ИНФЕКЦИОННЫЕ БОЛЕЗНИ И МИКРОБИОЛОГИЯ

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O. Rybal'chenko, O. Stepanova, O. Orlova,
A. Astafiev, V. Kapustina, E. Pariyskaya,
L. Zacharova, O. Vishnevskaya, A. Kudryavtsev

DESTRUCTIVE EFFECT OF ATMOSPHERIC PRESSURE PULSED CORONA DISCHARGE PLASMA IN AIR ON BIOFILMS OF OPPORTUNISTIC BACTERIA

St. Petersburg State University, 7–9, Universitetskaya nab., St. Petersburg, 199034, Russian Federation

Pulsed corona discharge (PCD) of moderate frequency in air has a bactericidal and bacteriostatic effect on *Escherichia coli* M17 cultures at both cellular and population levels. PCD exposure inhibits forming a microbial community and results in the destruction of formed biofilms. The paper presents data of electron microscopy investigations of cells' and biofilms' ultrastructure after PCD treatment over a 90 second period. The morphological properties of opportunistic bacteria *E. coli* M17 cells altered after sub-lethal and lethal thermal exposure. Refs 32. Figs 6.

Keywords: pulsed corona discharge, atmospheric pressure discharge source, non-thermal air plasma, opportunistic bacteria, *Escherichia coli*, bacterial biofilm, cell destructive mechanisms.

ДЕСТРУКТИВНОЕ ДЕЙСТВИЕ ИМПУЛЬСНОГО КОРОННОГО РАЗРЯДА В ВОЗДУХЕ НА БИОПЛЕНКИ УСЛОВНО-ПАТОГЕННЫХ БАКТЕРИЙ

О. В. Рыбальченко, О. М. Степанова, О. Г. Орлова, А. М. Астафьев, В. В. Капустина,
Е. Н. Парийская, Л. Б. Захарова, О. Н. Вишневская, А. А. Кудрявцев

Санкт-Петербургский государственный университет,
Российская Федерация, 199034, Санкт-Петербург, Университетская наб., 7–9

Импульсный коронный разряд (ИКР) средней частоты в воздухе способен оказывать бактерицидное и бактериостатическое действие на культуру *Escherichia coli* M17 как на клеточном, так и на популяционном уровне. Действие ИКР препятствует формированию микробного сообщества и приводит к деструкции уже образованных биопленок. В работе представлены данные электронно-микроскопических исследований ультраструктуры клеток и биопленок после их обработки ИКР в течение 90 секунд. Изменения морфологических свойств клеток *E. coli* M17, вызванные обработкой ИКР, подобны изменениям после сублетального и летального теплового воздействия. Библиогр. 32 назв. Ил. 6.

Ключевые слова: импульсный коронный разряд, низкотемпературная воздушная плазма, условно-патогенные бактерии, *Escherichia coli*, бактериальная биопленка, механизмы деструкции клеток.

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1. Introduction

Applications of gas and liquid electric discharge in biomedicine have been attracting the careful attention of many researchers. In particular, contemporary electric discharge technologies for water bactericidal processing [1, 2], medical instrument sterilization [3] and surgical procedure [4, 5] already exist. The potential to generate "cold" non-equilibrium plasma of atmospheric pressure gas discharge are being intensively studied [6–8]. The latter is an urgent issue in relation to the disinfection of living and temperature sensitive materials [9, 10].

The applications mentioned above for the most part are based on generation of pulsed electric discharges with the frequencies from several tens of Hz to units of MHz. Their low-current stage is a corona. It is the source of a non-equilibrium plasma with a gas temperature less than 400 K [8, 11]. Due to low discharge currents and in the case of low energy density deposited into a gas volume, a pulsed corona discharge can be considered as a potential tool for plasma antimicrobial treatment of human skin and materials sensitive to heat and electric loads. However, a corona discharge is unstable and it can instantaneously transition to a high-current spark/arc. Hence, the durations of supply pulses must be shorter than the time necessary for the streamer breakdown of the whole discharge gap.

