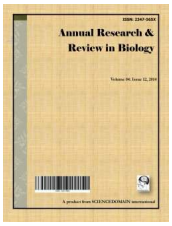

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Influence of Dielectric-barrier Discharge (DBD) Cold Plasma on Water Contaminated Bacteria

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NSR and HHM designed the study and wrote the protocol. Author FSM performed the statistical analysis wrote the first draft of the manuscript. Authors FSM and NKA managed the analyses of the study. Authors NSR and HHM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aim: Dielectric Barrier Discharge (DBD) cold plasma has efficient antibacterial activity on bacterial contamination at a short period of time and confirms its potential for routine used in clinical environment biodecontamination as an alternative to conventional disinfectant methods of water treatment.

The goal of this study is to evaluated *in vitro* effectiveness of DBD cold plasma on clinically important opportunistic pathogens that identified from water samples which were: *Legionella E.meningosepteca* and *S. paucimobilis* growth and to study DBD effectiveness on survival bacterial cell after treatment.

Methodology: 100-200 cfu/ml of Seven different environmental bacterial isolates belong to *Legionella* (4 isolates) *E.meningosepteca*(one isolate) and *S. paucimobilis* (two isolates) at species were subjected to eleven different exposure time of DBD cold plasma treatment rang from 15Sec to 300 Sec, and plated on suitable agar media. Results were quantified by viable count.

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Results: It was found that using of different time of DBD plasma against different bacterial species give increase reduction rate until reaching lethal time of each species, this reduction time was species and isolates dependent, that the two *L. pneumophila* isolates have elimination time between 90 sec and 120 sec, while, *S. paucimobilis* two isolates need (75 -120) sec for total reduction and *E. meningoseptica* need only 45 sec for total destruction. The effect of sub-lethal dose of nonthermal DBD plasma on surviving bacterial cell on some virulence factors was studied for one isolate from each of *L. pneumophila* and *S. paucimobilis*. Results show that the ability of isolates for biofilm production was strongly reduced by these two isolates and the ability of tested isolates to produced protease, lipase and acid phosphatase enzymes were eliminated after exposed to sub-lethal dose of DBD plasma.

Conclusion: Nonthermal DBD plasma is effective in treatment of tested isolates for relatively short exposure time. These activity is strain depending and survival cell after plasma treatment loss it is ability to exhibit some virulence property. From these investigations, it was proven that the DBD method was an effective and alternative application to conventional disinfectant methods of water treatment.

Keywords: Cold plasma; L. pneumophila; S. paucimobilis; E. meningoseptica.

1. INTRODUCTION

Cooling water and water supply can contaminated with biofilms containing pathogen and opportunistic pathogens [1]. Among the most clinically relevant gram negative healthcare associated clinically important pathogens that identified in water samples were *Legionella* spp. and *Sphingomonas paucimobilis.*

The genus *Legionella* is primarily associated with Legionnaires' disease [2] *L. pneumophila* was first isolated by McDade *et al.* [3], and it was the only known species at that time, now there are more than 60 species and subspecies, about half of them are associated with Legionnaires' disease [4-6].

Sphingomonas paucimobilis was formally known as CDC Group IIk, biotype 1, then receiving its taxonomic place in 1977, when it named *Pseudomonas paucimobilis* [7].This bacterium placed in the genus *Sphingomonas* in 1990 [8]. *S. paucimobilis* can be widely distributed in natural environment. It has been isolated from soil, plant, water and other sources [9].

In medicine and biology plasma is known as the non-cellular fluid component of blood, while in physical sciences, ''plasma'' refers to the forth state of matter [10]. Plasma can be classified according to its temperature into: High temperature plasma and low temperature plasma (cold plasma). There are many schems for cold atmospheric plasma production, clinical applications of dielectric barrier discharge (DBD) plasma have been assessed in different areas, Joshi et al. [11] conclude that DBD plasma application can be a valuable in decontamination technique for removal of planktonic and biofilm-embedded bacteria like methicillin-sensitive *S. aureus* and *E. coli*. In the environment, the contaminated surfaces play an important role in microorganisms transmission causing health associated infections [12]. Cahill et al. [13] highlights DBD cold plasma efficient antibacterial activity on bacterially contaminated surfaces at a short period of time and confirms its potential for routine used in clinical environment biodecontamination.

