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ORIGINAL ARTICLE

Disinfection of an ambulance using a compact atmospheric plasma device

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Abstract

Aims: The worldwide spread of the coronavirus SARS-CoV-2 has highlighted the need for fast and simple disinfection processes, amongst others for ambulance cars on site. To overcome current drawbacks regarding room disinfection, the use of cold atmospheric plasma in remote operation represents a promising alternative for the disinfection of larger volumes. In this study, a compact plasma system was evaluated regarding its disinfection efficiency inside an ambulance car.

Methods and Results: The developed plasma device is based on a dielectric barrier discharge (DBD) and operates with ambient air as process gas. The humidified afterglow from the plasma nozzle was introduced into an ambulance car with a volume of approximately 10 m^3 while *Bacillus atrophaeus* endospores, *Staphylococcus aureus* or Phi 6 bacteriophages dried on different surfaces (PET-films, glass slides or aluminum foil) were exposed to the reactive gas inside the ambulance vehicle at eight different positions. Reductions of spores by more than 4 orders of magnitude were found on all surfaces and positions within 2 h. Due to their higher susceptibility, Phi 6 bacteriophages and *S. aureus* counts were reduced by at least 4 orders of magnitude within 30 min on all surfaces.

Conclusion: The results show that different microorganisms dried on variable surfaces can be inactivated by several orders of magnitude inside an ambulance by plasma gas from of a compact DBD plasma nozzle.

Significance and Impact of the Study: Plasma gas generated on site by a DBD plasma nozzle proved to be highly efficient for the disinfection of the interior of an ambulance car. Compact plasma systems could be a viable alternative for the disinfection of vehicles or rooms.

KEYWORDS

ambulance, dielectric barrier discharge, disinfection, endospores, gas plasma, phages, room disinfection, virus

INTRODUCTION

The SARS-CoV-2 pandemic has highlighted the need for fast, effective, automated, readily available and reliable room disinfection processes. Proper disinfection of areas used by patients colonized or infected with pathogens can reduce or eliminate the risk of onward transmission to others. Besides clinical areas, also ambulance vehicles require thorough disinfection after transportation of highly contagious patients. Manual disinfection with appropriate sanitizers is time consuming, often limited to easily accessible surfaces, and the disinfection success always depends on the operator (Otter et al., 2020). Currently applied systems for automated room disinfection are mostly based on aerosolized hydrogen peroxide, hydrogen peroxide vapor, ozone, ultraviolet C light or pulsed xenon UV (Otter et al., 2020). The interior of ambulance cars usually cannot be disinfected on site, which results in considerable effort and downtime. The German Robert Koch Institute (RKI) in Germany proposes the use of formaldehyde for the disinfection of rooms at a concentration of $5\,g\,m^{-3}$ with an air humidity of at least 70% RH and a dwell time of 6 h. This can be achieved by vaporization of 50 ml of a 12% solution of formaldehyde per m^3. Subsequent neutralization should be done with 10 ml of a 25% solution of ammonia per m^3. However, formaldehyde is not considered as a sporicidal agent under ambient conditions. Hydrogen peroxide is also suggested for room disinfection by the RKI, but in this case, an individual validation for each specific application is required (RKI, 2017). Consequently, new technologies are needed which provide a preferably high microbicidal efficiency, short cycle times, low costs and a high degree of automation while being safe and easy to use.

Cold atmospheric plasma generated by dielectric barrier discharge (DBD) has proven to exhibit considerable microbicidal activity. All kinds of microorganisms including bacteria, viruses as well as spores have been shown to be susceptible towards gas plasma, which makes it a promising candidate for disinfection applications (Sakudo et al., 2019). Plasma generated from air comprises reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as atomic oxygen (O), singlet oxygen ($^1O_2$), superoxide anion (O_2^-), ozone (O_3), hydroxyl radicals (·OH), atomic nitrogen (N), excited nitrogen N_2 (A), nitric oxide (NO) or NO_2 (Bourke et al., 2017; Scholtz et al., 2015). These oxygen- and nitrogen-based reactive species have strong oxidative effects on microbial structures like lipids, proteins, and DNA (Scholtz et al., 2021). Direct plasma treatment of surfaces within the glow discharge is known to be highly efficient due to the impact of charged particles as well as ultraviolet radiation. However, the suitability of direct plasma for the disinfection of large areas with complex geometries appears to be limited. On the other hand, it has been demonstrated that inactivation of microbes is also possible when samples are located spatially separated from the plasma volume or in an adjacent chamber. Since charged particles usually recombine before arriving at the substrate and short-lived reactive species are also neutralized quickly, remote plasma usually requires longer treatment times (Misra et al., 2011) but offers a more gentle treatment of complex targets (Müller et al., 2018). In a few studies, the surface to be disinfected was placed in the flowing afterglow of the plasma source (Moisan et al., 2014; Müller et al., 2018) but studies on the application of plasma sources for room disinfection are scarce. In a previous work, we demonstrated that *B. atrophaeus* spores deposited on different surfaces can be inactivated by several orders of magnitude within few minutes in a chamber of 300 L volume which is flushed with the plasma afterglow from a DBD plasma nozzle (Kramer et al., 2020). In a similar way, Ki et al. (2019) demonstrated a uniform disinfection of contaminated glass plates in a laboratory chamber of 175 L volume using a DBD Plasma source and *Staphylococcus aureus* as test microorganisms (Ki et al., 2019).