Despite this drawback, many trials have been devoted to sterilization of liquids and solid surfaces using corona discharge. Mechanisms of bactericidal effect can be greatly different versus the properties of a treated medium and the supply parameters of an electric discharge setup. Main agents causing inactivation of microorganisms at the electric discharge in gas or water are an electric current [12, 13], chemical reactive species including bactericides appearing as a result of the electric current passing through a medium containing oxygen and/or nitrogen [14] and ultraviolet radiation accompanying excitation and ionization acts [15].

The way in which a bacterial cell dies as current passes through is defined by the type of current (DC/AC/pulsed), its magnitude and impact duration. The authors [16] have given a comparative analysis of the antimicrobial potential of different types of current. They have noticed that the mechanisms of microorganism inactivation with the electric current are still not completely understood and often seem to be controversial. Nevertheless, some distinguishing events can be identified: 1) damage of the cell membrane and blocking of cell division due to a direct impact of the electric current, and 2) electric current passing through the medium causes an increase in the temperature of the treated substance and electrolysis which, in turn, results in the production of chemical reactive species. It is obviously that all of the mentioned physical and chemical processes should be expected under the treatment of a substance with corona discharge.

Therefore, pulsed corona discharge (PCD) initiated close to a surface invaded by microorganisms in a O_2-containing medium (air) can be expected to lead to its disinfection. The effect of PCD on a bacterial community called a biofilm is of particular interest. Biofilm is a principal medium that allows microorganisms to survive under the natural conditions of a human organism and in an ambient environment [17].

Biofilms strongly protect microorganisms from negative factors in the surroundings. Bacterial communities are known to be resistant to antibiotics 100–1000 times stronger than a therapeutic dose for inhibiting vital functions of planktonic cells [18].

Biofilms might consist of a single type of microorganism or several ones. They can be formed within different cavities of their macroorganism host, including mucous membranes of gastrointestinal, respiratory and urogenital tracts, and also on skin surfaces [19]. Bacteria being causative agents of acute and chronic infections form biofilms into tissues of an affected macroorganism. Surface film, a membrane-like structure, separates a biofilm from the ambient environment. The main component of a surface film is a three-layered membrane, which has an ultrathin structure like the universal plasma membrane [20].

Besides a surface film, all of the cells in a bacteria community are surrounded by a glycan intercellular matrix. It probably gives bacterial cells an additional protection from antimicrobial germicides. This in turn can promote the chronization of an infection and torpid development of a disease, which cannot be affected by the baseline therapy, natural immunity and etiotropic medicines [20].

Therefore, nowadays the development of new ways to destroy biofilms including antimicrobial and probiotic drugs, bacteriocins and other physicochemical impacts is one of the most important medical trends [21, 22]. Pulsed corona discharge is proposed to be able to have a bactericidal effect on a microbial biofilm and destroy cells inside it and its additional protective layers. Thus, the current article is devoted to the investigation of the PCD effect in air on opportunistic bacteria *E. coli* M17 biofilms.

2. Materials and Methods

2.1. Microorganism culturing

The biofilms of gram-negative bacteria *E. coli* M17 (separated from probiotic colibacterine strain of *E. coli* M17) were chosen as study objects. To obtain the biofilms, 24 hour broth cultures (nutrient broth with 1 % of peptone M244, HiMedia, India) of 10^8 CFU/ml were inoculated into a culture medium (nutrient agar with 1 % of peptone MO12, HiMedia, India) with a tampon. A bacterial lawn was grown during 24 hours on Petri dishes at the temperature of 37 °C.

2.2. Pulsed corona discharge set-up

To generate a PCD in air, an original high-voltage pulsed power source was developed and fabricated. It runs according to a single-step scheme and includes a high-voltage pulsed changer (HVPC) and a high-voltage pulse transformer (HVPT) (Fig. 1).

