

Efficiency of Ozone generated by Dielectric Barrier Discharge on Oxacillin susceptibility and biofilm formation of *Staphylococcus aureus* clinical isolates

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Abstract: *Staphylococcus aureus* is a leading cause of severe infections in both hospitals and community with high morbidity and mortality rates. Add to that, the extensive use of antimicrobial drugs has led to the widespread emergence of resistant bacterial strains and appearance of multidrug resistant bacteria like methicillin resistant *S. aureus* (MRSA). The shortage of antimicrobial strategy as a therapy necessitated to search for an alternative therapeutic tools. In the present study, we examined the antibiotic effect on 20 staphylococcal clinical isolates combined with Sub-Lethal Doses (SLD) of ozone. Ozone (O₃) was generated using Dielectric Barrier Discharge (DBD) system. The discharge system was found to be efficient in ozone generation where ozone was generated in the discharge filaments via electron impact. Ozone concentrations from 0.5 to 45 gm/cm³ were detected at very low consumed electric power from 1 to 20 W. Almost no survival of bacterial cells were detected after being treated with O₃ concentration 4 g/m³, flow rate 0.1 l/min at 37 °C for 2.5 min. we used O₃ SLD for MRSA and methicillin sensitive *S. aureus* (MSSA) treatment and investigated the interrelation for MSSA isolates and inconsequent for MRSA. We also studied the effect of SLD on bacterial biofilm formation by investigating ultra-structure with scanning electron microscope. Our study prove a synergistic effect between O₃ exposure and antibiotic treatment for MSSA infection as well as attenuation of biofilm formation and we speculate our primary investigation to be a core for new strategy for staphylococcal infection therapy.

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Key words: MRSA; DBD; Ozone therapy; biofilm formation; antibiotic susceptibility

1. Introduction

Staphylococcus aureus is an invasive pathogen which is normally a benign human commensal but becomes a deadly pathogen upon penetration into host tissues. This multitasking bacterium can promote disease in almost any tissue and cause a variety of illnesses, including skin infections, toxic shock syndrome, endocarditis, and food poisoning^[1]. *S.aureus* is one of the most frequent causes of a wide range of both hospital- and community acquired infections, from superficial skin and other soft tissue infections to life threatening toxic shock, pneumonia, endocarditis, and septicemia. Emergence of drug resistance staphylococci, notably methicillin resistant *S. aureus* (MRSA), has endowed its infection more problematic because of limited choice of effective antibiotics. Furthermore, the emergence of strains with reduced susceptibility to vancomycin, which has become the mainstay of therapy worldwide, has been reported since the late 1990s^[2]. MRSA not resistant only to methicillin but also most of the commonly used antimicrobial agents e.g. aminoglycosides, macrolides, chloramphenicol, tetracycline, and cephalosporin^[3,4]. *S. aureus* have the capability to live

in a wide variety of environments. It also has an inherent ability to form biofilms on biotic and a-biotic surfaces^[5, 6]. Biofilm is a multi-cellular complex structure can attach to host surfaces and has a dual function in bacterial pathogenicity as it can protect bacteria from host immune defense as well as responsibility to antimicrobial agents^[7,8].

Ozone is a unstable gas, triatomic of oxygen, easily to decompose in short half-life to a nontoxic product, oxygen, without residues in surrounding and capable to destroy all forms of microorganisms at low concentrations^[9,10]. Advantage of using O₃ instead of antibiotic in bacterial treatment is inability to enhance bacterial resistance and non-toxic effect to environment. FDA was approved using of ozone for foods processing, storage and treatment since 2001 that was motivated more research about ozone utilization^[11]. Ozone is a powerful oxidant and easily to be introduced for many application in different states as a gas phase, ozonized-aqueous form or ozonized oil form to fit the purpose of using e.g. disinfection^[12], cleansing effect^[13,14] and treatment of wounds, ulcers and burns respectively^[15]. Ozone is usually generated in one of three ways:

Electrochemical generation, ultraviolet rays generation and non-thermal plasma produced by electrical discharges^[16,17]. The aim of this study was to evaluate first the effect of ozone generated by on-thermal plasma produced with Dielectric Barrier Discharge (DBD) as a co-therapy with antibiotic treatment for staphylococcal clinical isolate survival rate either when bacteria are MRSA or MSSA also to determine attenuation of SLD of ozone on staphylococcal biofilm formation.

2. Experimental set up

2.1. Experimental apparatus

The schematic diagram of ozone generator is shown in figure (1). A cylindrical dielectric barrier discharge (DBD) cell has been used as an ozone generator. The inner electrode is made from brass rod with diameter of 0.6 cm, and the outer electrode is made from graphite painted on the outside surface of the dielectric material (pyrex glass tube) with diameter of 0.12 cm, the thickness of the glass tube is 0.15 cm and the gap space between the inner electrode and the dielectric material is 0.15 cm. The length of the outer electrode (the length of the discharge volume) is 35 cm. The used gas is oxygen with purity of 99.9 %. The oxygen gas has flowed in the discharge cell with different flow rates controlled by to needle valves the inlet and the outlet of the discharge cell where the gas pressure (p) is kept at atmospheric pressure.