The goal of this study is to evaluated *in vitro* effectiveness of DBD cold plasma on *Legionella* and *S. paucimobilis* growth and survival.

2. METHODOLOGY

2.1 Bacterial Isolates

Seven different bacterial isolates were isolated during this study, these isolates were as follow: two *L. pneumophila* isolates (Lp44, L77); Two *S. paucimobilis* isolates (S8, S148) and one *E. meningoseptica* isolate (E 11). All these isolates have the ability to form biofilm to produce acid phosphatase, protease and lipase enzymes.

2.2 Cold Plasma Effectiveness

2.2.1 DBD System

The DBD system used in this work, shown in Fig. 1 was designed and constricted locally by Dr. Hamid H. Murbat, in College of Science for women, Baghdad University, Department of Physics, its consist of two electrodes, each of

them is made of copper rod with (50 mm) diameter surrounding by Teflon for isolated. One millimeter thickness quartz sheet was used as a dielectric material between two electrodes, the two electrodes were connected into high voltage transformer that Its voltages can vary between (1-15 KV). In our experiment the applied voltage was fixed at (6 KV). The samples were putted in the lower electrodes and the distance between the samples upper electrode and the samples was fixed at (1 mm).

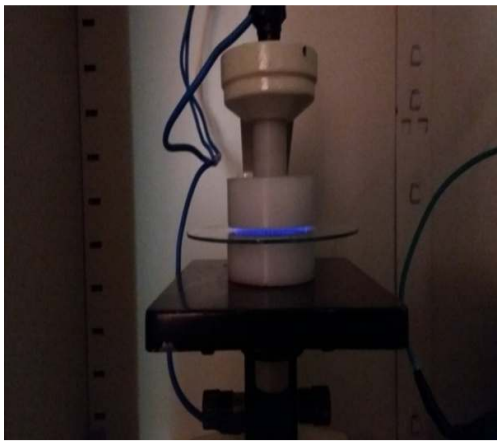

Fig. 1. Locally design DBD plasma generator used in this study

Eleven different exposure time of DBD cold plasma were applied *in vitro* on different bacterial species which were *E. meningoseptica*, *Legionella* spp. and *S. paucimobilis* with bacterial concentration range between(1×10^2-2×10^2)cfu /ml . Bacterial suspension was adjusted spectrophotometrically. This experiment performed by place 100 microliter from each isolate on sterile Petri dish, this plates were exposed to DBD cold plasma generator shown in Fig. 1. Samples were exposed to plasma for (0,15, 30,45, 60,75, 90, 105,120,180, 240and 300) seconds, the exposure time was chosen according to previous study [14]. After that warm proper medium for each species (BCYEα for *Legionella* species , Muller-Hinton agar for other species was pour on Petri-dish over bacterial suspension, mixed well and leave to solidified at room temperature, three dishes leaved without exposure that served as control, all plates were incubated at 36±1^{o}C for 48-72 hours. All operations were done at sterile condition. Results were quantified by viable count. All experiments were performed with three replicate.

The treatment effectiveness calculated by two values, the first one by the calculation of the percentage of cfu observed on agar for treated sample relative to cfu for control at each isolate where survival was observed. The second value is by estimation the percentage of reduction in standard time. This value is determined by comparing the reduction in bacterial concentration of the treated sample with that of control sample. Percentage of reduction (R) was calculated using the following formula [15]:

$$R = \frac{No - Nt}{No} \times 100$$

Where: N_o is initial number of bacteria before exposure to plasma. N_t is number of bacteria recover after exposure to plasma at desired time(t).

