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The Use of an Atmospheric Pressure Plasma Jet to Inhibit Common Wound-Related Pathogenic Strains of Bacteria

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ABSTRACT: The Plaz4 electrosurgical generator produces a nonequilibrium atmospheric pressure plasma jet (APPJ) at 26°C. This APPJ was tested for its antibacterial capabilities on common wound-related pathogens. The inhibition zone (IZ) of bacterial growth and surviving colony-forming units (CFUs) within the IZ were determined for 4 common clinical isolates: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Agar was inoculated at a high density with a bacterial culture medium (10^7 CFUs/mL). The strains' susceptibility to the APPJ was tested at exposure times between 30 and 140 seconds. A positive correlation between plasma exposure time and bacterial growth IZ size, and a negative correlation between exposure time and the number of surviving CFUs inside the IZ, were observed. It was possible to achieve a round IZ, 4 cm in diameter, for all strains after 70 seconds of exposure, with less than 10 CFUs/cm^2 surviving within the zone. *P. aeruginosa* was more resistant to plasma and required a longer exposure to achieve an IZ similar to that of other strains. However, the number of CFUs surviving inside the IZ was smaller for this strain for a majority of test conditions. Intraoperative contamination is a significant cause of perioperative infection. Drug-resistant bacteria are endemic to hospitals and are a significant public health concern. With the increasing risk of infections related to drug-resistant bacteria, it is crucial to look for alternative treatments. Electrosurgical generators are routinely used in surgical cases. Therefore, Plaz4 applied to surgical sites or debrided wounds could result in a clinically meaningful reduction in tissue bioburden.

KEY WORDS: cold plasma, atmospheric pressure plasma jet, antibiotic resistance, antibiotic alternative, superbugs

I. INTRODUCTION

Intraoperative contamination is a significant cause of perioperative infection and is the rationale for perioperative antibiotic prophylaxis. During perioperative surgical care, the use of topical and/or systemic antibiotics is common practice to manage and/or prevent wound- and implant-related infections.[1] In part because of near-ubiquitous perioperative antibiotic use, drug-resistant bacteria are now endemic to hospitals and are a significant public health concern. The US Department of Health and Human Services and Centers for Diseases Control and Prevention estimate that, in the United States, more than 2 million people are affected every year with bacterial infections that are resistant to one or more antibiotics typically prescribed to treat these infections. In some cases no acceptable treatment options exist, or the antibiotics that would be effective against

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these resistant strains are highly toxic. Moreover, at least 23,000 cases of drug-resistant bacterial infections have been fatal. Survivors have significantly longer hospital stays, delayed recuperation, or other conditions that may be complicated by an antibiotic-resistant infection (e.g., long-term disability).[2]

Among all bacteria-resistant infections, the gram-negative strains are especially concerning because of their resistance to nearly all available drugs; this threat will worsen without ongoing public health monitoring and prevention activities.[2] This issue has reached levels that many experts believe are a potential existential threat to the human population.

The most common gram-negative pathogens related to clinical infections in the health care arena are *Pseudomonas aeruginosa*, *Acinetobacter* and *Enterobacteriaceae* (e.g., carbapenem-resistant *Escherichia coli*). In the case of the pathogen *Acinetobacter*, ~63% of infections are considered multidrug-resistant, with a high proportion of infections occurring among critically ill patients. *P. aeruginosa* is responsible for 13% of severe health care–associated infections that become resistant to multiple drugs.[2,3]

Some of the gram-positive bacterial strains, such as *Staphylococcus*, are causing public health concerns as well, especially in the postsurgical patient population. Methicillin-resistant *Staphylococcus aureus* can cause a range of illnesses, from skin and wound infections to pneumonia with empyema to bacteremia (blood-borne hematologic infections) that may cause sepsis and death.[3]

With the increasing risk of infections related to drug-resistant bacteria (often termed "superbugs"), it is crucial to look for alternative treatments to help minimize antibiotic use without increasing the rates of perioperative infections[3,4] or furthering the development of bacterial antibiotic resistance.