The high-voltage pulsed power source generates a signal of AC voltage with the amplitude of 30–60 kV and the pulse frequency of 250–400 kHz. The output voltage pulse is applied to a high-voltage electrode which is a multifilament wire with the total cross sectional area of 2.5 mm^2. The diameter of the discharge region is about 4–6 mm. The experimental setup is shown in Fig. 1.

Discharge current was detected via the measurements of voltage drop across a resistor of 30 ohms. Fig. 2 presents signal forms versus the distance between the high-voltage electrode and the agar surface *d*. The agar layer was 3 mm high. The amplitude of discharge current signal falls down at the decreasing of *d*. Fig. 2a–b illustrate a signal waveform at the regime of corona affecting the agar surface at the distance *d* of 16 and 4 mm. Peak-to-peak amplitude of the current discharge is 0.5 and 0.27 A, correspondingly. And current pulse frequency is slightly decreasing from 250 to 208 kHz. Decrease in discharge

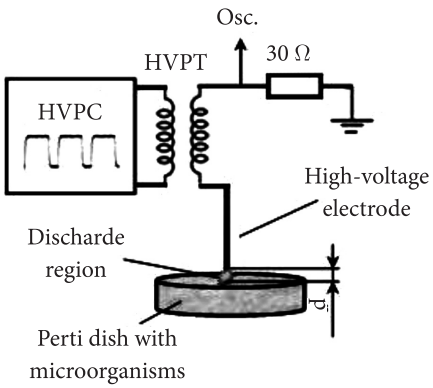

Figure 1. Schematic of the experiment

current at shortening of the distance between the electrode and agar surface can be explained as a consequence of increase in resistance of the discharge gap, which is equivalent to a reactive load.

The oscilloscope pattern in figure 2b corresponds to a pre-breakdown point that is followed by a sudden corona to arc transition at $d = 2$ mm (Fig. 2c). This leads to the changing of the current signal waveform. Small current spikes highlighted by circles which are typical for streamer coronas almost disappear. The pulses become infrequent, and their frequency decreases to ~170 kHz; the maximal peak-to-peak amplitude remains at a level of 0.25 A.

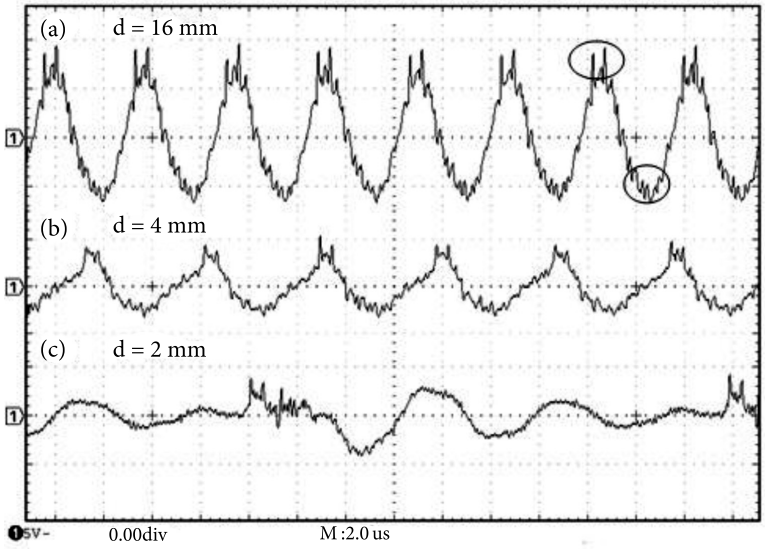

Figure 2. Oscillogram of the discharge current for corona (a), (b) and arc (c) at various distance *d* between the high voltage electrode and agar surface (a vertical division corresponds to 5 V; a horizontal division — 2 μs; the curves show 30 ohm resistance drop; current spikes due to streamer-type discharge are marked by circles)

The measured currents are, in fact, in a secondary winding of the transformer and despite their relatively high values the density of current deposited on the treated surface is rather low, as part of it is lost by air coronas without reaching the object. We cannot tell anything about space distribution of current density lost by air. This point requires additional investigations. The microorganisms have been treated at PCD regime presented in Fig. 2b. The corona-arc transition was controlled.

