

Plasma Medicine in Dermatology: Basic Antimicrobial Efficacy Testing as Prerequisite to Clinical Plasma Therapy

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ABSTRACT: Plasma medicine has become an emerging field in medical sciences since cold plasma has demonstrated significant antibacterial properties *in vitro* and *in vivo*. However, systematic antimicrobial plasma testing against bacteria, fungi, and parasites is still lacking. Chronic wounds, fungal skin and nail infections, and colonization with multidrug-resistant pathogens like *Staphylococcus aureus* (MRSA) often pose significant therapeutic and economical problems and new therapeutic concepts are strongly warranted. The challenge of worldwide increasing resistance problems including different pathogens like methicillin-resistant MRSA, vancomycin-resistant enterococci, and gram-negative species producing beta-lactam hydrolysing enzymes (extended spectrum β -lactamases) constitute the need for alternative antimicrobial treatments. Cold plasma therapy with a completely different mode of action compared to conventional antimicrobials may offer an alternative to conventional external antibiotic and antiseptic therapies. In order to estimate the possible role as physical antiseptic, basic susceptibility data from plasma are needed. To provide such data, the plasma susceptibility of representative skin and wound pathogens against low-temperature atmospheric pressure plasma jet (APPJ device) and dielectric barrier discharge plasma treatment was tested *in vitro* on agar. The same plasma sources were used to test clinical fungal isolates *in vitro* and isolates of the parasite demodex folliculorum *ex vivo*. Plasma treatment proved to be highly effective in eradicating all treated strains and species including *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* group (*K. pneumoniae* ssp. *pneumoniae*, *K. oxytoca*), *Staphylococcus aureus*, hemolysing Lancefield *Streptococci* (group A and B), *Proteus* group (*P. mirabilis*, *P. vulgaris*), *Acinetobacter* spp., *Stenotrophomonas* spp., *Enterococcus faecalis*, *Candida albicans*, and *Staphylococcus epidermidis*. Plasma irradiation of *Trichophyton rubrum*, *Trichophyton interdigitale*, *Microsporon canis*, and the yeast *Candida albicans* was able to kill >90% of the organisms during 30 s *in vitro* with no isolate exhibiting resistance. The APPJ-plasma killed *Demodex folliculorum* after exposure time of 2 s. Our data suggest plasma as a possible new option in the treatment of microbial, fungal, and parasitic dermal infections.

KEY WORDS: atmospheric low-pressure plasma jet (APPJ), dielectric barrier discharge (DBD), antiseptis, dermatomycosis, *Tinea pedis*, fungi, plasma medicine, *Trichophyton mentagrophytis* var. *interdigitale*, *Trichophyton*, *Microsporon*, *Candida* spp., MRSA, skin and wound pathogens, *Demodex folliculorum*, skin infection, folliculitis

I. INTRODUCTION

Plasma medicine is an expanding focus and offers new therapeutic aspects combining potent physical partial efficacies like UV, IR, ozone, reactive nitrogen (RNS), and oxygen (ROS) species. Reactive OH groups N_xO_x , and HNO_x are generated and nowadays many successful treatments of different illnesses have been described. Non-thermal atmospheric pressure plasma has been introduced in medical and biologic applications since it demonstrates well-characterized antimicrobial *in vitro* efficacy as well as medically important biochemical effects.¹⁻³ In recent years, first results of clinical plasma applications were undertaken to treat diverse skin and soft tissue infections like bacterial dermatitis, chronic ulcer wounds, and eyelid infections, but also severe pulmonary tuberculosis.^{4,5}

A. Skin and Wound Pathogens

In our previous work, we were able to show that two plasma sources, the APPJ and the DBD, were highly effective in reducing bacterial and fungal species.^{3,6} The plasma sources killed most species on agar after 3 s up to 30 s without exception. Plasma irradiation produced large and distinct inhibition zones depleted of bacterial and fungal growth. Together with similar published data by other groups and in combination with noncritical data from risk assessment,⁷⁻⁹ it can be deduced that plasma as first “physical antiseptic” could also be effective in hospital hygiene and wound management to disinfect skin and contaminated, colonized, and infected skin and wounds. Especially chronic wounds like venous and decubital ulcers are important often harbor MRSA and other multiresistant pathogens.^{10,11} Actually, the hypothesis of plasma as wound antiseptic is under investigation and meanwhile a clinical trial to treat chronic venous leg ulcers with DBD plasma has been initiated at the Department of Dermatology, Venerology, and Allergology in Göttingen, Germany (see www.clinicaltrials.gov). In another study, Morfill and colleagues treated chronic ulcer wounds with cold plasma and found a significant decrease of bacterial load after treatment.¹² Predominantly, these data seem to support the antiseptic role of plasma. However, any clinical use of an antimicrobial agent requires systematic testing of clinical important isolates and species using a suitable test model and the prediction of antimicrobial susceptibility on the basis of adequate statistical data represents an imperative necessity in modern antimicrobial treatment. Thus, when plasma is intended for local antiseptic or antibiotic use, this application has to be based on validated reproducible and standardized efficacy testing.

Up to now, these data are lacking. Clinical efficacy of antiseptics necessitates at least three decadic logsteps of antibacterial reduction of a given bacterial bioburden, which has to be evaluated in standardized *in vitro* tests using a defined set of representative species. Therefore, as primary tests to prepare the implementation of such standardization, we systematically tested the plasma susceptibility of different sets of clinical relevant bacterial and fungal strains *in vitro*.

B. MRSA

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of skin and soft tissue infections including severe infections like wound infections, pneumonia, and sepsis^{13–15} acquired in hospitals or in the community.^{13,16–18} Successful MRSA management strongly relies on the implementation of standard infection-control measures; despite some progress in some EU regions, however, MRSA containment still remains a significant challenge worldwide.^{19,20} Even though the proportion of MRSA among *S. aureus* is still above 25% in eight out of 28 countries, the occurrence of MRSA is stabilizing or even decreasing, as observed in Austria, France, Ireland, Latvia, the UK, and Cyprus.²¹ In the international ranking, Germany holds a middle position with a proportion of MRSA among *S. aureus* of ~20%.²¹ The highest MRSA contaminations are typically found in intensive care units where patients are at high colonization risk due to, e.g., invasive medical devices, antibiotic treatments, and multi-morbidity.²² Wounds, especially chronic wounds like venous and decubitus ulcers, represent another important risk factor for MRSA colonization.^{10,11}

Single-room isolation, screening of potential carriers, decontamination (sanitation) of colonized patients and medical staff, and the implementation of evidence-based guidelines together with a functioning multibarrier hygiene system belong to the most important measures in MRSA containment.^{19,20} Among these, the safe eradication of MRSA is crucial for the success of MRSA containment management. Following German recommendations, standard decolonization consists of antiseptic washings of the whole body combined with nasal antibiotic decontamination over seven days.^{21–23} The main substances for eradication of MRSA are antibiotics (mupirocin) and antiseptics, i.e., polihexanide and octenidine dihydrochloride,^{21–25} which however can pose some problems for MRSA eradication.^{24,25} Alternatives are therefore warranted and since cold plasma already has demonstrated high efficacy against many microbial pathogens *in vitro*^{1–3,6,26} and *in vivo*,¹² and appears to be well tolerated,^{7,27–29} plasma treatment may serve as an alternative antiseptic. However, systematic susceptibility data of clinical MRSA isolates to plasma treatment are rare and have yet neither been compared to MSSA susceptibility nor correlated to *in vivo* conditions. Therefore, we tested the efficacy of two different cold plasma sources (DBD and APPJ) against a representative set of clinically relevant strains of MRSA including hospital-associated (HA-MRSA), community-associated (CA-MRSA), and livestock-associated (LA-MRSA) strains in comparison with MSSA susceptibility.

C. Fungal infections in dermatology

Tinea pedis and onychomycosis represent the most frequent fungal infections worldwide and may cause significant clinical distress such as secondary bacterial infections, discomfort (pain, itching), and esthetic disfigurement and easily lead to recalcitrant infection. In addition, every unsuccessfully treated tinea lesion serves as a potential reservoir to further germ transmission. Nearly 30% of Europeans suffer from tinea pedis, often

combined with nail infection (onychomycosis);^{30,31} both diseases are increasing worldwide, in part due to the growing prevalence of older people and the progress of modern medicine using more and more immunodepressing therapies (chemotherapy, irradiation, biologics). Other risk factors include genetic disposition, humid environment (bathing, sports), diabetes mellitus, psoriasis vulgaris, and vascular insufficiency. Recalcitrant tinea pedis and onychomycosis pose substantial individual but also socioeconomical problems.^{32,33} Most common causative fungi of tinea pedis and onychomycosis are the dermatophytes *Trichophyton rubrum* and *Trichophyton interdigitale*, to a lesser extent *Candida albicans*, and seldom molds (e.g., *Aspergillus niger*).

D. Therapeutic Dilemma

Tinea pedis and onychomycosis are treated with topically applied antifungal drugs (i.e., terbinafine) in combination with systemic therapy in the case of more severe disease. However, despite long-lasting combined therapies, clinical improvement often cannot be achieved. The main reasons for this therapeutic dilemma are reinfections in a highly contaminated environment, the humid milieu (interdigital humid chamber phenomenon) promoting fungal growth, the synergistic role of invading bacteria (*S. aureus*) and yeasts (*C. albicans*) and the thickness of the invaded corneocyte layer harboring inaccessible reproductive fungal hyphae. Another fact is the impossibility for the antimycotic to reach inhibitory concentrations (minimal inhibitory concentration) in the affected skin or nail tissue. The treatment of tinea pedis and onychomycosis can take one year or more and is accompanied by possible adverse effects of the applied drugs.³⁴ Another problem is the lack of adequate options for environmental decontamination procedures. This is of crucial importance since tinea pedis is a contagious infection disease that is propagated mainly from human to human, and via environment by many animals like pets (cats and dogs). Typical transmitting objects are contaminated towels, sponges, and clothes. Arthrospores of dermatophytes can survive in the environment for several years, representing a long-term source of infection.

1. Plasma Option

In the treatment of fungal diseases, it can be supposed that plasma can overcome the basic problem of pharmacological therapy by physical means, the inactivation of the fungal reproductive element, spores, and hyphae without affecting the neighboring tissue, avoiding multiple treatment sessions.

2. *Demodex Folliculorum*

Rosacea is a frequent and often disfiguring and chronic dermatologic disease mainly of the mid face causing central facial erythema, telangiectasia, papules, and pustules. In the context of a complex pathogenicity, *Demodex folliculorum* plays an important role showing significant density in the skin of patients with papulopustulous rosacea. Rosacea belongs to the hard to heal diseases and new approaches for treatment are strongly required.

With the exception of leishmania treatment (*in vitro*), the knowledge of plasma effects on parasites is rather scant.³⁵ If plasma also demonstrates efficacy against other parasites, this would be of particular relevance, e.g., in dermatology. Therefore, we investigated the plasma susceptibility of *Demodex folliculorum*, a parasite with involvement in the pathogenesis of rosacea, a widespread disease of the facial skin. Accordingly, we irradiated clinical isolates of *Demodex folliculorum* from a patient with chronic pustulous rosacea by low-temperature atmospheric pressure plasma *ex vivo*.

II. MATERIALS AND METHODS

The plasma sources, the treatment of wound and skin pathogens, the antifungal treatment, and the demodex treatment were described in former contributions of our group (for wound bacteria, see Ref. 36, for antifungal treatment, see Ref. 6, and for demodex treatment, see Ref. 27). Biofilm and MRSA treatment are new contributions.