Cold atmospheric plasma may offer a promising alternative to the topical use of antibiotics, and in the treatment of superficial skin and wound infections; encouraging results and numerous advantages in biomedical applications have been summarized in recent reviews.[5–9] Early preclinical and human clinical trials are showing promising results, with reduced wound bioburden and improved wound healing.[10–12] Several methods for producing cold atmospheric plasmas can suit different applications. Two common approaches are dielectric barrier discharge and atmospheric pressure plasma jets (APPJs), each with their own advantages and disadvantages for different biomedical applications. For the treatment of wounds, which present irregular surfaces and sensitive tissues, APPJs may offer certain advantages: they can be applied at a greater distance with less risk of inadvertent contact to sensitive/painful tissue, and they may better accommodate the variable topography of a clinical wound.

One of the aforementioned advantages of cold plasma is bacterial susceptibility to plasma upon exposure, resulting in bacterial death, inactivation, or sterilization.[5,13–18] Moreover, research indicates that bacteria do not build up resistance to cold plasma treatment.[19,20] While many studies have looked at the bacteriostatic/bactericidal effects of plasma application, few have tested multiple wound isolates at different exposure times with the same device.[21] Daeschlein et al.[21] exposed established colonies on a culture

media, rather than freshly plated cultures, to better represent endemic wound bioburden. Such data are necessary to develop guidelines to assist clinicians in determining the minimum effective "dose" of plasma for a given clinical infection.

The Plaz4 cold plasma generator used in this study is a nonequilibrium APPJ delivery system that is designed to meet all applicable international standards for an electrosurgical unit (Figs. 1 and 2). Given the wealth of literature suggesting that cold plasmas can kill pathogenic bacteria, the use of the Plaz4 for coagulation during surgical cases (e.g., on surgical site incisions; after debridement of chronic, infected wounds) may have the added benefit of reducing low-density inocula that are often introduced during surgery, which could subsequently reduce infection-related complications. Similarly, it could reduce the remaining wound bioburden following debridement of chronic or infected wounds.

FIG. 1: Plaz4 nonequilibrium atmospheric pressure plasma jet delivery system.

FIG. 2: Applicator (hand-held gun) for plasma delivery.

II. MATERIALS AND METHODS

In this study the Plaz4 APPJ was tested for its antibacterial capabilities on 4 common wound pathogens: *E. coli*, *S. aureus*, *P. aeruginosa*, and *Acinetobacter baumannii*. Tests were repeated at different exposure times to create dose–response curves for each strain.

The Plaz4 system, as provided by the manufacturer (Plasmology4, Inc., Scottsdale, AZ), was used to deliver a nonequilibrium helium–oxygen (99%:1%) gas plasma jet for all plasma treatments (Figs. 1 and 2). The device was preset by the manufacturer to generate a 460-kHz radiofrequency signal at 36 kVp-p. The high voltage is pulsed at 550 Hz, with a pulse duration of approximately 50 μs as the waveform decays before the next pulse. The ionization chamber contains a stack of planar electrodes around which the gas flows and is ionized before exiting the outflow port. The target substrate acts as the ground electrode. Detailed information on the structure of the plasma source can be found in a published patent.[22] The feed gas is controlled by the system at a flow rate of 11.8 L/min, and plasma exits the applicator through a 9-mm-diameter port (Fig. 2).

The plasma temperature was measured by placing a laboratory-grade alcohol thermometer directly into the plasma stream, approximately 15 mm from the applicator tip. The plasma jet was maintained with the thermometer in this position until no further increase in temperature was observed. The bulk plasma temperature was determined to be 26°C using this methodology. Because of the thermal mass of the thermometer bulb, the plasma temperature may be slightly higher than the recorded value, but this value is representative of the surface temperature expected of the target substrate during plasma exposure.