2.3. Transmission electron microscopy (TEM)

Microbiologic specimens prepared with the ultrathin section method (see section 2.4) and the method of positive staining (see section 2.5) were observed by the transmission electron microscope JEM 100C (JEOL, Japan) at the accelerating voltage of 80 kV.

2.4. Ultrathin section method

Ultrathin section method was used to analyze an internal structure of bacterial biofilms with a transmission electron microscope. The specimens were selected from the border areas of inhibition of *E. coli* M17 cells including the areas of visually undetected microorganism growth. The feature of the specimen preparation provides the maintenance of an original structure of the investigated objects and their spatial distribution. Areas of bacterial growth were cut out with agar, preliminary fixed in 2.5 % solution glutaraldehyde in a Hanks buffer (pH 7.2) at 4 °C. It should be noted that the fixing solution was placed under agar plate. This method of fixation was used to preserve the integrity of the surface structures of microbial communities. After 24 h 0.5 % aqueous agarose solution (30°C) was placed on the surface to maintain biofilm structure. Securing agarose's biofilms were washed with distilled water, and then fixed in 1.0 % OsO_4 aqueous solution for a day at 4°C. For dehydration samples were entirely incubated in solutions of increasing alcohol concentration in a standard procedure and embedded in resin. For clear location and orientation of sample analysis, a Pyramytome LKB-11800 was used (LKB, Sweden), which allowed correct focusing and an estimation of region location for further analysis. From regions of bacterial growth, ultrathin sections were obtained. Subsequently, preparations were analyzed employing a transmission electron microscope JEM-100C (JEOL, Japan).

3. Results and Discussion

PCD effect was observed for both fresh-inoculated lawns of *E. coli* M17 and its 24 hour biofilms. So, in the case of fresh-inoculated lawns we investigated the influence of corona discharge on bacterial growth, and the inhibition of bacteria treated between 90 and 180 seconds was measured. And, in the case of biofilms, changes in morphological properties of the formed biofilm after PCD-treatment over 90 s were analyzed. The distance between an agar surface and the high-voltage electrode was fixed at 4 mm for all experiments.

3.1. Evaluating inhibition of bacteria in biofilms

Fresh-inoculated cultures of *E. coli* M17 after PCD-treatment were cultured over 24 h at the temperature of 37 °C under thermostating conditions. Photos of Petri dishes with a 24 hour biofilm of *E. coli* M17 are presented in Fig. 3. Zones of inhibition formed as a result

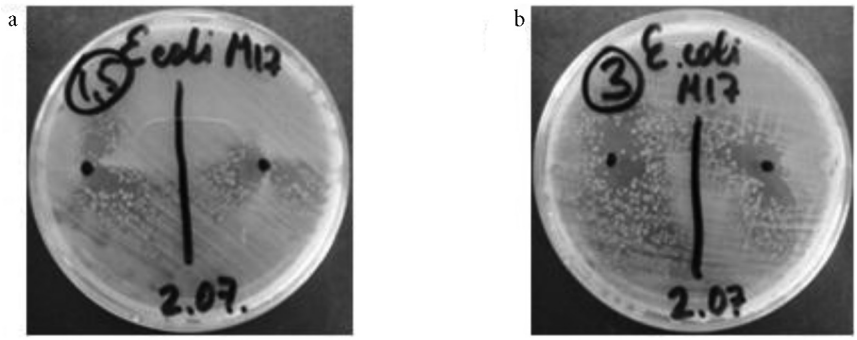

Figure 3. Petri dishes with a 24-hour biofilms and colonies of microorganisms cultured after PCD treatment during 90 (a) and 180 (b) sec.

of PCD-effect on growing cells can be seen in the centers. Their sizes are 10 and 13 mm across diameter at the treatment duration of 90 and 180 s, correspondingly (Fig. 3a–b). Zones of total cell inactivation were at first surrounded by individual colonies of bacteria.

