Control of methicillin-resistant *Staphylococcus aureus* in planktonic form and biofilms: A biocidal efficacy study of nonthermal dielectric-barrier discharge plasma

Suresh G. Joshi, MD, PhD,[a,b] Michelle Paff, BS,[a] Gary Friedman, PhD,[b] Greg Fridman, PhD,[b,c] Alexander Fridman, PhD,[b] and Ari D. Brooks, MD[a]
Philadelphia, Pennsylvania

Background: Bacterial contamination of surfaces with methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious problem in the hospital environment and is responsible for significant nosocomial infections. The pathogenic contaminants form biofilms, which are difficult to treat with routine biocides. Thus, a continuous search for novel disinfection methods is essential for effective infection control measures. This demonstration of a novel technique for the control of virulent pathogens in planktonic form as well as in established biofilms may provide a progressive alternative to standard methodology.

Methods: We evaluated a novel technique of normal atmospheric nonthermal plasma known as *floating-electrode dielectric-barrier discharge* (FE-DBD) plasma against a control of planktonic and biofilm forms of *Escherichia coli*, *S aureus*, multidrug-resistant methicillin-resistant *S aureus* (MRSA) -95 (clinical isolate), -USA300, and -USA400, using widely accepted techniques such as colony count assay, LIVE/DEAD BacLight Bacterial Viability assay, and XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay.

Results: Exposure of free living planktonic forms of *E coli*, *S aureus*, and MRSA were rapidly inactivated by DBD plasma. Approximately 10^7 bacterial cells were completely (100%) killed, whereas 10^8 and 10^9 were reduced by approximately 90% to 95% and 40% to 45%, respectively, in less than 60 seconds (7.8 J/cm^2) and completely disinfected in ≤120 seconds. In established biofilms, the susceptibility of MRSA USA400 was comparable with USA300 but less susceptible than MRSA95 (clinical isolate), *S aureus*, and *E coli* ($P < .05$) to FE-DBD plasma, and plasma was able to kill MRSA more than 60% within 15 seconds (1.95 J/cm^2). The killing responses were plasma exposure-time dependent, and cell density dependent. The plasma was able disinfect surfaces in a less than 120 seconds.

Conclusion: Application of DBD plasma can be a valuable decontamination technique for the removal of planktonic and biofilm-embedded bacteria such as MRSA -USA 300, -USA 400, methicillin-sensitive *S aureus* (MSSA), and *E coli*, the more common hospital contaminants. Of interest, *E coli* was more resistant than *S aureus* phenotypes.

Key Words: Dielectric-Barrier Discharge plasma; dielectric barrier discharge; nonthermal plasma; *Escherichia coli*, *Staphylococcus aureus*; methicillin-resistant *S aureus*; MRSA; infection control.

Copyright © 2010 by the Association for Professionals in Infection Control and Epidemiology, Inc. Published by Elsevier Inc. All rights reserved. (Am J Infect Control 2010;38:293-301.)

From the Surgical Infections Program, Department of Surgery, College of Medicine,[a] Division of Plasma Biology & Medicine, A.J. Drexel Plasma Institute,[b] and School of Biomedical Engineering, Science, & Health Systems,[c] Drexel University, Philadelphia, PA.

Address correspondence to Suresh G. Joshi, MD, PhD, Program on Surgical Infections Research, Department of Surgery, Drexel University College of Medicine, 245 N 15th St, Mail 413, NCB-7150, Philadelphia, PA 19102. E-mail: Suresh.Joshi@Drexelmed.edu.

Supported through the award from Defense Advanced Research Projects Agency (DARPA), Department of Defense of the United States Government, and the Telemedicine and Advanced Technology Research Center (TATRC).

Conflicts of interest: None to report.

0196-6553/$36.00

Copyright © 2010 by the Association for Professionals in Infection Control and Epidemiology, Inc. Published by Elsevier Inc. All rights reserved.

doi:10.1016/j.ajic.2009.11.002

Hospital-acquired infection is major challenge for clinicians today, and contamination of hospital environments in one way or other is one of the most prominent reasons. In the United States, out of 2 million annual nosocomial infections, approximately 50% of the patients suffer from nosocomial infection with drug-resistant bacteria, of which methicillin-resistant *Staphylococcus aureus* (MRSA) is a major component every year. The overall annual direct medical cost of such infections to US hospitals ranges from $35.7 to $45 billion (after adjusting to 2007 dollars using the consumer price index adjustments for inpatient hospital services), and, after adjusting the range of effectiveness of possible infection control interventions, the benefit of prevention ranges from $25.0 to $31.5 billion (approximately 70% of infections preventable for inpatient hospital services).[1,2] It is evident that opportunistic pathogens arriving in their planktonic forms

contaminate inanimate and animate surfaces by forming mature biofilms.[3,4] Biofilm is not just a secured shelter but a defense mechanism and a nutrition depot for pathogens. Thus, it comprises a functional association of cells developed within an extracellular polysaccharide biomatrix and allows organisms to persist in an area for a longer time with a slower growth rate, in addition to providing mechanical protection and nutrients.[3-5]

Among the nosocomial pathogens, 2 are of particular interest because of their high prevalence in the hospital environment. These are *S aureus* and *Escherichia coli*. MRSA is a leading cause of nosocomial bacteremia in the United States.[6] As per current reports, the MRSA strains USA300 and USA400 are particularly resistant to traditional biocides when present within biofilms.[5,6] Biofilm-embedded organisms are most likely responsible for medical device-related infections, skin and soft tissue infections, surgical site infections, catheter-associated bacteremia and urinary tract infections, pneumonia, and prosthetic implant-associated infections.[7] MRSA embedded in biofilms shows different phenotypic responses than their planktonic counterpart, and this property is found to be associated with virulence. A recent report showed that powerful biocides used in hospitals were not able to decontaminate efficiently, and approximately 3% to 80% of the pathogens survived in biofilms.[5] In addition, bacterial contamination of the surfaces of hospital objects is also responsible for colonization of health care workers and leads to transmission of nosocomial infection by indirect mechanisms.[8,9] Therefore, effective disinfection of potentially contaminated surfaces is recommended. Thus, there is a desperate need to control contamination of surfaces, medical equipment, and invasive devices and to find a suitable and powerful method for infection control that will eradicate or strategically limit these reservoirs of infection.

