to deactivate all the CFUs in MRSA colonies (Figure 10).

With longer treatment times, reactive species can reach beyond the plasma-treated area in gaseous phase as well as penetrate more into the agar gel along the surface and at deeper levels. Figure 11 represents the increasing diffusion of nitric oxide in the agar as treatment time increases. It can be seen that the nitric oxide diffusion area becomes broader as treatment time increases. This indicates that with increased treatment time, more nitric oxide penetrates into the solid agar, and this can increase the area of decontamination.

V. CONCLUSION

Antibiotic resistance in *S. aureus*, one of the most common musculoskeletal pathogens, is a serious problem. New treatment methods are needed to combat these pathogens. The effect of cold plasma treatment on the decontamination of MRSA and *E. coli* in planktonic cultures and colonies was studied. Cold plasma treatment is a bactericidal treatment and is an effective way for inactivating *E. coli* and MRSA strains ATCC BAA-1556, ATCC BAA-1717, and ATCC 33591 in planktonic phase and in colonies. Complete sterilization occurs after 60 seconds for all the strains in planktonic phase. Longer treatment times are needed to observe a uniform distribution of treatment effect for colonies on the plasma-treated area. For a given strain, longer treatment time is needed to completely inactivate bacteria in colonies than in planktonic phase. With longer treatment times, the reactive species, such as nitric oxide, diffuse more into the agar and can widen the area of decontamination.

VI. ACKNOWLEDGMENT

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