3.2. Electron microscopy investigations of morphological properties of cells and biofilms

Figure 4 shows cells of the biofilm ultrathin sections without PCD-treatment (Fig. 4a) and after corona discharge exposure (Fig. 4b). As a result of cytoplasm destruction typical global inclusions appear in treated cells (shown by an arrow).

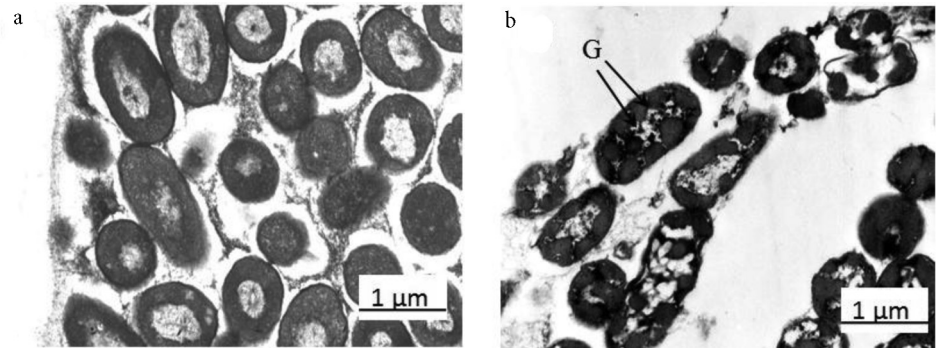

Figure 4. TEM images of ultrathin sections of *E. coli* M17 cells in biofilms: a — control; b — after PCD-treatment during 90 s. Scale bar –1 μm

The structure of *E. coli* M17 biofilms was investigated by TEM. In contrast to the control specimen (Fig. 4a), a treated one has areas with completely destroyed cells which are replaced by hollows (H) and areas of cell fusion into a single symplast (S) (Fig. 5).

Electron microscopy images obtained at higher magnification allow us to estimate the integrity of a surface film (SF) — one of the biofilm protective structures. Some areas of biofilm under PCD-treatment have almost undamaged structure, but a three-layered membrane entering into the composition of a surface film (SF) remains not visible (Fig. 6). Figure 6 shows a destroyed area of the surface film (SF), which has the broken structure.

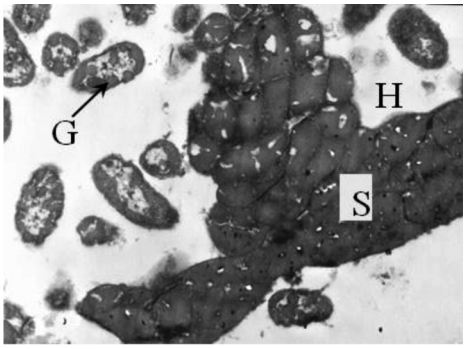

Figure 5. TEM images of ultrathin sections of *E. coli* M17 biofilm with hollows (H) and cell symplasts (S) after PCD treatment. Scale bar –1 μm

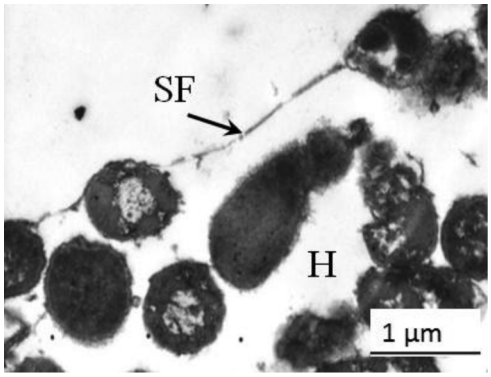

Figure 6. Surface film of biofilm (SF) after PCD treatment: undestroyed area and destroyed cells. Scale bar –1 μm

Most of the cells inside biofilms after PCD treatment demonstrate destructive changes. A nucleoid has disappeared from cytoplasm, DNA strands cannot be seen (Fig. 5). Such cytoplasm has become homogeneous and electron-dense, and thus nonfunctional. One part of the destroyed cells formed a symplast, a structure from several cells coupled together. An intercellular matrix inside the biofilm invisible. Another section of the destroyed cells did not form a symplast, but they contain globular inclusions (G) in their cytoplasm (Fig. 4b, 5).