A. Plasma Sources

1. Atmospheric Pressure Plasma Jet (APPJ)

The schematic setup of the atmospheric pressure plasma jet (APPJ) device, kINPen 09 (INP Greifswald e.V., Greifswald, Germany), used in this study is depicted in Fig. 1. For a detailed characterization of the APPJ, see Ref. 37. Briefly, in the center of a quartz capillary (inner diameter 1.6 mm), a pin electrode (1 mm diameter) is mounted. Argon as the feed gas flows through the capillary (gas flow rate of about 6 l/min). A radio frequency (RF) voltage (1–5 kV, 1.5 MHz) is coupled to the center electrode. The plasma is generated from the top of the center electrode and expands to the surrounding air outside the nozzle. The axial temperature profile of the plasma was obtained by fiber optic temperature measurement (FOT Lab Kit Fluoroptic Thermometer, Luxotron model 755). A temperature-dependent fluorescent signal of luminescent magnesium fluorogermanate that was excited with a Xe flash lamp was monitored. To avoid thermic effects on skin, a pulsed electronic regulation holds the temperature at the tip of the beam $\leq 37^\circ\text{C}$. By optical emission spectroscopy (OES) using a fiber spectrometer (StellarNet EPP2000-UVN), emission lines of excited argon in the VIS/NIR region between 700 and 1000 nm and nitrogen emission lines between 320 and 400 nm were found. Excited OH radicals at 309 nm supply the most significant emission.

There was no detectable emission in the UV-C range between 200 and 280 nm.¹ The emission spectra recorded at different axial positions of the plasma jet at 3 W and an Ar gas flow rate of 5 slm in the continuous working mode are shown in Fig. 2.

2. Dielectric Barrier Discharge (DBD)

The dielectric barrier discharge device (DBD, (CINOGY GmbH, Duderstadt, Germany) generates high voltage pulses (14 kV) across small gaps with the electrode covered by

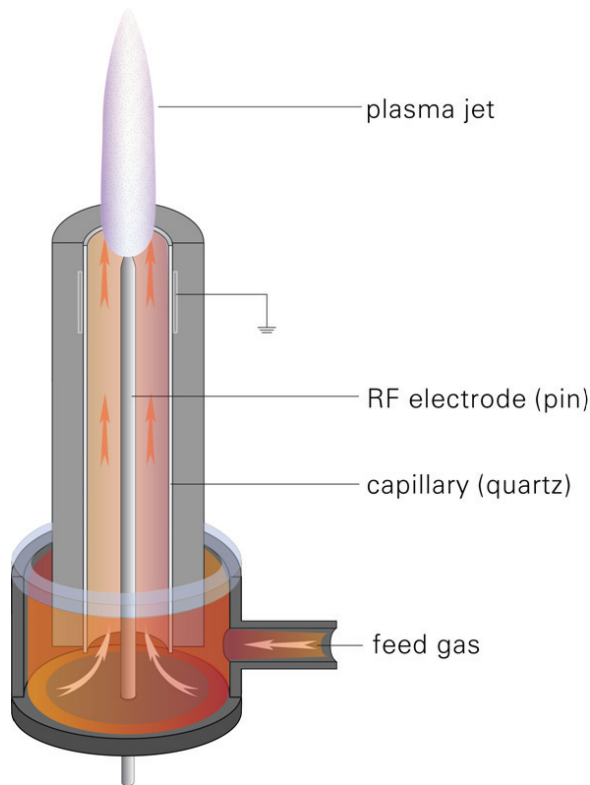


FIG. 1: Schematic setup of the APPJ device¹

a dielectric barrier made of macor (Fig. 3). The diameter of the glass ceramic electrode measures 20 mm. The pulse repetition rate is adjustable between 100 and 400 Hz, which leads to electric power dissipated in the gas discharge in the range of 167–237 mW. Hereby, transferred electric energy per pulse was measured by the Lissajous method and power was calculated according to frequency.^{38,39}

The UV emission intensity and spectral distribution were measured by arranging the DBD electrode (Fig. 4) parallel to a plate of indium tin oxide coated with fused silica that hereby acts as the floating counter electrode. The spectral distribution of the plasma radiation was measured with the spectrometer STE-EPP2000 (StellarNet, Inc., Tampa, Florida). With a calibrated gauge head between 250 and 400 nm UV-3719-4a and an X1-Optometer (Gigahertz-Optik GmbH, Türkenfeld, Germany) that rested on the surface of the uncoated side of the silica plate, the irradiation intensity of the plasma emission could be measured after passing through the absorbing counter electrode. With known spectral transmission properties of the counter electrode and the spectral distribution of the plasma emission, we calculated the radiation intensity prior to absorption, thus, allowing the measurement of the irradiation intensities that are comparable

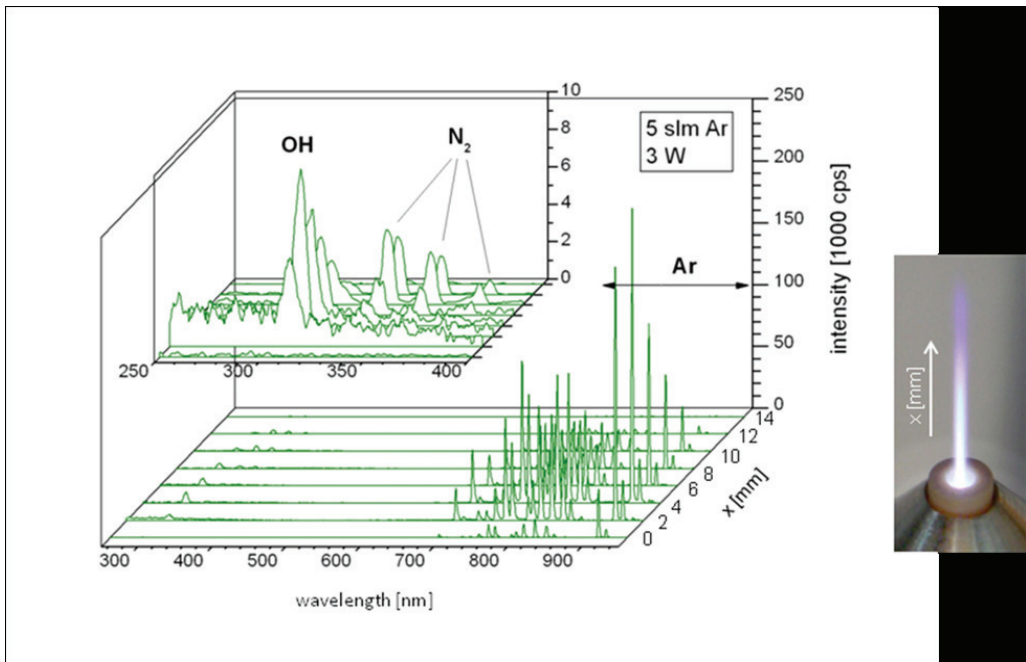


FIG. 2: Optical emission spectra measured at different axial positions of the Ar-plasma jet¹

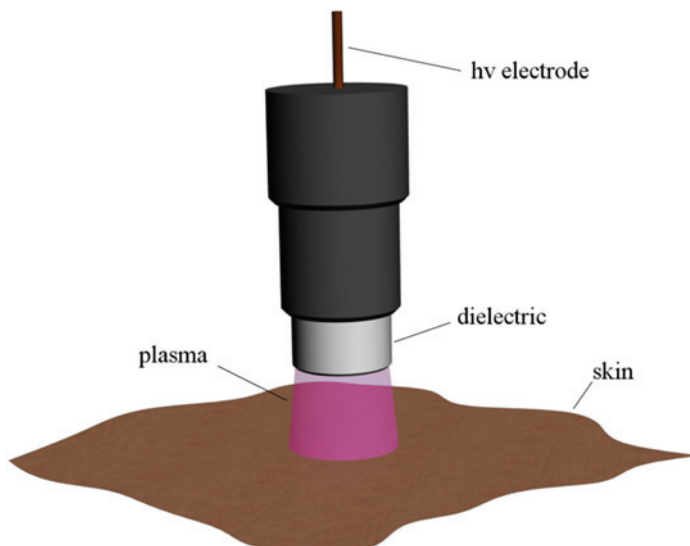


FIG. 3: Schematic setup of the DBD device

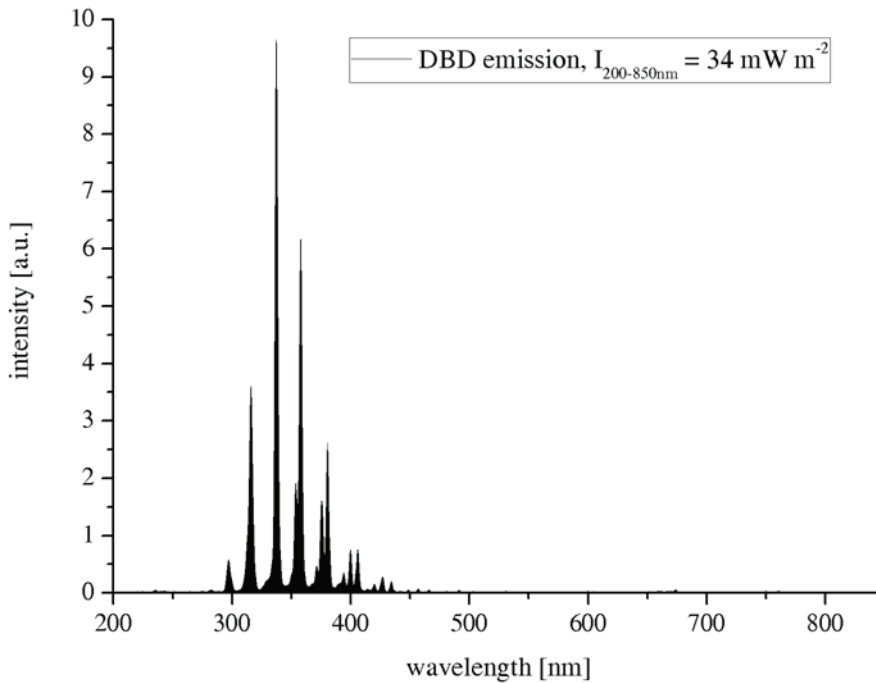


FIG. 4: Spectral distribution of DBD emission comprising of second positive system and first negative system of electronically excited N_2 and N_2^+ microbial strains and isolates under investigation.

with direct gas discharges on living tissue. With this method, an irradiation intensity of 34 mW/m^2 was determined for the DBD device in a 2 mm distance from the dielectric. The results in Fig. 4 show typical emission lines for DBD discharges in air operated at these experimental conditions originating from electronically excited nitrogen neutrals and ions. Most of the radiation lies in the UV-A range, low emission is found in the UV-B range, and virtually no radiation is emitted in the UV-C range.

3. Skin and Wound Pathogens

In total, 105 clinical isolates of 11 different species including most relevant wound pathogens were tested (10 isolates of each species except *Stenotrophomonas spp.* with five isolates): *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Klebsiella* group (*K. pneumoniae ssp. pneumoniae*, *K. oxytoca*) (KLEBS group), *Staphylococcus aureus* (SA), *hemolysing Lancefield Streptococci* (group A and B) (HS), *Proteus* group (*P. mirabilis*, *P. vulgaris*) (PROT group), *Acinetobacter spp.* (ACI), *Stenotrophomonas spp.* (STENO), *Enterococcus faecalis* (EF), *Candida albicans* (CA), and *Staphylococcus epidermidis* (SE).

All strains were isolated from acute or chronic wounds of patients of our dermatologic clinic during routine microbiology (microbiologic laboratory of the dermatologic clinic in Greifswald) after suspected infection of a wound or in the course of microbial surveillance cultures (MRSA screening, admission screen for multidrug resistant strains). Each strain was isolated from one patient (no double testing). In addition, nine reference strains were tested (Table 1).