A. Microbiological Methods

APPJ exposure was tested on 4 common wound pathogens: *E. coli*, *S. aureus*, *P. aeruginosa*, and *A. baumannii*. All tested pathogens were clinical isolates of common bacteria related to hospital infections:

- *E. coli* (ATCC 25922): Clinical isolate, registered with the US Food and Drug Administration; a gold standard in pharmaceutical tests for antibiotic sensitivity
- *P. aeruginosa* (ATCC 27853): Clinical isolate from blood, control strain and testing of antibiotic sensitivity; commonly used by the pharmaceutical industry
- *A. baumannii* (ATCC BAA-1605): Clinical isolate, sputum of US military service member returning from Afghanistan in 2006; resistant to ceftazidime, gentamicin, ticarcillin, piperacillin, aztreonam, cefepime, ciprofloxacin, imipenem, and meropemem; sensitive to amikacin and tobramycin.
- *S. aureus* (ATCC 6538): Clinical isolate from human lesion, registered with the US Food and Drug Administration; used for testing bactericides, sanitizers, disinfectants, inhibition, and efficacy, and for quality control.

B. Direct Plasma Exposure

Stock cultures of bacterial strains were grown aerobically at 37°C in tryptic soy broth for a minimum of 1 week, with a daily change of nutrient media to achieve mature bacterial cultures before starting the experiment. Further, these cultures were diluted and plated (100 µL) onto growth medium plates supplemented with nonspecific agar, with a seeding density of 10^7 colony-forming units (CFUs)/mL. Plates were set aside for 30 min before exposure to plasma to allow absorption of the broth and initial attachment of the bacteria to the surface. Plates were then exposed to the APPJ system, which delivered a plasma plume 9 mm in diameter (Fig. 3), at a distance 25 mm from the agar surface, for different durations (30, 60, 70, 90, 120, and 140 seconds). The plasma plume was directed vertically downward at the center of the plate and was held in a fixed position for the duration of the treatment. Plates were incubated at 37°C for 18 hours before being analyzed.

The zone of inhibition of bacterial growth on agar plates after plasma exposure was verified in a quantitative manner by measuring the area/diameter of the inhibition zone (IZ) and number of CFUs inside the IZ using ImageJ image processing software.[23]

Data sets were statistically analysed using analysis of variance (ANOVA) for strain and exposure time versus IZ and surviving CFUs per square centimeter, with *t*-tests used for post hoc testing where appropriate. Derived P values <0.05 were considered significant. The experiment was performed on 3 plates per tested condition and was repeated 3 times, for a total of 9 plates per test condition. The data represent average values and standard deviations.

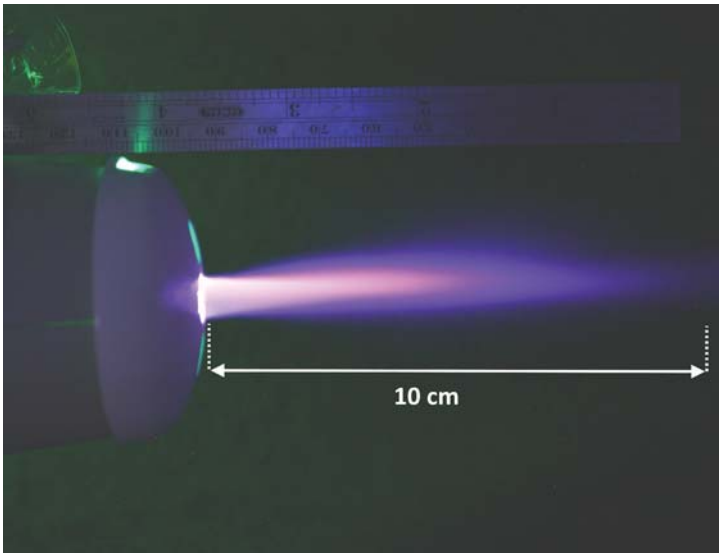

FIG. 3: Photograph of the plasma plume generated by the Plaz4 system. The plume is emitted through the applicator (gun) with and operating gas mixture (99% helium, 1% oxygen) and a gas flow rate of 11.8 L/min.