2.3 Effect of Sublethal Dose of Cold Plasma Exposures on Surviving Bacteria

The effect of sublethal dose on survival bacteria ability to produce acid phosphatase, protease and lipase as described in [16] and to form biofilm as described in [17] were tested for two isolates (L77and S148) as describe above.

3. RESULTS AND DISCUSSION

It was found that using of different time of DBD plasma against different bacterial species give increase reduction rate until reaching lethal time of each species, this reduction time is species dependent, from Table (1) it seen clear that two isolates of *L. pneumophila* need DBD different time to reach total destruction. That Lp44isolate was eliminated at 90sec while Lp77 isolate eliminated at 120sec, this was true about *S. paucimobilis* two isolates where S8 need 75sec while S148 need 120sec for total destruction.

Sixty seconds were needed for total eradicated of *L. longbeachae* and *E. meningoseptica* isolates, that 99.7% reduction of *E. meningoseptica* initial number occur at 45 sec which represent the lowest time of eradication. These results were come in accordance with Joshi et al. [11] who demonstrate that, DBD killing responses were dependent on cell density and plasma exposure-time. These results clearly point out that sterilization by plasmas has very striking features in terms of practicality and effectiveness [18]. Kvam et al. [19] treat three antibiotic resistant microbial strains which exhibited rapid declines in intracellular ATP levels as a function of plasma duration,

Table 1. The effect of DBD plasma treatment time on different bacterial isolates at bacterial number range between 100-200 cfu representing by Reduction percentage value (R%). Where Lp44 and Lp77: *L. pneumophila*; Ll 63: *L. longbeachae* and Lm86: *L. micdadei*; E11: *E .meningoseptica* and S148 and S8: *S. paucimobilis* isolates

Isolates	R% value at 15s	R% value at 30s	R% value at 45s	R% Value at 60s	R% Value at 75s	R% value at 90s	R% value at 105s	R% value at 120s
Lp44	29.43	65.16	81.38	94.89	98.5	100	100	100
Lp77	37.88	75.76	84.85	96.97	96.97	99.49	99.75	100
Ll 63	73.76	87.40	97.11	100	100	100	100	100
Lm86	37.54	64.43	75.91	96.92	100	100	100	100
E11	87.34	92.29	99.7	100	100	100	100	100
S 8	37.66	62.12	71.64	92.64	100	100	100	100
S 148	80.99	95.6	96.42	97.8	98.07	99.17	99.73	100
Chi-Square (χ^2)	10.39 **	8.502 **	8.761 **	4.0255 *	1.927 NS	0.038 NS	0.036 NS	0.00 NS

*** (P<0.01), (P<0.05), NS: Non-significant*

microscopically, observed that plasma rapidly inactivates bacterial cells and permeability of cell surface occur within few second after treatment, followed by loss of membrane integrity and leakage of intracellular components like ATP, protein and nucleic acid.

Statistic significant different was seen among reduction percentage by DBD plasma of different isolates at the same exposure time which sugust that bacterial killing by cold plasma is strain dipending process.

At time of writing this paper, there is no available local reported work concern cold plasma effectiveness on *Legionella* species, *E. meningoseptica* and *S. paucimobilis.* Other researcher work with another gram negative bacteria indicate cold plasma effectiveness like Laroussi [20] who reported cytoplasm leakage before total cell fragmentation of *E. coli* planktonic cells exposure to plasma discharges exceed 30 seconds. Park et al. [21] indicate bacterial distraction after 20 seconds of exposure to argon plasma discharge and Purevdorj et al. [22] observed similar results for *Bacillus pumilus* spore-forming bacteria. In another study found that cell walls of treated cells are damaged forming spheroplasts which are smaller in size and these spheroplasts are still alive but it non-culturable since they can retain an intact cell membrane [18]. Also Ziuzina et al. [23] observed that treatment of *Salmonella*, *L. monocytogenes* and *E. coli* by atmospheric cold plasma (ACP) for 30s reduced planktonic bacterial cells

