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ORIGINAL ARTICLE

Inactivation of bacterial opportunistic skin pathogens by nonthermal DC-operated afterglow atmospheric plasma

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Abstract

Aims: Multidrug-resistant opportunistic pathogens are clinically significant and require the development of new antimicrobial methods. In this study, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells were exposed to atmospheric plasma on agar plates and *in vitro* on porcine skin for the purpose of testing bacterial inactivation.
Methods and Results: Microbial inactivation at varying exposure durations was tested using a nonthermal plasma jet generated with a DC voltage from ambient air. The observed reduction in colony forming units was quantified as $\log_{10}$ reductions.
Conclusions: Direct plasma exposure significantly inactivated seeded bacterial cells by approx. 6 $\log_{10}$ on agar plates and 2–3 $\log_{10}$ on porcine skin. On agar plates, an indirect 'bystander' inactivation outside the plasma delivery area was also observed. The reduced inactivation observed on the skin surface was most likely due to cell protection by the variable surface architecture.
Significance and Impact of Study: Atmospheric plasma has potential for clinical application as a disinfectant of patient skin and medically relevant surfaces.

Introduction

Plasma describes an ionized gaseous state that can be generated by an electrical discharge. Depending on the gas (e.g. noble gases or molecular gases, such as air) and the application, plasmas may be associated with high temperatures and often require operation at lowered pressures in addition to excitation with oscillating high voltages. Conversely, plasmas that can be generated at atmospheric pressures into air with inherent low temperatures are therefore tolerable for tissues and medical instrumentation. Early work demonstrated that regardless of the operating conditions, 'cold' plasma discharge types kill vegetative bacterial cells or the spores of several *Bacillus* spp. (Chau *et al.* 1996; Laroussi 1996; Hury *et al.* 1998; Kelly-Wintenberg *et al.* 1998; Moisan *et al.* 2001). Subsequently, many different plasma systems and operating conditions and parameters have been tested, while applications have been focused on the inactivation of organisms, primarily on surfaces and in aqueous fluids.

Specific practical antibacterial applications include applications in food, such as the decontamination of *Escherichia coli* O157:H7 in apple juice (Montenegro *et al.* 2002) and *E. coli* in dry almonds (Deng *et al.* 2007). Several studies targeted clinical applications. *Pseudomonas aeruginosa* was inactivated on biomedical plastic (Yang *et al.* 2009), and adherent oral micro-organisms were inactivated on dentin slices (Rupf *et al.* 2010). Inactivation of *Ps. aeruginosa* and *Staphylococcus aureus* on the surface of porcine corneas *in vitro* was more effective than disinfection by antiseptics licensed for use in the eye (Hammann *et al.* 2010). Finally, in a clinical trial testing the treatment for chronic wounds, a significant reduction in the load of miscellaneous bacteria was demonstrated (Isbary *et al.* 2010).

In a recent study (Ermolaeva *et al.* 2011), inactivation of planktonic bacterial by nonthermal argon application *in vitro* and in a wound infection model was studied. On agar plates, a 5-min plasma application completely inactivated 10^5 Gram-negative bacterial cells of several types.

This application also inactivated 83–100% of 10^5 Gram-positive bacterial cells. In a rat superficial infected slash wound model, a 10-min plasma application tended to reduce *Ps. aeruginosa* surviving colony forming unit (CFU) and significantly inactivated *S. aureus* cells.

Almost every study on microbial inactivation with 'cold' plasmas thus far relies on a plasma that is generated from a noble gas such as argon or helium. To increase inactivation efficiencies, sometimes oxygen or other gases are admixed. A discharge in these gases is generally achieved with the use of oscillating high voltages with frequencies of several kilohertz and amplitudes of a few kilovolts. In this study, a plasma jet generated from ambient atmospheric pressure air by applying a moderate DC high voltage of 2 kV in a microhollow cathode geometry was used (Kolb *et al.* 2008).