C. Pretreatment of Agar Plates

To ensure that the plasma was not simply modifying the agar surface so that it was incapable of sustaining bacterial growth, we performed 2 investigations of the effects of plasma pretreatment of the agar on bacterial growth and the presence of an IZ. The first used a single strain (*E. coli*) to assess whether the time between pretreatment of the agar and inoculation of the agar with bacterial broth had any influence on bacterial growth. The second confirmed that all tested bacterial strains responded in a similar way to agar pretreatment.

For the first experiment, all agar plates were exposed to plasma for 70 seconds. Subsequently, after 30, 60, and 120 seconds, 4 hours, and 24 hours, 100 μL of *E.coli* broth (seeding density, 10^7 CFUs/mL) was plated on the plates exposed to plasma. The plates were incubated at 37°C for 18 hours before being analyzed. Three sample plates per test condition were assessed.

For the second experiment, agar plates were exposed to plasma for 70 seconds, and all tested bacterial strains (*E. coli*, *S. aureus*, *P. aeruginosa*, and *A. baumannii*) were inoculated (seeding density, 10^7 CFUs/mL) onto the pretreated agar plates after 30 seconds and 24 hours, which represented the shortest and longest wait times from the first experiment. Plates were incubated at 37°C for 18 hours before being analyzed. Three sample plates per test condition per strain were assessed.

D. Gas-Only Exposure

Although plates were allowed to sit in ambient conditions for 30 minutes after inoculation and before plasma treatments, a study of only gas flow was conducted to ensure that the inoculum was not being affected solely by gas flow. In this substudy the high-voltage power supply was disabled and system-delivered gas was dispensed from the applicator at the same distance and flow rate as the plasma test conditions. A stock culture of *E.coli* broth was plated onto agar plates with a seeding density of 10^7 CFU/mL, then set aside for 30 min before gas-only exposure, as in the plasma direct test condition. Plates then were exposed for 70 seconds to gas flow only. Plates were incubated at 37°C for 18 hours before being analyzed.

III. RESULTS

Results show a positive correlation between the duration of plasma exposure and the size of the bacterial growth IZ, as well as a negative correlation between exposure duration and the number of surviving colonies inside the IZ (Figs. 4–6).

The overall trend for all strains indicates that by increasing the plasma exposure time, the diameter of the IZ for all strains increases from a mean of 2.8 cm at 30 seconds to 4.3 cm at 140 seconds (Fig. 5). ANOVA of the IZ demonstrates a significant difference between strains until 140 seconds of exposure time, at which point all strains have a substantially equivalent IZ. Post-hoc testing reveals that *P. aeruginosa* is the sole outlier

