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Evaluation of High-frequency equipment efficacy in the inhibition of multidrug-resistant Pseudomonas aeruginosa strain

Avaliação da eficácia de equipamento de alta-frequência na inibição de *Pseudomonas aeruginosa* multidroga-resistente

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Abstract

Introduction: The emergence of antibiotic resistance, mainly due to the misuse of antibiotics, has brought forth the need for studies on new approaches to treating bacterial infections and the decontamination of surfaces. High-frequency equipment has been shown to have antibacterial effects due to the formation of ozone during sparking. **Objective:** This study aims to evaluate the efficacy of High-frequency equipment against clinically isolated multidrug-resistant (MDR) or extensively drug-resistant (XDR) *Pseudomonas aeruginosa*. **Methods:** A clinically isolated XDR *P. aeruginosa* strain and a standard ATCC (American Type Culture Collection) 27853 strain were spread-plated, divided into three groups, and treated with high-frequency equipment for 0, 5, and 10 minutes prior to incubation. Inhibition was measured through ImageJ, and statistical analysis was performed using the GraphPad Prism statistics software through ANOVA with Tukey post-test, considering $p<0.05$ as statistically significant. **Results:** Both strains were successfully inhibited using high-frequency equipment for exposure times of 5 and 10 minutes (p-value < 0.05). Between 47.2% and 61.44% of the surface area covered by the electrode was inhibited. No differences between exposure times (5 and 10 minutes) or resistance profiles were found. **Conclusion:** XDR *P. aeruginosa* strain resistance mechanisms do not seem to impact its susceptibility to high-frequency treatment, as it was equally inhibited compared to a standard ATCC strain. Significant inhibition was detected after only 5 minutes of exposure.

Keywords: *Pseudomonas aeruginosa;* Drug Resistance, Multiple; Drug Resistance, Microbial; Disinfection

Introduction

Antibiotic resistance is becoming an increasingly common issue. Many factors, such as its indiscriminate use and prescriptions, can result in the emergence/selection of antibiotic-resistant strains. Furthermore, the formation of biofilms, associated with pathogenicity, also constitutes a factor in the resistance of microorganisms to antimicrobials[1,2]. The emergence and spread of multidrug-resistant (MDR) or extensively drug-resistant (XDR) bacteria have been observed in the community, the hospital, and the environment in the last few decades[1]. Antimicrobial resistance substantially influences public health, mainly by increasing the rate of difficult-to-treat healthcare-associated infections (HAIs)[2].

Pseudomonas aeruginosa has a high rate of antimicrobial resistance and is an opportunistic pathogen responsible for a large number of HAIs worldwide. This bacterium can colonise and form biofilms in many medical devices, such as catheters and endotracheal tubes[4]. This ability to form biofilms on inanimate surfaces makes their control in these environments a fundamental condition for mitigating contamination and/or cross-infection. These challenges raise the need for new alternative methods of controlling bacterial infections that do not rely on antibiotics.

High-frequency equipment has been used in many applications over the years, including in treating bacterial infections. However, after the arrival of the "age of antibiotics", this treatment method has seen little to no use against infections, mostly used by physiotherapists and aesthetics professionals due to their anti-inflammatory activity and ability to facilitate healing[5]. This equipment can inhibit bacteria through the formation of ozone, as well as increase vasodilation and facilitate immune response[6]. This equipment can be equipped with different glass electrodes filled with gas or rarefied air. Once the electric current reaches the electrode, that gas is ionised, forming ozone on the surface of the electrode[7,8].

High-frequency plasma devices have shown promising antimicrobial activity in various studies[5,6,7,8]. Historical devices like the violet wand demonstrated efficacy comparable to modern cold atmospheric pressure plasma sources, such as the kINPen 09 and dielectric barrier discharge. Modern High-Frequency equipment remains relatively unchanged and is more affordable than modern CAPP

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sources while remaining effective in eradicating various wound pathogens and biofilms in vitro[5].

