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RESEARCH ARTICLE OPEN ACCESS

Effect of cold plasma on some virulence factors in *Pseudomonas aeruginosa* isolated from clinical cases

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Abstract
Objective: *Pseudomonas aeruginosa* infections pose a significant challenge due to their ability to resist various antibiotics and form biofilms. Excessive antibiotic use accelerates the development of multidrug-resistant *P. aeruginosa*, which is further enhanced by its ability to form biofilms. Therefore, alternative treatment options are needed to control antibiotic resistance and biofilm formation. Cold plasma contains a mixture of reactive oxygen and nitrogen species, which directly kill bacteria, modify virulence factors, and enhance innate immune responses. This study aimed to evaluate the effect of cold plasma on *P. aeruginosa* antibiotic resistance and ability to form biofilms.
Methods: Clinical isolates of *P. aeruginosa* were exposed to cold plasma for different periods (3, 6, and 9 minutes). Changes in susceptibility to several antibiotics were assessed using the disk diffusion method, and biofilm formation was examined using crystal violet staining.
Results: The results demonstrated the clear effectiveness of cold plasma against antibiotic resistance and the biofilm-forming ability of *P. aeruginosa*. The effectiveness of the tested antibiotics was increased, and the bacteria lost their ability to form biofilms by 100%. The results support the idea of using cold plasma technology as an alternative to antibiotics to eliminate pathogenic and antibiotic-resistant bacteria, thereby treating diseases associated with these bacteria.
Conclusions: The results demonstrated the potential of using cold plasma technology as an alternative to antibiotics to treat diseases associated with this bacterium.

Keywords: Cold plasma, Antibiotic resistance, Biofilms, *P. aeruginosa*, Clinical isolates

Plain English Summary

P. aeruginosa is an opportunistic pathogen notable for its biofilm-forming ability and antibiotic resistance. This study aimed to assess the effects of cold plasma, a non-thermal physical agent rich in reactive species, on clinical isolates of *P. aeruginosa* for varying exposure durations. Associated with these results was a remarkable enhancement in biofilm formation inhibition and antibiotic susceptibility. These findings emphasise the promise of cold plasma technology for the treatment of infections caused by multidrug-resistant bacteria.

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Introduction

Cold plasma has recently emerged as a promising technology that offers high efficiency in inhibiting microbes and their toxins, including bacteria, fungi, and viruses, with minimal adverse effects and environmental friendliness compared to chemical disinfectants (1). Cold plasma is a partially ionised, active gas and has a relatively low temperature, ranging from 30–50°C (2, 3). It contains a mixture of reactive oxygen and nitrogen species, which directly kill bacteria, modify virulence factors, and enhance innate immune responses (4). Cold plasma is typically produced by heating a gas, which generates free radicals, reactive oxygen species (ROS-O, O2, O3, OH-), and reactive nitrogen species (RNS-NO, NO2), combined with UV radiation and an electric field, which together produce a combined effect (5). These species interact either with the cell membrane or with intracellular functions (6). They induce cell death through multiple mechanisms. These mechanisms include activating the mitochondrial pathway, triggering endoplasmic reticulum stress, generating reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing DNA damage, arresting the cell cycle, and modifying signalling pathways.

In addition, oxidative stress induced by cold plasma can lead to significant changes in RNA and DNA within the nucleus (7). Cold plasma technology relies on the production of reactive species at low temperatures, making it an alternative to antimicrobial therapy while minimising thermal damage to biological tissues (8, 9). Its applications are primarily in biomedicine and therapy (10). Cold plasma is widely used in oral therapy, tissue regeneration, wound healing, and cancer treatment (11). Cold plasma selectively targets cancer cells, inducing apoptosis and inhibiting tumour growth while sparing healthy cells, making it an attractive option for treating localised cancer with minimal side effects (12). Breast, lung, and skin cancers have been successfully treated with cold plasma (13). It is also used to sterilise dental and surgical equipment used in hospitals (14). It is also used to treat dental diseases and whiten teeth, as it is highly effective in inhibiting bacteria, sterilising oral diseases, and is painless before cavity preparation, root canal treatment, and treating disturbed tissues (15).

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, motile, aerobic bacterium. It is widely found in nature and isolated from soil, plants, water, and animals (16). It is an opportunistic bacterium that causes a wide range of diseases, including pneumonia, wound infections, burn infections, endocarditis, and meningitis, in addition to numerous nosocomial infections, which have been associated with high rates of morbidity and mortality among elderly and immunocompromised patients (17). These high rates are linked to the rapid development of multidrug resistance and the resulting difficulties in treating *P. aeruginosa*.

This resistance is achieved through several factors, including reduced membrane permeability, the use of efflux pumps, specialised enzymatic activity, horizontal resistance-gene transfer, mutations, and biofilm formation (18, 19). Biofilm formation can facilitate the survival of *P. aeruginosa* in the body by protecting it from the host immune system and antimicrobials. It also acts as a reservoir for antibiotic resistance genes, leading to the development of multidrug-resistant *P. aeruginosa* strains (20). Thus, treating *P. aeruginosa* infections poses a significant challenge due to their ability to resist various currently available antibiotics (21). This has necessitated the need for alternative therapeutic options to antibiotics to control biofilm formation in clinical settings. Cold plasma may be an alternative to antibiotics (22). Therefore, the current study aims to find an environmentally friendly method with minimal side effects to control *P. aeruginosa* instead of antibiotics.

