Antibacterial impact of nonthermal atmospheric plasma on catheter-based biofilms of *Staphylococcus epidermidis* and *Klebsiella pneumoniae* isolated from small ruminants *in vivo*

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Background
Nonthermal atmospheric pressure sterilization is one of the suggested and efficient techniques to hinder the spread of illnesses. Reactive species such as oxygen, hydroxyl, and other radicals play a prime role in the mechanism of plasma sterilization. *Staphylococcus epidermidis* is the most prevalent cause of primary bacteremia and infections of indwelling medical instruments. The ability to induce disease is related to its natural niche on the skin and capability to adhere and form a biofilm on foreign surfaces. *Klebsiella pneumoniae* is a zoonotic pathogen frequently isolated from infections related to the presence of bacterial biofilm on devices, such as catheters, which are responsible for loss of patients' health.
Objective
S. epidermidis and *K. pneumoniae* are being combated due to their high frequency of occurrence and ability to form biofilms as survival and virulence characteristics. These particular benefits impose a significant financial burden on hospitals.
Materials and methods
In this study, the nonthermal plasma treatment induced by surface dielectric-barrier discharge was used to destruct the developed biofilm formed by clinical *S. epidermidis* and *K. pneumoniae* isolated from clinical cases of small ruminants. The biofilms were induced *in vivo* by catheter-based rat model preparation. The biofilms were examined before and after the treatment using a scanning electron microscope.
Results and conclusion
The produced nonthermal plasma degenerated and reduced the number of adherent and aggregated viable bacteria.

Keywords:
biofilm, goat, plasma, surface dielectric-barrier discharge, sheep

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Introduction

Nowadays, numerous devices are commonly used in the diagnosis and treatment of patients. They are, to a great extent, constructed from materials that are intended for contact with the live tissue of the patient, and hence are termed to 'biomaterials.' However, the implantation of the biomaterial by surgical intervention may share in the initiation of the process of microbial adherence to the surface. Relying on the time of formation, species merits and environmental circumstances, the size of such biofilm may be from a few microns to several millimeters in thickness. Portions of mature biofilm may separate from the surface and migrate through blood vessels to distant body places, and resulting in the formation of new biofilm layers in other locations causing generalized infections [1,2]. The most prevalent reasons of nosocomial infections are biofilms formed on urological and vascular catheters, various kinds of artificial heart valves, peritoneal dialysis catheters, as well as artificial ventilation equipment and infusion pumps.

Staphylococci are common bacterial colonizers of the skin and mucous membranes of humans and other mammals. *Staphylococcus epidermidis* stands first among the causative agents of nosocomial infections, representing the most prevalent cause of infections on medical devices [3].

Klebsiella pneumoniae is one of the zoonotic pathogens that exhibit a very high ability to form biofilms, which frequently occupy the form of mucoid, cohesive slime.

The ability of *K. pneumoniae* to change the pH of urine (by means of urease) is of high significance in the pathogenesis of urinary tract infections (UTIs) [4,5].

UTIs are one of the most prevalent infections in humans and certain animals as pets and as much as 80% of hospital urinary infections are accompanied with the administration of a urinary catheter. According to statistical data, 30% of bacterial UTIs cases lead to generalized inflammatory process and/or to urosepsis [6]. Before the pivotal role of biofilm in UTI pathogenesis was known, the acquisition and spread of resistance mechanisms among urinary pathogens were looked as the greatest challenges in UTI therapy.

Klebsiella spp. are characterized by exceptional quickness in acquiring the plasmid-encoded enzymes with inactivating β-lactams, extended spectrum β-lactamases, metallo-β-lactamases, and carbapenem-resistant. Moreover, *K. pneumoniae* is implemented in 6–17% of opportunistic UTIs, which is primarily related to the formation of biofilms inside the urinary catheter or at the catheter insertion parts [7].