Staphylococcus aureus (David and Daum 2010), *Acinetobacter baumannii* (Perez *et al.* 2007; Peleg *et al.* 2008; Kunz and Brook 2010) and *Ps. aeruginosa* (Kunz and Brook 2010) are clinically relevant, opportunistically pathogenic bacteria that can develop partial, or even complete, antibiotic resistance (Talbot *et al.* 2006). Owing to the ineffectiveness of available antibiotics, development of alternative methods of inactivation, including nonantibiotic methods, is necessary. After testing plasma inactivation of bacterial lawns of these organisms grown on agar plates, inactivation was tested *in vitro* using porcine skin as a model for human skin (Barbero and Frasch 2009).

Materials and Methods

Plasma system

Ambient air was flowed through a microhollow cathode discharge geometry with a diameter of 0·8 mm, where a discharge was operated with an applied DC voltage of 2·0 ± 0·1 kV and current of 30 mA (Kolb *et al.* 2008). At a flow rate of eight standard litres per minute, the flow conditions were turbulent and an afterglow plasma plume with a luminous length of approx. 15 mm was expelled. For this distance, inactivation studies were conducted by mounting the plasma jet on a *xyz*-stage and moving it twice over a 2×2 cm^2 area in perpendicular raster patterns 10 mm above the agar surface. Exposure times of the direct treatment area were adjusted by changing the speed for each pass, the distance between the raster lines, and the total number of passes.

Plasma application on agar plates

Atmospheric plasma was applied as described in each experiment to lawns of *S. aureus* (ATCC 25923), *A. baumannii* (ATCC 19606) and *Ps. aeruginosa* (ATCC 27853) cells on trypticase soy agar plates in triplicate. After overnight incubation at 37°C, the surviving colonies were quantified (Flash & Go Automated Colony Counter; IUL Instruments, Barcelona, Spain). Numbers were recorded for the immediate 2×2 cm^2 treatment area and for adjacent concentric rings of increasing diameter and approx. 10 mm width. The initial cell number inoculated was determined from serial dilutions, and the $\log_{10}$ reduction in colony forming units (CFUs) was calculated.

Plasma application on porcine skin

Porcine skin was acquired from a local abattoir. After storage at −80°C, the remaining skin flora organisms present were moderate numbers of nonhaemolytic staphylococci and rare alpha haemolytic streptococci (data not shown). Square pieces with an area of 4·2 ± 0·2 cm^2 were inoculated with bacterial cells, allowed to dry and treated with the plasma jet. Control pieces received no plasma exposure. Each tissue piece was suspended in 1 ml of sterile PBS and homogenized using a Stomacher 80 lab blender (Tekmar Co., Cincinnati, OH, USA). The homogenates were serially diluted and plated on mannitol salt agar for *S. aureus* and MacConkey agar for *A. baumannii* and *Ps. aeruginosa.* Colonies were quantified, and the $\log_{10}$ reduction in CFUs was determined as described above.

Statistical analysis

The statistical significance between the groups was determined by one-way ANOVA with post-test (GraphPad Software, La Jolla, CA).

Results

For the cultures on agar plates, nearly complete inactivation of $1–3 \times 10^6$ cells was observed in the delivery area for each bacterium at the initial 215-s exposure parameters (Table 1). Therefore, to determine the minimum required exposure time for inactivation, the raster rate and meander width were adjusted (Table 2). Significant activation was observed with exposures of 165, 135, 111 and 87 s ($< 0·01$). At the 40- and 60-s exposures, *S. aureus* cells were significantly inactivated, while a large variation in *Ps. aeruginosa* inactivation was observed. At the 40-s exposure, no inactivation of *A. baumannii* was observed.