The two electrodes were connected to a high voltage AC power supply of 50 Hz frequency and a variable voltage of 0-10 kV. A limiting resistance of 250 kW was used to limit the discharge current. The applied voltage was measured via a resistive potential divider (PD) 500:1. The discharge current was measured by measuring the potential drop across a resistance of 1 kW. The accumulated charge on the electrodes was measured by measuring the potential drop across a capacitor of 14 mF. The waveforms of the voltage, current and charge was measured by a digital storage oscilloscope (Model HM-407). The concentration of ozone formed inside the DBD system was measured using ozone detector (Model HI-AFX-Instrumentation, USA), which also measures the flow rate, the pressure and temperature of the gas inside the cell of the detector. The detector is placed adjacent directly after the ozone generator. The temperature of the output gas is in the range of 298–305 °K.

The output gas from the DBD cell containing a mixture of oxygen and ozone. The ozone concentration in the mixture has been controlled by changing the discharge voltage and hence the discharge current. The gas mixture has been injected to the treated samples at different ozone concentrations and different treatment time.

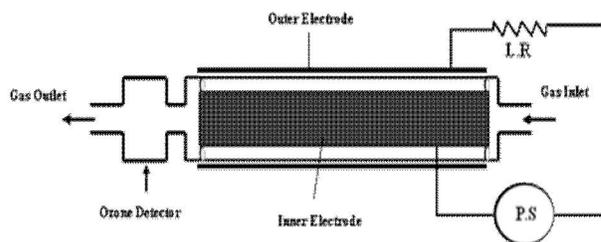


Figure 1. schematic diagram of the ozone generator

Oxacillin susceptibility assays

1. Disc-diffusion method

The standardized Kirby-Bauer disc-diffusion method was performed on Mueller-Hinton agar using antibiotics oxacillin (1 µg) for testing susceptibility of *S. aureus* to oxacillin as a derivative of methicillin antibiotic^[20]. In brief, the Trypticase soy agar (TSA) was poured into sterile petri plates and was allowed to solidify. A suspension equivalent to 0.5 McFarland was prepared from each isolate. A swab was dipped and streaked on the surface of a TSA plates. Standard antibiotic discs were introduced on the upper layer of the seeded agar plate. The plates were incubated at 37°C for 24h. The experiment was carried out three times and the mean values are presented. The antimicrobial activity was evaluated by measuring the diameter of zone of inhibition in mm. the interpretative ranges, per NCCLS as follow Zone sizes Oxacillin Susceptible (S) >13 mm, Oxacillin Intermediate (I) 11-12 mm and Oxacillin Resistant (R) <10 mm^[21].

2. Broth method

To assist a method suitable for susceptibility examination before and after ozone treatment, we used tube-dilution method^[22,23] but with some modification, we used fixed dose from Standard antibiotic disc of oxacillin 1 µg (Bioanalyse ®) in a Trypticase soy broth (TSB) medium dispensed in Eppendorf tubes 1.5 ml. The antibiotic containing tubes were inoculated with a standardized bacterial suspension of 1–5×10⁵ CFU/mL following overnight incubation at 37°C and growth was measured at OD₆₀₀.

Biofilm Formation

Quantitative determination was carried out by using micro titer plates of 96 flat bottomed wells^[24]. Briefly, each well was filled with 0.2 ml of 10⁵ CFU/ml of a bacterial suspension in TS broth. After 24h incubation in aerobic condition at 37°C, the contents were aspirated and plates were washed twice with phosphate buffered saline (PBS, pH: 7.2). The wells were stained with 0.1% crystal violet for 2 min

then washed three times with PBS to remove the excess of stain. The plates were read in an enzyme-linked immunosorbent assay (ELISA) reader (BioTecan, ELx808) to 492 nm. Sterile TS broth was used as a negative control. All the experiments were repeated at least 5 times, and the values of optical density (OD) were then averaged.

Scanning electron microscope

The samples were fixated by gluteraldehyde 2.5% and dehydrated by serial dilution of ethanol with agitation using automatic tissue processor (Leica EM TP, Leica Microsystems; Austria), then samples were dried using CO₂ critical point drier (Audosamri-815, Tousimis; Rockville, Maryland, USA). After that, samples were coated by gold sputter coater (SPI-Module, USA) and observed by scanning electron microscopy (JSM-5500 LV; JEOL Ltd – Japan) by using high vacuum mode at the Regional Center of Mycology and Biotechnology, Cairo, Egypt.