4. Microbiology

Samples were processed following the national guidelines for microbiologic diagnostics. Identification and susceptibility testing was performed using the automated VITEK compact system (Biomérieux, Nürtingen, Germany), selective culture conditions (elevated salt concentrations, lowered incubation temperature for staphylococci), and real-time PCR (Light cycler, Roche diagnostics, Basel, Switzerland) for *mecA* detection (MinervaBiolab, Berlin, Germany). After identification and susceptibility testing, the cultures of the defined strains were stored at -70°C .

5. In Vitro Susceptibility Test Model

Before starting plasma treatment, the strains were thawed and cultured aerobically at 36°C overnight on blood agar (5% sheep blood, Oxoid, Wesel, Germany). From these colonies, suspensions in sterile broth were made and diluted until nearly confluent growth was obtained after another overnight culture on blood agar. Directly after plating the suspensions on agar under sterile conditions, plasma treatment was initiated. The APPJ device and the DBD device were fixed in an adjustable rack to secure a 2 mm distance between the tip of the plasma jet of the APPJ or the DBD device. Plasma treatment was performed punctually for 3 s. The temperature did not

TABLE 1. Tested reference strains for comparison

Tested species*	Reference strain
<i>Escherichia coli</i>	ATCC 25922
<i>Pseudomonas aeruginosa</i>	ATCC 15442
<i>Klebsiella</i> group	ATCC 700324
<i>Staphylococcus aureus</i>	ATCC 1924
<i>Staphylococcus epidermidis</i>	ATCC 12228
<i>Proteus</i> group	ATCC 6380
<i>Enterococcus faecalis</i>	ATCC 29212
<i>Candida albicans</i>	ATCC 10231
Hemolyzing Lancefield Streptococci (group A and B)	ATCC 27956

surpass 37°C as measured by the digital thermometer ScanTemp 485 (Dostmann electronic GmbH, Wertheim, Germany). All treatments were accomplished on the same day time under controlled room climate with $T = 22.01 \pm 0.89^\circ\text{C}$ and relative humidity $rH = 41.9 \pm 3.7\%$. Directly after each treatment, the agar plates (Columbia sheep blood agar, Oxoid, Wesel, Germany) were incubated at 36°C aerobically and the growth of bacteria after 24, 48, and finally 72 h was controlled visually. For calculation of susceptibility to plasma treatment, the diameters of the areas without vegetative growth (D_{test} : mean of two measurements at 90 deg angle) were measured and divided by the reference diameter D_{ref} (deduced from former experiments, data not shown). The results are expressed as a susceptibility index QR_{PL} following the equation $QR_{\text{PL}} = D_{\text{test}} (\text{mm})/D_{\text{ref}} (\text{mm})$. To state the susceptibility, characters of the single isolates the obtained QR_{PL} values were classified as follows: $QR_{\text{PL}} > 0.95$ are defined as susceptible (S) to the plasma treatment, between 0.90 and 0.94 as intermediary susceptible (I), and values < 0.89 as resistant (R) to plasma treatment. The method was created referring to agar diffusion method (Bauer-Kirby) testing bacteria susceptibility against antibiotics (Daeschlein *et al.*, publication in preparation).

6. In Vitro Biofilm Testing

To assess the efficacy of cold plasma against biofilms, we developed a biofilm model to treat biofilms on two different surfaces: V4 stainless steel plates (1 cm diameter) and polyethylene plates (1 cm diameter). To produce biofilms, a suspension of CASO-bouillon with overnight grown *Staphylococcus aureus* (ATCC 4020) was incubated for three days together with the plates under gentle shaking at 37°C. After this period, the plates were overgrown by multiple layer biofilms as proven by microscopic examination and detachment experiments demonstrating non-soluble films on the plate surface (data not shown). The ability of the plasma to eradicate the *S. aureus* biofilms was examined by irradiating freshly prepared biofilms on the plates that had been rinsed in sterile saline directly before treatment to remove all soluble bacteria. The irradiation was realized with the DBD device fixed in a rack assuring a distance of 1 mm between plasma electrode and plate surface. After treatment, the plates first were pressed against contact agar and second (after rinse) were sonified (50 cycles/s) for 20 s to disrupt all viable germs that consecutively were plated quantitatively onto blood agar. After 48 h of incubation (aerobe at 37°C), the grown colonies were visually enumerated and compared to controls (nontreated plates). As positive controls, we used antiseptic solutions (polihexanide, 70% ethanol, acetic acid 2, and hypochlorite) that were incubated with the biofilm pates over 1, 2, and 3 min. An eradication of a biofilm on a plate was diagnosed when no growth was found after sonifying of plates and three days of aerobe incubation. We tested a noncommercial DBD plasma (CINOGY, with an electrode of 2 cm diameter) and the PlasmaDerm DBD (CINOGY) with an electrode of 1 cm diameter and different power mode and exposure times of 1, 2, and 3 min.

7. MRSA and MSSA Treatment

In total, 55 MRSA and 50 MSSA isolates from skin or wounds from dermatologic patients at the dermatology clinic in Greifswald were included in the tests. Fifty of the MRSA isolates were HA-MRSA (hospital-associated), 4 CA-MRSA (community-associated, USA300), and one LA-MRSA (livestock-associated, STD 1398).

8. Antifungal Treatment

a. Species under Investigation

The following fungi were investigated. Yeasts: *C. albicans*, $n =$ five strains. Dermatophytes (each five strains): *T. rubrum*, *T. interdigitale*, and *M. canis*. All strains were isolated in our clinic from patients with mycotic lesions (tinea pedis, tinea corporis, onychomycosis).

b. Testing Punctual Antifungal Effects on Agar

Fresh suspensions of clinical fungal isolates (10^6 cfu/ml) in sterile saline (0.9 %) were diluted until nearly confluent growth of the colonies on Sabouraud dextrose agar (4% glucose, Heipha, Germany). Taplin agar (4% glucose, Heipha, Germany) was obtained. Dermatophytes were cultured on Taplin or Sabouraud dextrose agar over three to seven days at 30°C until full growth developed. *C. albicans* was cultured on Sabouraud dextrose agar over three days at 36°C.⁴⁰

For plasma treatment, the APPJ device was fixed in a rack to assure a constant distance between the tip of the plasma jet and the surface of the agar with the plated microorganisms, allowing direct contact of the tip of the jet with the agar surface over definite time spans (0, 3, 6, 9, 12, 16, 20, 24, 28, and 30 s). After plasma treatment, the agar plates were incubated and visually screened for inhibition zones at the irradiated areas after three to seven days. In order to evaluate the kinetics of the antifungal efficacy over time for each species, we measured the diameters of the inhibition zones resulting after increasing irradiation time and calculated the amount of inactivated colony forming units at each time on agar (corresponding to the eradication zone).

c. Irradiation of Fungal Colonies Grown on Agar

To approximate clinical use with the need to effectively eradicate larger areas of mycotic skin lesions by the plasma, fungal suspensions on whole agar plates (56.7 cm²) were treated by the APPJ. One isolate of *T. rubrum* and two of *T. interdigitale* were tested. Suspensions of the respective fungal strains were streaked onto the Sabouraud dextrose agar or the Taplin agar before being treated with the jet. The treatment differed from the former (punctual) test by passing the jet over the agar plate following meandric lines over 10 min (5 × 2 min) according to the experimental setup described in Ref. 3. The

irradiated culture dishes were incubated directly after irradiation aerobically at 30°C over seven days (dermatophytes) and 36°C over three days (*Candida*) and compared to untreated control plates.

d. *Irradiation of Fungal Elements Embedded in Dandruffs from Tinea Lesion*

Skin samples (dandruffs) of a tinea patient were taken from untreated lesions by scalpel scrubbing. Samples (every sample size about 1 × 1 mm) were placed on agar plates and immediately irradiated over 2 min by plasma (APPJ). Nonirradiated dandruffs from the same lesions served as controls. After irradiation of the dandruffs, the agar plates were aerobically incubated over four weeks at 30°C and the number of grown colony forming units (cfu) of *T. interdigitale* compared with controls (nonirradiated dandruffs). Microbiologic examination was performed by treating the samples with KOH for solubilization, consecutive microscopic evaluation (staining with lactophenol cotton blue at 40 × 10 magnification), and cultivation on Sabouraud dextrose agar (with 4% glucose, heipha Dr. Müller GmbH, Eppelheim, Germany) over four weeks at 30°C. In total, we tested 15 series of sampling dandruffs from patients before antifungal treatment of tinea pedis caused by *T. interdigitale*. 12 (minimum) to 32 (maximum) dandruff samples (Table 2) were taken at each serial sampling, half the samples were plasma treated (2 min punctual) and the remaining half served as (not irradiated) control. Cultured colonies were biochemically and microscopically differentiated following the national guidelines for good laboratory practice. After culturing, the number of positive samples (cultural fungal growth) of irradiated and not irradiated dandruff samples was compared and the percent reduction calculated.

e. *Treatment of Environmental Arthrospores*

Three pairs of shoes (one pair of sports shoes, two pairs of leather shoes) of a patient with chronic tinea pedis of both feet were examined for spores of *T. interdigitale*, which was the causative microorganism of the tinea. The insoles of all shoes were cultural positive for *T. interdigitale* resulting in 1–5 cfu/swab and shoe on Sabouraud dextrose agar (data not shown). Directly after swabbing, the insole of every shoe was irradiated by the APPJ by moving the jet over the complete inner surface over 3 min. Thereafter, again, the insoles of all shoes were quantitatively swabbed (one insole, one swab) with culturing on Sabouraud dextrose agar. The agar was incubated aerobically over four weeks at 30°C with weekly visual control for fungal growth.

f. *Treatment of Demodex Folliculorum Ex Vivo*

From a 54-year-old rosacea patient who suffered from typical symptoms (teleangiectasia, itching with erosions), hair samples from both temple sites were taken on two different days during ambulant therapy. Single hairs were plucked by forceps to be im-

TABLE 2. Comparison of bactericidal activity and some characteristics of plasmas used in this study

Sample	<i>n</i> dandruff samples evaluated	<i>n</i> plasma treated dandruff samples	<i>n</i> dandruff samples with fungal* growth after APPJ	<i>n</i> dandruff samples nontreated with fungal* growth (controls)	reduction efficacy by plasma treatment (%)
22/09	14	7	0	3	100
23/09	20	10	0	2	100
24/09	24	12	0	0	—
25/09	28	14	0	14	100
26/09	32	16	0	0	—
27/09	20	10	0	7	100
28/09	26	13	0	0	—
29/09	16	8	0	0	—
37/09	16	8	0	8	100
41/09	24	12	0	0	—
68/09	12	6	0	0	—
884/08	16	8	0	0	—
891/08	14	7	0	0	—
12/08	16	8	0	8	100
30/08	16	8	0	3	100

* *T. interdigitale*

mediately microscopically analyzed for parasites in the microbiological laboratory of our dermatologic clinic.

For visualization and preanalytics, the hairs were taken from the tube and embedded in sterile saline (0.9%) on a glass slide (76 × 23 mm) under a cover slide (20 × 30 mm) for microscopic examination using 400× (binocular microscope LABOVAL 3 Carl Zeiss Jena) and 20×–40× magnification (digital microscope Coolscope, Nikon). Before irradiation tests, the moving of the parasites was monitored by video recording in order to define the normal pattern of motility under test conditions. The spontaneous motility of parasites was found nearly continuously with slow movements of body and legs (approximately one movement of at least one part of the body/s could be defined as control).