Outside the 2×2 cm^2 delivery areas, an indirect inactivation was observed for *Ps. aeruginosa* and *S. aureus* but not for *A. baumannii* (Fig. 1). Distinct but incrementally reduced inactivation ($P < 0·001$) of *S. aureus* occurred across the entire area of each plate (Fig. 2). At exposure

Table 1 Effect of exposure duration on inactivation of 1–3 × 10^6 inoculated cells within the 4-cm^2 delivery area. Values represent mean ± standard error of the mean of 3–5 independent experiments

	Log_{10} reduction		
Exposure duration (s)	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
40	0·0 ± 0·0	2·0 ± 1·2	5·4 ± 0·2***
60	4·1 ± 0·5***	3·4 ± 1·1	5·2 ± 0·2***
87	5·0 ± 0·2***	5·3 ± 0·2**	5·4 ± 0·5***
111	5·2 ± 0·3***	5·9 ± 0·5***	5·4 ± 0·4***
135	5·6 ± 0·2***	5·6 ± 0·5***	6·1 ± 0·4***
165	5·7 ± 0·3***	5·5 ± 0·2***	5·9 ± 0·4***
215	5·4 ± 0·3***	5·5 ± 0·1***	6·1 ± 0·2***

***$P < 0·001$; **$P < 0·01$ with respect to control inoculum.

Table 2 Raster variables for plasma delivery

Exposure duration (s)	Meander width (mm)	Rate (mm s^{-1})
40	5	6·0
60	2	8·0
87	2·5	4·6
111	1	7·9
135	1	6·5
165	1	5·3
215	0·5	7·8

durations of 87 s or less, a significant ($P < 0·01$) inactivation of *Ps. aeruginosa* cells extended outside the delivery area to a radius of 2·08 cm.

Bacterial inactivation on porcine skin was tested using the longest exposure time of 215 s that was used for cultures on agar plates and at a shorter exposure of 111 s. A 100- to 1000-fold bacterial inactivation was observed at both 111- and 215-s exposures (Table 3).

Discussion

In this study, cells from each of these organisms were inactivated by plasma exposure up to 6 log_{10}, the maximum

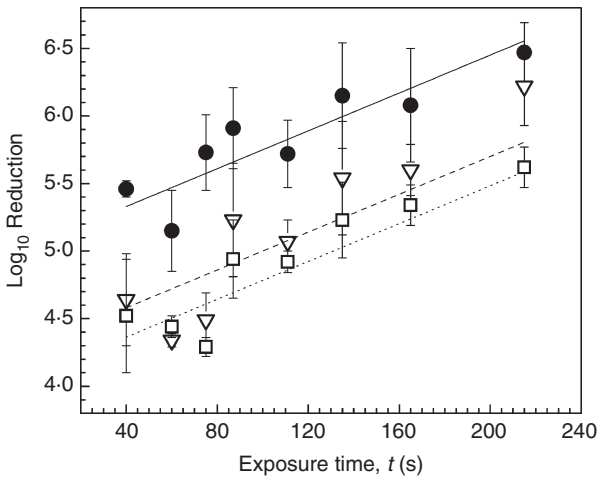

Figure 2 Indirect effect of plasma exposure on *Staphylococcus aureus*. Log_{10} CFU reduction in concentric rings surrounding exposure area. (●), 2·08-cm ring surrounding direct delivery area; (▽), 2·08- to 3·17-cm ring; and (□), 3·17- to 4·25-cm ring.

inoculated onto the delivery area of each agar plate. *A. baumannii* was most resistant organism to direct exposure. Theoretically, this confirms the environmental resilience of this bacterium (Perez *et al.* 2007). *Ps. aeruginosa* showed an intermediate sensitivity, while the cells of the Gram-positive organism *S. aureus* were the most sensitive. Potentially, this could be due to the differences between Gram-negative and Gram-positive cell wall composition. Several investigations have compared plasma inactivation of Gram-negative and Gram-positive vegetative bacteria. Some studies tended to demonstrate that Gram-negative organisms were more effectively inactivated by plasma (Chau *et al.* 1996; Kelly-Wintenberg *et al.* 1998; Rahul *et al.* 2005; Lee *et al.* 2006; Daeschlein *et al.* 2010), while others did not (Marsili *et al.* 2002; Scholtz *et al.* 2007; Feng *et al.* 2009; Hammann *et al.* 2010; Rupf *et al.* 2010; Sohbatzadeh *et al.* 2010; Ermolaeva *et al.* 2011). The observed differences in these studies may be due to differences in the employed plasmas, the medium in which the inactivation