In this study, we used a DBD plasma nozzle for the disinfection of the interior of an ambulance car. The disinfection process was evaluated using *B. atrophaeus* endospores, *S. aureus* cells as well as Phi6 phages in order to take into account a potential sporicidal, bactericidal as well as virucidal effect. Bacterial endospores exhibit high resistance to various disinfectants, which makes them a suitable candidate for the evaluation of disinfection treatments. *Staphylococcus aureus* is of high relevance regarding nosocomial infections and bacteriophage Phi6 represents a well known surrogate for enveloped viruses. All microorganisms were dried on either PET-film, aluminum foil or glass slides and samples were positioned on eight different locations inside the ambulance car. A series of count reduction tests was performed whereat the vehicle with a volume of $10\,m^3$ was flushed with the plasma afterglow for different times. According to DIN EN 17272:2020, an automated room disinfection process for medical facilities can be regarded effective when the initial microbial counts are reduced by four orders of magnitude (bacterial spores and viruses) and five orders of magnitude (bacteria)2020.

MATERIALS AND METHODS

Plasma source and treatment conditions

A portable plasma system developed by the company Plasmatreat GmbH (Steinhagen, Germany) was used for

all test trials. Gas plasma was generated with a plasma nozzle based on a DBD equipped with an air cooling system. Ambient air at a pressure of 6 bar was used as process gas (14 L min^{-1}). The input power was approximately 200 W and the discharge frequency was 13,500 Hz. Previous studies have demonstrated that the supply of water is crucial for the sporicidal action of the gas plasma (Kramer et al., 2020). Therefore, the plasma afterglow was humidified by introducing deionized water (3 ml min^{-1}) via an airstream (0.5 L min^{-1}) and an evaporator (150°C). The water vapor was directed into the afterglow at the outlet of the plasma nozzle approximately 10 cm from the plasma discharge. A HPLC-pump (Gynkotek M 300) was used for water injection. The plasma system was placed in the driver's cabin of an ambulance car (volume: ~10 m^3) and the outlet of the nozzle was directed into the transport cabin. The generated ozone concentration at the nozzle outlet measured with a GM-6000-RTI Ozomat (Anseros, Germany) was about of 4.5–5 g m^{-3}.

Test strains, spore and sample preparation

Bacillus atrophaeus DSM 675, *S. aureus* DSM 346 and *Pseudomonas* phage Phi6 DSM 21518 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Preparation of *B. atrophaeus* spore suspensions was done as previously described by Muranyi et al. (2007) using manganese sulfate for induction of sporulation. Prior to harvesting, spore suspensions were checked by phase-contrast microscopy for sporulation by at least 80%. Spore suspensions were pasteurized (10 min, 75°C) to remove vegetative bacteria and then stored at 5°C. The colony count of the spore suspension was 2×10^9 CFU per ml.

Bacteriophage Phi6, an enveloped dsRNA virus of Cystoviridae with a RNA genome of 13.5 kbp and a size of 75 nm has been discussed previously as a potential surrogate for Ebola virus or Coronavirus in different scenarios (Gallandat & Lantagne, 2017; Prussin et al., 2018; Vatter et al., 2020). *Pseudomonas syringae* was initially grown in 100 ml trytptic soy broth (TSB, Oxoid, UK) at 25°C for 24 h in a shaking bath. Tryptic soy agar (TSA, Oxoid) was subsequently inoculated with the bacterial suspension by use of an inoculation loop, incubated for 24 h at 25°C and stored at 5°C. Propagation of Phi6 phages was done by lysis on plates using *P. syringae* DSM 21482 as host bacterium. An overnight culture of *P. syringae* (TSB, 24 h, 25°C) and the phage suspension from the DSMZ were added to 200 ml soft-agar (TSB, 0.5% Agar-Agar, 3 mM $MgSO_4$) in a 1:1 ratio. Subsequently, 3 ml of this mixture was added to bottom-agar plates (TSA, 3 mM MgSO4) and incubated at 25°C for 10 h. For phage harvest, 2 ml SM-buffer (0.1 M NaCl, 8 mM $MgSO_4$, 50 mM Tris–HCl, pH 7.5) was added to each bottom-agar plate (40–60 plates in total) before shaking for 4 h on an orbital shaker. The phage suspension was obtained by collecting and pooling the supernatant of all plates. After filtration through a 0.45 µm filter, the phage suspension was stored at 5°C. The final titer was 1.4×10^{10} PFU per ml.

Staphylococcus aureus was initially grown in 100 ml TSB at 37°C for 16–18 h in a shaking bath. TSA was subsequently inoculated with the bacterial suspension by use of an inoculation loop, incubated for 24 h at 37°C and stored at 5°C. Working cultures were made by inoculating 100 ml of tryptic soy broth with cell material from the agar surface and following incubation for 16–18 h at 37°C in a shaking bath until early stationary phase. 20 ml of the cell culture were subsequently centrifuged at 9000 *g* for 10 min and washed twice with sterile deionized water in order to remove all solutes.