Therefore, prevention and sterilization techniques become urgent requirements to evade the spread of the illnesses; one of the efficient sterilization methods is nonthermal atmospheric pressure plasma sterilization [8,9]. Several techniques have been used to overcome the limitation to induce nonthermal plasma at atmospheric pressure; one of them is using dielectric-barrier discharges [10], pulsed power source [11], microhollow cathode system [12], as well as a segmented cathode [13]. Moreover, some researchers used gliding arc discharge and some others use several kinds of plasma jets [14]. It is believed that a corporation of the various techniques can be used to achieve the requirement for inducing nonthermal atmospheric plasma (APP) with possessing high rate of reactive species generation on a large scale. The generation of nonthermal APP conquers many applications in various fields including sterilization [15], medical therapy [16], and other biomedical applications [17].

Recently, nonthermal APP technology has emerged to overcome the barriers of thermal treatments [18,19]. On the other side, nonthermal plasma technology has not only been used to decontaminate and extend the shelf life of food products, but it is also utilized to improve the bioactive components of food products [20]. The reactive radicals produced during nonthermal plasma treatment may share in the elevation of nutritious and bioactive compounds [21].

In this study, surface dielectric barrier discharges (SDBD) were used to prohibit the growth of *S. epidermidis and K. pneumoniae* biofilm. The degenerative effects of SDBD plasma on bacteria biofilms were examined by an scanning electron microscope (SEM).

Material and methods

Construction of the plasma system

The electric circuit for SDBD (Fig. 1a) was set in a Teflon box (Fig. 1b), and the plasma treatment was performed in a honeycomb configuration (Fig. 1c) as previously established [22]. The creation of plasma criteria was performed according to the described physical circumstances [23–25].

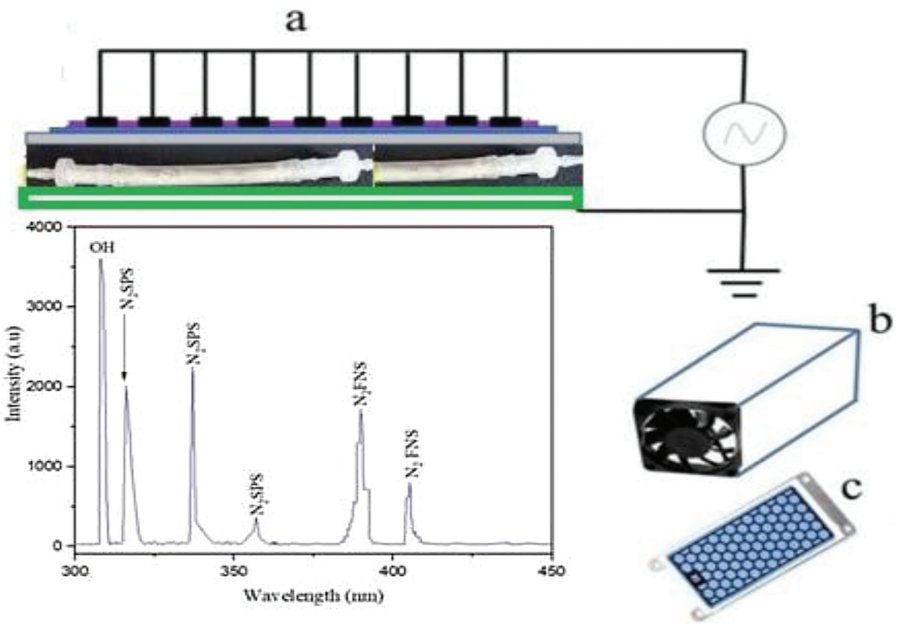
Ethical approval

All experimental procedures were carried out according to the following protocol approved by the Medical Research Ethics Committee, National Research Centre, number (1179102021).

Biofilm formation *in vivo* by catheter-based model preparation from *S. epidermidis* and *K. pneumoniae* biofilm.