High-frequency equipment has demonstrated significant antimicrobial effects against various bacterial strains. Studies have shown that high-frequency plasma devices can effectively inhibit the growth of both Gram-negative and Gram-positive bacteria, such as Enterobacter aerogenes and Staphylococcus aureus[6]. *Antimicrobial efficacy is dependent on intensity and exposure time, with higher intensities and longer durations generally producing stronger effects*[6]. *These devices have also demonstrated effectiveness against biofilms, outperforming traditional antiseptics like ethanol and polyhexanide*[5].

While there have been positive accounts from studies on the antibacterial effect of high-frequency equipment against bacteria such as Staphylococcus aureus, Klebsiella aerogenes, and P. aeruginosa, the effect of this therapy against multidrug-resistant bacteria hasn't been as thoroughly explored[6,7,8].

This study aims to evaluate the efficacy of high-frequency equipment as a tool for treating a clinical isolate of XDR P. aeruginosa. The results were compared with P. aeruginosa ATCC 27853 to determine if the sensitivity to high-frequency treatment changes in the presence of antibiotic resistance mechanisms.

Methodology

Bacterial strains

The ATCC 27853 strain of P. aeruginosa, as well as an XDR clinical strain called PA350, previously phenotypically and genetically characterised according to its antibiotic resistance, were stored at -70ºC in Tryptic Soy Broth (TSB) supplemented with 15% glycerol. Before experimentation, the two strains were reactivated and amplified in a shaker incubator at 150 RPM, 35ºC, for 24h. The optical density of the bacterial suspension was measured by spectrophotometer and adjusted to 1,5x10^{10} CFU/ml. 100 µL of bacterial suspension was added to 90 mm diameter glass Petri dishes, each containing 15 mL of Pseudomonas F Agar (Difco™) and disseminated with a Drigalski spreader. For each strain, nine plates were divided into three groups labeled "Control," "5min", and "10min", according to the duration of high-frequency treatment to be applied (0, 5, and 10 minutes, respectively).

Phenotypic assays of antimicrobial resistance:

The antimicrobial susceptibility test was performed through a disc diffusion test and interpreted according to the Brazilian Committee for Antimicrobial Susceptibility Testing (BrCAST/EUCAST, 2023) (http://brcast.org.br). Strains were considered XDR when they appeared non-susceptible to at least one agent in all antimicrobial categories except ≤2[9].

Detection of carbapenemase was phenotypically analysed by mCIM/eCIM assays[10,11] *and screened for polymyxin resistance by the polymyxin drop test.*

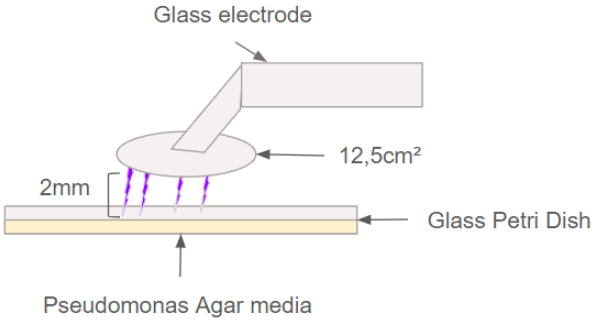
Genotypic Antimicrobial Resistance Assays:

DNA was extracted by boiling, and the β-lactamases genes (blaIMP, blaKPC, blaSPM-1, blaVIM) were detected by conventional PCR performed according to protocols previously described[12,13], *as well as efflux pump genes MexA, MexE, and MexX according to previously described methods.*

High-frequency challenge

The five- and 10-minute experimental groups were treated with high-frequency equipment (Ibramed, São Paulo, Brazil) coupled to a 12.5 cm² glass electrode positioned 2mm from the center of the Petri dishes (Figure 1). The high-frequency equipment was activated at maximum potency for 5 and 10 minutes in the respective experimental groups, during which sparks were observed, indicating ozone production. After the high-frequency treatment concluded, the plates were incubated at 35ºC for 24 hours in a microbiological incubator.

Figure 1 - *Visual representation of inhibition experiments*.



Source: This study.

Inhibition area measurement

After incubation, the petri dishes were photographed using a colony counter as a support. Inhibition measurements were done using the ImageJ software, adapting the analysis protocols of previous studies[14,15,16]. *Images were imported into ImageJ, where the grid pattern from the colony counter served as a calibration scale. For each petri dish, inhibited areas were manually selected using the polygon tool, and pixel counts within these areas were converted to square units.*

Statistical analysis

Statistical analyses were performed using GraphPad Prism® 5.0 software. They involved ANOVA with a Tukey post-test; a P value of less than 0.05 was considered statistically significant[14].