PET-film (50 µm), glass slides (1 mm, Paul Marienfeld GmbH & Co. KG) or aluminum foil (150 µm, Goodfellow, UK) were used as carriers for the disinfection trials in order to take into account different materials which typically can be found in an ambulance. Pre-cut parts of 4×4 cm of PET-film were wiped with 70% ethanol before use, while glass slides and aluminum foil (4×4 cm) were wiped with 70% ethanol and then autoclaved at 121°C for 15 min. Two pieces of each specimen were adhered to quadratic petri dishes respectively. Inoculation was done by either spraying (*B. atrophaeus* spores) or spot inoculation (Phi6, *S. aureus*). Spores were washed twice with deionized water and finally diluted to approximately 5×10^7 CFU per ml in deionized water before 10 µl were sprayed on an area of 2×2 cm with a two-substance nozzle (Schlick, Germany) using nitrogen (2 bar) as process gas as previously described by Muranyi et al. (2007). In case of spot inoculation, the Phi6 stock suspension as well as the freshly prepared working culture of *S. aureus* were diluted 1:10 using sterile deionized water before 50×1 µl were inoculated on each test specimen respectively. Inoculation density was approximately 1×10^7 CFU per sample in case of *S. aureus* and 7×10^7 PFU per sample in case of bacteriophage Phi6. The inoculated carriers were immediately dried at 20°C and 20% RH for up to 30 min until optical dryness.

Disinfection trials in an ambulance car

The disinfection trials were carried out in a patient transport ambulance provided by the Bavarian Red Cross (BRK). The plasma system was placed inside the driver's cabin and the outlet of the plasma nozzle was connected to the trunk compartment with a flexible hose. Quadratic petri dishes with two individually inoculated test specimen

were placed at eight different positions (left window, right window, stretcher, ceiling, floor, backdoor, side door, under back seat) inside the ambulance in each disinfection trial (Figure 1). Additionally, three inoculated and dried test specimen were placed in the driver's cabin in every trial during the treatment time and served as references to quantify the initial microbial count. After the samples were attached to the different spots, all doors were closed before the plasma system was operating for 30, 60, 90 or 120 min. The humidity and temperature inside the chamber were recorded with a Basetech BTTH-1014 Thermo-/Hygrometer (Conrad Electronics) at the beginning and at the end of each treatment cycle. Due to the season in which all the trials were performed, the initial temperature and relative humidity ranged between 0–15 °C and 60–80% RH respectively. The temperature in the ambulance car increased by 1–2°C per hour of treatment time while the relative humidity reached 90–99% RH after 30 min of treatment. After the respective treatment time, the plasma system was turned off and all doors were opened. The samples were removed and the number of cfu or pfu per sample were immediately determined as described below.

Sample handling and determination of colony forming units and plaque forming units

Directly after exposure of samples to the gas plasma inside the ambulance, the inoculated specimen were either transferred to stomacher bags with 50 ml of sterile 1/4 strength ringer solution (Sigma Aldrich) supplemented with 0.1% Tween 80 (*B. atrophaeus, S. aureus*) or 10 ml of sterile 1/4 strength ringer solution (Phi6 bacteriophage). All samples in stomacher bags were manually rubbed for 1 min to detach microorganisms from the sample surface and to suspend them homogeneously. The effectiveness of detachment regarding bacteria and spores was checked by light microscopy. The number of colony forming units of the sample suspensions was determined by the pour plate technique using TSA. 1 ml of undiluted or serially (10-fold) diluted sample suspension in 1/4 strength ringer solution was transferred to petri dishes in triplicates (3 × 1 ml) respectively. After solidification, all plates were incubated at 30°C (*B. atrophaeus*) or 37°C (*S. aureus*) for 48 h before the number of colony forming units was counted manually. Plaque forming units of sample suspensions were determined by the agar overlay technique using TSA plates with 0.75% soft agar. 100 µl of an overnight culture of *P. syringae* DSM 21482 (TSB, 25°C, 24 h) was added to 5 ml of the tempered (45°C) soft agar, together with 100 µl of diluted or undiluted sample suspension. After mixing, the soft agar was poured on top of solid TSA plates and incubated for 15 h at 25°C before the number of plaque forming units was counted manually. The numbers of colony forming units or plaque forming units per sample were calculated on the basis of mean values from three replicate plates and the respective dilution factor.