Materials and Methods

Bacterial Isolates

One hundred clinical samples were collected from various sources, including burns, wounds, ears, tonsils, and urine, from patients aged 1–60 years for both genders, males and females, at Al-Diwaniyah Teaching Hospital, Afak General Hospital, and the Specialised Burns Centre during the period from October 2024 to January 2025.

Diagnosis

All bacterial isolates were initially identified using Gram staining and biochemical tests (oxidase, catalase, citrate uptake test, and growth at 4 and 42°C). Diagnosis was confirmed using the VITEK2 device and PCR by detecting the presence of the 16S rRNA gene. Primers for this gene were designed using the NCBI-GenBank Database and the Primer3plus primer design software. These primers were prepared by the Korean company Macrogene, as shown in Table 1.

Antibiotic susceptibility test (AST)

Susceptibility testing was performed for the antibiotics Piperacillin, Amoxicillin/clavulanate, Ceftriaxone, Cefotaxime, Imipenem, Gentamicin, Amikacin, Ciprofloxacin, and Levofloxacin using the Kirby-Bauer disk diffusion method according to

the recommendations of the Clinical Laboratory Standards Institute (CLSI). The bla OXA-10, *aac(6')-Ib*, and *gyrA* genes were also screened for resistance to antibiotics, β-lactams, aminoglycosides, and fluoroquinolones, respectively, using PCR. Primers for each gene were designed using the NCBI-GenBank Database and the Primer3plus primer design software. These primers were prepared by the Korean company Macrogen (Table 1).

Detection of Biofilm

The ability of *P. aeruginosa* to form biofilms was detected phenotypically by crystal violet staining using a 96-well microtiter plate, and molecularly using PCR to detect the presence of the lasR gene, a diagnostic marker for biofilm production by this bacterium. The primers for this gene were designed using the NCBI-GenBank Database and the Primer3plus primer design software. These primers were prepared by the Korean company Macrogen (Table 1).

Table (1): Shows the names of the primers, their nitrogenous base sequences, and amplification product size

Primers		nitrogenous base sequences (5'-3')	amplification product size	sources
P. aeruginosa detection	F	GAGTACGGTAGAGGTGGTAGAA	559 bp	(23)
	R	GTAAGGGCCATGATGACTTGA		
LasR	F	AAGTGGAAAATTGGAGTGGAG	130 bp	(24)
	R	GTAGTTGCCGACGATGAAG		
bla_{OXA-10}	F	TCCTGCGCTACCAATGACTT	760 bp	This study
	R	TGCGACACCAGGATTTGACT		
aac (6')-Ib	F	GGAACAGTACTTGCCAAGCG	502 bp	This study
	R	GATCACCGCTTCCCTCATGA		
GyrA	F	CCTCAACAACCTCTATGCCC	511 bp	This study
	R	GCCGATCAGGTTGAGGATTT		

lasR/ Legionella activator of system Regulation; bla OXA/ Beta-lactamase OXA-type gene; *aac(6')-Ib*/aminoglycoside 6'-N-acetyltransferase type Ib; *gyrA* / DNA gyrase subunit A gene

Plasma technology

A Plasma Jet cold plasma device powered by argon gas was used. It was locally designed in the Physics Department laboratory, College of Education, Al-Qadisiyah University. The device's operating principle relies on a high-voltage electrical discharge to generate plasma (20) (Figures 2, 3).

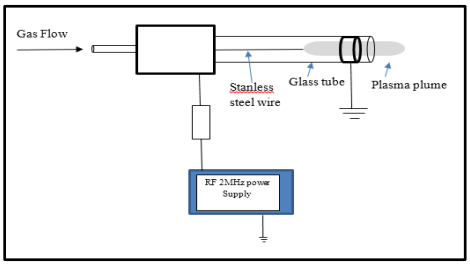

Figure 1: Cold plasma device (20)

Figure 2: is a photograph of the cold plasma device

Exposure

Bacterial suspensions were prepared at a concentration of 0.5 McFarland, with three replicates for each isolate and a control group. Using the disk diffusion method, the bacteria were cultured on Muller-Hinton agar medium, and the plates were incubated for 24 hours at 37°C (21). After bacterial growth appeared, each plate was exposed to cold plasma using an argon-powered Plasma Jet device at 25°C, with an 11 kV power source, a frequency of 12 kHz, and a gas flow rate of 4 litres per minute. The nozzle of the device was directed directly at the centre of the plate, maintaining a distance of 1 cm between the nozzle and the plate surface (20). Different periods (3, 6, and 9) were applied to each isolate.

Evaluation of the Antibacterial Effect of Cold Plasma

After exposing the bacterial isolates to cold plasma for different periods, the plates were left at room temperature for 10 minutes to allow the plasma-treated surface to stabilise physically and chemically and prevent unwanted interactions. Bacteria were then taken from the exposure area, and antibiotic susceptibility testing was repeated using the disk diffusion method and crystal violet staining using the microtiter plate method to evaluate the effect of cold plasma on antibiotic resistance and biofilm formation.