At day one of the investigation, two parasites of *Demodex folliculorum* could be isolated from the patient. One did not show any motility during the whole test period and was excluded from the study. At day two (two months later), we were able to detect four parasites of *Demodex folliculorum* on hairs (Fig. 5), two with nearly

constant movements near the hair follicle and two parasites embedded in secretlike material around the hair follicle showing slow movements with interruptions of up to 25 s. All parasites to be treated were transferred from their original slides and hair to a second “treatment” slide with fresh sterile saline where plasma irradiation with APPJ was performed.

For plasma treatment, in total, five parasites were irradiated by APPJ at two investigation days. Irradiation by the jet was pointed directly against the parasite at first under the cover slide and thereafter without the slide, with the tip of the jet (visible beam) at a distance of 3 mm to the parasite at an angle of 45 deg. During irradiation, a lifetime observation with a microscope (LABOVAL 3 Carl Zeiss Jena) was realized. A second person monitored and filmed the motility of the parasites (C-5050 digital camera Olympus, Nikon) under irradiation. Three irradiation periods of 4×15 s were performed with 30 s of interruption. After 3 min, the same irradiation procedure as described was undertaken without cover slide. At the second investigation day, plasma treatment was performed directly without cover slide starting with 1, 5, 15, 30, and 60 s of irradiation of every parasite. Up to their own irradiation, every nonirradiated parasite served as control and was examined by microscopy like the irradiated one during as well as after treatment. After each treatment, a period without irradiation of 10 min was introduced to monitor potential delayed plasma effects.

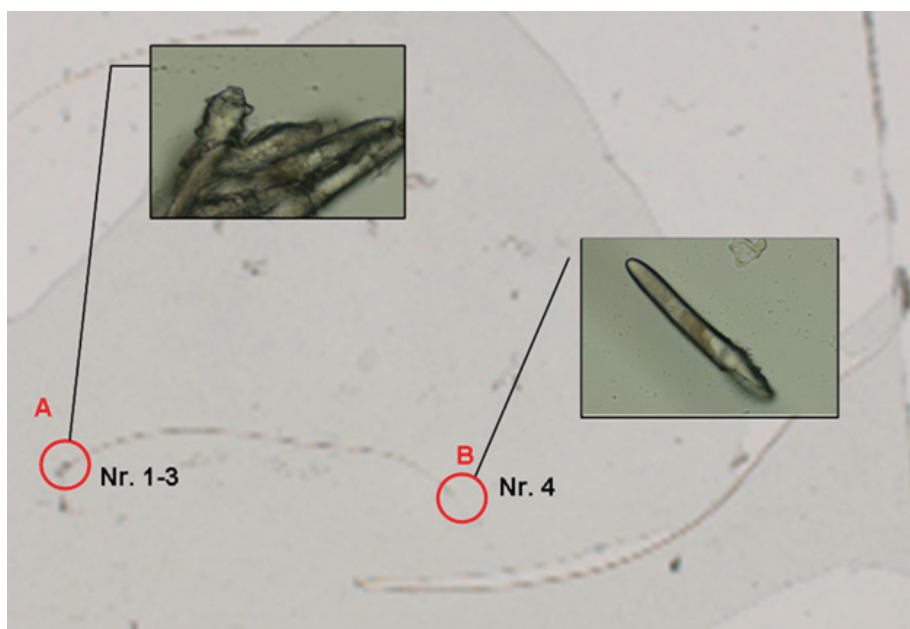


FIG. 5: *Demodex folliculorum* in the hair of the 52-year-old patient with rosacea—magnified 20–40×

III. RESULTS

A. Skin and Wound Pathogens

1. Agar Test Model

Treatment by both plasma sources resulted in bacteria-free zones (circular inhibition zones) in the growth zone of the agar surface as measured 72 h after irradiation corresponding to a significant decline of the bacterial growth. The resulting diameters of the inhibition zones differed between species. The corresponding areas without bacterial growth after plasma treatment are given in Table 3. Figs. 6(a)–6(c) show the box plots and confidence intervals of the obtained diameters of selected (clinically most important) species [*Staphylococcus aureus* (SA), *hemolysing streptococci* (HS), *Pseudomonas aeruginosa* (PA), *Escherichia coli* (EC), and *Candida albicans* (CA)]. Altogether, most

TABLE 3. Ranking of areas without vegetative growth (SD) after *in vitro* treatment of clinical strains with DBD, APPJ pulsed and APPJ nonpulsed ($n=10$, STENO $n=5$)

Ranking	Species	DBD (mm ²)	Species	APPJ nonpulsed (mm ²)	Species	APPJ pulsed (mm ²)
1	HS	389.9 (26.9)	PA	40.4 (3.2)	EC	21.5 (2.0)
2	SE	358.9 (19.8)	CA	30.7 (2.6)	CA	20.9 (2.0)
3	EF	344.8 (21.2)	KLEBS group	30.2 (2.5)	STENO	20.0 (0.9)
4	CA	305.8 (22.8)	STENO	26.0 (2.3)	PA	18.9 (1.6)
5	STENO	272.0 (23.8)	EC	21.7 (2.2)	EF	17.1 (1.6)
6	KLEBS group	271.6 (21.6)	EF	21.7 (1.7)	HS	12.4 (1.4)
7	SA	269.9 (12.7)	SA	19.2 (0.9)	KLEBS group	9.4 (0.6)
8	PA	263 3 (23.1)	SE	18.7 (1.6)	PROT group	7.2 (0.4)
9	PROT group	246.4 (23.3)	HS	14.0 (1.5)	SA	7.0 (0.4)
10	EC	232.5 (15.2)	PROT group	13.4 (1.8)	SE	6.8 (0.5)
11	ACI	211.9 (12.1)	ACI	13.2 (1.2)	ACI	3.2 (0.3)

homogenous median values can be found after treatment by pulsed APPJ [box plot, Fig. 6(c)]. The medians of the diameters varied between 3.0 mm (SA) and 5.1 mm (EC) after treatment by pulsed APPJ, 4.3 mm (HS) and 7.2 mm (PA) after treatment by non-pulsed APPJ [box plot, Fig. 6(b)], and 17.3 mm (EC) and 22.0 mm (HS) after treatment by DBD [Fig. 6(a)]. In comparison to the nonpulsed APPJ, except EC, the diameters of the inhibition zones of SA, PA, HS, and CA were significantly smaller after treatment with pulsed APPJ ($p < 0,05$).

This means that the best efficacy obtained by DBD was found against HS, by non-pulsed APPJ against PA, and pulsed APPJ against EC. Accordingly, the second rank was found treating SE by DBD, CA by nonpulsed APPJ and pulsed APPJ. Accordingly, the third rank was found treating EF by DBD, KLEBS group by nonpulsed APPJ, and STENO by pulsed APPJ. In summation, we can conclude that DBD was most effective against HS, nonpulsed APPJ against PA, and pulsed APPJ against EC.

2. Susceptibility Index Evaluation

The mean \pm Sd QR_{PL} obtained after treatment with the DBD, the APPJ ranked from mean 0.98 ± 0.0 (*Proteus group*, nonpulsed APPJ) to 1.04 testing the four species *Klebsiella group* ± 0.03 , *Staphylococcus epidermidis* ± 0.04 , *Stenotrophomonas spp.* ± 0.02 , and *Staphylococcus aureus* ± 0.03 (all pulsed APPJ, data not shown). According to the definition of susceptibility evaluation, for every species $>95\%$, (100%) all isolates were susceptible to both sources. When the values for S, I, and R were compared between the different sources and species, no significant differences were observed (data not shown). The results of selected (the most relevant) pathogens and colonizers of acute and chronic wounds and skin infections *Staphylococcus aureus* (SA), *hemolyzing streptococci* (HS), *Pseudomonas aeruginosa* (PA), *Escherichia coli* (EC), and *Candida albicans* (CA) are presented as box plots and confidence intervals in Figs. 7(a)–7(c). Except for SA, the indices obtained by pulsed APPJ [box plot, Fig. 7(c)] and nonpulsed APPJ [box plot, Fig. 7(b)] were more homogeneous compared with DBD [box plot, Fig. 7(a)], these differences were statistically not significant ($p > 0.05$). Furthermore, the tested clinical species showed no difference compared with the reference strains ($p > 0.05$, data not shown).

3. QR_{PL} of Gram-Positive, Gram-Negative Species and Fungi

Mean $QR_{PL} \pm SD$ are shown in Table 4. Comparing QR_{PL} of gram-positive, gram-negative species, and fungi, the indices ranked from 0.99 ± 0.05 (fungi) to 1.03 ± 0.04 (gram-negative bacteria), as obtained by irradiation with the pulsed APPJ (Table 4). Except for fungi showing significant difference to gram-negative bacteria ($p = 0.02$, after irradiation with pulsed APPJ), no significant differences were observed.

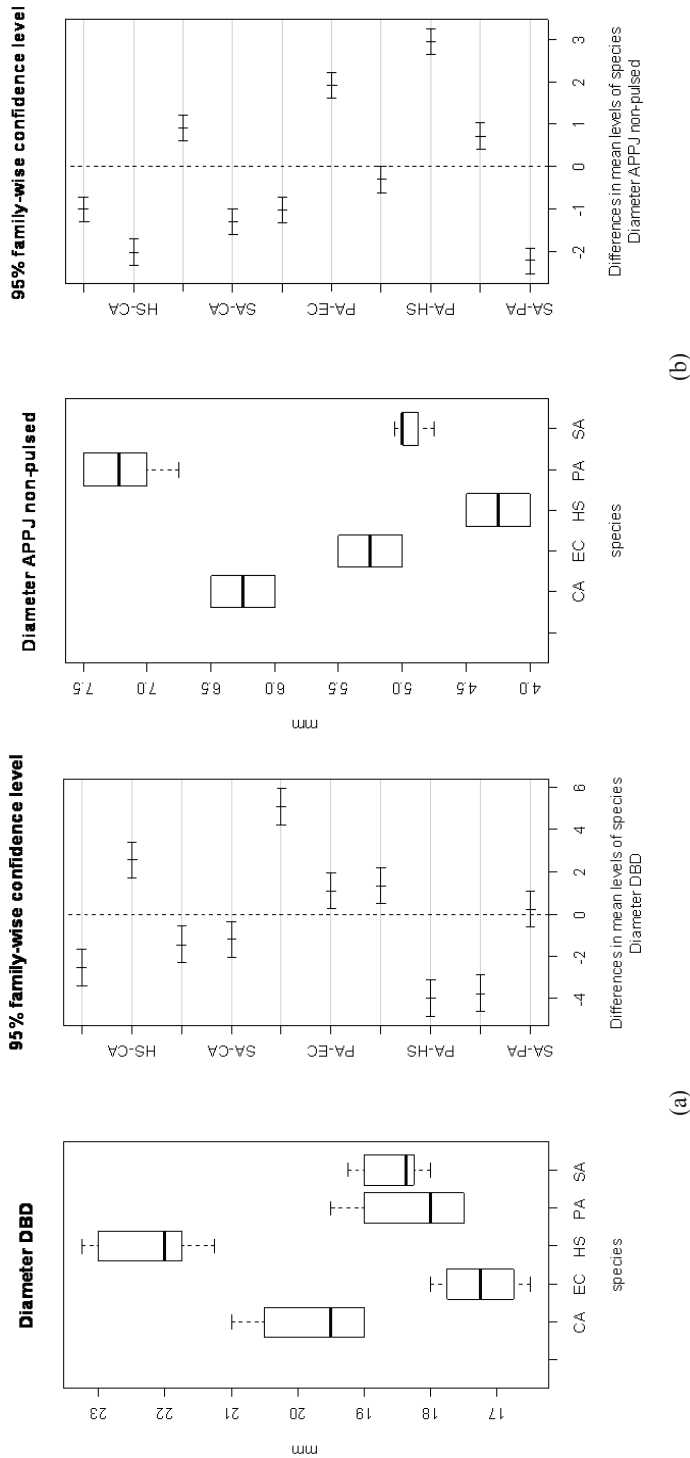
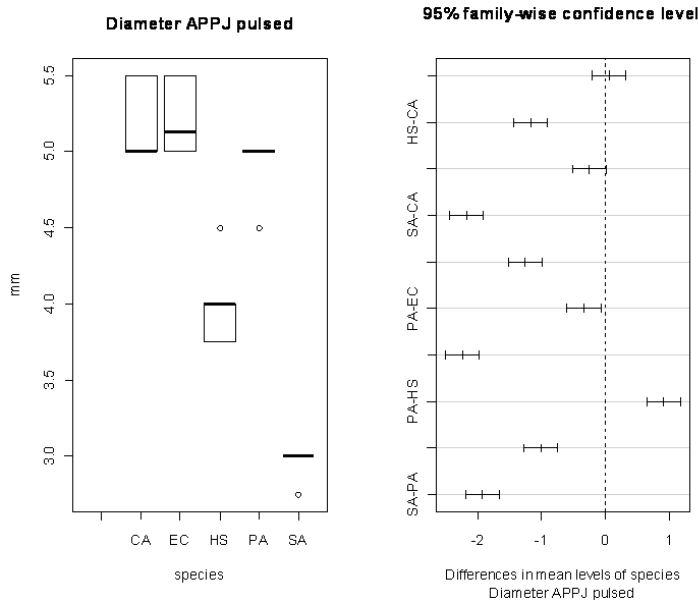