The dielectric-barrier discharge (DBD) plasma generating probe was designed by our collaborating authors at the A.J. Drexel Plasma Institute (Philadelphia, PA).[10] The apparatus generates microsecond-duration, high-voltage pulsed cold plasma between the quartz surface covered electrode and the sample surface, which serves as a second electrode. The high-voltage electrode is completely covered with a dielectric barrier, which makes it safe for sterilization applications, and the nature of the applied microsecond pulses do not elevate the surface temperature above 38°C to 40°C. Our earlier findings demonstrated that this floating-electrode dielectric-barrier discharge (FE-DBD) plasma technique can be safely applied to delicate surfaces and to animal skin, such as mouse and rat.[10] Plasma generates many charged and neutral active species, which are responsible for plasma's biocidal activity. The most prominent active species in plasma—which will interest bacteriologists—are ozone, nitric oxide, superoxide, hydrogen peroxide, singlet oxygen, OH-radicals, ultraviolet, electrons, and other charged species.[11] Considering the properties of these species, an antimicrobial effect can be expected. Indeed, thermal plasma is used to generate these active species, which are then blown onto a surface for sterilization in commercial use. Our preliminary findings on in vitro susceptibilities of *E coli* to direct exposure to nonthermal FE-DBD plasma also demonstrated that this technique may prove ideal for surface decontamination.[12] These findings led us to study the responses of *E coli*, and *S aureus* and its virulent phenotypes such as MRSA -USA300 and -USA400 (the common nosocomial contaminants) in their planktonic as well as established biofilm forms to this nonthermal plasma. In this study, we evaluated the efficacy of the FE-DBD plasma technique and its direct and indirect methods of application against both forms of bacteria, as well as the surface. Most widely accepted evaluation techniques such as colony count assay, LIVE/DEAD bacterial viability assay, safranin microtiter plate assay, and XTT assay are used to determine the percentage of viable cells and biofilm quantification.

MATERIALS AND METHODS

Culture and isolates of bacterial pathogens

E coli, *S aureus* (also referred to as *methicillin-sensitive S aureus* [MSSA], occasionally), MRSA USA300 (BAA-1680), and MRSA USA400 (BAA-1683) strains were purchased from American Type Culture Collection (ATCC; Manassas, VA). Clinical isolates of MRSA (referred to as *MRSA95*) were isolated from Hahnemann University Hospital's clinical laboratory from patients with skin and soft tissue infection and identified by API and VITEK 2 automatic systems (BioMérieux, Inc., Durham, NC). All strains were maintained and used as overnight cultures in trypticase soy broth (TSB) for primary inoculations. Hydrogen peroxide or 70% ethyl alcohol was used as known biocide agents and either TSB alone or phosphate-buffered saline (PBS) alone as negative controls, as appropriate.

DBD plasma generating device, parameters, and conditions

The DBD plasma generating probe was designed by our collaborating authors at Drexel Plasma Institute.[10] A FE-DBD plasma, which is generated just in room air (no separate gases are used or added) at normal atmospheric conditions, is cold to touch. Here, the first electrode is a dielectric protected powered electrode, and the second electrode is a sample-carrying surface.

The second electrode is not grounded and remains floating, having potential to ignite discharge plasma when the powered (first) electrode approaches the surface to be treated. The diameter of plasma generating probe was 25 mm (sufficient for 15-mm diameter area under treatment). A low-frequency alternating current (120 V) is generated, and the desired output voltage and frequency (hertz) can be obtained through a step transformer across the interface. A typical plasma power of 0.13 W/cm^2 was used for variable times of exposures to deposit desired amount of plasma energy (joule/square centimeter) to the sample to be treated. The micropulse mode has fair uniformity, and, therefore, uniform deposition of plasma energy is possible. The application of direct plasma here implies directly exposing a biologic sample or article-surface of interest to plasma being discharged, and there is just an air column of ~3 mm between the first electrode and the sample. The concept of indirect plasma treatment is the one wherein 100 μL of fluid of interest, eg, PBS (150 mmol/L sodium chloride and 150 mmol/L sodium phosphate, pH 7.2, at 25°C), is exposed to plasma being discharged, and 80 μL of treated PBS immediately applied (no delay time) to the surface or suspension of interest. All bacterial samples were either directly exposed to cold plasma discharge or PBS-mediated plasma energy was applied to bacteria-contained samples/surfaces. Upon application, plasma-generated short-lived and long-lived species exert their effects on bacteria. The plasma energy (joule/square centimeter) levels that were used in the present research (otherwise stated) are as follows: 0 (0 seconds), 0.39 (3 seconds), 0.78 (6 seconds), 1.56 (12 seconds), 1.95 (15 seconds), 3.12 (24 seconds), 3.9 (30 seconds), 7.8 (60 seconds), 11.7 (90 seconds), 15.6 (120 seconds), and 19.5 (150 seconds), and others.