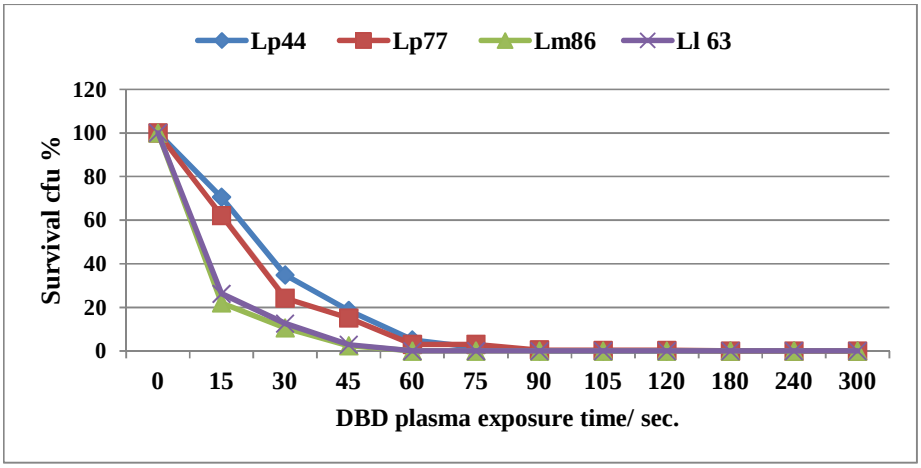

Fig. 2. Percentage of survival bacterial number relative to time of DBD exposure of four *Legionella* isolates, where Lp44 and Lp77: *L. pneumophila*; Ll 63: *L. longbeachae* and Lm86: *L. micdadei*

suspended in broth to undetectable levels, while biofilm attach bacteria need 300 seconds to reduced bacterial number to a maximum of 5 log_{10} CFU/sample depending on conditions and bacterial type. While Yost and Joshi [24] suggested that plasma-treated solution contains superoxide and hydrogen peroxide like reactive species and their products which cause oxidative changes in cell components, and lead cell death.

In Iraq, Humud et al. [25] found that, Gram-negative species used in this experiment more susceptible to cold plasma needle treatment than Gram-positive bacteria. Hamad and Mahmood [26] used *E. coli* and *S. aureus* as a model system to optimize the conditions for bacterial treatment, they found that protraction of treatment time promote destruction effectiveness.

Khalaf et al. [27] treat *E. coli* and *Staphylococcus* spp bacteria for a period of time as sterilization process. They demonstrated that these two species were affected by operation conditions (increasing or decreasing) according to the produced plasma properties. Hussain et al. [28] indicated that when non-thermal argon needle plasma used against two species of Gram-positive bacteria, plasma inactivated all bacterial population during 40 seconds. To our knowledge this is the first local study of the effect of cold plasma upon *Legionella* species, *Sphingomonas paucimobilis* and *Elizabethkingia meningoseptica*.

3.1 Effect of Sublethal Dose of Dbd on Survival Bacteria

Whilst the antibacterial action of non-thermal plasma is well characterized, relatively little attention has been paid to investigating the consequences of short plasma exposures on surviving microorganisms and their biomolecules [29]. The effect of sub-lethal dose of nonthermal DBD plasma on surviving bacterial cell was studied by test its ability to form biofilm on cover slid and glass tube and production of enzymes (acid phosphatase, protease and lipase). These tests were perform for two isolates Lp77 and S148 depending on DBD effectiveness results.

3.1.1 Biofilm formation assay

3.1.1.1 Coverslipe method

Results show that the ability of tested isolates for biofilm production was strongly reduced by these two isolates (Fig. 4) after exposure to sublethal dose of DB Dplasma.

3.1.1.2 Glass tube method

Result show strongly reduction in isolates ability to form biofilm in glass tube, that was confirm by the former result of coverslipe method (Table 2).

3.1.2 Enzyme production

Results show that the ability of tested isolates to produced protease, lipase and acid phosphatase was eliminated after exposed to sub-lethal dose of DBD plasma (Fig. 5).