3. Results and Discussion

3.1. Establishing Dielectrical Barrier Discharge generation system

3.1.1. Voltage and current waveforms

Voltage and current waveforms of the DBD cell has been measured and shown in figure 2. From the figure, it can be seen that a streamer discharge was formed, which is characterized by discrete current spikes. These spikes were related to the formation of microdischarges (filaments) of tens of nanosecond duration in the gap space [25]. They start when the breakdown field is reached locally and extinguish (turn off) when the field is reduced to such an extent that electron attachment and recombination dominate over ionization. These microdischarges are randomly distributed in the discharge area. The peak of each individual spike is related to the number of instantaneous microfilaments that were formed at this instant, and hence a high current spike indicates that a high number of microdischarges are initiated almost simultaneously. It can be noticed that, when the external voltage rises, additional microdischarges are formed at new positions because the presence of residual charges on the dielectric has reduced the electric fields at positions where the microdischarges have already occurred. Because high voltages at low frequency tend to spread the microdischarges and increase the number of instantaneous filaments [26], the peaks of the spikes increase by increasing the peak of the applied voltage.

3.1.2. Consumed power

The consumed power has been measured using Lissajous method [27] where the voltage difference between the two electrodes has been measured as a

function of the charge on the electrodes (Lissajous curve). Figure 3 shows the consumed power in the DBD cell as a function of the discharge voltage. The consumed power in the DBD cell has been found to be very low, even at high voltage (around 8 kV) the consumed power is less than 20 W. The low consumed power can be referred to the characterized filamentary discharge behavior. In the filamentary discharge the time of the filament is very short, few tens of nanoseconds [28].

This short discharge time limits the dissipation of the power in unwanted processes like heating. The gas temperature under the conditions of the present work varies from 298–305 °K.

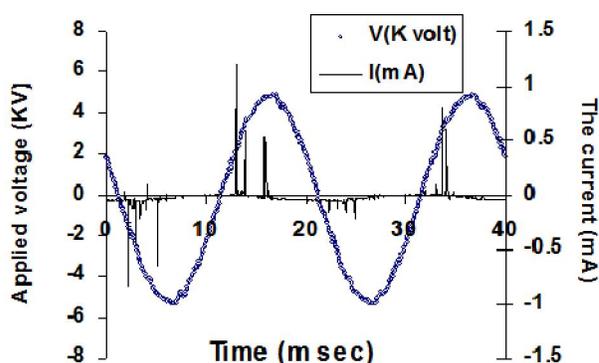


Figure 2. Voltage and current waveforms in dielectric barrier discharge

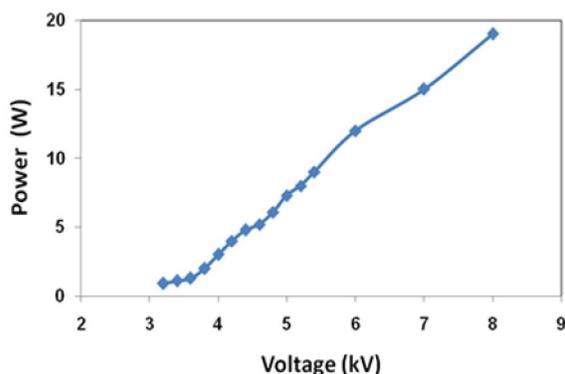


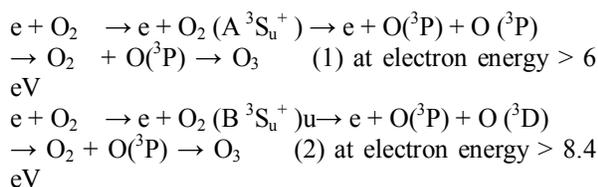
Figure 3. The consumed power in the DBD cell as a function of the applied voltage

3.1.3. Ozone concentration

Figure 4 shows the variation of ozone concentration with the applied voltage (V) at different gas flow rates. It can be noticed that, the curves of the variation of the ozone concentration with the applied voltage show two different slopes.

At applied voltage lower than 5 kV the ozone

concentration increases linearly with the applied voltage with low slope. At applied voltages higher than 5 kV the ozone concentration increases linearly with the applied voltage with higher slope. This behavior is recorded at different gas flow rates. To discuss this behavior the mechanism of ozone formation must be taken into account. The first region, $V = 3 - 5$ kV, starts at the onset voltage (around 3 kV) where breakdown in oxygen takes place and dissociation of oxygen molecules occurs, producing atomic oxygen, which recombines with oxygen molecules to produce ozone, and hence ozone is detected. The ozone concentration can be noticed to increase at a slow rate, up to a certain applied voltage (around 5 kV), and then it tends to increase at a higher rate in the second region, at applied voltage higher than 5 kV. It has been reported in the literatures that [28 - 30], ozone is being produced through two different mechanisms in pure oxygen:



It is believed that ozone is generated at a slow rate by the first reaction in the first region, while it is generated by both reactions afterwards in the second region, and this explains the enhancement of the rate of ozone generation. From figure 4 it can be concluded that; the ozone concentration decrease as the flow rate increase which can be referred to the fact that; as the flow rate increases the residence time of the gas in the discharge cell decreases and hence the probability of ozone formation, due to the above two reactions, decreases.