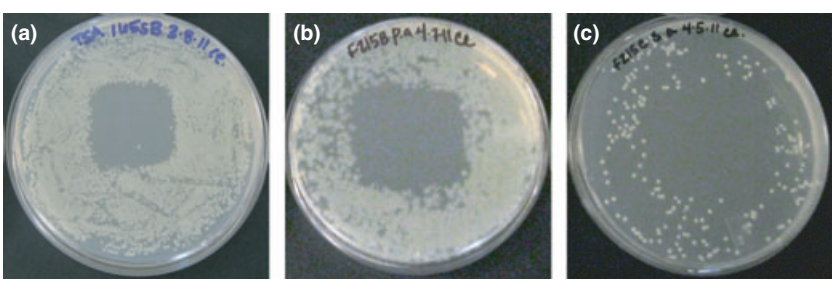

Figure 1 Differential effect of plasma exposure. Trypticase soy plates were inoculated with equal number of organisms and plasma applied at the 215-s exposure duration. Plates are shown after overnight incubation at 37°C. (a) *Acinetobacter baumannii*; (b) *Pseudomonas aeruginosa*; and (c) *Staphylococcus aureus*.

Table 3 Bacterial inactivation on pig skin pieces ($n = 3$)

Organism	Calculated inoculated $\log_{10}$ CFU	$\log_{10}$ CFU recovered from control	Plasma exposure duration in seconds	$\log_{10}$ CFU reduction after plasma delivery
Acinetobacter baumannii	6·17	6·09 ± 0·03	215	3·76 ± 0·06***
			111	3·59 ± 0·08***
Pseudomonas aeruginosa	6·83	6·08 ± 0·06	215	2·41 ± 0·04***
			111	2·61 ± 0·01***
Staphylococcus aureus	6·46	6·08 ± 0·02	215	3·28 ± 0·12***
			111	3·21 ± 0·12***

***$P < 0·001$ with respect to control CFUs recovered.

was performed or other specific characteristics of the organisms tested.

Several groups have specifically demonstrated that oxidative stress is the mechanism by which plasma inactivates bacteria (Hury *et al.* 1998; Moreau *et al.* 2000; Philip *et al.* 2002; Kim and Kim 2006; Lu *et al.* 2008). *A. baumannii* and *Ps. aeruginosa* are strict aerobes, while *S. aureus* is a facultative anaerobe. Each of these organisms produces directly protective enzymes to inactivate reactive oxygen species, including catalase, superoxide dismutase and several peroxidases, along with oxidative damage repair enzymes (Storz *et al.* 1990). *E. coli* cells lacking the *soxS* gene are more sensitive to plasma application than wild-type cells (Perni 2007), indicating that these enzymes are important protection against plasma inactivation. Nitric oxide may be the most common ionic species in this plasma (Kolb *et al.* 2008), and each of these organisms has a demonstrated sensitivity to nitric oxide (Mihu *et al.* 2010; Privett *et al.* 2010; Friedman *et al.* 2011). Several Gram-positive organisms, including *S. aureus*, synthesize nitric oxide in part as protection against oxidative damage (Crane *et al.* 2010). As *A. baumannii* and *Ps. aeruginosa* do not produce nitric oxide synthase, it is interesting that they are more resistant in this case.