FIG. 6: (a) Box plot and confidence level of the diameters of areas without vegetative growth of the most important species of wound and skin infections after plasma treatment with DBD. (b) Box plot and confidence level of the diameters of areas without vegetative growth of the most important species of wound and skin infections after plasma treatment with nonpulsed APPJ. (c) Box plot and confidence level of the diameters of areas without vegetative growth of the most important species of wound and skin infections after plasma treatment with pulsed APPJ.



(c)

FIG. 6: (Continued)

4. Diameters of Gram-Positive, Gram-Negative Species, and Fungi

Mean diameters are shown in Table 5. Comparing diameters of gram-positive, gram-negative species, and fungi, the diameters ranked from 3.6 ± 0.7 mm (gram-positive bacteria) as obtained by irradiation with pulsed APPJ (Table 5) to 20.8 ± 1.5 mm (gram-positive bacteria) as obtained by irradiation with DBD. Between all compared groups are significant differences except gram-positive compared with gram-negative bacteria treated with pulsed APPJ ($p = 0.59$).

5. In Vitro Biofilm Testing

Conventional antiseptics as positive controls allowed up to three decadic logstep reductions of *S. aureus* biofilms on PE plates after 2 min of co-incubation. Only 3 min of antiseptic treatment with polihexanide and 70% ethanol were able to completely kill *S. aureus* biofilms in our model in 50% of treated plates (PU or V4a stainless steel), indicating that complete biofilm eradication can be expected from ≥ 5 RF. Treatment of PE plates with noncommercial DBD (2 cm diameter electrode) over 1 min generated RF up to five decadic logsteps ($RF \leq 5$) and therefore did only sporadically eradicate the biofilms. Comparable efficacies were reached with the PlasmaDerm (1 cm diameter electrode) source only at full power mode. Longer plasma treatment regardless of the power mode did not generate more anti-biofilm activity (data not shown). The obtained reduction after treatment with PlasmaDerm (30% and full power mode) was comparable with conventional antiseptics

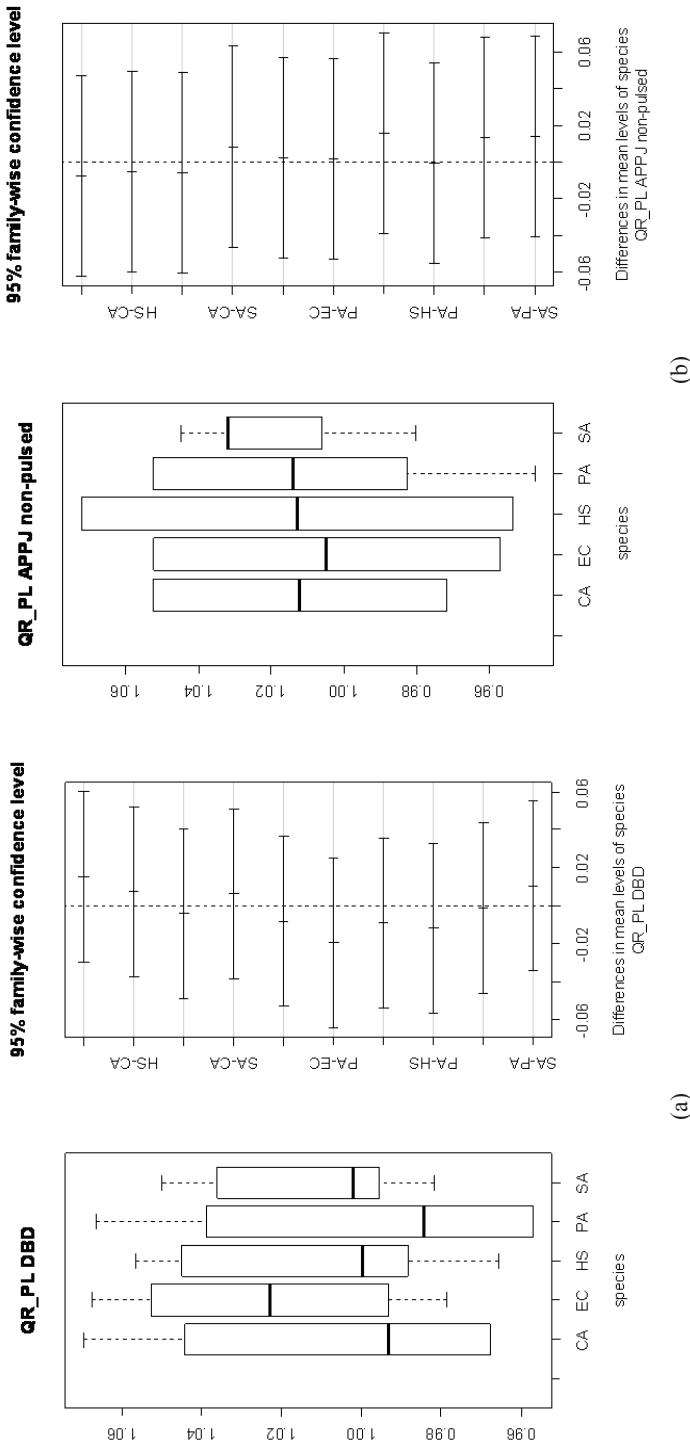
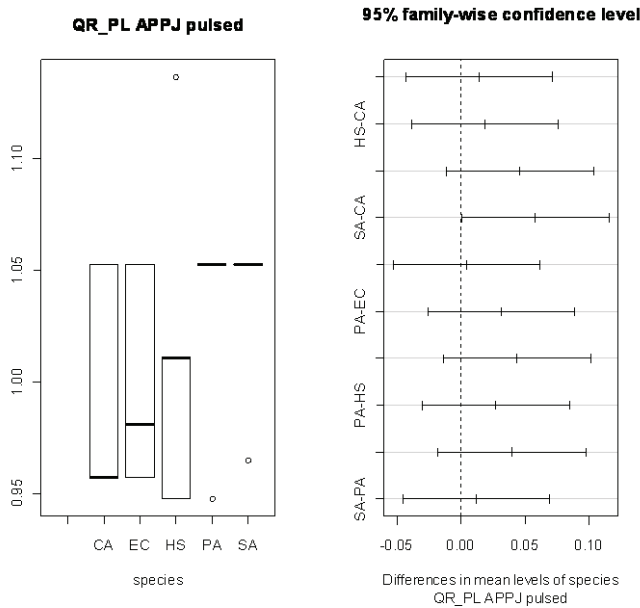


FIG. 7: (a) Box plot and confidence level of the susceptibility index (QR_{PL}) of the most important species of wound and skin infections after treatment with DBD. (b) Box plot and confidence level of the susceptibility index (QR_{PL}) of the most important species of wound and skin infections after treatment with nonpulsed APPJ. (c) Box plot and confidence level of the susceptibility index (QR_{PL}) of the most important species of wound and skin infections after treatment with pulsed APPJ.



(c)

FIG. 7: (Continued)**TABLE 4.** Differences between the QR_{PL} of gram-positive, gram-negative bacteria and fungi obtained by three plasma devices (nonpulsed/pulsed APPJ and DBD) after 3 s treatment

Plasma source	Mean QR_{PL} (SD)		
	Gram-positive	Gram-negative	Fungi
DBD	1.02 (0.03)	1.01 (0.04)	1.01 (0.04)
APPJ nonpulsed	1.02 (0.04)	1.00 (0.05)	1.01 (0.04)
APPJ pulsed	1.02 (0.05)	1.03* (0.04)	0.99* (0.05)

* Significance ($p = 0.02$)

(2–3 RF after 2 min exposure time). Treatment of V4a stainless steel plates with both plasma sources allowed complete inactivation of biofilms in all treated cases after 1 min exposure indicating ≥ 5 RF logstep reduction power of the DBD (data not shown).

B. MRSA and MSSA

1. Agar Test Model

Treatment of MRSA (all isolates HA-MRSA) with the DBD and APPJ resulted in inhibition zone diameters of 22.97 ± 1.56 mm and 5.59 ± 0.40 mm, causing areas without

TABLE 5. Diameters (mean + SD) of gram-positive, gram-negative bacteria and fungi obtained by three plasma devices (nonpulsed/pulsed APPJ and DBD) after 3 s treatment

Plasma source	Mean diameter (SD) [mm]		
	Gram-positive	Gram-negative	Fungi
DBD	20.8 (1.5)*	17.4 (2.6)*	19.7 (0.7)*
APPJ nonpulsed	4.8 (0.4)*	5.4 (1.2)*	5.3 (0.2)*
APPJ pulsed	3.6 (0.7)	3.8 (1.2)	5.2 (0.2)*

* Significance ($p = 0.02$)

vegetative growth of $407.72 \pm 81.28 \text{ mm}^2$ and $24.2 \pm 4.96 \text{ mm}^2$ (data not shown). The mean value of the inhibition zone diameters of the LA-MRSA (three replications) were $23.71 \pm 0.51 \text{ mm}$ with the DBD and $5.70 \pm 0.17 \text{ mm}$ with the APPJ. The corresponding areas without vegetative growth were $441.55 \pm 18.87 \text{ mm}^2$ with the DBD and $25.52 \pm 1.54 \text{ mm}^2$ with the APPJ. Treatment of the four CA-MRSA (three replications) resulted in inhibition zone diameters of $24.79 \pm 0.97 \text{ mm}$ using the DBD and $5.77 \pm 0.18 \text{ mm}$ using the APPJ, with corresponding areas without vegetative growth of $482.94 \pm 37.92 \text{ mm}^2$ (DBD) and $26.14 \pm 0.98 \text{ mm}^2$ (APPJ). Testing MSSA, the DBD caused areas without vegetative growth of $460.90 \pm 14.23 \text{ mm}^2$ with diameters of $24.23 \pm 0.37 \text{ mm}$ and the APPJ produced zones of $27.98 \pm 2.66 \text{ mm}^2$, corresponding to diameters of $5.96 \pm 0.28 \text{ mm}$.

2. Comparison of Obtained Diameters

The inhibition zone diameters of HA-, LA-, and CA-MRSA obtained by both plasma types were compared and showed no significant differences ($p > 0.05$). Comparing diameters of MSSA and all types of MRSA showed significantly ($p = 0.001$) smaller diameters of MRSA after treatment indicating less susceptibility of MRSA in comparison with MSSA regardless of the used plasma source (data not shown).

