Rapid inactivation of bacterial spores using plasma activated water: development, species identification and sporicidal mechanism

C. Tsai[1], L. Kienast[1], S. Kalghatgi[1], D. Petrak[1], E. Bogovich[1], J. Louis[1], J. Redfearn[2], S. Pecnik[3], C.J. Woolverton[3], R. Gray[1] and D. Pappas[1]

[1] *EP Technologies LLC, Akron, OH, U.S.A.*
[2] *Department of Biological Sciences, Kent State University, Kent, OH, U.S.A.*
[3] *Department of Environmental Health Sciences, Kent State University, Kent, OH, U.S.A.*

Abstract: This study demonstrates that rapid inactivation (> 6 log reductions in 10 seconds) of *C. difficile* spores on dry surfaces can be achieved using plasma activated water (PAW) generated in situ or in a form of fine droplets. The concentration of peroxynitrite and pH level in the PAW likely play a critical role in the rapid sporicidal efficacy, leading to spore coat damage and membrane integrity loss.

Keywords: *C. difficile* spores, plasma activated water (PAW), peroxynitrite

1. Introduction

Clostridium difficile (*C. difficile*) is a bacterium that causes illnesses ranging from persistent diarrhea to severe inflammation and damage of the colon. *C. difficile*-associated diarrhea results in increased lengths of hospital stay, costs, morbidity, and mortality among adult patients [1]. According to the Center for Disease Control (CDC), *C. difficile* is linked to 14,000 deaths each year in the United States. U.S. hospitals treat 165,000 cases of hospital-acquired, hospital-onset *C. difficile* per year, which add $1.3 billion in excess costs.

It is notoriously difficult to eliminate *C. difficile* because it can form bacterial endospores. Bacterial spores can exist in a dormant state for many years in non-favorable environments and re-germinate upon favorable stimuli from the external environment. *C. difficile*-infected patients shed these spores in their local environment, which includes their feces, skin, and clothing. This frequently results in contamination of abiotic, contact surfaces, devices, and materials. These environments serve as a reservoir for the *C. difficile* spores and can facilitate re-infection within the original host patient or spread to others within a localized setting. The bacterial spores are typically highly resistant to many chemical and physical agents. Most of common fungi, virus, and bacteria (e.g., *E. coli*) can be readily inactivated by alcohol-based sanitizers. However, to destroy bacterial spores, more caustic, harsh chemicals such as bleach and peracetic acid need to be used since alcohol has no sporicidal activity. To achieve a greater than 6 log reduction (> 99.9999%) of the *C. difficile* spores on inanimate surfaces, these chemicals must be applied for intervals that range from 3-5 minutes. Therefore, a new technology which can achieve more rapid disinfection of inanimate surfaces will be highly beneficial to healthcare environments since it can help prevent the spread of *C. difficile* infection, further reducing *C. difficile*-caused morbidity and mortality.

This work developed two rapid surface disinfection methods which can inactivate *C. difficile* spores on inanimate surfaces within 15 seconds using plasma activated water (PAW). The sporicidal efficacy of a direct dielectric barrier discharge (DBD) reactor and a mini-plasmatron system was also compared. The key sporicidal species (e.g., peroxynitrite) produced in PAW were identified. The spore inactivation mechanism was also investigated in this work.

2. Experimental Setup and Methods

2.1. DBD and Mini-plasmatron Reactors

The DBD plasma reactor and the mini-plasmatron system used in this work are shown in Fig. 1(a) and Fig. 1(b), respectively. The DBD reactor consists of a copper rod (2.54 cm in diameter) that serves as a high voltage electrode and utilizes a polyetherimide (PEI) shell as the insulating housing and a quartz disc as the dielectric material. A high voltage wire was used for electrical connection to a high voltage AC burst power supply. Stainless steel disks of 1 cm in diameter contaminated with *C. difficile* spores were mounted at a distance of 2 mm from the DBD reactor on a ground metal plate. This configuration enables the DBD reactor to create plasma using ambient air on top of the treated object. The air plasma was driven by continuous high voltage bursts with a burst repetition rate of 3.5 kHz and a peak-to-peak voltage amplitude of 20 kV. Each voltage burst is composed of a decaying sinusoid with a frequency of about 32 kHz.