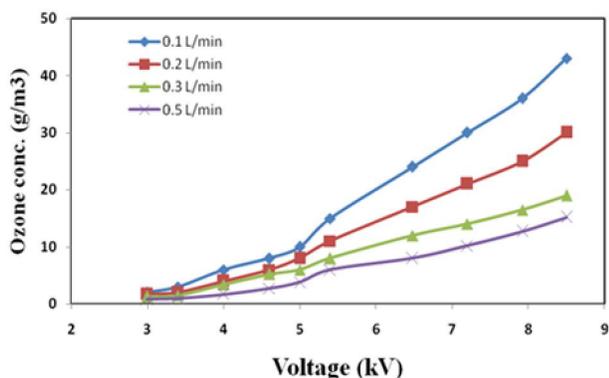


Figure 4. Ozone concentrations as a function of the applied voltage at different gas flow rates

3.2. Oxacillin sensitivity of *Staphylococcus aureus* clinical isolates

Twenty clinical isolates belonging to *Staphylococcus aureus* sp were examined for oxacillin, semisynthetic derivative of methicillin, susceptibility test. To classify our isolate to Methicillin-Resistant *Staphylococcus aureus* (MRSA) or Methicillin-Sensitive *Staphylococcus aureus* (MSSA), we established a modified method using broth culture for susceptibility test by using oxacillin (1 µg/ml) to eligible our assay for easily comparative data. Thirteen isolates, MA12, MA14, MA15, MA16, MA17, MA18, MA110, MA113, MA114, MA116, MA117, MA118 and MA119, after treatment with amoxicillin for 24 h were classified as MRSA. This classification based on growth pattern related to untreated isolates that were about ≥ 0.8 (Fig. 5). Five isolates, *S. aureus* ATCC 29213, MA13, MA19, MA115 and MA120, were responded to amoxicillin treatment for 24 h as the growth pattern were shifted about ≤ 0.7 related to untreated isolates and considered as MSSA (Fig. 5). MA111 and MA112 were showed intermediate shifting in growth pattern after treatment with amoxicillin to be > 0.7 and < 0.8 related to untreated isolates (Fig. 5). These classification were assumed based on the result of disk-diffusion method assay (data not shown).

3.2. Effect of ozone treatment on deactivation of MSSA and intermediate *S. aureus* clinical isolates

After antibiotic susceptibility test, we categorized our clinical isolates to mainly three groups; MRSA, MSSA and intermediate response isolate. In this experiment we aimed to study the attitude of MSSA and intermediate regarding different doses of ozone treatment. 29213, MA13, MA19, MA115 and MA120 as MSSA in addition to MA111 and MA112 that showed intermediate activity were exposed to different doses of ozone with different concentration and different flow rates as well as exposure time and finally we were able to adjust conditions to be represented in dose-response manner as follow; 4 g/m³ concentration with flow rate 0.3 l/min. at 37°C for 0, 0.5, 1, 1.5, 2, 2.5 and 3 min treatment (Fig. 6-A). we can observe from investigated result that Inhibitory Concentration for 50% survival rates (IC₅₀) for all isolates were exposing to 4 g/m³ ozone concentration with flow rate 0.3 l/min at 37°C for 1 min and IC₉₀ = 2 min. when increasing concentration of ozone were added, the survival cells were decreased until active lethal dose with exposure time for 2.5 min which enough for deactivation for all cells which indicating sensitivity of isolates was directly proportional to ozone concentration (Fig. 6-A).