Plasma bactericidal activity and planktonic forms

The cultures of interest were inoculated as 10 μL primary cultures into 10 mL TSB medium and incubated at 37°C for 24 hours at 180 rpm and then appropriately diluted to obtain the desired number of cells (colony-forming units (CFU)/milliliters). Concurrently, the optical densities at 580 nm and colony count assays were performed in parallel to select the desired cell densities. In some of the early experiments, overnight cultures were used to prepare bacterial lawns on trypticase soy agar (TSA) plates, and, after plasma treatment or nontreatment, plates were incubated at 37°C up to 48 hours and observed periodically at 18 hours, 24 hours, and 48 hours for zone of inhibition of growth. For colony count assays, 50 μL of cell suspension (in PBS) of the given cell densities were used to expose to plasma treatments and then appropriately diluted to spread on TSA plates. For indirect plasma treatments, 25 μL of PBS was exposed to plasma and then immediately mixed with 25 μL of 2X cell suspension (to have final 1X cell suspension in PBS), held for 5 minutes before serial dilution for colony count. The plates were observed periodically as above, and colonies were counted to calculate total CFU/milliliter.

Plasma-mediated inactivation of pathogens on dry surfaces

A known amount of cell suspension of *E coli* or *S aureus* was inoculated as 20 μL drop on glass surface, spread in a defined area (15-mm diameter concavity or monocavity glass slides; Electron Microscopy Sciences, Hatfield, PA), and allowed to dry on the surface before treatment by exposure to plasma over time. Upon treatment, the bacterial cells were harvested by applying 50 μL PBS to get a homogenous suspension, and the suspension was appropriately diluted to process for colony count assay. An air-dried sample that was not plasma treated served as a positive control, and values were normalized to the controls to calculate net percent surviving cells.

Bacterial pathogens as biofilms

Biofilms were developed for *E coli*, *S aureus* (MSSA), MRSA95, MRSA -USA300 and -USA400, essentially as described.[5,7] For direct plasma application, biofilms on sterile 15-mm circular glass coverslips (in 12-well plates) were developed and, for indirect application, in sterile 96-well plates (all Costar, Corning Inc., Corning, NY). In brief, an overnight culture was diluted to 1:100 with sterile TSB, and 200 μL of suspension was applied to either wells containing a coverslip or to 96-well plates, in triplicates to incubate for 24 hours at 37°C without shaking. The next day, fluid from the biofilm containing coverslips or 96-well plates was gently aspirated and washed 3 times with PBS and either left untreated or treated with plasma by direct or indirect application. In the direct plasma application experiment, the coverslip was held under discharge plasma for a given time, and the coverslip-biofilm was washed 2 times with PBS to remove any planktonic form or loosely attached pathogen before appropriate staining or detection of metabolic marker. For indirect plasma application experiments, the 96-well plates bearing biofilms were washed 3 times with PBS before treatment and 2 times posttreatment and then used for appropriate staining or detection of metabolic activity.

Safranin assay

Biofilms developed in a 96-well plate were washed as above and dried briefly by holding at 37°C (20 minutes) to stain by safranin microtiter plate method.[13] The biofilms were stained with 200 μL of 0.1% aqueous solution of safranin for 15 minutes. The excess stain was removed by washing 3 times with PBS. After addition of 70% ethanol, the biofilm-containing wells were held at room temperature for 15 minutes. The plate was read in microtiter plate reader (Synergy; BioTek, Winooski, VT) after brief shaking (3 seconds) at 550 nm. The well containing TSB alone plus safranin was used as negative control to normalized readings. The safranin assay is used for semiquantification of biofilms and is often the test used for detection of biofilms in clinical isolates.

Quantification of viable cells in the biofilm by LIVE/DEAD assay

The LIVE/DEAD BacLight bacterial viability assay kit is routinely used for detection of efficacy of bactericidal agents, and quantification of biofilms.[5] Biofilms were established and plasma treated as above before staining by BacLight bacterial viability kit (Molecular Probes, Invitrogen, Carlsbad, CA), as recommended by the manufacturer. To prepare a fresh working solution of stain, 1.5 μL of solution A (SYTO9 dye) and 1.5 μL of solution B (propidium iodide) were added to 997 μL of sterile deionized water and, after mixing thoroughly, 700 μL was added to each well carrying biofilm on a glass coverslip in the dark. The plates were incubated in darkness at room temperature for 15 minutes. Each coverslip was inverted and mounted using antifade mounting medium on microscopic slide and viewed using a Leica DMRX fluorescence microscope (Germany) with attached Leica DG300FX digital camera system, using fluorescein and Texas red bandpass filters (for corresponding SYTO9 green dye and propidium iodide). The images were captured from 5 randomly selected areas for 3 different sets of experiments, saved as TIFF file and edited using Adobe Photoshop CS3 (Adobe Inc, San Jose, CA) using the ''ImageJ'' (NIH, Bethesda, MD) program to calculate mean number of green and red pixels in each area.

Quantification of viable cells by XTT assay

For each assay, fresh XTT reagent solutions were prepared as described earlier.[14] From the aliquots, 0.5 mg XTT (Molecular Probes) and 1 μmol/L Menadione (Sigma Chemical Co, St. Louis, MO) working solution was made up in 1X PBS. After appropriate treatment or no treatment, the microtubes containing samples of planktonic form of pathogen were spun at 8000 rpm/6 minutes, and supernatant was discarded. The cells were resuspended in 200 μL of XTT reagent, mixed thoroughly, and tubes incubated at 37°C/2 hours. After centrifugation, the supernatant (100 μL) containing orange-colored XTT metabolic product was measured by reading absorption at 492 nm using a microtiter plate reader (Synergy Mx; BioTek). The readings were normalized, and percent surviving cells were calculated against untreated samples. Similarly, the XTT assay for biofilms was carried out either for coverslips placed in 12-well plates (700 μL reagent in each well) or 96-well plates (200 μL for each well), and no spinning was involved.