3. Susceptibility Index

Treatment of HA-MRSA with the DBD and the APPJ caused mean QR_{pi} of 1.02 ± 0.07 and 1.02 ± 0.07 , respectively. The corresponding data for MSSA were 1.00 ± 0.02 for the DBD and 1.01 ± 0.05 for the APPJ. Testing of the LA-MRSA strain resulted in a QR_{pi} of 1.06 for the DBD and 1.04 for the APPJ. CA-MRSA strains were tested fully susceptible with both plasma devices (mean QR_{pi} of 1.11 ± 0.02 with DBD, 1.05 ± 0.01 with APPJ). According to the definition of susceptibility valuation, 88% and 82% of the HA-MRSA isolates were tested fully susceptible (Table 6) when treated with the DBD and the APPJ, respectively. Twelve percent of strains were intermediately susceptible when treated with DBD and 18% with APPJ. The difference between “intermediary” and “full” susceptible strains was statistically

significant ($p < 0.05$) for both plasma types. Neither MRSA (including LA- and CA-MRSA) nor MSSA isolates exhibited resistance to the plasma treatment, which means that all QR_{pt} were found in the susceptible or in the intermediate range (≥ 0.95 , Table 6).

4. Comparing Susceptibility between HA-, CA-, and LA-MRSA

When the susceptibility data of CA-MRSA and LA-MRSA were compared with HA-MRSA, no significant differences were observed ($p > 0.05$). Also, no differences were observed between CA-MRSA and LA-MRSA ($p > 0.05$) either (data not shown).

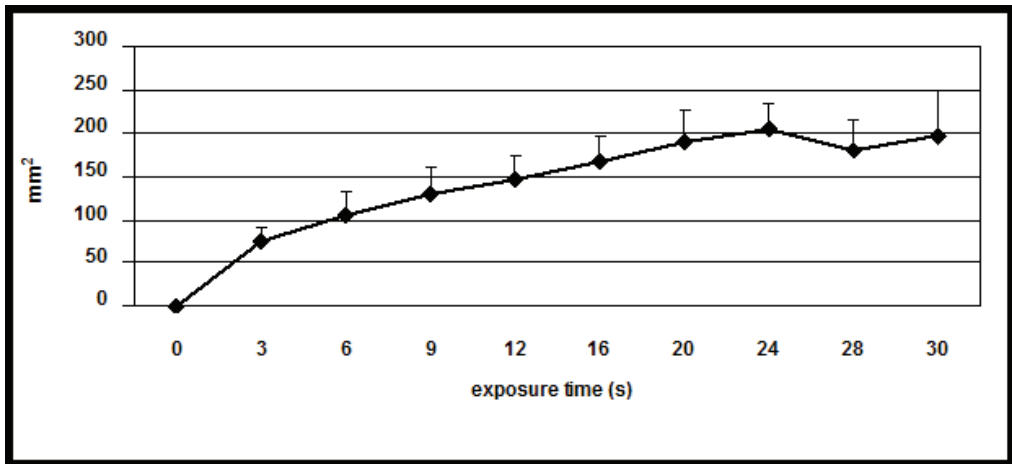
5. Antifungal Treatment

a. Effects of Plasma Treatment

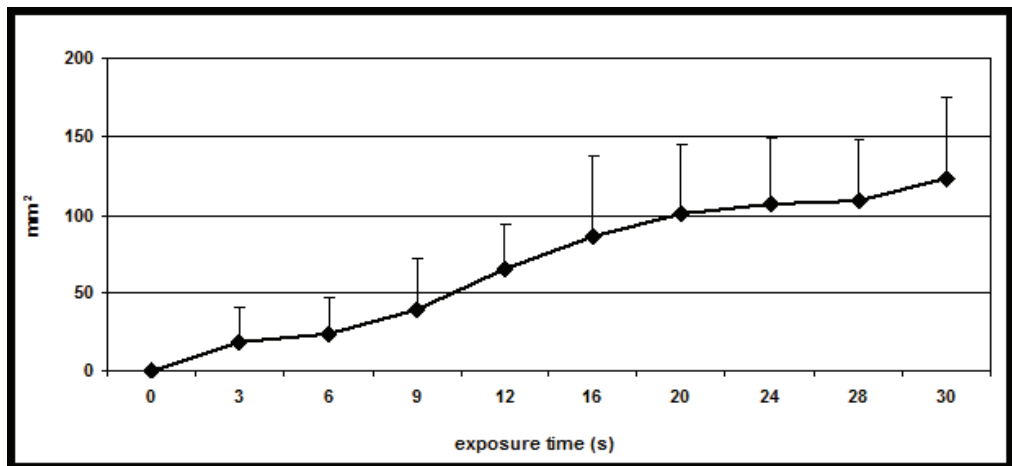
Punctual plasma treatment of the fungal suspensions on agar was followed by circular inhibition of the growth of any tested species and isolate *in vitro*. The plasma irradiation produced a growth-free circle around the point of contact with the plasma jet during irradiation. The inhibition zones were analyzed for mycotic growth by swabbing the irradiated areas and cultivating the samples over eight weeks on selective media. Because no more growth could be obtained on all visually fungus-free plasma-treated areas (data not shown), the term inhibition was replaced by eradication (mycocidal effect). The eradication zones varied between isolates and species and increased with treatment time [Figs. 8(a)–8(d)]. The maximal inhibition zones were obtained with *C. albicans* with 200 mm², followed by *T. rubrum* (123 mm²) and *T. interdigitale* (107 mm²). The highest progression of the fungus-free area over treatment time was found with *C. albicans* [Fig. 8(a)] with 50% of reduction area obtained after 6 s of plasma treatment, the lowest with *M. canis* [Fig. 8(d)] with a delay of 28–30 s until 50% maximal area was reached (Table 7). The lowest reduction was found treating *M. canis*

TABLE 6. Classification of the plasma treated isolates. As reference diameter, the median of diameters of all HA-MRSA (for MRSA testing) and absolute diameters of all MSSA (for MSSA testing) was chosen. The transition from S to I is defined with the 5%-quantile of the relative diameters < 1 .

Tested diameter of isolate	R	I	S
HA-MRSA DBD ($D_{ref} = 22.5$), $n = 50$	0	6 (12%)	44 (88%)
HA-MRSA APPJ ($D_{ref} = 5.5$), $n = 50$	0	9 (18%)	41 (82%)
LA-MRSA DBD ($D_{ref} = 22.5$), $n = 1$	0	0	1 (100%)
LA-MRSA APPJ ($D_{ref} = 5.5$), $n = 1$	0	0	1 (100%)
CA-MRSA DBD ($D_{ref} = 22.5$), $n = 4$	0	0	4 (100%)
CA-MRSA APPJ ($D_{ref} = 5.5$), $n = 4$	0	0	4 (100%)
MSSA DBD ($D_{ref} = 24.3$), $n = 50$	0	0	50 (100%)
MSSA APPJ ($D_{ref} = 5.9$), $n = 50$	0	0	50 (100%)



(a)



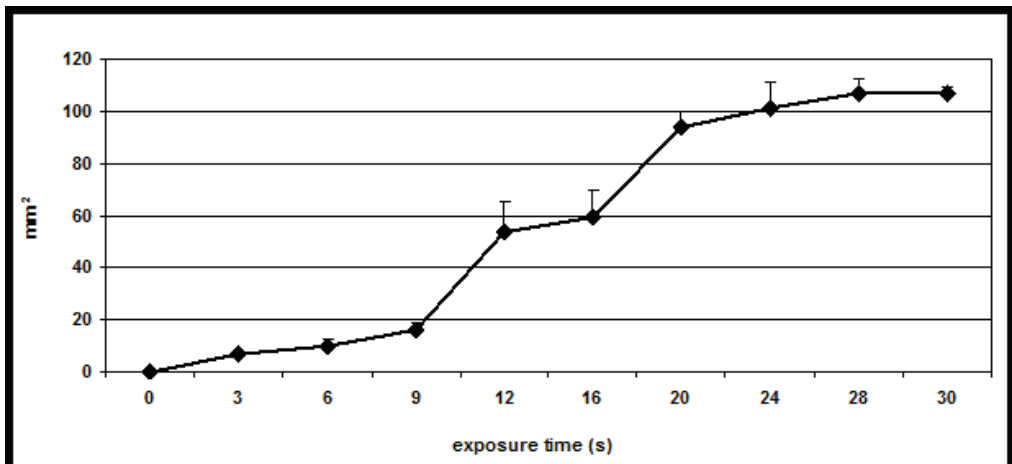
(b)

FIG. 8: (a) Inhibition zones of irradiated strains of *C. albicans* ($n = 5$). (b) Inhibition zones of irradiated strains of *T. rubrum* ($n = 5$). (c) Inhibition zones of irradiated strains of *T. interdigitale* ($n = 5$). (d) Inhibition zones of irradiated strains of *M. canis* ($n = 5$).

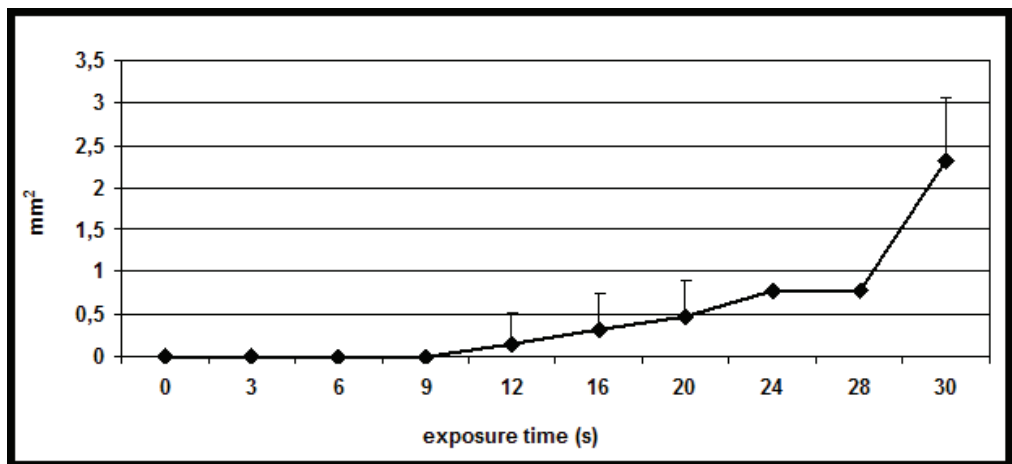
(2.3 mm²). Compared with the other species, treatment of *M. canis* was related to the lowest number of killed fungal cfu (0.3 cfu/cm², Fig. 9).

b. Treatment of Fungal Suspensions on Larger Areas (Agar Plates of 56.7 cm²)

To verify if the time necessary to effectively eradicate larger areas by a moving jet can be derived from our data, obtained with punctual treatment (see above), we tested the



(c)



(d)

FIG. 8: (Continued)

plasma jet to eradicate three clinical strains ($1 \times T. rubrum$ and $2 \times T. interdigitale$) on 56.7 cm^2 of agar (corresponds to surface of conventional agar plates). Because 15 s were shown to be able to eradicate $>50\%$ of the fungal growth on agar, we chose 10 min of treatment for one plate. To test the influence of larger growth ($>1000 \text{ cfu/plate}$) on the eradicating plasma potency, we additionally irradiated one plate with excessive growth (1572 cfu/plate on control plate). A 10 min ($5 \times 2 \text{ min}$) irradiation of the agar plates was followed by complete eradication of the fungal growth. The excessive amount of fungal cfu on a plate with $>1500 \text{ cfu}$ on its surface did not shrink the eradicating potency (Table 8).