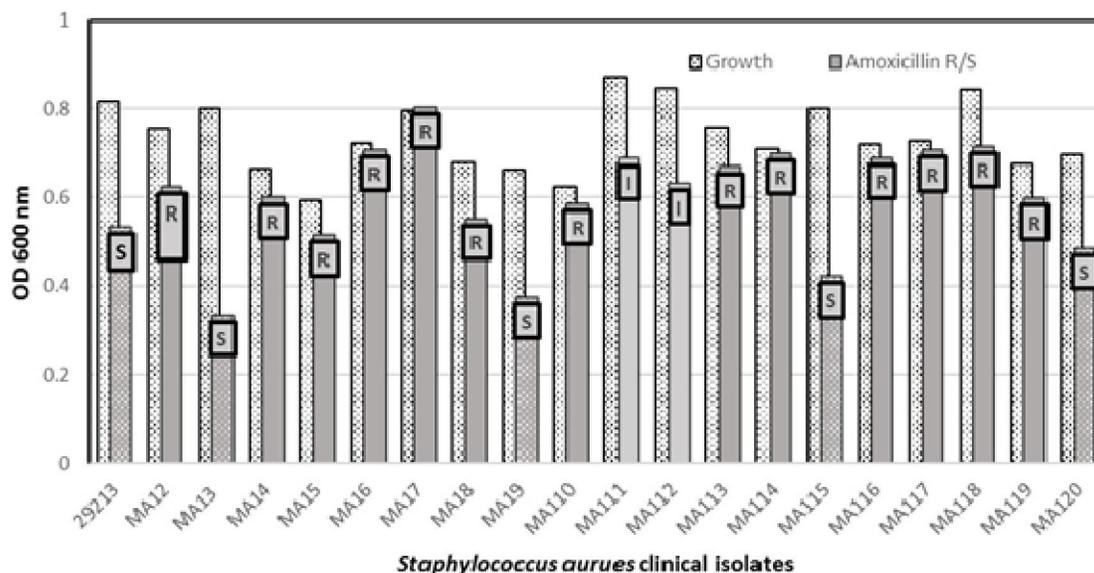


Figure 5. Amoxicillin susceptibility of *S. aureus* clinical isolates in presence and absence of amoxicillin at $1\mu\text{g/ml}$. R; resistant isolate when treated/untreated ≥ 0.8 , S; sensitive isolate when treated/untreated ≤ 0.7 , I; intermediate isolate when treated/untreated > 0.7 and < 0.8 . Data are mean of three independent experiment

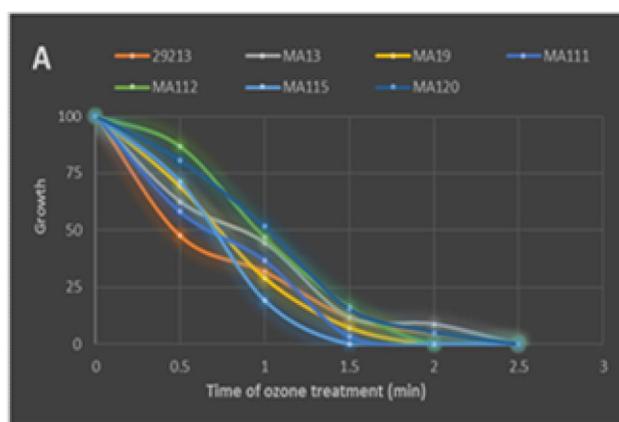


Figure 6-A. Effect of ozone treatment on MSSA and methicillin Intermediate *S. aureus* isolates. Ozone concentration was 4g/m^3 with flow rate 0.3 l/min . at 37°C for 0, 0.5, 1, 1.5, 2, 2.5 and 3 min. Growth was measured at 0 time exposure and represented as 100 value. Data are mean of three independent experiment and represented as a mean

3.3. Effect of ozone treatment on deactivation of MRSA clinical isolates

In this experiment, we aimed to investigate ozone deactivation for MRSA isolates e.g. MA12, MA14, MA15, MA16, MA17, MA18, MA110, MA113, MA114, MA116, MA117, MA118 and MA119. The survival rate of MRSA isolates were decreased as ozone doses increased same like MSSA (Fig. 6-B). Although survival rate of MRSA isolated were completely varied,

but finally the lethal dose for all viable cells was occurred when exposed to O_3 concentration 4 g/m^3 for 2.5 min (Fig. 6-B).

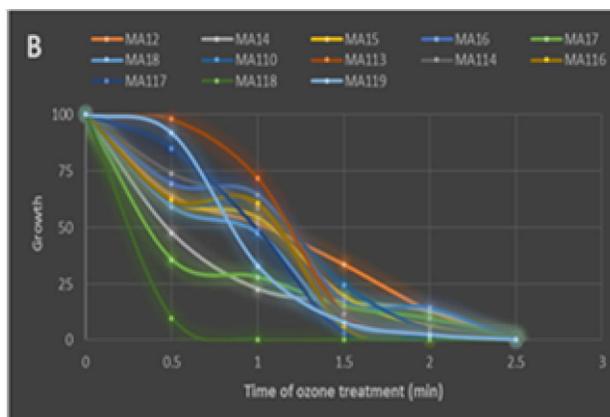


Figure 6-B. Effect of ozone treatment on MRSA isolates. Ozone concentration was 4 g/m^3 with flow rate 0.3 l/min . at 37°C for 0, 0.5, 1, 1.5, 2, 2.5 and 3 min. Growth was measured at 0 time exposure and represented as 100 value. Data are mean of three independent experiment and represented as a mean

3.5. Combined action of ozone exposure and amoxicillin treatment for MRSA and MSSA strains

Deactivation of MRSA and MSSA cells by ozone treatment were obtained by exposing to 4 g/m^3 for 2.5 min (Fig. 6 – A and B). In this experiment, we studied the combined action between sub-lethal doses of ozone

in presence and absence of amoxicillin 1 $\mu\text{g/ml}$. we speculated this combined condition might be work synergistically to decrease lethal dose of ozone treatment or increase antibiotic susceptibility to amoxicillin. MSSA isolate, 29213, interestingly showed shift in IC_{90} from exposing to O_3 4 g/m^3 for 2.3 min to less than 1 min (Fig. 7-A). Also MA113 was confirmed the evidence of synergistic relation between exposing to O_3 and antibiotic treatment (Fig. 7-B), while MA120 did not show notable attenuation for this double action (Fig. 7-C). Unfortunately, when MRSA isolates i.e. MA110, MA115 and MA117 (Fig. 7 – D, E and F) respectively were investigated under the same condition, cell survival rates did not respond to double action effect.