A potential residual microbicidal effect coming from the test specimen after plasma exposure was assessed

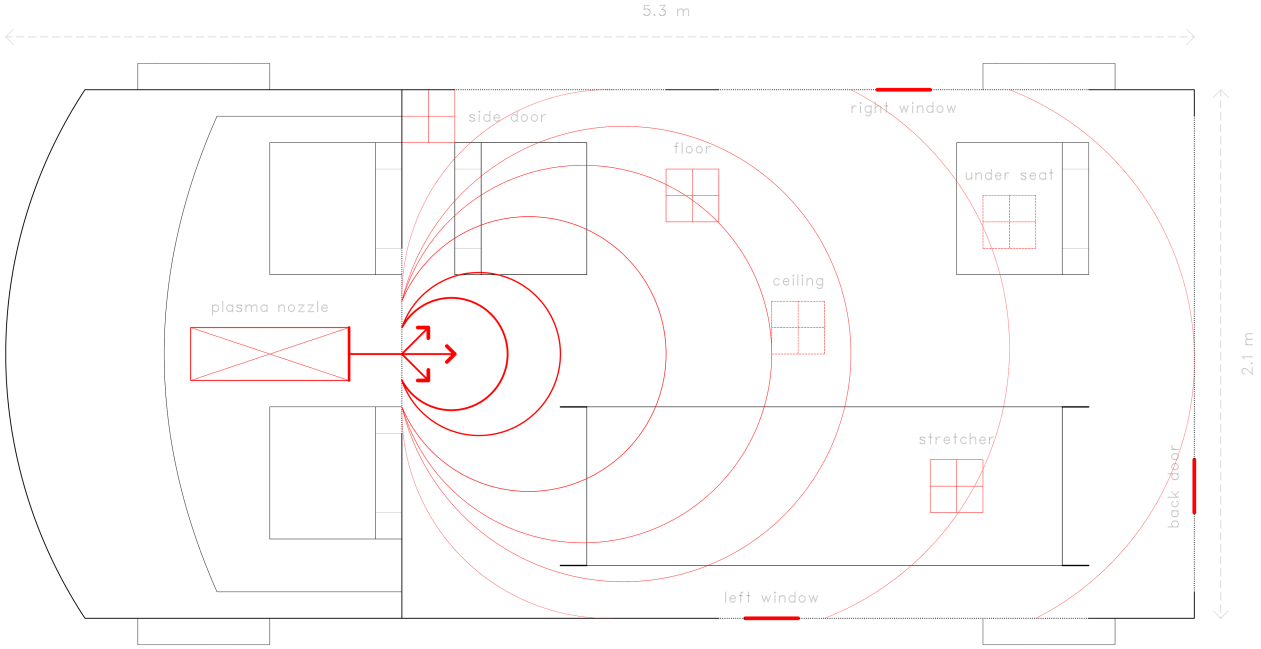

FIGURE 1 Experimental setup in the ambulance: The plasma system including the DBD plasma nozzle was placed inside the drivers cabin and connected to the trunk compartment with a flexible hose. The respective sample positions are indicated

separately. For this purpose, sterile test specimen were placed inside the ambulance during the plasma treatment. The plasma exposed test specimen were then transferred to the respective rinsing solution, which was previously inoculated with a defined number of the respective microorganism. After manual rubbing of the plasma exposed test specimen for 1 min, the inoculated rinsing solution was sampled as described above.

Statistical analysis

Mean values of pfu or cfu were calculated from the duplicate samples at every spot in the ambulance car (N) and from three reference samples which were placed in the drivers cabin during plasma treatments (N_o) in every trial. Log_{10} reductions ($\log_{10}[N_o N^{-1}]$) were calculated for every spot inside the ambulance car within each independent test trial. Log_{10} reductions are presented as mean values with standard deviations from three independent trials which were performed at different days. Significant differences of mean values were assessed by a one way analysis of variance with pairwise multiple comparison (Holm-Sidak method) using SigmaPlot 14.0 (Systat Software Inc) on a significance level of $p < 0.05$. The percentage recovery of microorganisms from dried test specimen was calculated for each trial from the determined numbers of pfu or cfu from reference samples and the applied inoculum. It is presented as mean values with standard deviations from three independent trials.

RESULTS

The percentaged recovery of viable microorganisms from the reference samples is shown in Table 1. Due to the high resistance towards desiccation, *B. atrophaues* spores were recovered to a high degree from all surfaces. Recovery of *S. aureus* was distinctively lower and showed significant variation among the different test specimen. Although *S. aureus* is considered to exhibit comparably high resistance under dry conditions, desiccation of vegetative bacteria is likely to be the reason for the lower recovery. Bacteriophage Phi6 showed the lowest recovery, which is in line with previous findings. Wood et al. (2020b) found similar recovery rates of Bacteriophage Phi6 from glass, stainless steel or ceramic tile within 2 h after drying. It is difficult to identify the exact reason for the variable recovery of *S. aureus* and Bacteriophage Phi 6 from the different surfaces. However, the wettability of glass and aluminum is considerably higher compared to the PET-film, resulting in larger droplets after inoculation. This in turn reduced