Results

Antimicrobial susceptibility of the PA350 clinical strain

The clinical strain XDR PA350, challenged with high-frequency treatment, was resistant to all tested antibiotics except aztreonam, which was susceptible to increased exposure ("I") (Table 1).

Table 1 - *Antimicrobial susceptibility profile of the clinical P. aeruginosa isolate-XDR PA350.*

Strain	AMI	ATM	CFM	CAZ	CZA	CIP	IMP	LVX	MER	PPT	TOB
PA350	R	I	R	R	R	R	R	R	R	R	R

Resistance against AMI (Amikacin), CFM (Cefixime), CAZ (Ceftazidime), CZA (Ceftazidime-avibactam), CIP (Ciprofloxacin), IMP (Imipenem), LVX (Levofloxacin), MER (Meropenem), PPT (Piperacillin-Tazobactam), and TOB (Tobramycin) was tested through disc diffusion, according to Br-cast. The response to each antibiotic is indicated with R (Resistant) and I (Susceptible with increased exposure).

Source: Research data.

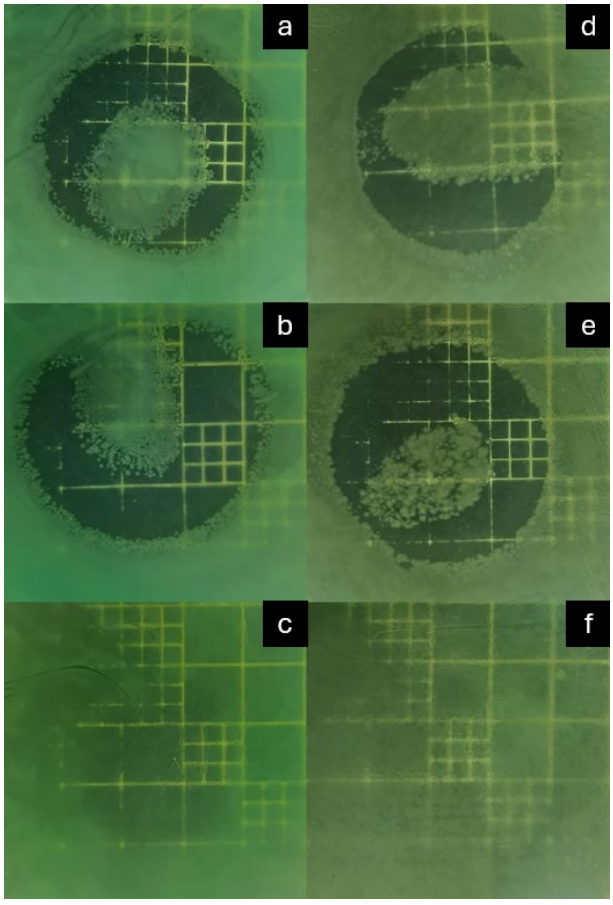
The results of the mCIM and eCIM tests were positive, indicating metallo-β-lactamase production. Out of all the antimicrobial resistance genes screened, only the SPM-1 gene was detected. This result suggests that the resistance to β-lactams in strain PA350 was conferred by the metallo-β-lactamase SPM-1.

The PA350 clinical strain used in the experiments was classified as an XDR strain as it is non-susceptible to ≥1 agent in all but ≤2 antimicrobial categories[9]. The Metallo-β-lactamase SPM-1 was responsible for resistance to all β-lactams tested, and the resistance to other classes indicates many resistance mechanisms in this specific strain. These results justify using this strain as an experimental model in this study.

High-frequency inhibition challenge

Compared to the control group, an average inhibition area of 7.68 cm² was observed in strain ATCC 27853 after 10 minutes of treatment, and 5.9 cm² after 5 minutes of treatment ($p < 0.0001$ and $p=0.0002$, respectively). There was no statistically significant difference between the two exposure times. Regarding the XDR-PA350 strain, the average area of inhibition was 6.43 cm² after 10 minutes ($p=0.0002$) and 6.74 cm after 5 minutes ($p=0.0023$) compared to the control. The inhibited areas fall between 47.2% and 61.44% when compared with the total area covered by the electrode. For this strain, inhibition areas were also similar after the two treatment times, with no statistically significant difference (Figure 2 and Figure 3).