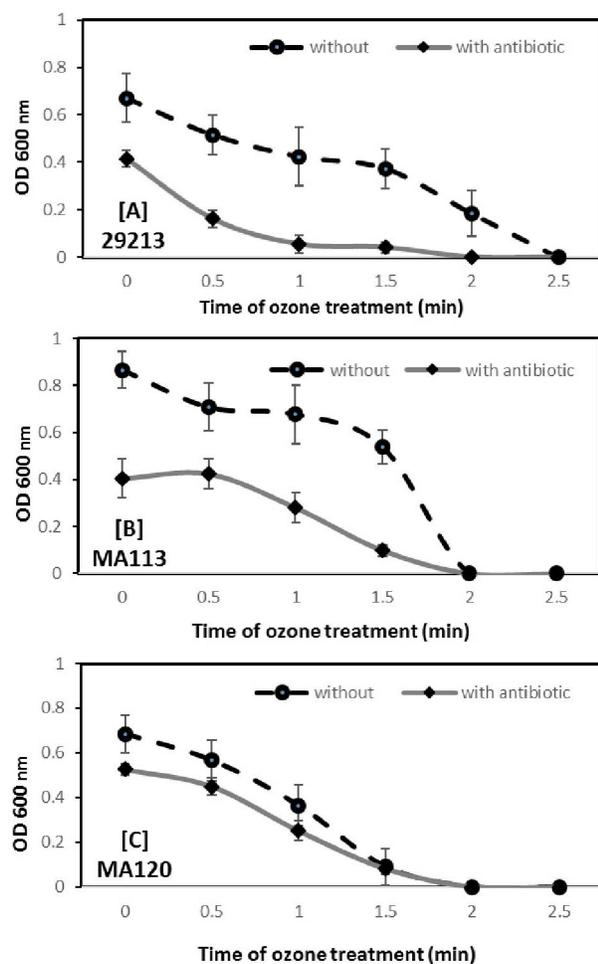


Figure 7 - A, B and C. Effect of combined action of ozone and oxacillin treatment on MSSA isolates A) 29213, B) MA113 and C) MA120. Ozone concentration was 4 g/m^3 with flow rate 0.3 l/min. at 37°C for 0, 0.5, 1, 1.5, 2 and 2.5 min and oxacillin concentration (1 $\mu\text{g/ml}$). the experiment performed in triplicate and data represented as an average \pm standard error

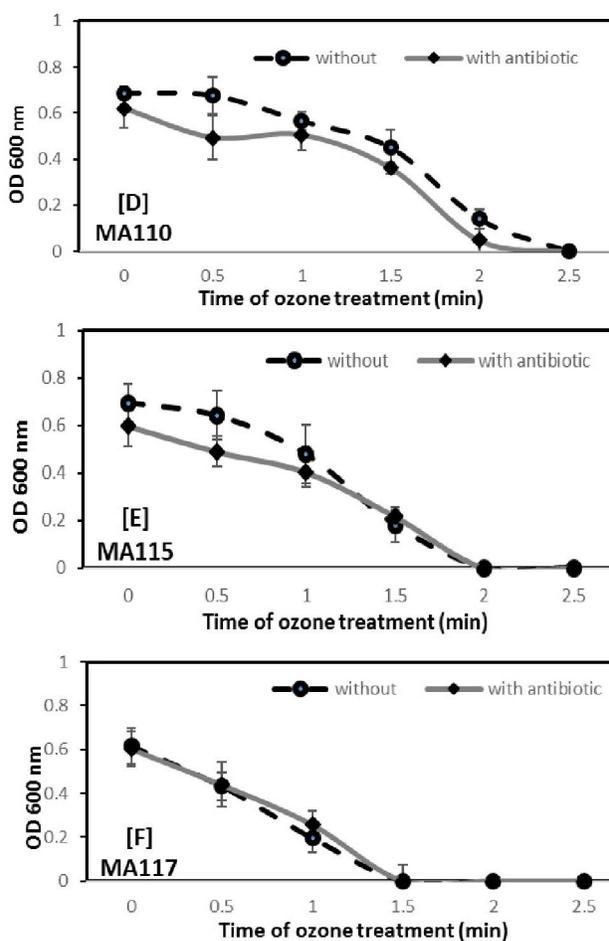


Figure 7 - D, E and F. Effect of combined action of ozone and oxacillin treatment on MRSA isolates D) MA115, E) MA110 and F) MA117. Ozone concentration was 4 g/m^3 with flow rate 0.3 l/min. at 37°C for 0, 0.5, 1, 1.5, 2 and 2.5 min and oxacillin concentration (1 $\mu\text{g/ml}$). The experiment performed in triplicate and data represented as an average \pm standard error