TABLE 1 Recovery of microorganisms from the control samples

	***Bacillus atrophaeus* spores**			***Staphylococcus aureus***			**Bacteriophage Phi6**		
Material	**Inoculation (CFU per sample)**	**CFU per sample**	**Recovery (%)**	**Inoculation (CFU per sample)**	**CFU per sample**	**Recovery (%)**	**Inoculation (CFU per sample)**	**CFU per sample**	**Recovery (%)**
PET	7.0E + 5	4.93E + 5 ± 5.27E + 4	70.4 ± 7.5	1.0E + 7	7.41E + 5 ± 1.83E + 6	7.4 ± 1.8	7.0E + 7	7.37E + 6 ± 1.87E + 6	10.5 ± 2.7
ALU	7.0E + 5	4.60E + 5 ± 9.82E + 4	65.7 ± 14.1	1.0E + 7	2.83E + 6 ± 1.89E + 6	28.3 ± 18.9	7.0E + 7	2.22E + 6 ± 1.02E + 6	3.2 ± 1.5
Glass	7.0E + 5	6.33E + 5 ± 1.21E + 5	90.4 ± 17.3	1.0E + 7	3.46E + 6 ± 1.15E + 6	34.6 ± 11.5	7.0E + 7	1.46E + 6 ± 7.41E + 5	2.1 ± 1.1

TABLE 2 Inactivation of *Bacillus atrophaeus* spores by plasma gas in an ambulance at eight different spots. Data are presented as mean ± SD log reduction obtained from 3 individual test trials performed with duplicate samples respectively

Material	Treatment time (min)	*Bacillus atrophaeus* spores								
		1-stretcher	2-roof	3-window	4-window	5-floor	6-back door	7-side door	8-under seat	Mean
PET	30	1.39 ± 0.18	1.70 ± 0.37	1.09 ± 0.82	0.90 ± 0.65	0.78 ± 0.25	3.14 ± 0.57	0.78 ± 0.14	1.89 ± 0.29	1.46 ± 0.86[a]
	60	**4.43 ± 0.04**	**4.43 ± 0.04**	4.23 ± 0.39	4.09 ± 0.57	4.04 ± 0.11	**4.43 ± 0.28**	4.30 ± 0.28	4.17 ± 0.25	4.27 ± 0.28[a]
ALU	30	0.23 ± 0.30	0.18 ± 0.05	0.18 ± 0.13	0.09 ± 0.10	0.18 ± 0.17	0.38 ± 0.33	0.12 ± 0.07	0.27 ± 0.19	0.20 ± 0.18[b]
	60	1.78 ± 0.59	1.34 ± 0.38	1.10 ± 0.59	0.74 ± 0.58	1.63 ± 0.51	1.24 ± 0.54	1.27 ± 0.46	1.15 ± 0.33	1.28 ± 0.52[b]
	90	4.14 ± 0.52	3.52 ± 0.31	3.00 ± 1.21	3.56 ± 1.11	3.62 ± 0.57	4.01 ± 0.48	3.44 ± 0.79	3.49 ± 0.68	3.60 ± 0.72[a]
	120	**4.40 ± 0.19**	4.30 ± 0.16	**4.40 ± 0.19**	4.13 ± 0.43	**4.40 ± 0.19**	**4.40 ± 0.19**	**4.40 ± 0.19**	4.30 ± 0.20	4.34 ± 0.22[a]
GLASS	30	0.08 ± 0.10	0.18 ± 0.26	0.11 ± 0.10	0.10 ± 0.10	0.06 ± 0.04	0.18 ± 0.19	0.07 ± 0.05	0.03 ± 0.03	0.10 ± 0.12[b]
	60	1.68 ± 0.78	0.51 ± 0.08	0.32 ± 0.13	0.26 ± 0.04	0.91 ± 0.24	0.86 ± 0.22	0.75 ± 0.26	0.64 ± 0.15	0.74 ± 0.50[c]
	90	3.52 ± 0.82	2.89 ± 0.69	2.35 ± 0.70	2.30 ± 0.82	2.36 ± 0.62	3.96 ± 0.80	2.87 ± 0.76	2.37 ± 0.74	2.78 ± 0.96[b]
	120	**4.54 ± 0.07**	4.02 ± 0.57	4.26 ± 0.22	3.44 ± 0.65	4.34 ± 0.27	4.41 ± 0.28	4.49 ± 0.16	4.20 ± 0.29	4.21 ± 0.45[a]

Note: Results in bold indicate that the numbers of cfu on all six coupons were beneath the detection limit of 16.7 cfu/sample.

Different superscript letters of mean values for a given treatment time indicate significant differences ($\alpha = 0.05$) among the surfaces.

TABLE 3 Inactivation of Staphylococcus aureus by plasma gas in an ambulance at eight different spots. Data are presented as mean ± SD log reduction obtained from 3 individual test trials performed with duplicate samples

Material	Treatment time (min)	*Streptococcus aureus*								
		1-stretcher	2-roof	3-window	4-window	5-floor	6-back door	7-side door	8-under seat	Mean
PET	30	**4.45 ± 0.17**	**4.45 ± 0.17**	4.14 ± 0.67	4.43 ± 0.22	4.25 ± 0.51	4.25 ± 0.45	4.35 ± 0.18	4.37 ± 0.26	4.34 ± 0.35[a]
ALU	30	4.70 ± 1.08	**5.16 ± 0.32**	4.91 ± 0.24	4.76 ± 0.61	5.02 ± 0.38	**5.16 ± 0.32**	4.87 ± 0.30	**5.16 ± 0.32**	4.97 ± 0.46[b]
GLASS	30	5.12 ± 0.32	5.24 ± 0.26	5.08 ± 0.20	5.04 ± 0.35	4.94 ± 0.36	**5.30 ± 0.16**	4.97 ± 0.47	**5.30 ± 0.16**	5.12 ± 0.29[b]

Note: Results in bold indicate that the numbers of cfu of all six coupons were beneath the detection limit of 16.7 CFU per sample.