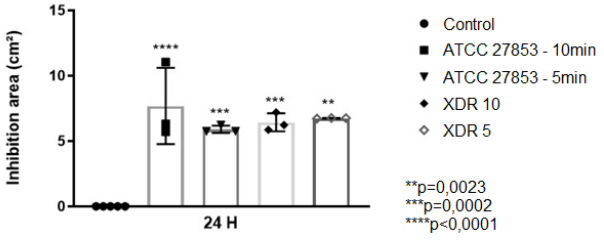
Figure 2 - *Representative images of the inhibited area of each experimental group.*



A square area of around 25cm² from each experimental group, with a single sample of each triplicate representing each group, can be seen in the figure above. Plates seeded with P. aeruginosa strain ATCC 27853 can be seen on the left (a, b, and c), while the clinical strain XDR PA350 is shown on the right (d, e, and f). Experimental groups 5M (a and d), 10M (b and e), and control (c and f) can be differentiated by the lettering in the upper right corner of each figure.

Source: This study.

Figure 3 - *Average inhibition area (cm²) for each treatment group*



The inhibited areas, in cm², were compared between plates seeded with P. aeruginosa ATCC 27853 and clinical strain XDR PA350, having been exposed to high-frequency treatment for 0, 5, and 10 minutes. Inhibition areas were manually selected and calculated through ImageJ software. These data do not show a statistically significant difference between the inhibition areas of experimental groups, but they do show a statistically significant variation between control and experimental groups ($p<0.05$).

Source: This study.

Discussion

Petri dishes to which high-frequency currents were applied prior to incubation exhibited significant inhibition areas in their center, where the electrode was positioned. This indicates that the method was effective in inactivating even multidrug-resistant strains, with no statistically significant difference between standard and resistant strains.

Similar areas of inhibition across five and 10-minute treatments indicate that antimicrobial activity was limited by electrode area and did not appear to become more effective with increasing treatment duration, with shorter treatment times potentially being more effective over larger areas. This result corroborates the findings of LOVATO et al.[6], which found that a 30-second treatment was effective in controlling low concentrations of E. aerogenes and Staphylococcus aureus, while also expanding the known efficacy of the method to MDR Pseudomonas aeruginosa against which inhibition with high-frequency equipment had not yet been fully explored, as well as the efficacy of high frequency equipment against higher concentrations of bacterial cells. In the aforementioned study, lower bacterial concentrations (10^1 CFU) were challenged with the technique, while in the present study, a total CFU count higher by several logarithmic scales ($1{,}5x10^9$ CFU) was sown onto the agar plates.

This treatment method remained effective against high bacterial concentrations, indicating robust antimicrobial activity against a high bacterial load and potential benefit against biofilms. However, as can be seen in Figure 2, the inhibition area appears to be limited by the size of the electrode. Future studies could investigate a quantitative approach to the antibacterial effects of high-frequency ozone generators against MDR bacteria and their efficacy against biofilm cultures.

Although the use of ozone as an antibacterial agent has been widely documented, few studies have been conducted on the efficacy of high-frequency ozone generators against bacteria, and even fewer have investigated their effect on drug-resistant strains[18].

Exposure of Pseudomonas aeruginosa to antibiotics and oxidising agents, such as those used in hospital settings, can induce significant changes in the microorganism's gene expression and stress response mechanisms. These strains might encounter reactive oxygen species (ROS) and reactive chlorine species (RCS) in these hospital environments, which can damage cellular components and lead to cell death. XDR P. aeruginosa strains found in hospital settings, such as the one investigated in this study, might, therefore, have various adaptations, helping the bacterium withstand oxidative stress and enhancing its ability to survive in hostile environments, including those involving disinfection with oxidising agents[19]. As a result of this adaptive response, P. aeruginosa enhanced antioxidant mechanisms may reduce its susceptibility to ozone-based inhibition methods, such as high-frequency ozone-generating equipment. The strain's robust antioxidant defenses can neutralise the oxidative damage caused by ozone, making it less effective as an inactivation method[19]. This reduced susceptibility could potentially mitigate the effects of high-frequency equipment.