Somolinos et al. [30] observed that sub-lethal injury cells are higher sensitive to stress conditions after treatment and my lose viability during subsequent storage under unfavorable conditions and they found that the occurrence of sub-lethal injury in plasma for treated bacteria is depending on treatment conditions, they also

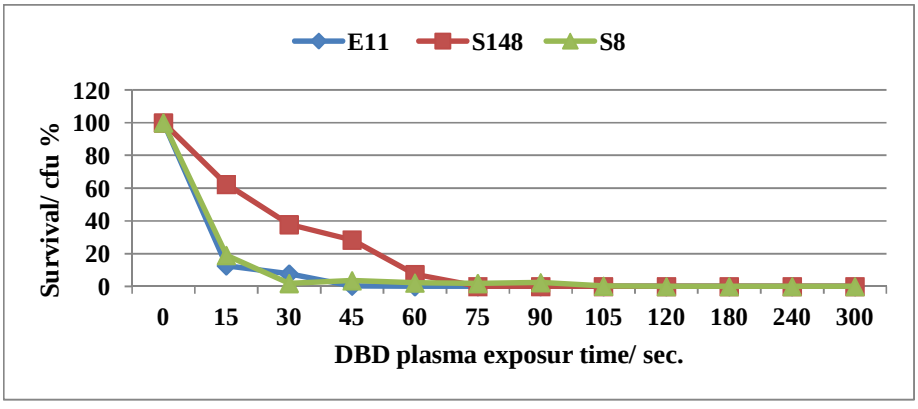

Fig. 3. Percentage of survival bacterial number relative to DBD exposure time of *E .meningoseptica* (E11) and *S. paucimobilis* (S148, S8) isolates

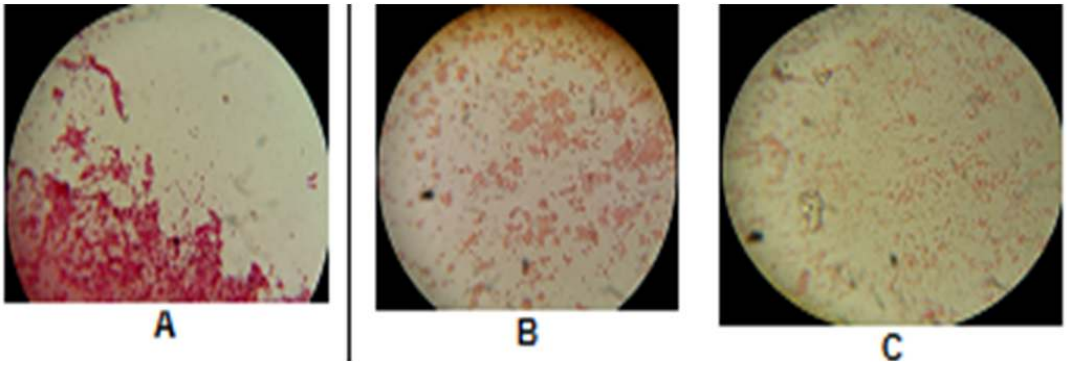

Fig. 4. Biofilme formation after exposure to sublethal dose of DBD cold plasma (×1000); A: control; B: S148 isolates of *S. paucimobilis*; C: Lp77 isolate of *L. pneumophila*

Table 2. Detection of biofilm production by two method for *L.pneumophila* Lp77 isolate and *S. paucimobilis* S148 isolates after exposed to sub-lethal dose of DBD cold plasma

Bacterial Isolates code	Biofilm Production by	
	Coverslips method	Tube method Based on intensity of color by OD measured at 570 nm ± SD
Control	+	0.251
Lp77	-	0.224 ± 0.0552
S148	-	0.191± 0.0125