Statistical analysis

The data from the experiments were analyzed using Prism software 4.03 for Windows (GraphPad, San Diego, CA), and standard deviations were calculated from minimum 3 sets of experiments. All the experiments were repeated in triplicate. The *P* value was derived using pair comparisons between 2 bacterial groups using Student *t* test and 1-way analysis of variance for multiple comparisons wherever applicable (*indicates *P* value as a statistically significant [$P < .05$]).

RESULTS

Plasma rapidly kills bacteria: A proof of concept

Our earlier research established a novel working technique of FE-DBD plasma (plasma) and demonstrated that the plasma generated by this device is nonthermal, normal atmospheric, safe to animal and human skin, and absolutely easy to apply on surfaces.[10,11] The purpose of the present study was therefore to evaluate the potential of plasma to inactivate bacterial pathogens, such *E coli* and *S aureus*, and virulent strains such as MRSA USA300 or USA400 and achieve disinfection or sterilization of contaminated surfaces. During our preliminary experimental data analysis, we realized that plasma inactivates *E coli* and *S aureus* when present on agar surface and develops a zone of inhibition of growth. When fluid such as PBS (a physiologic solution widely used in cellular and microbiologic studies) was exposed to plasma discharge, it appears able to capture active species produced in plasma or forms products out of reactive species and application to bacterial lawns (a heavy bacterial load) on agar resulted in the development of a clear zone of inhibition. The area of zones of inhibition was proportionate to plasma exposure time (amount of net energy deposition) (data not shown because of space constraints). The findings encouraged us to undertake concurrent PBS-mediated (indirect) plasma application.

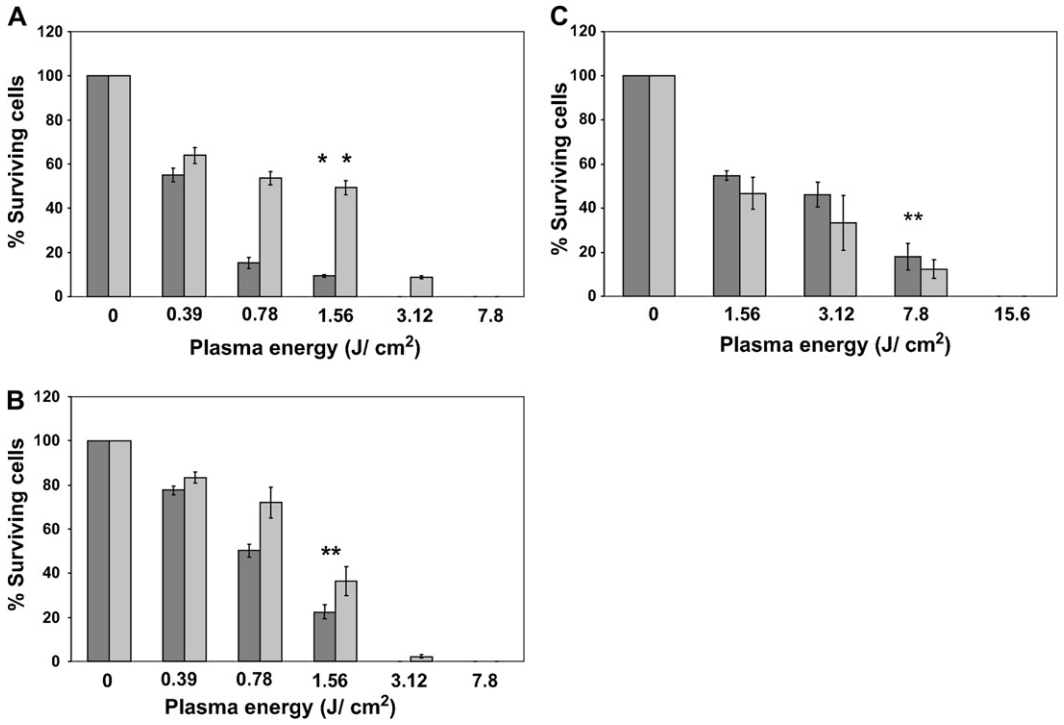


Fig 1. The colony assays demonstrating a rapid inactivation of *E coli* and *S aureus* (at 10^7 CFU/mL), by both the direct (A) and indirect (B) plasma application. The killing effect was plasma exposure time dependent in both the cases. A highly significant inactivation was observed in less than 60 seconds (7.8 J/cm^2). (C) Sterilization of *E coli*- and *S aureus*-contaminated glass surfaces is possible by exposure to plasma. Approximately 10^9 CFU/mL of bacteria was dried over glass surface and immediately exposed to plasma over time. In <120 seconds, both the contaminants were completely inactivated. Darkly shaded column indicates *E coli*; lightly shaded column indicates *S aureus*; bar, standard deviation; *indicates statistically significant ($P < .05$) inactivation as compared with the corresponding plasma nontreated cells.

Dose-dependent responses of planktonic cultures and surface contaminants to nonthermal plasma

A colony count assay is a commonly used quantification assay in bacteriology to demonstrate inactivation of bacterial pathogens. We compared the efficacy of plasma applications on representative bacteria (1 each from gram-positive and gram-negative groups). The findings of the colony count assay were highly reproducible when carried in 3 to 5 sets, each in triplicate. Figure 1A and B demonstrates that comparatively more inactivation is observed upon direct plasma application than using the indirect route ($P < .05$ for 6 seconds onwards). In both the direct and indirect plasma application, *E coli* was significantly more susceptible as compared with *S aureus* (which took little more plasma energy for comparable inactivation ([$P < .05$]). Sterilization of both the organisms (at $\sim 10^7$ CFU/mL) was observed in 60 seconds.