3.6. Effect of ozone sub-lethal doses on biofilm formation of *Staphylococcus aureus* clinical isolates

Biofilm formation differ from bacterial isolates to each other. Among *S. aureus* spp, some strains like 29213 has no ability to perform biofilm formation, we used it as a negative control for our experiment, while capability of other strain differ in their attitude like MA17 and MA114 (Fig. 8). To evaluate the effect of sub-lethal doses of ozone treatment on biofilm formation we used 2 g/m^3 concentration with flow rate 0.1 l/min. at 37°C for 0, 0.5 and 1 min treatment. The mentioned doses showed non-significant changes in growth pattern that mainly proportional to biofilm formation. It was easy to note that, MA17 vigorously form biofilm after 24 h incubation (Fig. 8). Biofilm

formation ability was decrease as ozone concentration increase for MA17 isolate and it was found that no significant effect in case of MA114 isolate (Fig. 8).

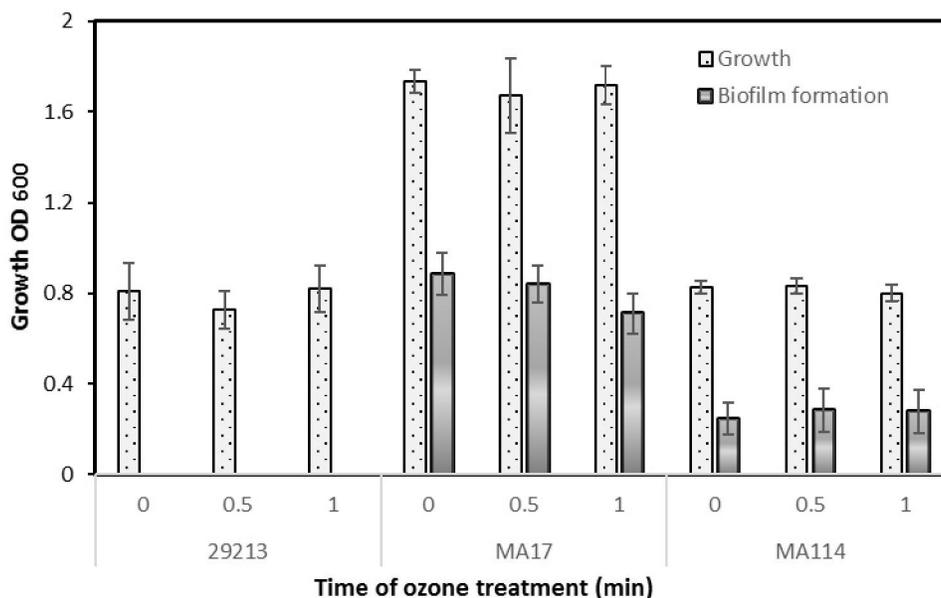


Figure 8. Normal growth and biofilm formation by *S. aureus* ATCC 29213 (negative control), Isolate MA17 and MA114 treated with ozone sub-lethal dose (2 g/m^3) with flow rate 0.1 l/min. at 37°C for 0, 0.5 and 1 min. the experiment performed in triplicate and data represented as an average \pm standard error

3.7. Scanning Electron Microscope images for *Staphylococcus aureus* M117 under sub-lethal dose of ozone treatment

To study the fine structural changes in *Staphylococcus aureus* M117 cells according to treatment of sub-lethal ozone dose, we used scanning electron microscope (SEM) images before and after treatment of assigned dose of ozone that allow normal growth pattern, ozone concentration 2 g/m^3 with flow rate 0.1 l/min for 1 min. However phenotype result did not show any changes in cell viability (Fig. 8), treated cells with ozone sub-lethal dose showed observable changes in cell shape and cell aggregation as well as intracellular spaces (Fig. 9 - B1). In addition, ozone sub-lethal dose affect cells surface to become roughly and malformed (Fig. 9 - B2). All commented changes in fine structure of staphylococcal cells positively affect biofilm formation process and pathogenicity related host infection.

4. Discussion

The main purpose of this study was to evaluate using ozone treatment generated from DBD for staphylococcal infection caused by MRSA or MSSA isolates under treatment with antibiotic. At the first, we studied lethal dose condition for both staphylococcal types and we found the exposure for 2.5 min with 4 g/m^3 is enough for killing or deactivate all microbial cells. Effect of ozone on bacterial cells is a complicated

process due to numerous targets could be easily oxidized including protein, unsaturated lipid, enzymes and nucleic acids [31, 32]. The second experiment performed to see how well ozone at different Sub-Lethal Doses (SLD) could be used for attenuation of microbial cells in water under stress of amoxicillin antibiotic $1 \mu\text{g/ml}$. This result is significant in terms of the fact that combined action has a synergistic effect for MSSA isolates e.g. 29213 and MA113 while in case of MRSA there is no clear evidence for any kind of interference action although both of antibiotic and ozone have their own targets. Ozone decomposition giving rise to one molecule of oxygen and to the very reactive oxygen atom which explain its high reactivity. It has been supposed that ozone may have a pertinent therapeutic role in various types of infections due to generation of reactive oxygen species such as anion superoxide, hydroxyl radicals and hydrogen peroxide [33 - 35]. The second part of current study dealing with effect of O₃ SLD on Bacterial biofilm. Biofilm formed in a successive steps in host or surrounding environment and play a vital role in bacterial resistance mechanisms [36]. Our result prove a correlation between SLD = 2 g/m^3 which did not show in changes in bacterial cell survival rates although many changes in cell ultra-structure were illustrated in SEM images i.e. cell aggregation, surfaces and interspaces between sells. In conclusion, MRSA and MSSA isolates can be inactivated by lethal ozone doses