TABLE 7. APPJ treatment of clinical fungi. Maximum occurring cleared areas A_{\max} (mm²), corresponding treatment time t_{\max} (s) and corresponding treatment time to 50%, 90% reduction.

Pathogen	A_{\max} (mm ²) /SD	t_{\max} (s)	$t_{50\%}$ (s)	$t_{90\%}$ (s)
<i>T. interdigitale</i>	107/5.3	28	12	20
<i>T. rubrum</i>	123/52.3	30	12	28
<i>M. canis</i>	2.3/0.7	30	28–30	28–30
<i>C. albicans</i>	204/29.7	24	6	16–20

* After treatment of maximum 30 s; SD standard deviation

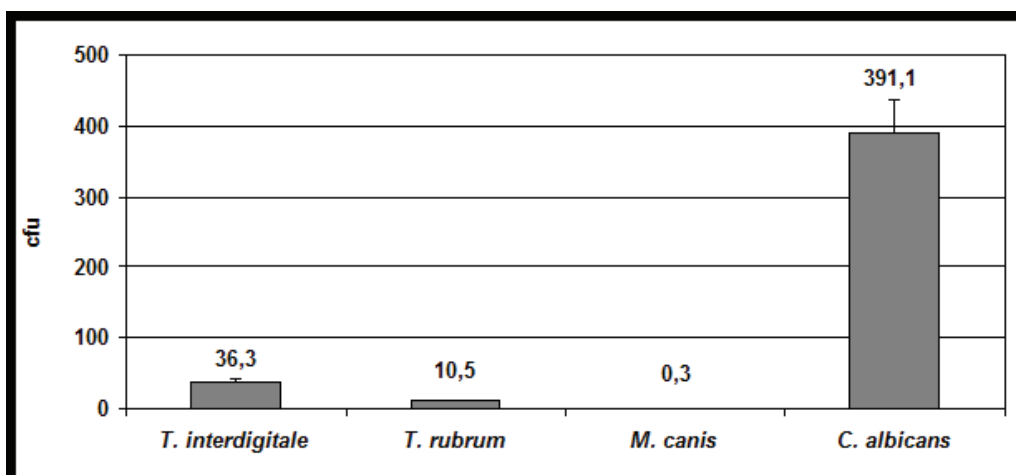


FIG. 9: Maximum of killed fungal cfu (mean, $n = 5$) obtained during plasma treatment of 30 s

TABLE 8. APPJ treatment of clinical fungi. Maximum occurring cleared areas A_{\max} (mm²), corresponding treatment time t_{\max} (s) and corresponding treatment time to 50%, 90% reduction.

Pathogen	Control plate (cfu/plate)	cfu after APPJ (cfu/plate)	Treatment time (min)	Reduction
<i>T. rubrum</i> (Taplin)	277	0	5 × 2	100%
<i>T. interdigitale</i> (Sabouraud)	1572	0	5 × 2	100%
<i>T. interdigitale</i> (Sabouraud)	352	0	5 × 2	100%

c. Irradiation of Dandruffs Ex Vivo

Seven of the 15 sample series showed fungal growth of *T. interdigitale*, the highest positivity was found with 14 culture positive from 28 dandruff samples (sample series 25/09, Table 2), the positive ranking scaled from 2 to 14 positive samples per series. All irradiated dandruff samples failed to exhibit cultural growth of *T. interdigitale* after eight weeks of cultivation compared to not irradiated control dandruffs. Two minutes of plasma treatment was shown to effectively eradicate reproductive fungal elements in dandruffs of patients with tinea pedis (*T. interdigitale*).

d. Irradiation of Shoes

All plasma-treated six insoles from three pairs of contaminated shoes failed to exhibit any fungal (or bacterial) growth after six weeks of incubation the agar plates (data not shown). The plasma irradiation was shown to eradicate environmental fungal contamination in heavily contaminated shoes.

6. Treatment of *Demodex Folliculorum*

In the first experiment, *Demodex* was not killed after 3×60 s of APPJ irradiation through the cover slide and motility of the parasite only slightly decreased. After removing the cover slide, plasma irradiation of $4 \times 15 = 60$ s of treatment were necessary for a definite immobilization of the parasite (monitored with digital camera, Fig. 10). Consecutive control clips were not able to show any movement of the parasite up to 6 h, therefore, we concluded effective killing of the parasite by the APPJ treatment. At the second investigation day, we consecutively treated four parasites of the same patient. This time, we started irradiation directly without cover slide and observed complete immobilization after repeated treatments of 1 s with 10 min between first and second treatment (repetition of 1 s irradiation included because of significant loss of motility occurring immediately after treatment of 1 s).

During treatment and 10 min delay time, all control parasites continued their physiologic motility until they were also treated and consecutively killed after 2×1 s APPJ in the same way. Additional microscopic observations followed after 20, 30, 60, 75, and 90 min and finally every 60 min to a total time span of 6 h and did not show any more movement of all irradiated four parasites. After 6 h, even gentle pressure of the parasites (via glass slides) did not provoke any vital sign (movement). We conclude that all parasites were killed by APPJ treatment after 2 s of treatment.

IV. DISCUSSION

As a consequence of its unique bundle of biopotent species including UV, IR, electrons, reactive oxidative species, NO, electrical fields, current, ions, and particles,^{2,41,42} non-thermal atmospheric pressure plasma is now being introduced in medical and biologic science. In contrast to the number of reports regarding antimicrobial *in vitro* efficacy of

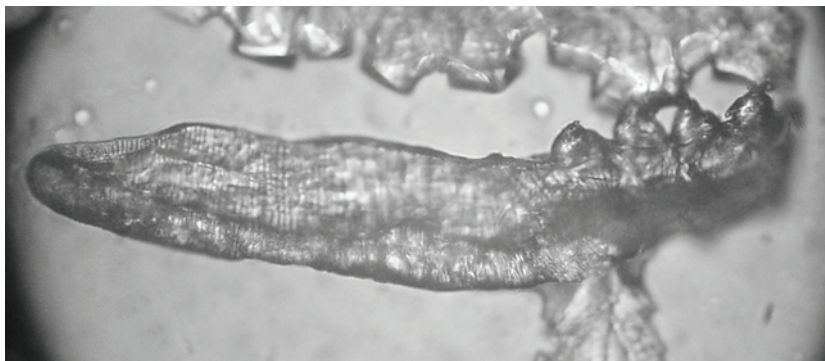


FIG. 10: *Demodex folliculorum* during plasma treatment—magnified 400×

plasma, clinical evaluations are rare and describe successful plasma treatment of specific infections, i.e., eyelid infections or pulmonary tuberculosis.^{4,5} In our previous work, we were able to show that two cold plasma sources, the APPJ and the DBD, were highly effective in reducing bacterial and fungal load *in vitro*.^{3,6} The plasma sources killed all tested species on agar after a 3–30 s treatment time.⁶ Together with similar published data by other groups and in combination with noncritical data from risk assessments,^{7,27–29,38} it can be deduced that plasma could also be effective in the antimicrobial treatment of focal superficial skin colonization and skin infections and also in hospital hygiene to eradicate MRSA with plasma as “first physical MRSA antiseptic” (Daeschlein *et al.*, submitted 2012⁴³). Up to now, the mode of action of plasma against bacteria was not completely understood. Therefore, only hypothetical approaches could be made, i.e., the membrane destruction by electrostatic force caused by charge accumulation on the outer surface membrane, which overcomes the tensile strength of the membrane. Furthermore, reactive species play an important role in plasma inactivation. Ozone produced by air plasmas causes germicidal effects by its interference with cellular respiration. Radicals can influence the membrane function by oxidizing fatty acids and other protein molecules.⁴⁴ Regarding the APPJ, the pulsed APPJ device was developed for best skin compatibility^{45,46} but it remained to be clarified how far the pulsing could influence the antimicrobial effect. The pulsing is the result of rhythmical interruption of energy input. Our results clearly show that in spite of larger differences of diameters of the obtained inhibition zones, no impairment of the antimicrobial net efficacy could be shown using the different plasma modes (no different QR_{PL} using pulsed or nonpulsed APPJ).

A. Skin and Wound Pathogens

The prediction of antimicrobial susceptibility on the basis of statistical data represents an imperative necessity in modern antibiotic treatment. Thus, when plasma is intended for local antiseptic use, this application has to be based on validated reproducible and

standardized test methods. Therefore, we investigated the antimicrobial plasma efficacy *in vitro* by two different plasma sources against a set of different clinical relevant strains including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, hemolyzing Lancefield Streptococci (group A and B), *Enterococcus faecalis*, and *Staphylococcus epidermidis*. Plasma treatment resulted in large inhibition zones for all tested species when irradiated within 3 s in our susceptibility test model using a DBD and a jet plasma source (APPJ). No isolate exhibited resistance to the plasma treatment, all QR_{PL} were found in the susceptible range (≥ 0.95).

The different species exhibited significant differences when the obtained diameters were compared. These differences were at first described when another test method was undertaken treating whole agar plates in a meandric movement by our group.³ These interspecies differences are important because the elucidation of their basis could clarify the mode of action exerted by the different plasma sources. In light of these data and in respect of the fact that already 3 s of treatment can result in bacteria-free zones, clinical antimicrobial plasma treatment seems realistic. An “atmospheric plasma dispenser,” a plasma device currently under investigation based on a corona plasma discharge technology, was recently presented by the group of Morfill *et al.*⁴⁷ and proved a convenient, rapid, and safe plasma disinfection of agar plates (five logsteps reduction of <10 s of treatment) and against relevant nosocomial bacterial gram-positive and gram-negative species including yeasts. This device proved also potent decontamination of textiles (socks).⁴⁷ The same group recently presented a study treating highly colonized chronic dermal wounds. Isbary *et al.*¹² showed that the plasma torch (MicroPlaSter) treatment was able to reduce the bioburden of bacterial colonization of the wounds significantly. Also, Fridman *et al.*^{2,35} already demonstrated skin decontamination on dead and living animals (without any relevant harm). Furthermore, a clinical trial to treat chronic venous leg ulcers with DBA plasma has just been initiated at the Department of Dermatology in Göttingen (see clinicaltrials.gov). Preliminary results support the concept of antimicrobial plasma efficacy on chronic wounds *in vivo* as well as the safety of skin plasma treatment.

In conclusion, plasma treatment proved high efficacy in killing the mostly occurring and most relevant clinical skin and wound pathogens. Our data provide the first systematic *in vitro* susceptibility data as a data base for the prediction of strain susceptibility to start successful calculated antimicrobial plasma decontamination, disinfection, and treatment *in vivo*. We conclude that cold plasma treatment can be a suitable alternative option to conventional antibiotics and antiseptics in the antimicrobial strategy in dermatologic infection management.

B. MRSA

MRSA *in vitro* susceptibility against plasma was demonstrated also by other groups^{47–51} and reduced susceptibility of CA-MRSA in comparison to USA400 and Newman strains to nonthermal plasma was reported.⁵⁰ Joshi *et al.*⁴⁹ showed eradication of MRSA on contaminated surfaces (and in biofilm) in 150 s. This difference, with longer exposure

time needed, could be due to the different plasma source he used. Closer to our time span was the disinfection described by Burts *et al.*⁵⁰ who effectively decontaminated MRSA on one-way pagers after 30 s. In contrast to our results, these authors found CA-MRSA more resistant than HA-MRSA. We compared HA-MRSA with CA-MRSA and LA-MRSA susceptibility and did not find a difference except for four CA- and one LA-MRSA isolate. Two other groups, Morfill *et al.*⁴⁷ and Kong *et al.*,⁵¹ reported MRSA *in vitro* susceptibility to plasma but did not give concrete numbers of tested isolates and possible differences in susceptibility. Up until now, no clinical data regarding plasma *in vivo* treatment of MRSA contamination, colonization, or infection have been reported.