Inactivation of planktonic forms is not equivalent to sterilization of heavily contaminated tough dry surfaces. Surface contamination by nosocomial pathogens is a common problem in the hospital environment. Therefore, in vitro experiments were undertaken wherein a heavy load of bacterial pathogens (1×10^9 CFU/mL) was applied to a glass surfaces, and, upon drying, plasma was applied either directly or indirectly (PBS mediated). Figure 1C shows increasing inactivation of *E coli* and *S aureus* in less than 120 seconds ($P < .05$). After treatment, the surface was sterile as detected by colony assay. The values (in Fig 1C) are normalized to the degree of natural inactivation by drying effect (which was ~50% of initial inoculums). The decontamination was proportional to the duration of plasma exposure. *S aureus* was slightly more susceptible to plasma when treated on a dry surface. The pathogens took between 24 hours to 48 hours to grow classical colonies after plasma treatment, and dormancy was ruled out by continued incubation of plates up to 3 days.

Time to plasma-mediated sterilization is proportional to bacterial load: The cell density-dependent responses

To find out whether the time required for sterilization is proportional to the amount of bacterial load,

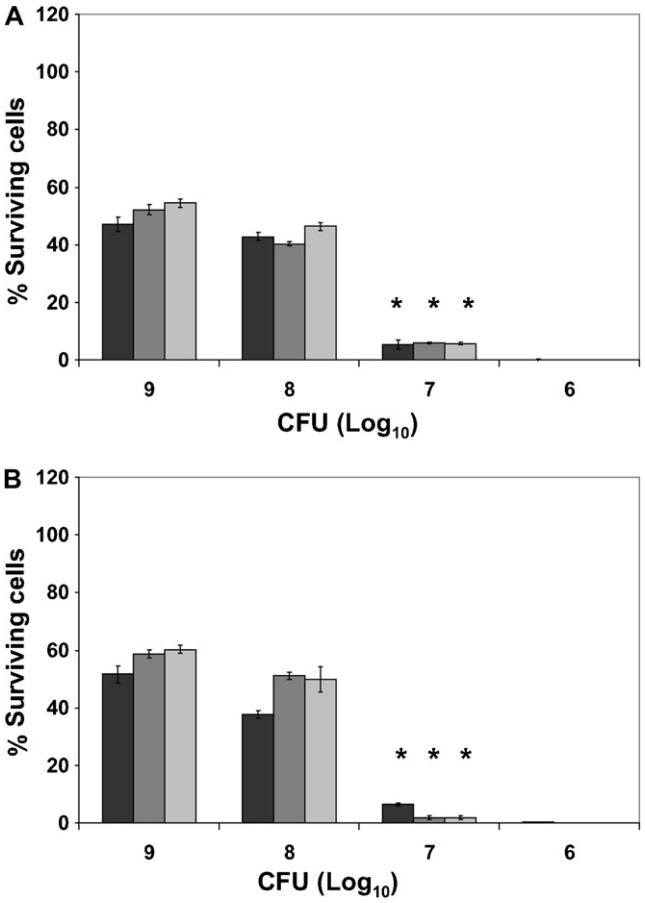


Fig 2. XTT assays demonstrating bacterial biomass-dependent responses towards direct (A) and indirect (B) plasma treatments. The planktonic cultures (10^6 to 10^9 CFU/mL) were exposed to 3.9 J/cm^2 (30 seconds) of plasma energy. Bar, standard deviation; *, $P < .05$ for given cell concentration as compared with plasma untreated cells; Solid column, *E coli*; darkly shaded column, *S aureus*; lightly shaded column, MRSA95.

we performed a series of experiments for both the direct and indirect plasma applications. We used 10^9, 10^8, 10^7, and 10^6 CFU/mL to evaluate plasma's antibacterial efficacy. The inactivation process took a longer time for all the organisms, viz. *E coli*, *S aureus*, and MRSA95 at higher loads (10^9 CFU/mL) versus sterilization at 10^6 CFU/mL when exposed for 30 seconds to either direct or indirect plasma. Figure 2 indicates that plasma can be safely applied to achieve 100% disinfection of these organisms (10^6 CFU/mL), including multidrug-resistant MRSA isolates in less than 30 seconds.

Biofilm producers, biofilms, and their response to plasma treatment

The organisms were tested for their biofilm formation ability, and biofilm formers were selected to test the effect of plasma on their biofilms. Figure 3A is representative safranin assay, which demonstrates that all the organisms, viz. *E coli*, *S aureus*, MRSA95, and USA300 and USA400, are excellent biofilm producers. Therefore, we developed biofilms for subsequent studies. A LIVE/DEAD assay using BacLight bacterial viability kit is a commonly accepted biofilm quantification method that detects and differentiates viable from nonviable bacteria.[5,15] Dying or near dead cells lose their membrane potential and develop a breach in continuity of the membrane that allows a cell impermeable dye (propidium iodide component) to get inside cells where it binds to DNA and makes cells fluoresce red. Figure 3B includes the representative microphotographs showing the increasing amount of dead/dying cells upon proportionate plasma exposure. Also seen is a micrograph of 70% ethanol-treated biofilm as control (+). The amount of dead cells was quantified as mentioned in the Materials and Methods section (data not shown) and found similar time-kill patterns as those seen with the XTT assay, showing decreasing percent of surviving cells as plasma exposure time increases (see following section).