A negative half-wave rectified sine wave with an amplitude of 6 kV was used to drive the mini-plasmatron system. In this case, the experimental samples contaminated with *C. difficile* spores were mounted at a distance of 2 mm from the mini-plasmatron for plasma treatment. In this study, both the DBD and the mini-plasmatron systems were employed to directly treat the *C. difficile* spores on dry surfaces.

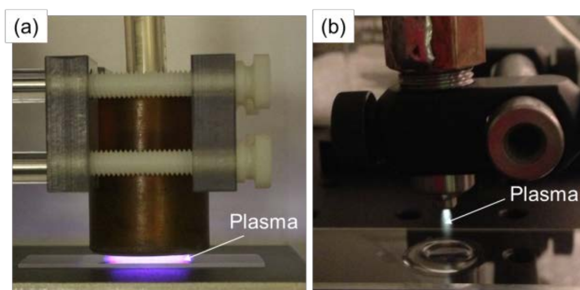


Fig. 1. Images of (a) DBD reactor and (b) mini-plasmatron used in this study

2.2. Method and System for PAW Generation

An in-situ PAW generation method was developed to inactivate the *C. difficile* spores on hard inanimate surfaces. To achieve the in-situ PAW generation, the surfaces contaminated with *C. difficile* spores were sprayed with water followed by the application of either the DBD or the mini-plasmatron. It should be noted that the water sprayed on the surface formed a < 500 µm thick water film. The water film thickness was proven to be critical for the sporicidal efficacy. In-situ PAW generation was employed in this work since it was found that preparing PAW using either the DBD or the mini-plasmatron and then applying the PAW to the *C. difficile* spores (ex situ) resulted in low efficacy (< 1.5 log reductions with 30 sec contact time; data not shown). This is likely due to the fact that before the PAW was applied to the samples some of the most active, but short-lived species (e.g., peroxynitrite) rapidly decomposed after the plasma was turned off. Therefore, creating PAW right on top of the *C. difficile* spores (in-situ PAW generation) is desired to ensure that the reactive, short-lived species can be produced in the vicinity of the spores.

In addition to the use of the DBD and the mini-plasmatron reactors to create PAW in situ, a DBD-based system which can deliver plasma-activated fine water droplets was developed. The schematic cross-sectional view of the plasma reactor is shown in Fig. 2. It had a cylindrical configuration with concentric electrodes. A metal rod at the center of the reactor served as the high-voltage electrode and was insulated by a dielectric barrier. A tubular dielectric material served as the device housing covered by a metal tube, which was connected to a ground potential. A spout with a reducing orifice was connected to the outlet of the plasma reactor to concentrate the flow as well as the species production. When the applied voltage exceeded the gas breakdown value, plasma was generated in the gap between the two dielectric barriers, as shown in the inset of Fig. 2. In this case, an AC sinusoidal voltage with a frequency of 22 kHz, a peak-to-peak voltage of 24 kV, and a duty cycle of 50% was used for plasma generation. Fine water droplets having an average size of about (1–3) µm were produced using an ultrasonic humidifier and were transported to the plasma reactor by air flow created by a DC blower. A reduction of the size of the water droplets was observed due to their exposure to the plasma. The operating parameters, such as droplet size, air flow rate, applied voltage, etc as well as the electrode design and length of plasma zone are expected to prolong the reactivity of the plasma generated actives. This system enables the highly reactive, short-lived species to be delivered onto the *C. difficile* spores before they decompose. To evaluate its sporicidal efficacy, the experimental samples were placed at a distance of 5 mm from the spout end.

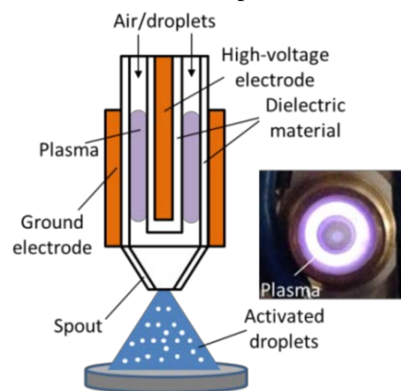


Fig. 2. Schematic cross-sectional view of the plasma setup for activated droplet generation.