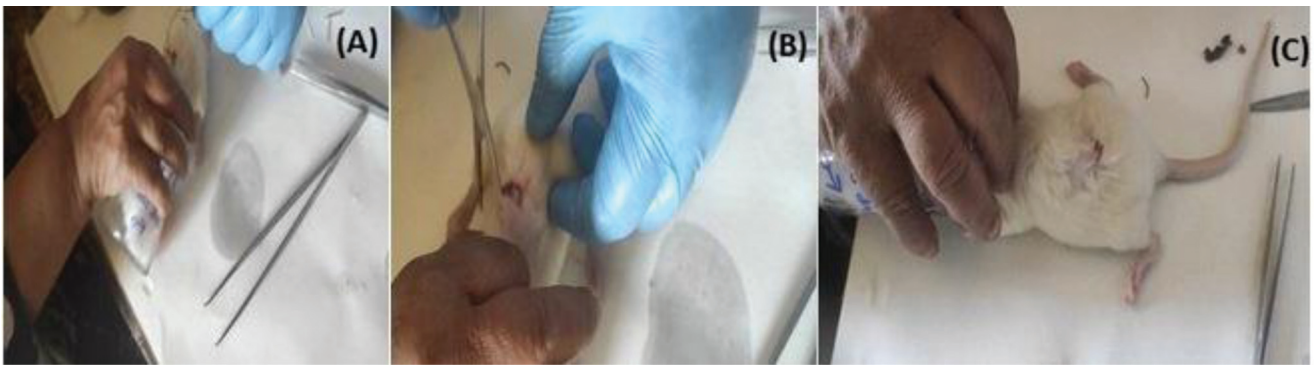
BALB/c female rats weighing average 125 g were anesthetized by injecting 0.4–0.75 mg of tribromoethanol/g of body weight intraperitoneally. The dorsal flanks of each rat were shaved and cleaned with betadine and alcohol. Fourteen-gage Teflon intravenous catheter was prepared, precut into 1 cm segments and sterilized by autoclaving. The biofilm-producing *S. epidermidis* and *K. pneumonia* isolates that were previously obtained from clinically diseased sheep and goat cases [26]. The isolates were refreshed in brain heart infusion broth, and adjusted 10^8 CFU was transferred to conical tubes (Falcon; Corning, Dokki, Giza, Egypt) containing 2 ml of Tryptic Soy Broth growth medium with 2% glucose. The tubes were incubated under constant stirring at 100 rpm/37°C for 24 h to permit bacterial growth. The flank of each tested rat was antiseptic with povidone–iodine pads, and a small s/c incision was made just above the hind leg using a sterile blade. A blunt probe is inserted to create an s/c pocket for applying the catheter as shown in Fig. 2. Note that catheters must be advanced to a sufficient distance from the primary incision site to avoid catheter extrusion from the rat over time. The sterile catheter

Figure 1



SDBD (a) electric circuit, (b) Teflon box, (c) honeycomb configuration [22]. SDBD, surface dielectric barrier discharge.

Figure 2



Insertion of catheter pieces in model rats (a, b, and c).

was inserted using forceps. After that, 5 µl suspension of 2×10^7 CFU of *S. epidermidis* and *K. pneumonia* in 20 µl of sterile PBS was injected through the skin into the catheter lumen [27]. After 10 days the catheter segments were removed and immersed in a 2.5% glutaraldehyde solution, fixed in an increasing alcohol series (15, 30, 50, 70, 90, and 100%) for 15 min each, dried in a vacuum centrifuge for 5 min, sputtered with gold and visualized under a SEM to examine biofilm formation [28].

Endpoint determination of bacterial colony counts

The catheter section was placed in 1 ml sterile saline; the sample was sonicated for 10 min and then placed in a suitable volume of saline and homogenized. Serial dilutions (1:10) of the catheter fluid were inoculated on triple sugar agar plates and incubated for 24 h at 30°C and bacterial colony counts were enumerated.