Plasma potential of sterilization of MRSA -USA300 and -USA400, in both planktonic and embedded biofilms forms

Most nosocomial infections by gram-positive organisms are mainly due to the methicillin-resistant phenotypes from clonal lineages of MRSA USA300 and USA400 in the United States.[16] We therefore selected a multidrug-resistant clinical MRSA isolate (MRSA95) and the most widely reported virulent biofilm-producing strains, the USA300 and USA400, for their comparative responses in the midst of biofilms. Figure 4 demonstrates the percent survival for cells of all 3 MRSA strains tested in this study during plasma exposure. For comparison, we included the biofilm-embedded *E coli* and *S aureus* (MSSA) strains. XTT assay is a gold standard to demonstrate viable cells. XTT assay findings from Fig 4 show that *E coli* in biofilm form offers more resistance to plasma inactivation than *S aureus* and USA400 and USA300 at various plasma energy levels. MRSA USA400 was slightly more resistant than MRSA USA300 in biofilm forms when plasma was applied directly (Fig 4A) or indirectly (Fig 4B). We also observed that direct plasma had more pronounced killing effect than indirect, but, nevertheless, both forms of plasma application sterilized all biofilm forms of all the pathogens (all, $P < .05$) in less than 120 seconds (15.6 J/cm^2). We found that the biofilms that are resistant to many of the powerful biocides were totally disinfected in a short time (<120 seconds/<15.6 J/cm^2). When we tested planktonic counterparts (Fig 4C) of MRSA

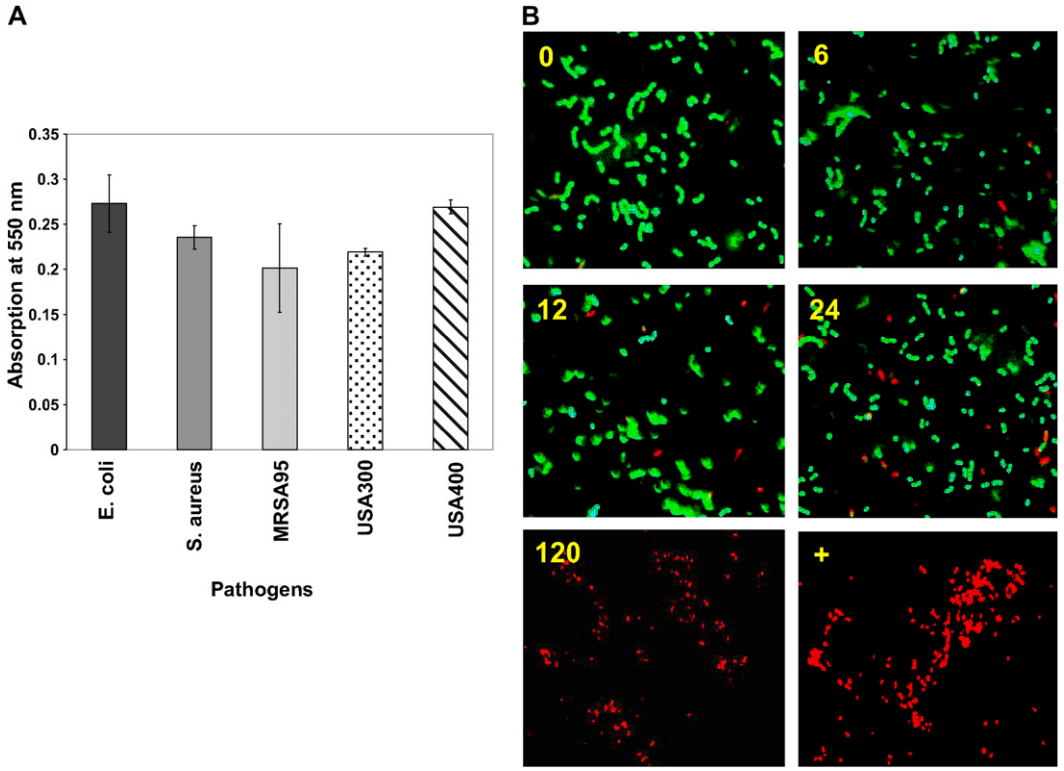


Fig 3. Quantification of biofilms. (A) Safranin microtiter plate assays showing stronger biofilm formation positivity by all study isolates by end of 24 hours (see under Materials and Methods for detail). (B) LIVE/DEAD Bacterial Viability assay showing decrease in MRSA95 viability upon indirect plasma application (number in photograph indicate plasma exposure time in seconds). Seventy percent ethanol treated biofilm (+) is seen as positive control. These assays were carried out for all study pathogens, and a similar pattern was seen, and the microphotographs are representative. Bar, standard deviation.

USA300 and USA400 (1×10^8 CFU/mL), we noticed relatively more resistance, and sterilization took approximately 150 seconds 19.5 J/cm^2) at the same settings (~30 seconds more exposure or 3.9 J/cm^2 more plasma energy).

DISCUSSION

These studies represent the first in-depth evaluation of the efficacy of nonthermal FE-DBD plasma for treatment of classical clinical pathogen, both in planktonic and biofilm forms. There are reports on bacterial responses to nonthermal plasma that demonstrated responses of saprophytic organisms[17] and oral commensals.[18] The type of plasma used in this report (FE-DBD) and effects on virulent pathogens such as MRSA have not been demonstrated previously. In addition, we present a new method of application of DBD plasma (ie, indirect method, mediated through fluid).