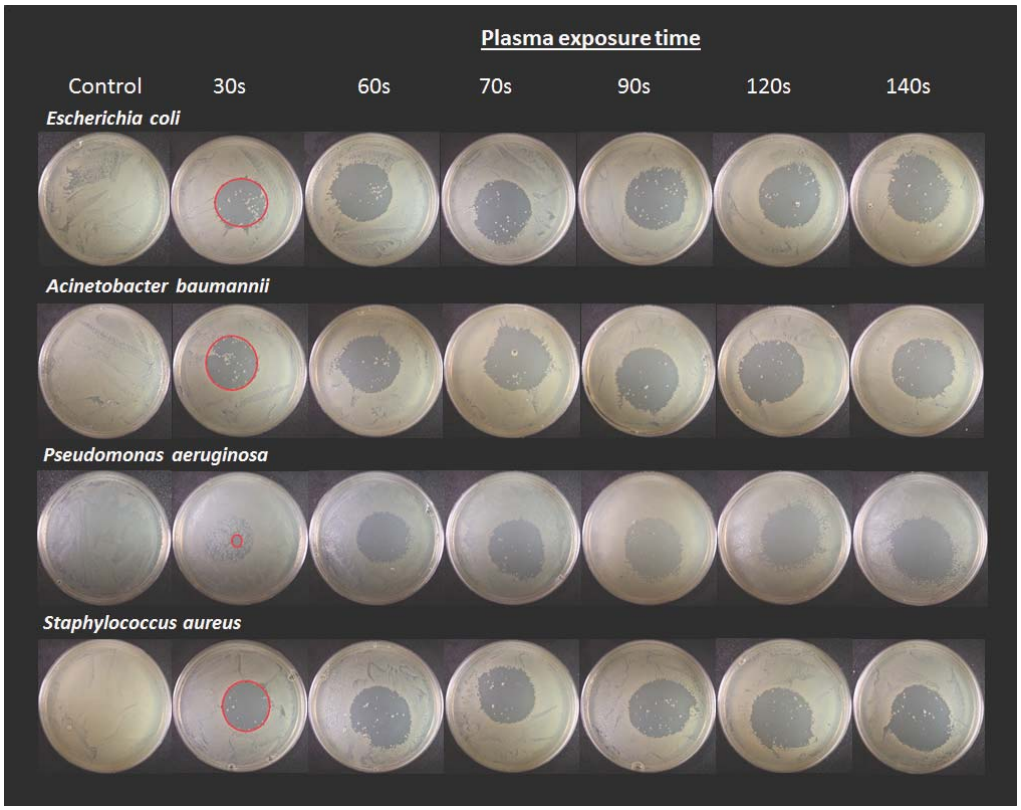

FIG. 4: Inhibition zone (IZ) of bacterial growth of selected pathogens in relation to atmospheric pressure plasma jet exposure duration (seconds). The interior circles in the second column represent examples of determined IZ .

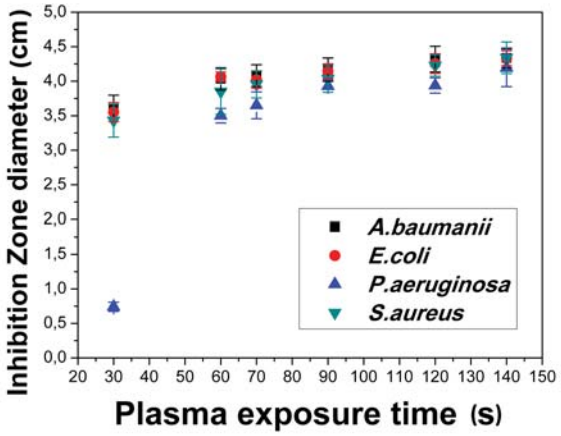

FIG. 5: Average diameter of the inhibition zone of various pathogenic strains after exposure to the atmospheric pressure plasma jet.

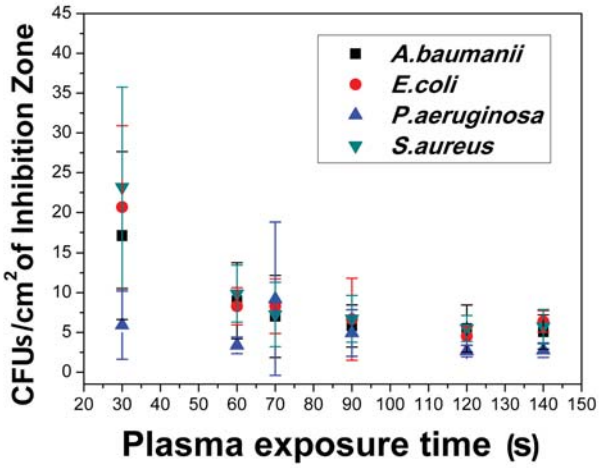

FIG. 6: Average colony-forming units (CFUs) per square centimeter of the inhibition zone after exposure to the atmospheric pressure plasma jet.

contributing to the significant difference in the IZ size. The difference between *P. aeruginosa* and other strains at 30 seconds is shown clearly in Fig. 4; it begins to approach other strains by 60–70 seconds but remains significantly different until 140 seconds.