that might be toxic for human, the available data proved attenuation of antibiotic susceptibility and bacterial biofilm formation for exposing for very short time and

we strongly recommend using of ozone as a co-therapy for severe infection or as a prophylaxis in SLD.

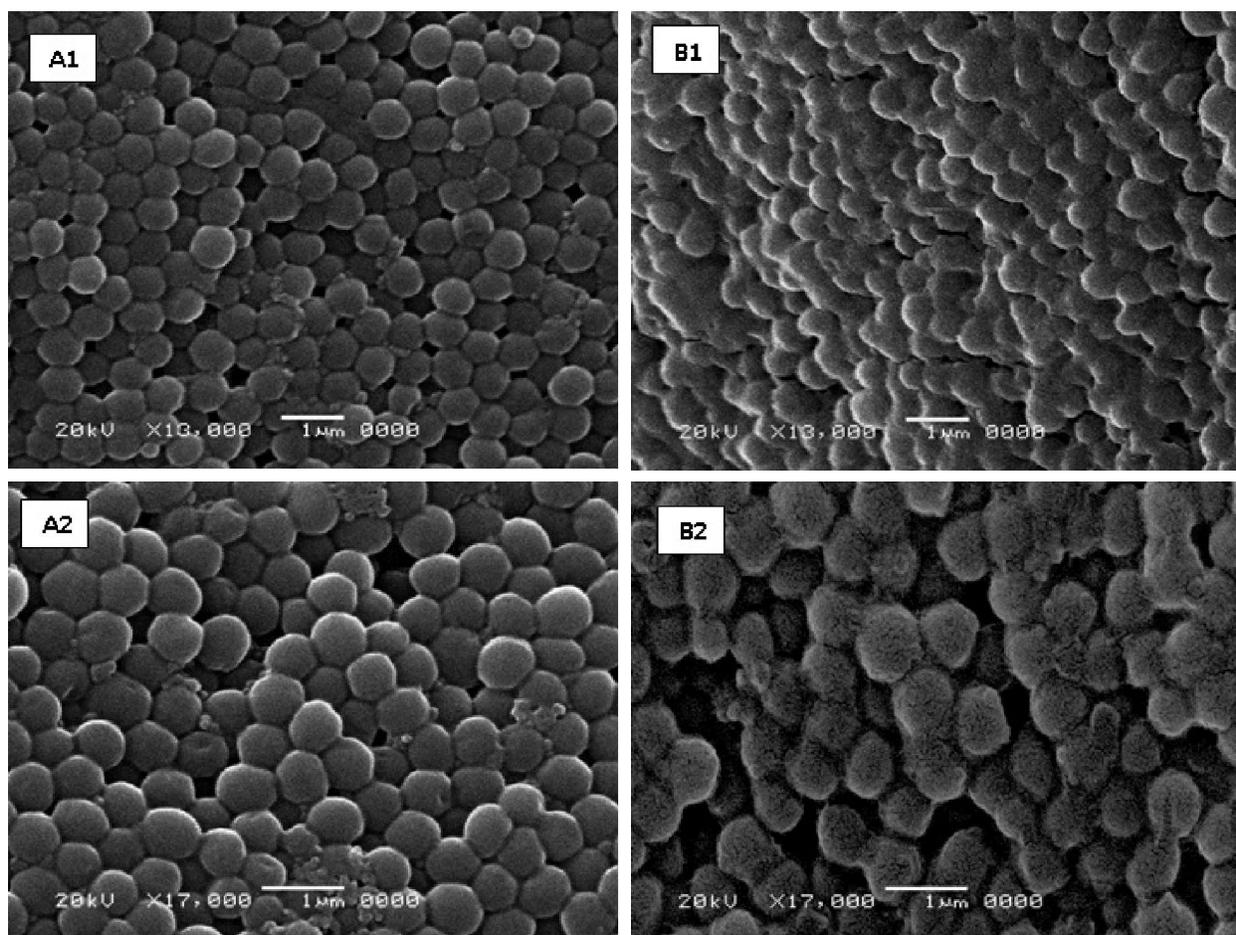


Figure 9. Scanning electron microscope micrograph of *Staphylococcus aureus* M117 cells. Untreated (A 1, 2) or treated (B 1, 2) with ozone. Ozone treated cells were exposed to sub-lethal dose (2 g/m^3) with flow rate 0.1 l/min. at 37°C for 1 min

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