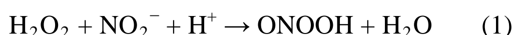
2.3. Sporicidal Testing Method

A quantitative disk carrier test method, modified based on EPA MB-31-03 SOP, was used to evaluate the sporicidal activities of plasma and PAW. A volume of 10 µl of *C. difficile* spores (~10^8 CFU/ml) in sterile water was added onto sterile stainless steel disks and left to dry for 30 min. The dry spores formed a visible contaminated area of about 3.5 mm in diameter on the stainless steel disk carriers. Then the contaminated surfaces were exposed to the plasma sources or PAW under the conditions described above for various treatment times. Following treatment, the disks were placed in test tubes filled with neutralizer (9.9 ml). The test tubes were sonicated for 1 minute and vortexed for 15 seconds to ensure removal of the spores from the stainless steel surfaces. The neutralizer solution containing spores was diluted and plated on Brain Heart Infusion Agar supplemented with 0.1% Sodium Taurocholate (BHIT). Anaerobic incubation for (36–48) hrs was then performed at 37ºC, followed by the estimation of surviving colony forming units (CFU).

2.4. Identification of Key Species

It has been reported that peroxynitrite is one of the key species in plasma activated water and it significantly contributed to the antimicrobial properties of PAW [2]. To evaluate the contribution of each type of species present in the plasma activated water, several different chemical solutions were prepared or synthesized to mimic the components (e.g., low pH, H_2O_2, NO_2^-, $ONOO^-$, and $OH^\bullet$) produced in the PAW for sporicidal testing with either 30 sec or 10 min contact time, as listed in Table 1. It should be noted that mixing H_2O_2 and $NaNO_2$ under acidic conditions (pH < 3.4) will produce yellow

peroxynitrite by reaction (1) (e.g., S5, S6, and S7) [3], which rapidly decomposes to nitrate in seconds in acidic environment via reaction (2) due to its short half-life [4]. Solutions prepared according to S5 and S7 were used for sporicidal testing 1 min after synthesis to ensure absence of the yellowish peroxynitrite for evaluation of the effect of acidified H_2O_2 and $NaNO_2$ only. S6 was prepared right on top of the spore samples so the effect of the mixture of acidified H_2O_2, $NaNO_2$, and $ONOO^-$ can be studied. S8 is an $ONOO^-$ stabilized solution by adding NaOH immediately after mixing the acidified H_2O_2 and $NaNO_2$ as the peroxynitrite is stable in alkaline solutions by reaction (3).



$$H_2O_2 + NO_2^- + H^+ \rightarrow ONOOH + H_2O \qquad (1)$$

$$ONOOH \rightarrow NO_3^- + H^+ \qquad (2)$$

$$ONOOH + OH^- \rightarrow ONOO^- + H_2O \qquad (3)$$

Table 1. Disinfecting solutions prepared in the sporicidal testing.

Sample label	Disinfecting solutions	pH	Contact time
S1	0.6 M H_2O_2	5.5	30 sec
S2	0.6 M $NaNO_2$	8.4	30 sec
S3	0.7 M HCl + 0.6 M H_2O_2 (acidified H_2O_2)	0.2	30 sec
S4	0.6 M H_2O_2 + 0.6 M $NaNO_2$ (H_2O_2 + NO_2^-)	6.9	30 sec
S5	0.7 M HCl + 0.6 M H_2O_2 + 0.6 M $NaNO_2$ (acidified H_2O_2 + NO_2^-)	0.5	30 sec
S6	0.7 M HCl + 0.6 M H_2O_2 + 0.6 M $NaNO_2$ (in-situ $ONOO^-$ production)	0.5	30 sec
S7	0.7 M HCl + 0.3 M H_2O_2 + 0.3 M $NaNO_2$ (similar to S5 but with lower H_2O_2 and NO_2^- concentrations)	0.45	30 sec
S8	Stabilized $ONOO^-$ (add 0.5M NaOH right after mixing 0.7 M HCl + 0.3 M H_2O_2 + 0.3 M $NaNO_2$)	12.6	30 sec
S9	10 mM H_2O_2	4	10 min
S10	10 mM H_2O_2 + 1 mM $Fe(ClO_4)_2$ (Fenton reaction to produce $OH^\bullet$)	> 4	10 min

2.5. Study of Spore Inactivation Mechanism

Fluorescence microscopy was used to examine the spore's membrane integrity with two fluorescent dyes – Syto®9 & Propidium Iodide (PI). Syto®9 is a cell permeable green-fluorescent dye while PI is a non-cell permeable red-fluorescent nuclear and chromosome counterstain. These two dyes can be utilized to detect whether the cells are live, dead or damaged. Living cells will take up Syto®9 and fluoresce green. Dead cells will take up both Syto®9 and PI, but quench Syto®9, so fluoresce primarily red. If a cell is damaged, both red and green fluorescence will be observed.