The average surviving CFUs per square centimeter within the IZ for all strains ranged from a maximum of 16.7 at 30 seconds to a minimum of 4.6 at 120 seconds. With increasing duration of treatment, the number of surviving colonies per area decreased (Fig. 6), even as the IZ increased. A significant difference between strains for the number of surviving colonies per square centimeter was detected by ANOVA at 30, 60, 120, and 140 seconds of exposure. Again, *P. aeruginosa* was the outlier driving the significant ANOVA, with fewer surviving colonies, even as the IZ size becomes equivalent to other strains. No significant difference was detected by ANOVA between strains for 70- to 90-second treatment durations.

To calculate the commonly reported log reduction, the actual number of CFUs within the IZ must be compared with the expected number of CFUs within the IZ from a control plate. For the average results across all strains at 120 seconds, a diameter of 4.2 cm and average of 4.6 CFUs/cm^2 was acheived. With seeding 100 µL of 10^7 CFUs/mL innoculum per plate, 2.4×10^5 CFUs would be expected for a control sample within a 4.2-cm-diameter area. For the samples treated for 120 seconds, 63 colonies were oberved in the IZs (4.6 CFUs/cm^2 × 4.2-cm-diameter area). Therefore, the ratio of the expected to observed CFUs is 0.9997, or a 3.6 log reduction. The maximum log reduction obtained was 3.8 for *P. aeruginosa* at 120 seconds.

No IZ of bacterial growth was observed on any of plasma-pretreated agar plates, regardless of the time interval between exposure and innoculation and regardless of strain (Fig. 7). Similarly, no IZ was noted on any inoculated agar plates treated with gas only (Fig. 7).

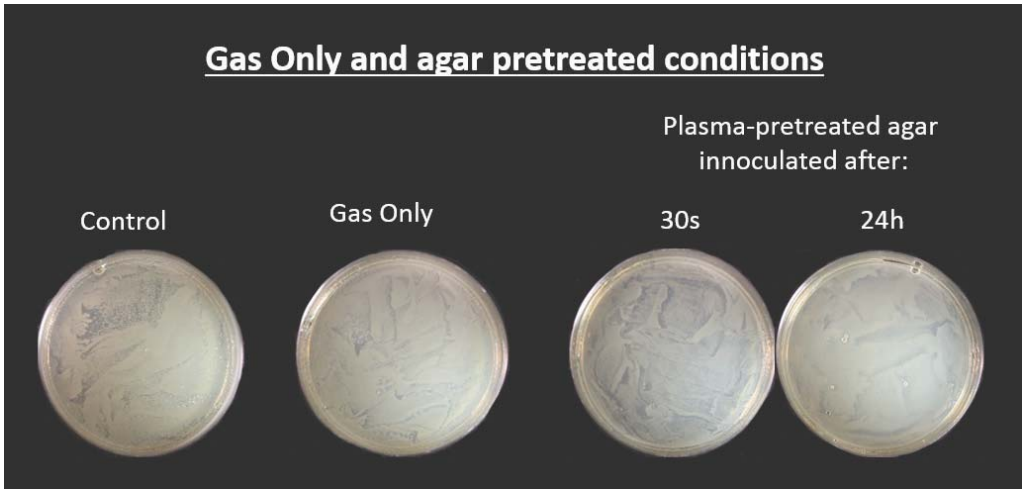

FIG. 7: Bacterial growth on an agar plate after 18 hours of incubation. "Gas Only" represents an agar plate inoculated with Escherichia coli and treated with only gas (no plasma). On the right, agar was pretreated with plasma and further inoculated with bacteria after 30 seconds and 24 hours.