Different superscript letters of mean values indicate significant differences ($\alpha = 0.05$) among the surfaces.

TABLE 4 Inactivation of Bacteriophage Phi6 by plasma gas in an ambulance at eight different spots. Data are presented as mean ± SD log reduction obtained from three individual test trials performed with duplicate samples

Material	Treatment time (min)	Bacteriophage Phi6							
		1-stretcher	2-roof	3-window	4-window	5-floor	6-back door	7-side door	8-under seat	Mean
PET	30	4.83 ± 0.77	5.00 ± 0.48	5.04 ± 0.18	5.12 ± 0.29	4.72 ± 0.63	**5.33 ± 0.11**	**5.33 ± 0.11**	**5.33 ± 0.11**	5.09 ± 0.24[a]
ALU	30	**4.79 ± 0.21**	4.11 ± 1.18	3.84 ± 0.71	4.13 ± 1.16	**4.79 ± 0.21**	**4.79 ± 0.21**	4.10 ± 1.19	4.69 ± 0.27	4.41 ± 0.40[b]
GLASS	30	**4.54 ± 0.35**	**4.54 ± 0.35**	**4.54 ± 0.35**	**4.54 ± 0.35**	**4.54 ± 0.35**	**4.54 ± 0.35**	**4.54 ± 0.35**	**4.54 ± 0.35**	4.54 ± 0.00[b]

Note: Results in bold indicate that the numbers of pfu of all six coupons were beneath the detection limit of 33.3 pfu/sample.
Different superscript letters of mean values indicate significant differences ($\alpha = 0.05$) among the surfaces.

the required drying time on aluminum and glass. This might be one reason for the higher recovery of *S. aureus* on aluminum and glass since the osmotic stress caused by deionized water is shorter. On the other hand, a slower drying process appears to be beneficial for the recovery of Phi6 phages, leading to a higher recovery on PET.

A potential residual microbicidal effect coming from the test specimen after plasma exposure was investigated separately. No antimicrobial effect due to carryover of residues on the test specimen was found for all microorganisms and all test coupons.

The results obtained from the disinfection trials are shown in Tables 2–4. Bacteriophage Phi6 and *S. aureus* showed a much higher sensitivity towards the plasma gas compared to *B. atrophaeus* spores. In average, Bacteriophage Phi6 was reduced by 5.09 ± 0.24 (PET), 4.41 ± 0.40 (ALU) and 4.54 ± 0.00 (Glass) orders of magnitude within 30 min. *S. aureus* was reduced by 4.34 ± 0.35 (PET), 4.97 ± 0.46 (ALU) and 5.12 ± 0.29 (Glass) orders of magnitude within 30 min. For both microorganisms, the reduction on PET was significantly different to those found on aluminum or glass carriers. However, the differences among the surfaces were most likely caused by the variable recoveries, which affect the maximum detectable log reduction.

Regarding *B. atrophaeus* endospores, the achievable count reduction was significantly affected by the respective substrate material. On PET, reductions by at least 4 orders of magnitude were obtained after a treatment time of 60 min on all spots in the vehicle. The average count reduction after 30 min was 1.46 ± 0.86 orders of magnitude, while a mean reduction by 4.27 ± 0.28 orders of magnitude was found after 60 min. On aluminum foil as well as glass slides, significantly longer treatment times were required to achieve a similar effect. While reductions by 1.28 ± 0.52 (ALU) and 0.74 ± 0.50 (Glass) orders of magnitude were determined after 60 min, the average count reduction increased to 3.60 ± 0.72 (ALU) and 2.78 ± 0.96 (Glass) orders of magnitude after 90 min. In order to demonstrate an average colony count reduction by at least 4 orders of magnitude, a treatment time of 120 min was necessary in case of ALU and Glass.

DISCUSSION

The presented results show that a high, uniform and reproducible reduction of the selected endospores, vegetative bacterial cells as well as bacteriophages dried on different surfaces was achieved with the DBD plasma system when it is operated in remote mode with the afterglow from the plasma nozzle being introduced into the