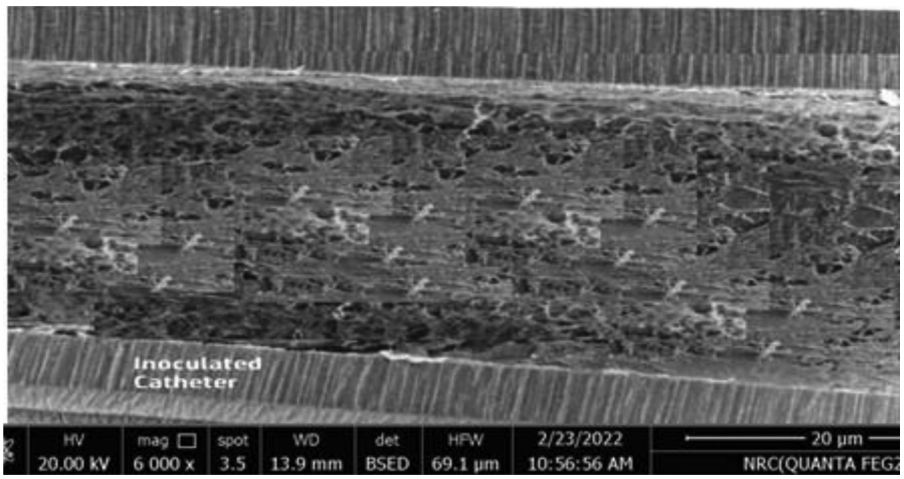
Results and discussion

The administration of an SDBD device as an antibacterial and safety agent was applied using human dermal specimens. The performance of biofilm prohibition using such plasma device was demonstrated *in vivo* [29]. Bacterial biofilms were formed, then catheters were removed from tested rats 10 days following bacterial inoculation, and processed for SEM analysis. These biofilms were composed from dense and uniformed cell masses. *S. epidermidis* cells were organized around a polymer matrix composed of exopolysaccharides, proteins,

and even nucleic acids. The smooth surface at the periphery of the figure represented the catheter with a biofilm visible on the internal face as shown in Fig. 3. Biofilm-forming cells and presence of the mucoid structure indicated the secretion of an exopolysaccharide matrix, characteristic to the biofilm of *S. epidermidis* grown on catheter segments. Slime formation was clearly apparent filling the space between the grape-like clusters colonies as displayed in Fig. 4.

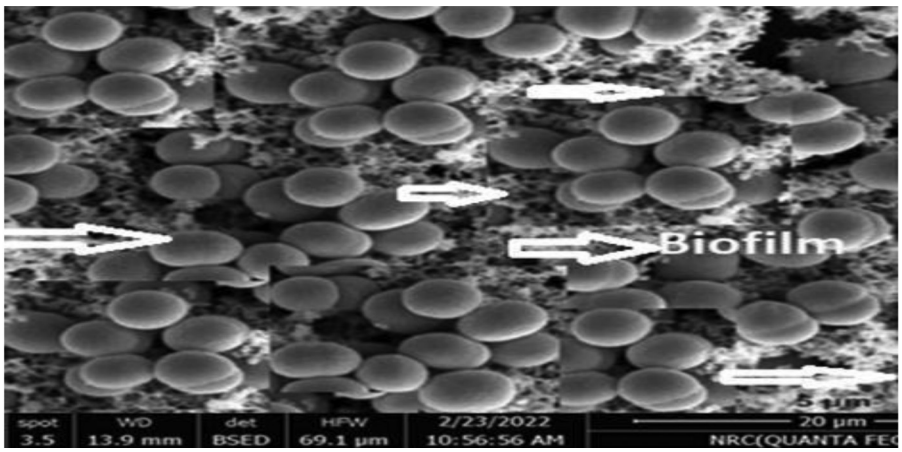
This appearance coincided with the previous biofilm criteria of Staphylococci [30]. Our study declared that the treatment with the nonthermal plasma emerged from SDBD minimized the number of adherent and aggregated bacteria cells in simple arrangements and prevented the agglomerates from forming a biofilm (Fig. 5). Also, failure of the bacterial cells to adhere to the catheter surface was observed. Similar to our results, the APP was capable to reduce the growth of microbial populations on any surfaces [31]. It is known that in the adhesion stage, there are fundamental processes that influence the appearance, motility, gene expression, and permanence of the cells that compose the biofilm. After that, the bacterium sets up a dense multiplication and cumulation of components that form the biofilm; teichoic acid and polysaccharide intercellular adhesin share in the viscous appearance and aggregation of colonies [32–35]. On the other side, *K. pneumoniae* exhibited normal rod-shaped cell structure with a smooth and regular surface without any shrinkage or cavity formation under SEM (Fig. 6). Exposure of *K. pneumoniae* cells to nonthermal plasma produced by SDBD showed multiple distortions and the cells displayed puckered or shrunken surfaces with a large number of bore