3. Experimental Results

3.1. Sporicidal Efficacy using Plasma Systems

Fig. 3 shows the sporicidal testing results against *C. difficile* spores using five different treatment methods: 1) direct DBD on dry spores, 2) mini-plasmatron on dry spores, 3) in-situ PAW generation on spores using direct DBD, 4) in-situ PAW generation on spores using mini-plasmatron, and 5) plasma activated fine water droplets. It should be noted that the DBD systems operated in an 'ozone mode', while the mini-plasmatron in an afterglow 'nitrogen oxides mode'. The testing results showed that when the dry spores were directly treated by the direct DBD or the mini-plasmatron device, 2 log reductions required exposure to the plasma for longer than 90 seconds. In-situ PAW generation using the mini-plasmatron also showed low efficacy (~ 2 log reduction after 120 seconds). However, in-situ PAW generation using direct DBD led to excellent sporicidal results (> 6 log reduction with 15-second plasma exposure time). Besides, the plasma activated water droplets produced by the DBD device were also able to reduce the microbial viability of *C. difficile* spores by 6 logs (99.9999%) in an even shorter application time (10 seconds).

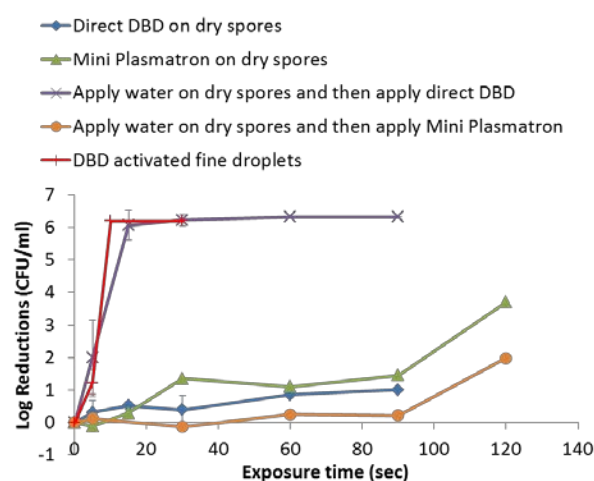


Fig. 3. Sporicidal testing results against *C. difficile* spores using different plasma treatment methods.

These results showed that PAW treatments are more efficacious than the dry plasma treatments. These also indicated that the aqueous chemical species are important for the rapid inactivation of *C. difficile* spores. It should be reiterated that preparing the PAW ex situ led to much poor efficacy than that by the in-situ PAW generation. This implied that there are very potent, but short-lived species (e.g., peroxynitrite), present in the PAW generated in situ. However, as shown in Fig. 3, the in-situ PAW generation using the mini-plasmatron device resulted in low efficacy. This may be due to the high pH (~5 after 30 seconds) of the plasmatron-activated water (lack of $H_3O^+_{aq}$ production in the afterglow configuration). It should be noted that the pH value of the PAW generated in situ using the DBD device was about 2 after 30 seconds and that of plasma activated water droplets was also about 2. As mentioned earlier, peroxynitrite production in a solution requires a pH lower than 3.4. The low pH in the PAW produced by the direct DBD and that in the plasma activated fine droplets may cause the production of a much higher concentration of the peroxynitrite than that in the plasmatron-activated water. Therefore, one may conclude that the rapid

inactivation of *C. difficile* spores is achieved when a sufficient number of powerful, short-lived aqueous species (e.g., peroxynitrite) is produced in the vicinity of the spores.