interior of an ambulance. As expected, *S. aureus* as well as Phi6 bacteriophages proved to be significantly more sensitive towards the plasma treatment compared to *B. atrophaeus* spores. The target for effective spore inactivation according to DIN EN 17272:2020, claiming a reduction by 4 orders of magnitude (medical area) or 3 orders of magnitude (veterinary area or food, industrial domestic and institutional area), was met after a treatment for 120 min. Inactivation of *B. atrophaeus* spores on aluminum foil and glass required significantly longer treatment times compared to the PET film. This is most likely attributable to the distinctively lower wettability of PET compared to aluminum and glass. The low surface energy of PET enabled a very homogeneous spray inoculation with small droplets, which in turn resulted in a fine distribution of spores on the inoculated surface (2×2 cm) after drying. In contrast, the high wettability of aluminum foil and glass slides resulted in large droplets that tended to coalesce. Spore agglomerates after drying were therefore observed in case of aluminum and glass but not on PET by light microscopy (not shown). It is likely that longer treatment times were consequently required to inactivate spores that were not directly accessible but covered by other spores. A reduced inactivation efficiency of plasma treatments towards microorganisms embedded in stacks or matrices has already been reported previously (Deng et al., 2005; Kramer et al., 2020). The target for effective inactivation of bacteria and bacteriophages according to DIN EN 17272:2020, claiming a reduction by 5 orders of magnitude (bacteria) and 4 orders of magnitude (bacteriophages), was met in case of bacteriophage Phi6 (all surfaces) and *S. aureus* (glass) after a treatment for 30 min. The reason why a reduction of *S. aureus* by at least 5 orders of magnitude could not be demonstrated on PET and ALU is most likely related to the lower recovery of viable bacteria from the control samples, which limits the provable logarithmic reduction.

To date, there are few published studies where a DBD was used for room disinfection. Previous investigations at which atmospheric plasma was applied in remote operation mainly focused on the disinfection of surfaces with the samples exposed to a flowing afterglow (Moisan et al., 2014; Roth et al., 2000; Yang et al., 2009). Müller et al. (2018) reported about the inactivation of *B. atrophaeus* endospores by remote plasma in different treatment volumes of 0.54 L, 1.8 L and 2.6 L by 4.4 $\log_{10}$ within 10, 20 and 30 min respectively, whereat a circulating plasma afterglow generated by a surface micro-discharge with humid air was used. Obtaining a similar inactivation efficiency, Moisan et al. (2013) studied the sporicidal effect of a flowing afterglow from a reduced-pressure N_2-O_2 discharge. Reductions of *B. atrophaeus*, *B. pumilus* and *G. stearothermophilus* by approximately 3–4 $\log_{10}$ were obtained within 15 min in a treatment chamber with a volume of 5.5 L (Moisan et al., 2013). Comparable reduction factors for *B. atrophaeus* spores were obtained in the present study within treatment times of 60–90 min, but in a considerably larger volume of approximately 10 m^3. Mok et al. (2015) introduced the afterglow from a corona discharge in a treatment chamber where a reduction of *Escherichia coli*O157:H7 by 3.5 orders of magnitude was demonstrated within 24 h. In a more recent study, Ki et al. (2019) investigated the disinfection capacity of a DBD plasma inside a laboratory chamber (0.175 m^3) or a van (2.43 m^3). A reduction of *S. aureus* by approximately two orders of magnitude within 30 min was reported inside the van. Furthermore, Ki et al. (2019) also showed that a high humidity of the process gas is mandatory in order to maximize the disinfection efficiency of the indirect plasma treatment. A high humidity increased the hydrogen peroxide and hydroxyl radical concentrations and reduced the ozone concentration, which is why Ki et al. (2019) concluded that not ozone but other water related reactive species like hydrogen peroxide or hydroxyl radicals are primarily responsible for the microbicidal action (Ki et al., 2019). Therefore, it appears likely that the high microbicidal efficiency of the DBD plasma nozzle in the ambulance was caused by reactive species which originate from water. In a previous study, we demonstrated that *B. atrophaeus* spores can be inactivated by several orders of magnitude within less than 5 minutes on different surfaces in a treatment chamber with a volume of 300 L by use of a DBD plasma system in remote operation (Kramer et al., 2020). In agreement with findings made by Ki et al. (2019), we found that humidification of the plasma gas is mandatory to enable a fast sporicidal effect and that ozone alone cannot be seen as the sporicidal component of the indirect plasma (Kramer et al., 2020). A similar conclusion was recently drawn by Kogelheide et al. (2020), who showed that ozone alone is not responsible for the sporicidal effect of a DBD discharge. The extent of spore inactivation was directly correlated with the humidity, which implied that reactive species formed from water molecules such as OH, H_2O_2, HNO_2, HNO_3, or HO_2NO_2, play an important role in spore inactivation (Kogelheide et al., 2020).

Considering the use of the compact DBD plasma system for room disinfection, its strengths clearly lie in the microbicidal efficiency, the required cycle times, the uniformity of treatments and the ease of use. Common cycle times for room disinfection systems based on hydrogen peroxide vapor or aerosolized hydrogen peroxide have been reported to be about 1.5–2.5 h and 2–3 h respectively (Weber et al., 2016) which is comparable or slightly longer than the required cycle times for spore inactivation in the present study. Regarding the disinfection

of an ambulance using aerosolized hydrogen peroxide, Andersen et al. (2006) reported that at least 3 cycles were necessary for a complete inactivation of *B. atrophaeus* on spore strips, which in sum took about 4–5 h. Significantly shorter cycle times of 1 h (PET) or 2 h (ALU, Glass) were required to reduce *B. atrophaeus* endospores by at least 4 orders of magnitude with the DBD plasma nozzle in the ambulance. Future stages of development with a higher process gas flow or multiple nozzles implemented in the plasma system could lead to a further reduction of the required process time.