Figure 3



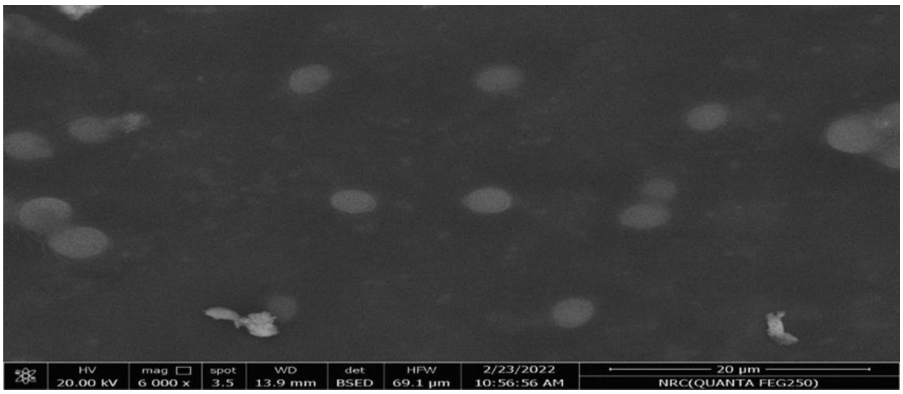
Catheter-associated *Staphylococcus epidermidis* biofilm growth in vivo under SEM. SEM, scanning electron microscope.

Figure 4



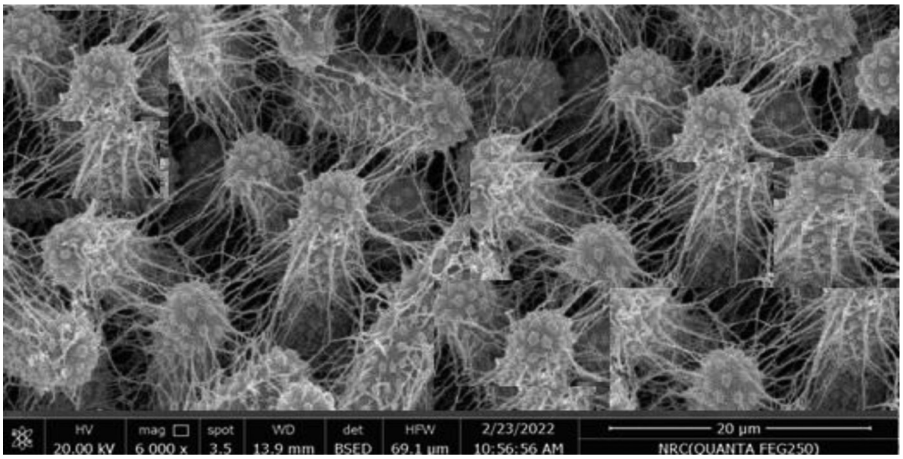
Staphylococcus epidermidis biofilm showed visible slime in the space between clusters.

Figure 5



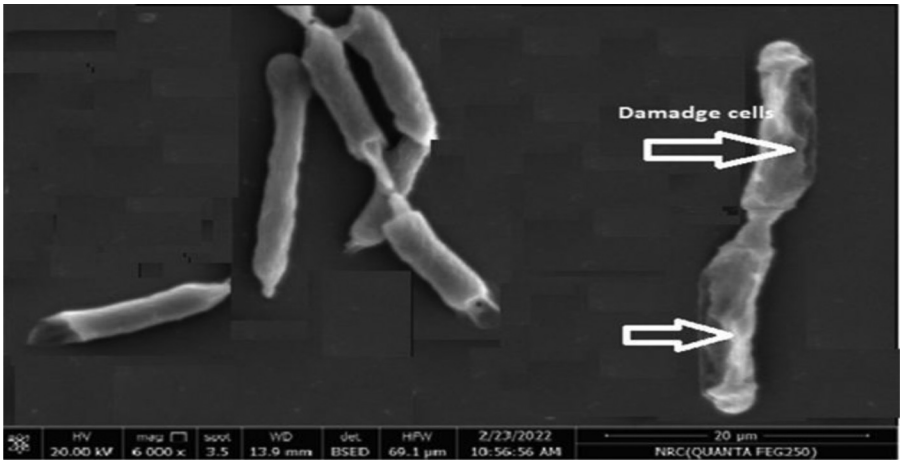
Biofilm of *Staphylococcus epidermidis* after exposure to nonthermal plasma.