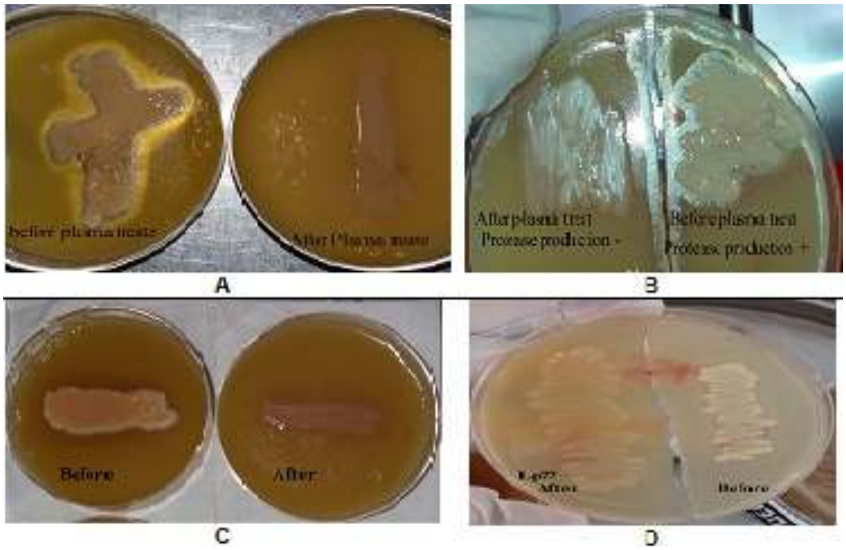

Fig. 5. Enzyme production before and after treating with sublethal dose of DBD plasma by *S. paucimobilis* S148 isolates A: Lipase production on Egg yolk agar; B: Protease production on Milk agar and for *L. pneumophila* Lp77; C: Lipase production on modified Egg yolk agar; D: Protease production on modified Milk agar. all plat incubate at 36±1℃ for 24-72 hours

demonstrated that combined treatment is essential to completely eliminate microorganisms. During this process conditions must be fit to avoid repair of cell damage by prevent synthesis of necessary components and energy, revealing that sub-lethally injured cells are capable of repairing its damage.

Garcia et al. [31] observed that the present of *E. coli* sub-lethal injury cell maybe due to the ability of these cells to repair cell injury, also Garcia et al. [32] indicated that reduce the time of treatment low electric field strengths don't produce extensive sub-lethal injury cell, whereas, Vaze et al. [33] found significant changes in cell

membrane and indicates that bacterial cells exposed to lethal dose completely inactivated while sublethal dose treated cells were re-grow. These researcher conclude that bacterial inactivation is not an-all or nothing- event case, and it may depend on the microorganism type and the treatment conditions [31,33,30]. Also Brelles-Marino [34] noted by scanning electron microscopic examination that these survival, treated-cells (injured cells), were also changed at the cellular level, therefore viability tests is needed before drawing conclusions, this may explain present study results, that bacterial ability to produce enzyme was effected by this treatment.

Flynn et al. [29] observed that nonthermal plasma treated *Agrobacterium tumefaciens* bacteria result in complete loss of pigment production gradually along with increase plasma exposure time. Pigment production is regulated by Quorum sensing (Qs) QS regulates many important cell functions in both Gram-negative and Gram-positive bacteria, including protein synthesis, metabolism, expression of virulence factors, biofilm maintenance and dispersal, biofilm formation, antibiotic resistance in addition to entry to stationary phase. Therefore, QS is a greatly attractive target for seek for new alternative antimicrobial [35]. Also Flynn et al. [29] found that non-thermal plasma can be used to attenuate bacterial Qs and interfere with Quorum sensing dependent virulence factor production in Gram-negative bacteria. This finding indicate the present of sub-lethal injured cell which exposed to low dose of cold plasma and lose their virulence factor gradually until they die, that was close to present study finding.

4. CONCLUSION

Nonthermal DBD plasma is effective on tested isolates treatment at relatively short exposure time. These activity is strain depending and survival bacterial cell after plasma treatment loss it is ability to exhibit some virulence property such as acidphosphatase, lipase and protease production in addition to loss its ability to form biofilm.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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