3.2. Sporicidal Efficacy using Mimicking PAW

Fig. 4 shows the sporicidal testing results against *C. difficile* spores using the mimicking PAW solutions shown in Table 1 to identify the species which primarily participate in the sporicidal activity. H_2O_2 alone (S1) and $NaNO_2$ alone (S2) led to about 0.3 and 0.6 log reductions with 30-second contact time, respectively. The efficacy of H_2O_2 was slightly increased to 0.6 log reductions with addition of HCl (S3). The mixture of H_2O_2 and $NaNO_2$ (S4) showed a similar efficacy as that with $NaNO_2$ alone (S2). A slight decrease in efficacy was observed by using the mixture of acidified H_2O_2 and $NaNO_2$ (S5, pH=0.5), compared with the acidified H_2O_2 (S3, pH=0.2). It should be noted that S5 is similar to the PAW after the plasma is turned off. This result indicated that even if the PAW has very concentrated H_2O_2 and NO_2^- with very low pH, it may still have no efficacy against bacterial spores. Creating peroxynitrite solution (S6) right on top of the *C. difficile* spores (in-situ peroxynitrite generation) resulted in > 3.34 log reductions. S7 with lower pH and H_2O_2 and $NaNO_2$ concentrations than those in S5 showed greater efficacy than S5. A comparison between S3 and S5 as well as that between S5 and S7 implied that the lower the pH the better the sporicidal efficacy with the presence of H_2O_2 and $NaNO_2$. Similar to S6, the stabilized $ONOO^-$ solution (S8, see sample image in Fig. 4) also exhibited excellent sporicidal property (> 3.11 log reductions). These results showed that peroxynitrite alone is a very powerful sporicidal species regardless of the solution pH. S9 and S10 were used to examine the role of $OH^\bullet$ in the sporicidal effect. In S10, $OH^\bullet$ was produced through the Fenton reaction. However, S9 appeared to be more efficacious than S10. This indicated that acidified H_2O_2 (S9) plays a more important role in *C. difficile* spores inactivation than $OH^\bullet$.

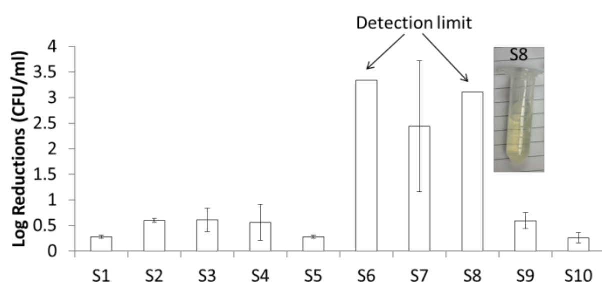


Fig. 4. Sporicidal testing results against *C. difficile* spores using prepared disinfecting solutions as listed in Table 1. The sporicidal detection limit in the testing was 3 log.

3.3. Fluorescence Microscopy Images

The fluorescence microscopy images of the *C. difficile* spores without treatment and treated by the plasma activated fine droplets can be seen in Fig. 5. Fig. 5(A) shows the image of living spores (without plasma treatment) fluorescing green, while in Fig. 5(B) several cells fluoresce red, indicating that the spores are dead after being treated by the activated droplets. Besides, this result demonstrated that the spore coat and membrane were damaged by the plasma treatment, and thus allowed PI (non-cell permeable dye) to be taken up. As a result, it can be concluded that the mechanism of *C. difficile* spore inactivation is likely due to the spore coat and membrane damage.

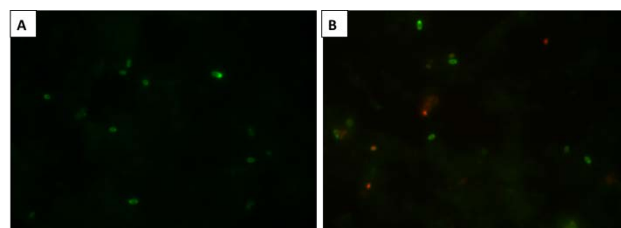


Fig. 5. Fluorescence microscopy micrographs (1000X total magnification) of LIVE/DEAD-stained *C. difficile* (A) without treatment and (B) treated by plasma activated fine droplets.

4. Conclusions

This work demonstrated that plasma activated water (PAW) can be used to achieve rapid inactivation of *C. difficile* spores on dry hard surfaces as long as the powerful, short-lived aqueous species can be produced in the vicinity of the spores. The experimental results showed that a higher than 6 log reduction of *C. difficile* spores can be achieved within a 10 second exposure time. It was found that the PAW treatments had a more rapid and efficacious sporicidal activity than the dry plasma methods (both the direct DBD and the mini-plasmatron) likely due to the synergistic action of the powerful, short-lived liquid-phase species (e.g., $ONOO^-$) and the low pH. Spore coat and membrane damage appeared to be the key sporicidal mechanism using activated droplets.

5. References

[1] N. Macleod-Glover, Canadian family physician Médecin de famille canadien, **56**, 417(2010).

[2] P. Lukes, Plasma Sources Science and Technology, **23**, 015019(2014).

[3] K. Robinson, Methods in Enzymology, **396**, 207 (2005).

[4] A. Saha, Free Radical Biology & Medicine, **24**, 653 (1998).