Figure 6



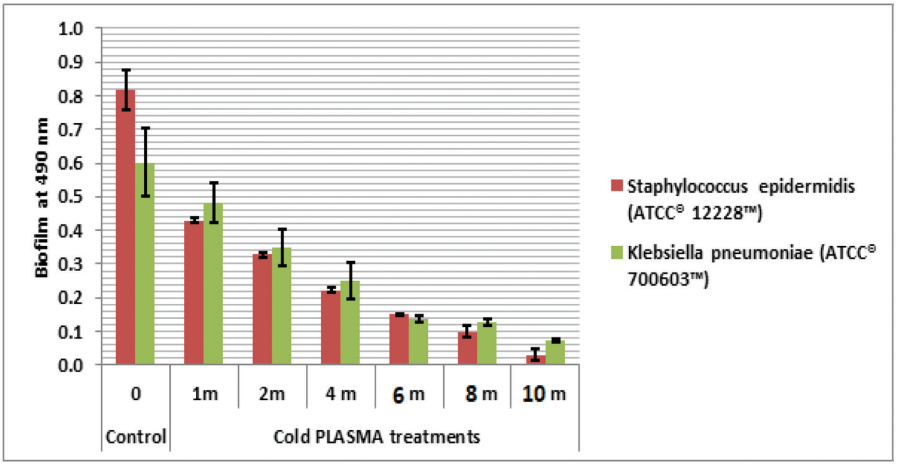
SEM of *Klebsiella pneumoniae* cells in mature biofilms formed on catheter *in vivo*. SEM, scanning electron microscope.

Figure 7



SEM of *Klebsiella pneumoniae* biofilm after exposure showed damaged bacterial cells. SEM, scanning electron microscope.

Figure 8



Correlation of biofilm formation of *Staphylococcus epidermidis* and *Klebsiella pneumoniae* with various time intervals of exposure to SDBD nonthermal plasma. SDBD, surface dielectric barrier discharge.

formation on the cell membrane constituted earlier criteria of bacterial cell damage (Fig. 7). Data shown in Fig. 8 declared great engagement between the time of exposure to nonthermal plasma and biofilm inhibition degree of examined bacteria on a catheter inserted in rats comparable with the control one. In *S. epidermidis,* the optical density of the biofilm declined linearly from 0.818, 0.430, and 0.030 with increasing the time of exposure to SDBD from 0, 1, and 10 min. Regarding *K. pneumoniae,* the optical density of the biofilm declined linearly from 0.602, 0.481, and 0.074 with increasing the time of exposure to SDBD from 0, 1, and 10 min.

In agreement with our results, the performance of low-temperature plasma therapy versus bacteria using the atmospheric-pressure plasma APP jet inhibited both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria on solid and porous surfaces [36]. The nonthermal atmospheric argon plasma was used to inhibit 78 genetically divergent *S. aureus* strains for clinical and epidemiological implications [37]. They indicated that a high complexity of microbial defence versus antimicrobial therapy and strain-relied susceptibility of *S. aureus* to plasma treatment. Moreover, the degree of microbial inactivation possibly depended on the type of microorganisms, the number and the physiological status of the cells, the inactivation medium, the operating gas mixture, and the flow rate [38,39]. The antibacterial efficacy of the nonthermal plasma was referred to the emergence of oxygen anions and hydroxyl radicals, which induce severe irreversible cell wall damage and destruction [40] and cell permeation [41]. The antimicrobial performance of charged particles, free radicals, and ultraviolet-emitting species was depicted in previous investigations [42–46]. In addition, the energized electrons and ions were able to collide with the organic molecules on the cells and disrupt their chemical bonds resulting in exerting perforations to the cell membrane [47].

Conclusion

As a novel tool of decontamination technology, the nonthermal plasma emerged from the SDBD system was found to be effective against biofilm layers produced by both Gram-positive (*S. epidermidis*) and Gram-negative bacterial (*K. pneumonia*) isolates.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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