Electron microscopy investigations have shown the significant morpho-functional changes of *E. coli* M17 cells of both cells and biofilms. The destroyed cell components of bacteria and particular rarefied cytoplasm is accompanied by seemingly globular inclusions (G) (Fig. 4b).

Since such globular inclusions are typical for sub-lethal and lethal thermal effects, the destructive mechanisms are probably based on ohmic heating of biological objects as a result of discharge current passing through them. Ohmic heating causes the destruction of thermal sensitive components of biofilms and cells.

As for the role of chemical airborne reactive particles, they can be rather high at much longer exposure times than the duration of treatment carried out in the presented work [27]. Moreover, to get a high efficacy of antimicrobial effect of a corona in air the additional sources of potential reactive species (e.g. water vapours, oxygen, hydrogen peroxide etc.) should be put into the discharge space [27, 28]. But this work did not suppose adding any other substances into the air discharge volume.

And the third probable mechanism for the inactivation of microorganisms is ultraviolet (UV) disinfection, which is less likely to play a role. In fact, corona discharge is a source of electromagnetic radiation, mainly in the ultraviolet region [29, 30]. And at low pressure UV radiation in the wavelength range from 160 to 220 nm plays the main role in the sterilization of surfaces [31] However, in the case of atmospheric pressure plasma source such as the developed gas discharge device UV contribution into inactivation of bacteria is negligible [32]. This can be evidently explained by intensive absorbing short-wave length radiation by the ambient air.

The influence of the PCD demonstrates the dominant role of current discharge in bactericidal effect on *E. coli* M17 biofilms which is revealed by the electron microscopy investigations of microbial cells and biofilms morphology.

4. Conclusion

The effect of pulsed corona discharge of moderate frequency on bacterial viability in *E. coli* M17 biofilms has been examined. To generate PCD in the ambient air, an original gas discharge device was developed. It is supplied by pulsed high-voltage power source with a voltage amplitude of 30–60 kV and pulse frequency of 250–400 kHz. The bactericidal effect of PCD on *E. coli* M17 cells as a part of the microbial community has been revealed.

The electron microscopy analysis of *E. coli* M17 cells and biofilms after PCD treatment has detected several destructive mechanisms. At the level of the population, the ratio of different morphological types of cells has been changed. It is detected as an increase in the numbers of destroyed cells and fully lysed bacteria. The destructive changes in cells are appeared with (i) the formation of focal destructions into cytoplasmic membrane, (ii) the spread of periplasmic space, (iii) the rarefied of cytoplasm, (iv) the formation of globular inclusions.

The observed biological effects in biofilms treated by PCD suggest that the dominant role in microorganism inactivation is played by the current discharge passing through the medium and resulting in its thermal damage.

The presented experimental data on changing ultrastructural organization of *E. coli* M17 biofilms after PCD treatment can help to reveal important details of bactericidal and bacteriostatic mechanisms and to compare impacts of other physicochemical factors of the external environment.

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Authors information:

Rybal'chenko Oksana V. — PhD, Professor; ovr@inbox.ru
Stepanova Olga M. — PhD; o.m.stepanova@spbu.ru
Orlova Olga G. — PhD, Senior Lecturer; oorlova18@mail.ru
Astafiev Alexander M. — astafev-aleksandr@yandex.ru
Kapustina Valentina V. — Post graduate student; kapustina.valeriya@list.ru
Pariyskaya Elena N. — PhD, Associate Professor; lenap9159@mail.ru
Zacharova Lidia B. — PhD, Associate Professor; zakharova.l@inbox.ru
Vishnevskaya Olga N. — Post graduate student; olga-vishn@yandex.ru
Kudryavtsev Anatoly A. — PhD, Associate Professor; akud@ak2138.spb.edu