Biofilm-mediated infections in the hospital environment have a negative impact on public health and are responsible for a huge financial burden on health care services.[1,2,5] The contamination of either inanimate or animate surfaces eventually leads to biofilm development if left untreated or unclean and becomes a risk factor. The fact that bacterial biofilms are responsible for more than 60% of total nosocomial infections is an indication that we need newer techniques to control these noxious biofilm producers.[7] These pathogens survive for a longer time embedded in a biofilm and grow with slower rates, and their metabolic and antimicrobial responses are often different from their planktonic counterparts.[19] Biofilm formation properties have been correlated to their virulence.[20] These organisms are expected to be more dangerous and resistant to disinfection, and, therefore, their control is one of the priorities of the hospital infection control team. There are many medical items, devices, tubing, and delicate surfaces that are likely to be damaged by application of physical or chemical methods of disinfection, and caustic chemicals can corrode these materials. Therefore, the health care industry and public health establishments are in a continuous search for newer techniques that are safe and easy to carry and eco-friendly. In this study, the efficacy of direct FE-DBD plasma has been evaluated, and, at

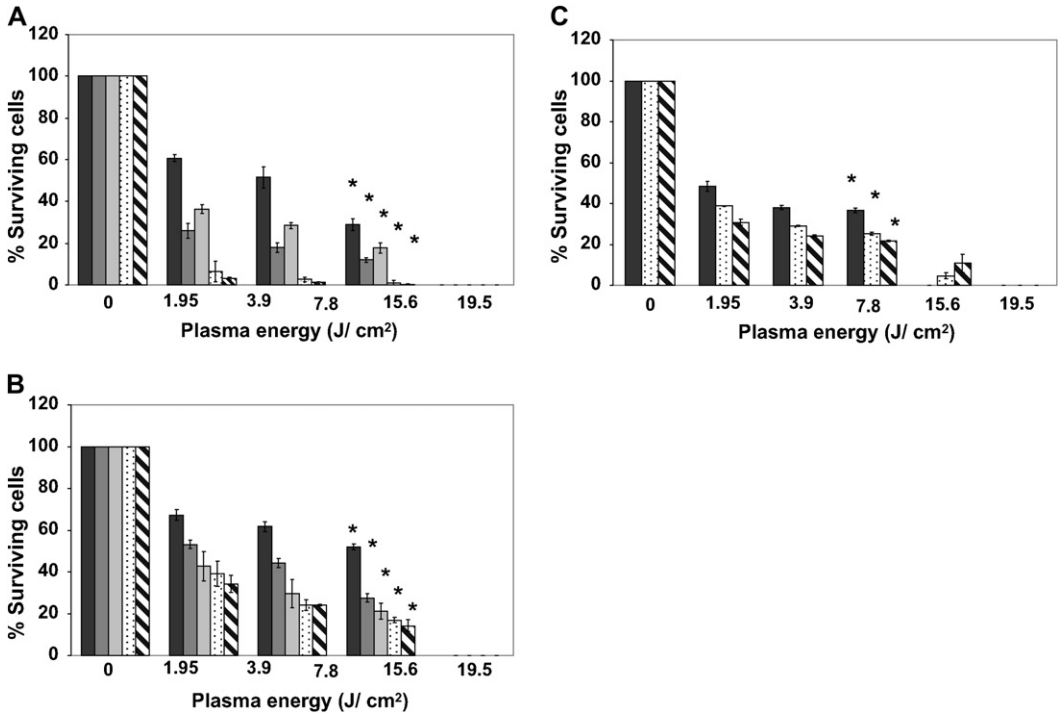


Fig 4. A comparative XTT assays showing a rapid inactivation of MRSA-USA300 and -USA400 when embedded in biofilms (A and B) and planktonic form (C), upon direct (A and C) and indirect (B) applications of plasma. All biofilm-embedded pathogens were inactivated in less than 150 seconds (required 19.5 J/cm^2 plasma energy). Bar, standard deviation; *significant inactivation ($P < 0.05$) as compared with plasma nontreated bacteria or biofilms; solid black column, *E coli*; darkly shaded column, *S aureus*; lightly shaded column, MRSA-95; lightly hatched column, MRSA-USA300; darkly hatched column, MRSA-USA400.

the same time, a concept of indirect application of plasma to sterilize contaminated surfaces is introduced.

Here, we have chosen *S aureus* and *E coli* as representative members, and both the organisms are important nosocomial pathogens. The study strains such as MRSA USA300 and USA400 clonal types of *S aureus* are of major concern to hospital infection control practitioners and are reportedly resistant to many biocidal agents when present in biofilms[5,7,21] and relatively resistant to other types of nonthermal plasma.[22] The findings presented here support that FB-DBD plasma is very effective in killing *E coli*, *S aureus*, MRSA95, MRSA -USA300 and -USA400 in their biofilm forms and inactivates them in less than 120 seconds (and needed less than 15.6 J/cm^2 of plasma energy). The planktonic forms of USA300 and USA400 were also inactivated in less than 150 seconds of direct plasma exposure (19.5 J/cm^2 of energy). These findings are highly comparable with other commercial surface disinfectants,[5] wherein a 10^3 to 10^5 reduction in CFU has been shown. The amount of bacterial load used in planktonic culture experiments (10^9 and 10^8 CFU/mL) are far more than used elsewhere (10^2-10^3 CFU/mL) and with better killing times,[20] compared with, when other types of nonthermal plasma techniques are used. The fact that plasma treatments applied to glass surfaces carrying heavy bacterial loads were able to sterilize effectively in less than 120 seconds is certainly encouraging.

In direct plasma treatment, a significant flux of charges reaches the surface of interest. In contrast, indirect plasma treatment employs mostly uncharged atoms and molecules that are generated in plasma and a small flux of charges to the surface. In indirect plasma, the active uncharged species are typically delivered to the surface via flow of gas through a plasma region.[11] Therefore, some degree of variation in antimicrobial effect is expected in these treatment regimes. In spite of this difference, both the methods were found highly efficient in killing bacteria on dry surfaces and in biofilms. It is likely that PBS was able to trap long-lived and some of the moderate to short-lived species, which exerted their antimicrobial effect on bacterial pathogens in this study. The experiments are in progress that will demonstrate which chemical species are predominantly trapped in such fluids versus active species generated during direct treatment by this plasma.