However, in this study, high-frequency equipment was shown to result in significant growth inhibition of clinically isolated XDR P. aeruginosa. The high-frequency treatment applied to the experimental groups appears to have effectively inhibited the growth of both P. aeruginosa ATCC 27853 and clinical strain XDR PA350, indicating that antibiotic resistance mechanisms do not affect susceptibility to high-frequency treatment. These results indicated that high-frequency therapy may be an effective tool for treating infections with MDR or XDR microorganisms, delaying or preventing the selection and emergence of new resistant strains due to the multi-targeted nature of ozone as a decontaminant, which indicates that this technique is unlikely to induce bacteria into expressing antibiotic resistance genes.

Other studies have investigated the use of high-frequency equipment in dentistry and demonstrated its effectiveness against Enterococcus faecalis, Staphylococcus aureus, and Staphylococcus epidermidis in disinfecting root canals[20], as well as in the treatment of acne vulgaris[21]. Other physical methods of antisepsis have also been investigated, such as high voltage, which has been shown to be effective in treating P. aeruginosa infections in experimentally induced burns in mouse models[22].

The ability to inhibit a high concentration of Pseudomonas aeruginosa, which is shown in literature to be one of the most frequent microorganisms that infect burn wounds[22], indicates that this method would be beneficial in their treatment, potentially reducing treatment costs and duration of hospital stay, as well as reducing the usage of antibiotics associated with these infections.

Due to the nature of the equipment involved, the high-frequency ozone generation could be used to treat accessible, superficial infections such as those afflicting burn injuries or skin ulcers. In addition, this method's ease of use and low cost allow it to be used in hospitals as a substitute or supplement to antibiotic treatment, helping to treat infections and prevent the spread of antibiotic resistance. Therefore, as healthcare-associated infections are often caused by MDR/XDR strains, especially those that lead to fatalities, the use of high-frequency equipment mediated ozone generation is a potentially useful alternative to chemical disinfectants, as microorganisms have shown resistance to disinfectants, as well as the expression of antibiotic resistance having been linked to exposure to disinfectants also prevent hospital-acquired infections through disinfecting surfaces and equipment.

Future studies should consider investigating the impact of varying ozone concentrations on a wider array of multidrug-resistant strains to explore further the potential of high-frequency treatment in combating bacterial infections. Understanding the dose-response relationship between ozone exposure and bacterial inhibition could

provide insights into optimising treatment parameters for different bacterial profiles. Additionally, studies comparing the efficacy of high-frequency treatment in liquid versus solid media could elucidate the influence of different environmental conditions on the treatment's effectiveness.

Another critical aspect to examine is the long-term impact of high-frequency treatment on the viability of biofilms formed by resistant strains. Due to their inherent resistance to antibiotics and disinfectants, biofilms pose a significant challenge in clinical settings[1. D]etermining whether high-frequency ozone generation can disrupt established biofilms or prevent their formation could be crucial in developing comprehensive infection control strategies. These studies would help assess the feasibility of incorporating high-frequency treatment into existing protocols for managing healthcare-associated infections.

Finally, the potential synergistic effects of combining high-frequency treatment with traditional antibiotic therapy warrant exploration. Given that high-frequency treatment targets bacteria through a different mechanism than antibiotics, it could potentially enhance the efficacy of antibiotic treatments or reduce the necessary dosage to achieve bacterial inhibition. This approach could help mitigate the development of antibiotic resistance while still providing effective treatment options for infections caused by multidrug-resistant bacteria.

Conclusion

High-frequency treatment effectively inhibited the growth of XDR P. aeruginosa in vitro, with noticeable inhibition after only 5 minutes of challenge. There was no statistically significant relationship between the antibiotic resistance profile of the strain and the efficacy of inhibition by the method tested here. This finding indicates that common antibiotic resistance mechanisms do not affect susceptibility to this technique, making it attractive as a potential tool for disinfecting surfaces and treating accessible or surface infections.

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