IV. DISCUSSION

Results showed that Plaz4 is able to inhibit all common wound isolates tested, but that strains require different exposure times to achieve large IZs with few remaining CFUs. Further, bacterial susceptibility to this APPJ is not selective to gram-positive or gram-negative bacteria. In general, all strains except *P. aeruginosa*, which required longer exposure times to achieve a comparable IZ size, showed very similar response curves upon exposure. By contrast, the CFU counts per square centimeter within the IZ were clearly lower for *P. aeruginosa*. Therefore it seems that this strain is more difficult to destroy and requires longer exposure times, but once the exposure threshold has been reached, there is a more uniform response by cells.

The difference in the response of *P. aeruginosa* requires further investigation. Among the tested strains, *P. aeruginosa* has the thinnest peptidoglycan layer (2.4 nm),[24] which is a stress-bearing component of the bacterial cell wall. Since the cell wall may react to stresses induced by electrostatic forces[25,26] and reactive species, certain cell wall–mediated responses to plasma may differ with cell wall thickness. Their slightly higher resistance when compared with other tested pathogens might be related to the major outer membrane—porin OprF—that is specific to this species. This difference slows the diffusion of various solutes across the membrane—in some cases a difference of 2 orders of magnitude compared with other bacterial species (e.g., *E. coli*). Low permeability contributes to their high level of intrinsic resistance to noxious agents[27] and may be contributing to their increased resistance to plasma exposure.

One of the promising outcomes of this study is that the multidrug-resistant clinical isolate *A. baumannii* strain did not show any signs of reduced susceptibility to plasma exposure compared with the other tested pathogens.

The experimental design described in this article, which exposed newly plated bacterial cultures to the APPJ, may not be representative of a well-established wound environment. As shown by Daeschlein et al.,[21] the stacking of bacteria in active colonies can limit the penetration and antibacterial effectiveness of certain APPJs. The limited clinical trials of APPJ effects on infected wounds[11] also demonstrate a reduction in antibacterial effectiveness (1–2 log reductions with repeated daily treatments) when compared with laboratory studies of simplified models.

Perioperative infections are generally caused by very low levels of innoculum that are not yet established within the host tissue bed, as the seeding of the surgical site generally occurs during the course of surgery. Therefore, when the surgical site is closed, it is not necessary to eradicate a high concentration of innoculum that is well-established on the tissue to prevent the development of a surgical site infection; rather, the goal is to kill sparsely distributed, poorly adhered, oportunistic pathogens. The ability to acheive inhibition over a large proportion of the surgical site without moving the applicator is clinically important since it is unknown whether or where pathogens may be within the surgical bed.

For chronic wounds or acute infections, debridement down to healthy, bleeding tissue is standard practice to remove dead tissue and established biofilm. Therefore, when used as an electrosurgical generator at the end of a wound debridement, an APPJ may be a clinically useful tool to reduce the overall wound bioburden.

V. CONCLUSIONS

The Plaz4 APPJ was able to inhibit all of the tested common wound isolates to a high degree. There were differences in the exposure time required to generate a clinically meaningful IZ, as well as differences in the number of colonies surviving within the IZ. In general, a 70-second exposure was able to achieve consistent zones and colony survival between tested strains.

If used as an electrosurgical unit on a surgical site or during a wound-related procedure, the Plaz4 system could reduce wound bacteria by $>10^3$ CFUs in an area up to 4.2 cm in diameter, depending on the strain present and the nature of the wound. The Plaz4 APPJ could be explored further as a postoperative surgical site treatment to potentially minimize, or potentiate, prophylactic antibiotic use and efficacy in preventing surgical site infections, and after the debridement of chronic or contaminated wounds.

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