The temperature of the plasma at delivery was 44·5 ± 4·2°C, slightly above classic biological heat shock. To test the contribution of temperature to inactivation, each bacterium was exposed to 46°C for the maximum plasma exposure time of 215 s in parallel experiments using either a water bath or a blow dryer ($n = 3$). This heat exposure did not significantly affect *A. baumannii* or *Ps. aeruginosa* growth. However, heat exposure produced a significant ($P < 0·05$) decrease of 0·2 $\log_{10}$ CFU number in this *S. aureus* strain. Although these time and temperature are within the decimal reduction time range of some *S. aureus* strains in liquid (Walker and Harmon 1966; Hassani *et al.* 2006), the strain tested here (ATCC 25923) is resistant to heat (Hassani *et al.* 2006). However, heat is implicated in sensitizing bacterial cells to oxidative stress (Knabel *et al.* 1990; Liu *et al.* 2005; Cebrian *et al.* 2007), and this combination could explain the differential plasma sensitivity of *S. aureus.*

'Bystander' or indirect activation was observed with *S. aureus* cells and to a lesser extent with *Ps. aeruginosa* cells. The same effect was observed using a similar plasma jet (Feng *et al.* 2009) and in other studies with helium-operated jets (Lu *et al.* 2008; Rupf *et al.* 2010). This inactivation outside the direct treatment area of 2×2 cm^2 suggests that long-lived species may be generated that are not necessarily in the plasma or the afterglow but potentially in subsequent reactions with ambient air or in the environment of the micro-organism. *S. aureus* cells in particular could be inactivated by both short-lived (and therefore short-range) agents directly expelled from the plasma and potentially to their longer-lived reaction products. Although the plasma was delivered in a square pattern, the indirect effect assumed a circular pattern, most likely due to gas flow dynamics. Nitric oxide in particular has a high diffusion capacity (Kelm 1999).

Using another physical inactivation method, pulsed electric fields, a sublethal effect on *Pseudomonas putida* cells was demonstrated. However, no pulsed field resistance was generated over 30 generations (Gusbeth *et al.* 2009). Conversely, generations of heat stress in *E. coli* induced acquisition of genomic mutations, resulting in the ability to grow at high temperatures (Rudolph *et al.* 2010). The 'bystander' effect may result in suboptimal plasma inactivation in peripheral areas, and surviving microbes may become more resistant to this physical inactivation method.

The level of inactivation on pig skin pieces, while significant ($P < 0·001$), was lower than that on agar plates ($P < 0·01$) for each organism. While the agar surface is relatively smooth, the skin's stratum corneum is composed of dead cells and includes hair follicles and sweat glands. An obvious hypothesis for this generally lower inactivation on skin was that its surface structure protected the organisms. Although the methods of detection were different (Benediktsdottir and Hambraeus 1983; Sullivan *et al.* 2004), the level of inactivation was similar

to that found in another study using an argon-operated plasma on infected wounds (Ermolaeva *et al.* 2011). Skin roughness is difficult to quantify (Cook *et al.* 1982; Van Neste 1991; Fischer *et al.* 1999; Bargo and Kollias 2010; Bloemen *et al.* 2011) and varies with age, anatomic site and the use of hand cream (Manuskiatti *et al.* 1998; Kampf and Ennen 2006), so skin roughness may remain a significant variable for clinical applications.

Interestingly, *A. baumannii* was the most sensitive to atmospheric plasma application on the skin surface. The difference in response between agar and skin surfaces might suggest differential chemical reactions of the plasma components with air, producing different reactive radicals and chemical compounds. Conceivably, depending on the chemical environment, some of the compounds generated on skin may be different or in different concentrations and could be more or less effective against specific organisms.

As the results show, the nature of the interaction of a nonthermal atmospheric pressure plasma jet with microorganisms hosted in different environments is apparently complex. An intricate and detailed understanding of chemical reactions and of the differences in membrane composition and biochemistry of cells seems necessary to obtain an understanding of the underlying mechanisms. Regardless, the results clearly demonstrate the potential of a nonthermal DC-operated air plasma jet against organisms with potential for multidrug resistance. While other plasma sources may be operated with noble gases at atmospheric pressure and can only tolerate admixtures of oxygen of a few per cent to avoid instabilities, this system is run with air and therefore potentially promotes higher concentration of reactive oxygen species, which may be an important factor in bacterial inactivation. With this plasma system, no thermal skin damage is detected histologically (Kolb *et al.* 2008), making the method a promising alternative for skin disinfection.

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