An important question is the practicability of plasma sanitation with the plasma devices with small application surfaces. Plasma devices with larger treatment surfaces include the “plasma dispenser,” which was created by the group of Morfill *et al.*⁴⁷ This apparatus allows the treatment of objects of the size of a hand; and up to now, clinical performance data are lacking.

C. Prevention Aspects

Our data strongly support a substantial clinical and economical potential of plasma treatment of contaminated, colonized, or infected skin harboring hygienically relevant pathogens like MRSA, VRE (vancomycin-resistant enterococci), or ESBL (extended spectrum beta-lactamase producer). Because >90% of all nosocomial (exogenously) transmitted infections are due to cross contamination via hands and a third of all nosocomial infections are considered avoidable by hygienic measures—hand hygiene and especially hygienic hand disinfection belong to the most important active and preventive hygienic measures to stop nosocomial infections.^{52,53} It is, therefore, of the utmost importance to identify a highly efficient and rapid disinfection procedure that has maximum bactericidal efficacy on both transient and resident hand flora. In light of unsatisfying decontamination of the body by conventional antiseptics, alternative skin and wound decontamination could open new horizons with plasma as an agent of a completely different mode of action in hospital hygiene. Our data clearly show excellent plasma susceptibility of MRSA and MSSA *in vitro* regardless of the type of MRSA. *In vitro*, no plasma resistant strain was detected. Contrary to antibiotics, plasma showed similar efficacy against MSSA and MRSA, indicating a completely different mode of action. This is a striking argument for further attempts to the introduction of plasma in preventive medicine in the face of worldwide increasing resistance of many pathogens.

D. Antifungal Treatment

At the moment, reports on antifungal plasma treatment are rare, and since the APPJ proved high activity against relevant wound pathogens and contaminants,⁵ a similar approach to inactivate dermatophytes on solid media appeared realistic. Data clearly show that representative species of the most commonly encountered fungal species in human mycology responsible for tinea corporis, tinea pedis, and onychomycosis could

effectively be inactivated by *in vitro* plasma treatment. From 3 to 30 s of treatment, all isolates exhibited increasing distinct zones completely free of fungal growth. It was remarkable and corresponds to our antibacterial results⁵ that for most species, the eradication zone significantly surpassed the zone of direct irradiation by the beam (direct plasma effect) indicating that other than direct irradiation effects were responsible for killing the fungal cells in this area (indirect plasma effect). The mycocidal effect was proven by failure of regrowth of fungal cells after treatment.

In our study, the enlargement of the eradication zone around the nozzle tip generated diameters of maximum 19 mm (*C. albicans*), 12 mm (*T. interdigitale* and *T. rubrum*), and 2 mm (*M. canis*). These data demonstrate potent but different antifungal effectiveness by the plasma with highest efficacy against *C. albicans*. Accordingly, a greater clearing effect can be expected by longer irradiation (not for *M. canis*). As a consequence independently from the area that could be cleared by the plasma treatment of individual fungus strain, the treatment in any case reliably achieves irreversible (mycocidal) inactivation of the complete fungal growth in a defined area, which differs from species to species and even in the case of relatively tiny eradication zones like those obtained with *M. canis* ($d = 2$ mm); this treatment was highly effective (all fungal growth eradicated). Interestingly, the relatively small diameters (of the corresponding area) obtained by irradiation of *M. canis* exactly reflect the geometry of the tip of the plasma beam (diameter 2 mm) and it can be hypothesized that in controversy to all other tested fungal species (also bacteria, data not shown), the inactivation of *M. canis* is restricted to direct plasma effects and can be designated as susceptible to direct and resistant to indirect plasma effects. The nature of the antimicrobial properties of plasma is still not fully understood. Based on the plasma characterization data given in Fig. 3 as well as in Ref. 37, temperature effects can be excluded as the main reason for the antifungal plasma activity just as UV radiation in the biologically active UV-C region around 254 nm. Because of the lack of substantial emission in the UV-C range, presence of excited NO can be widely excluded.¹ However, as demonstrated by the significant emission at 309 nm (Fig. 3), there is a substantial amount of OH radicals within the plasma that are produced by plasma chemical dissociation and excitation reactions from water molecules present in the feed gas as well as in ambient air above the wet agar plates. These OH radicals are supposed to be, together with other reactive oxygen species, the lethal plasma components toward living cells.^{1,3,54}

Because plasma treatment proved high effectiveness *in vitro* against the most relevant causative agents of human and partial veterinary fungal species, *T. rubrum*, *T. interdigitale*, *M. Canis*, and the worldwide most relevant yeast *C. albicans*, it can be assumed that *in vivo* treatment can eradicate these pathogens in mycotic lesions. Additionally, the reported plasma effects affecting wound healing like amelioration of tissue oxygenation as proved with laser doppler fluximetry (LDF) (Ref. 55) could further support the feasibility of antifungal therapy *in vivo*. A handicap of the APPJ is the unsuitability in the treatment of larger areas. As we could demonstrate that areas of 56.7 cm² can be effectively eradicated by the APPJ during 10 min, this time could be substantially shortened when plasma sources with larger application area can be applied.

Such treatment could also provide more homogenous and accurate irradiation. Sources with larger application areas are now under investigation [one source with application area of 18 cm² (DBD, Leibniz Institute of Plasma Science and Technology e.V. (INP Greifswald e.V.) and one with 3.14 cm² (DBD, CINOGY GmbH, Duderstadt)]. We were able to show cold plasma treatment proving high efficacy to kill the causing agent of tinea pedis. The efficacy and good performance of plasma treatment seems a future perspective to overcome the therapeutic dilemma of antifungal therapy of tinea pedis and onychomycosis and as a new treatment concept combines antifungal therapy with environmental transmission prevention.

E. Demodex Treatment

Rosacea is a common eruptive chronic facial dermatosis with various clinical presentations characterized by intermittent periods of exacerbation and remission on the basis of a genetic predisposition. The disease is typically characterized by transient or persistent central facial erythema, teleangiectasia, and often papules and pustules and can be classified into four subtypes: erythematous telangiectatic, papulopustular, phymatous, and ocular type. The pathogenetic background of rosacea yet is not fully understood. Current hypotheses include potential roles for vascular abnormalities, dermal matrix degeneration, environmental factors, the microorganisms *Propionibacterium acnes* and *Helicobacter pylori*,⁵⁶ and the parasite *Demodex folliculorum*.⁵⁷ Forton⁵⁸ demonstrated a statistically significant relationship between the presence of *Demodex* and perifollicular, and lymphohistiocytic inflammation in 69 biopsy specimens from rosacea patients. Erbagci⁵⁹ demonstrated that the density of *Demodex* was significantly higher in patients with papulopustular rosacea than in age-matched control subjects, where *Demodex* can also be found in hair follicles and sebaceous glands of the face, especially forehead, chin, eyelids, nose, and ear canal. These results were supported by further studies by Ruefli *et al.*⁶⁰ and Bonnar *et al.*⁶¹ In conclusion, the simple identification of *Demodex* is by no means proof of causative involvement but needs further support by assessing the density of mites.^{59,62} Mild forms of ocular rosacea respond readily to topical medications and eyelid hygiene, but more severe forms are to be treated with oral antibiotics like tetracyclines and Metronidazole, a synthetic, nitroimidazole-derived antibacterial and antiprotozoal agent.⁶³ The mechanism by which metronidazole reduces inflammatory lesions and erythema in patients with rosacea has not been fully elucidated and, like tetracyclines, it seems likely that the anti-inflammatory or immunosuppressive actions of metronidazole account for the therapeutic effect.^{64,65} Metronidazole has few adverse effects, mostly mild, including pruritus, skin irritation, and dry skin but also in rare cases epileptiform seizures, encephalopathy, and sensory neuropathy, and long-term use of metronidazole has been limited by concerns over adverse systemic effects and toxicity, underlining the need for alternative treatment.⁶⁶ In addition, many patients with rosacea and especially the teleangiectatic subtype are difficult to treat and the patients respond poorly to topical or oral medications. For this subtype, vascular laser, light therapy, surgery, and laser ablation have been increasingly utilized.⁶²

In conclusion, therapy of rosacea remains unsatisfactory because of the two main causes, i.e., the bad responsiveness to therapy, and the undesired effects of long-term therapy with antibiotics. Therefore, new therapeutic approaches are most welcome and since plasma treatment offers a multitude of biologic effects including anti-inflammatory and antimicrobial effects, it could be a promising option in the therapy of rosacea when it proves efficacy against the parasite *Demodex folliculorum*. This could be proven in our study. The data show that plasma (APPJ with argon as feeding gas) can rapidly inactivate adult *Demodex* parasites *ex vivo*. Surprisingly, we found in one case 2 s and another 60 s of plasma treatment necessary to kill the mites and this fact needs further clarification. Our plasma source generates substantially reactive oxygen species (ROS), which is indicated by significant emission of OH radicals at 309 nm (Fig. 2). There was no UV radiation emission in the UV-C range between 200 and 280 nm.³⁷ In the UV range between 260 and 360 nm, irradiance between 1 and 2 mW/cm² can be measured in the tip region of the plasma jet.³⁷ Consequently, it can be concluded that the killing of the mites is caused mainly by ROS activity that could be realized in a dual manner: by direct chemical action of OH radicals on the one hand, and by additional UV irradiation mainly at 309 nm on the other. However, detailed investigation of mechanisms of plasma action has to be a matter of future investigations.

In another setting, we were able to demonstrate the potent killing of *Propionibacterium acnes* *in vitro* (data not shown). Therefore, the treatment of *Demodex* in the lesions of patients with rosacea becomes realistic and clinical effects by eradicating Propionibacteria that are also involved in the pathogenesis of rosacea can be expected. In conclusion, plasma could be an alternative in the often frustrane therapy of rosacea since it provides anti-inflammatory, antiproliferative, broad antibacterial, and also anti-parasitic efficacy.

F. Potential *In Vivo* Plasma Application—Outlook

Before the application of cold plasma in humans, basic safety aspects must be taken into account. UV emission is one of these features and since both sources deliver UV in the range of 200–400 nm, it must be ensured that this irradiation does not exceed reference values for safe medical use. These recommendations are referenced by the International Commission on Non-Ionizing Radiation Protection (ICNIRP)⁶⁶ that does not recommend doses >3 mJ/cm² of UV irradiation in the range of 200–400 nm. In previous tests, both plasma sources were demonstrated to comply with this limit³⁷ allowing a potent antimicrobial efficacy during 3–60 s of treatment.^{6,26} These safety data are further supported by *in vivo* confocal laser scan microscopy (MAVIG GmbH, Munich, Germany) demonstrating an irradiation of 60 s without deteriorating skin integrity and architecture (no edema, infiltration of inflammatory cells, or aberrant cells).⁶⁸

Summing up, our results we were able to show that two different plasma sources, the APPJ and the DBD, were highly effective in eradicating bacterial, fungal, and parasitic burden *in vitro*. Together with published data by other groups and in light of noncritical risk assessments data,^{4,5,42,68} it can be deduced that both cold plasma sources are suitable

in the treatment of bacterial, parasitic, and fungal diseases *in vivo* but also used in hospital hygiene to eradicate infective pathogens including multi-resistant species like MRSA on contaminated, colonized, and infected wounds, skin, or other surfaces. Additionally, plasma could provide an effective device in the disinfection of fungal contaminations as a source of recalcitrant tinea in the environment (shoes, socks). The potent and fast (2 s, 60 s) antiparasitic efficacy of plasma *ex vivo* may encourage *in vivo* treatment of diseases with involvement of *Demodex spp.* like rosacea or demodicosis in veterinary medicine.

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