To demonstrate the efficacy of FE-DBD plasma, we used a combination of 3 commonly used techniques. The colony assay detects and quantifies only viable cells. Similarly, BacLight bacterial viability kit detects live versus dead cells (because of damage to cell membrane).[5] XTT assay is specifically designed to find out whether the cells are dead or live and respiring (only respiring cells convert XTT reagent into its orange-colored soluble metabolic product [act as a metabolic marker]), which can be quantified as described in the Materials and Methods section.[12] Thus, all evidence favors that FE-DBD plasma kills these bacteria upon exposure, leading to sterilization.

In our earlier publications, we demonstrated that there is no significant toxicity that can be observed when tested through a series of in vitro cell culture experiments as well as in vivo mouse and rat skin experiments.[10,11] The data generated from in vivo experiments on higher mammals also indicated no significant toxicity on intact and wounded skin (personal communications of Brooks laboratory). Therefore, there is a high potential of this device in the field of infection control and infected wound treatments. Further investigations are underway. In conclusion, the techniques (both direct and indirect plasma treatments) are novel, and plasma is easy to apply to various surfaces and requires extremely short duration of exposure time (less than 120 seconds and <150 seconds for MRSA -USA400 and -USA300 free living forms), making this technology a promising option for infection control in the clinical setting.

The authors thank funding agencies and Richard Sensenig and Kimberly Wasko for critical reading of this manuscript.

References

1. Lodise TP, Mckinnon PS. Burden of methicillin-resistant *Staphylococcus aureus*: focus on clinical and economic outcomes. Pharmacolotherapy 2007;27:1001-12.
2. Douglas S. The direct medical cost of healthcare-associated infections in US hospitals and the benefits of prevention. Available from: http://cdc.gov/ncidod/dhap/pdf/scott_CostPaper.pdf. Accessed August 12, 2009.
3. Olsen ME, Ceri H, Morck DW, Buret AG, Read RR. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res 2002;66:86-92.
4. Lauderdale KJ, Malone CL, Boles BR, Morcuende J, Horswill AR. Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material. J Orthop Res 2010;28:55-61.
5. Smith K, Hunter IS. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. J Med Microbiol 2008;57:966-73.
6. Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999-2005. Emerg Infect Dis 2007;13:1840-6.
7. Smith K, Perez A. Ramage, G, Lappin D, Gemmell CG, Lang S. Biofilm formation by Scottish clinical isolates of *Staphylococcus aureus*. J Med Microbiol 2008;57:1018-23.
8. Hota B, Contamination. disinfection, and cross-colonization: are hospital surfaces reservoirs for nosocomial infection? Clin Infect Dis 2004;39:1182-9.
9. Rutala WA, Weber DJ. How to assess risk of disease transmission to patients when there is a failure to follow recommended disinfection and sterilization guidelines. Infect Control Hosp Epidemiol 2007;28:146-55.
10. Fridman G, Peddlinghaus M, Ayan H, Fridman A, Balasubramanian M, Gusol A, et al. Blood coagulation and living tissue sterilization by floating-electrode dielectric barrier discharge in air. Plasma Chem Plasma Process 2006;26:425-42.
11. Fridman A. Plasma chemistry. New York: Cambridge University Press; 2008. p. 848-90.
12. James T, Shimellis A, Brooks A, Mukhin Y, Friedman G, Fridman A, et al. In vitro susceptibilities of *E coli* and *S aureus* in fluid to FE-DBD plasma. (Poster No. 21). Proceedings of Biomedical Talent and Technology Showcase held on November 19, 2008, at Drexel University, Philadelphia; 2008. p. 9.
13. Wu JA, Kusuma C, Mond JJ, Kokai-Kun JF. Lysostaphin disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms on artificial surfaces. Antimicrob Agents Chemother 2003;47:3407-14.
14. Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods 2008;72:157-65.
15. Filoche SK, Coleman MJ, Angker L, Sissons CH. A fluorescence assay to determine the viable biomass of microcosm dental plaque biofilms. J Microbiol Methods 2007;69:489-96.
16. Seybold U, Kourbatova EV, Johnson JG, Halvosa SJ, Wang YF, King MD, et al. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of healthcare-associated blood stream infections. Clin Infect Dis 2006;42:647-56.
17. Joaquin JC, Kwan C, Abramzon N, Vandervoort K, Brelles-Marino G. Is gas-discharge plasma a new solution to the old problem of biofilm inactivation? Microbiology 2009;155:724-32.
18. Sladek REJ, Filoche SK, Sissons CH, Stoffels E. Treatment of *Streptococcus mutants* biofilms with a nonthermal atmospheric plasma. Lett Appl Microbiol 2007;45:318-23.
19. Ursic V, Tomic V, Kosnik M. Effect of different incubation atmospheres on the production of biofilm in methicillin-resistant *Staphylococcus aureus* (MRSA) frown in nutrient-limited medium. Curr Microbiol 2008;57:386-90.
20. Souza RR, Coelho LR, Botelho AM, Ribeiro A, Rito PN, Vieira VV, et al. Biofilm formation and prevention of LukF-pv, seb, sec and tst genes among hospital- and community-acquired isolates of some international methicillin-resistant *Staphylococcus aureus* lineages. Clin Microbiol Infect 2009;15:203-7.
21. Brady A, Loughlin R, Deirdre G, Kearney P, Tunney M. In vitro activity of tea-tree oil against clinical skin isolates of methicillin-resistant and -sensitive *Staphylococcus aureus* and coagulase-negative staphylococci growing planktonically and as biofilms. J Med Microbiol 2006;55:1375-80.
22. Burts ML, Alexeff I, Meek ET, McCullers JA. Use of atmospheric nonthermal plasma as a disinfectant for objects contaminated with methicillin-resistant Staphylococcus aureus. Am J Infect Control 2009;37:729-33.