Regarding available UV based systems, it has been reported that significantly shorter cycle times in the range of 10–30 min are required for room disinfection. However, in case of UV-irradiation, the efficiency is known to be distinctively lower, especially due to the non-uniform treatments, which makes repositioning of the lamps necessary. This in turn requires additional time and intervention of the operator or a robot (Otter et al., 2020). Lindsley et al. (2018) investigated the use of Ultraviolet Germicidal Irradiation (UVGI) to disinfect the patient compartment in an ambulance. They found that the UV irradiation delivered to different surface locations varies tremendously and leads to long treatment times of up to 16.5 h when the required time is governed by the exposure time needed for the least-irradiated surface. Repositioning of the UV source or increasing the UV reflectivity of interior surfaces could reduce the disinfection time. However, the systems must be rigorously validated with the actual ambulance configuration before deployment (Lindsley et al., 2018).

With respect to ozone, it has been shown that a comparably slow sporicidal effect can be achieved, which also strongly depends on the air humidity. For instance, Aydogan and Gurol (2006) reported a 3 log reduction of *B. subtilis* on glass within 4 h at an ozone concentration of 3 mg L^{-1} at 90% RH. High humidity has been assumed to induce a swelling of spores, which allows diffusion of ozone into the spores (Mahfoudh et al., 2010). Preconditioning of *Bacillus atrophaeus* spores on glass coupons at 70% RH has been shown to reduce the required treatment time for a 6 log reduction with 18 mg L^{-1} ozone to only 1 h. However, the preconditioning itself took 15 h (Currier et al., 2001). Under similar conditions but without preconditioning, Wood et al. Wood et al., 2020a demonstrated a reduction of *B. subtilis* spores on glass by 4.51 orders of magnitude in a treatment chamber with a volume of 0.57 m^3 at 85% RH. A treatment time of 4 h at a comparably high concentration of 14 mg L^{-1} ozone was required therefor. A similar sporicidal effect was obtained in the present study on glass slides but in half the time (2 h) and in a volume that is almost 20 times larger. The DBD plasma system therefore appears clearly advantageous over pure ozone generators.

Hydrogen peroxide vapor systems are known for their high microbicidal efficiency and the homogeneous distribution. However, in case of aerosolized hydrogen peroxide, a homogeneous distribution of the active agent has been reported to be difficult, most likely due to the unidirectional nozzle and the gravity of particles (Otter et al., 2020). Fu et al. (2012) showed that aerosolized hydrogen peroxide exhibits a significantly lower homogeneity and efficiency compared to hydrogen peroxide vapor for room disinfection using *Geobacillus stearothermophilus* biological indicators at a cycle time of 2 h respectively (Fu et al., 2012). Despite the complex geometries of the interior in the ambulance, the disinfection success in the present study showed a high uniformity and reproducibility among the different spots. A high spatial uniformity of a DBD plasma for room disinfection was also reported by Ki et al. (2019).

It has to be kept in mind that the results of this study were all obtained at comparably low temperature and high relative humidity due to present weather conditions. This favors a high degree of condensation, which could be a crucial factor for the success of remote plasma disinfection as proposed previously by Jeon et al. (2014). Therefore, further studies will be required to address the impact of variable temperature and humidity in order to consider all relevant climatic conditions. Even though the microbicidal action of remote plasma appears to be related to reactive species coming from water, like hydrogen peroxide or hydroxyl radicals (Jeon et al., 2014; Ki et al., 2019; Kogelheide et al., 2020; Kramer et al., 2020), further effort should be made regarding the elucidation of the causative reactive species and their interaction. Since reactive species produced in a DBD discharge, including high levels of ozone, are powerful oxidants, the long term compatibility of relevant materials needs further investigation. Although we did not observe any negative effects on facilities in the ambulance car throughout this study, a potential impact of the plasma treatments on chemical and physical properties of selected plastics will be investigated in an upcoming project. With respect to safety precautions, UV systems are clearly advantageous over hydrogen peroxide, ozone or also plasma systems since no room sealing is necessary and immediate room entry is possible after the disinfection cycle. The necessity of appropriate safety precautions of plasma systems is expected to be comparable to those based on hydrogen peroxide or ozone. Regarding the ambulance vehicle used in this study, opening all doors after ending the disinfection treatments allowed re-entry after 20 min. Within this timeframe, the ozone concentration decreased to less than 0.1 mg m^{-3}. However, considering disinfection of closed rooms in buildings, appropriate measures must be taken, e.g. by catalytic converters. Regarding the cost-effectiveness of automated room

disinfection systems, no studies have been published yet. According to Otter et al. (2020), the relative purchase costs of equipment is likely to be UVC > H_2O_2 vapor systems > aerosolized hydrogen peroxide Otter et al. (2020). Upcoming development stages of the plasma system will be fully portable, requiring only ambient air and power supply, which enables the direct use on site in vehicles or also for room disinfection without the need for further consumables like hydrogen peroxide.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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