Results

The phenotypic and biochemical results showed that 9 (9%) of the isolates were *P. aeruginosa*, of which 5 were molecularly identified using PCR. Primer amplification results targeting the 16S rRNA gene showed that all bacterial isolates contained 100% of this gene, with a product size of 559 bp (Figure 3).

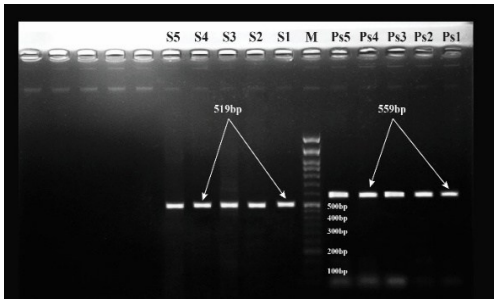

Figure 3: Primer amplification

The results of the disk diffusion antibiotic susceptibility test showed that all isolates were 100% resistant to the antibiotics. PCR results also showed that the isolates possessed the *aac(6')-Ib* gene, responsible for 100% resistance to aminoglycoside antibiotics, and the *gyrA* gene, responsible for 60% resistance to fluoroquinolones. None of the isolates possessed the *blaOXA-10* gene (Figures 4, 5).

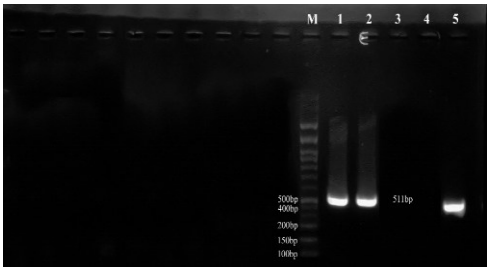

Figure 4: Agarose gel electrophoresis illustrating the PCR analysis of *gyrA* gene in *P. aeruginosa* isolates

M represents the marker ladder with a size of 100-1500 bp, and holes (1, 2, 5) show the isolates positive for the *gyrA* gene with a PCR product of 511 bp. Holes (3, 4) show the isolates negative for this gene. Electrophoresis conditions: agarose gel (1.5%), potential difference (100 V), current (80 A), time (1 h)

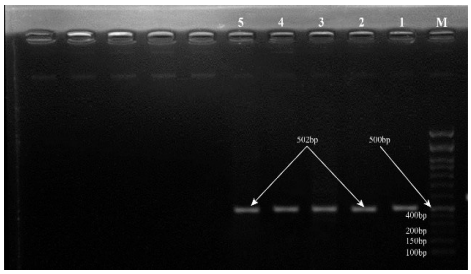

Figure 5: Agarose gel electrophoresis showing the PCR analysis of the *acc (6')-Ib* gene in *P. aeruginosa* isolates

M represents the marker ladder with a size of 100-1500 bp, and holes (1, 2, 3, 4, 5) show the isolates positive for the acc (6')-Ib gene with a PCR product of 502 bp. Electrophoresis conditions: agarose gel (1.5%), potential difference (100 V), current (80 A), time (1 h)

The results showed that these isolates had a 100% and robust ability to form biofilms. Molecular detection revealed the *lasR* gene responsible for biofilm formation in *P. aeruginosa*. The results showed that all isolates were biofilm-producing and possessed this gene at 100% expression (Figure 6).

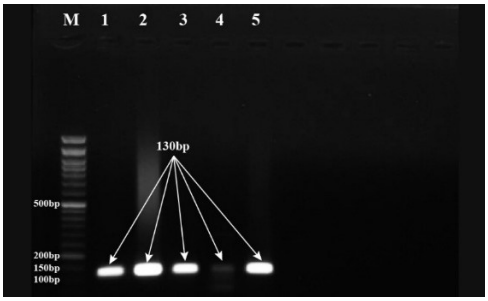

Figure 6: Agarose gel electrophoresis showing the PCR analysis of the *lasR* gene in *P. aeruginosa* isolates

M represents the marker ladder with a size of 100-1500 bp, and holes (1, 2, 3, 4, 5) show the isolates positive for the *lasR* gene with a PCR product of 130 bp. Electrophoresis conditions: agarose gel (1.5%), potential difference (100 V), current (80 A), time (1 h)

The results showed a decrease in the resistance of bacterial isolates to antibiotics as the exposure duration increased. Consequently, there was a significant increase in the effectiveness of antibiotics after exposure to cold plasma, as an increase in the diameters of inhibition was observed for most antibiotics (Table 2).

Table 2: Diameters of inhibition of antibiotics for *P. aeruginosa* after exposure to cold plasma

Antibiotics	Ear infections				Tonsillitis infections				Wound infections				LSD
	0	3	6	9	0	3	6	9	0	3	6	9	
Piperacillin	9Aa	10Aa	15Ab	15Ab	15Ab	15Ab	20Ac	20Ac	10Aa	18Ad	22Ae	24Af	1.46
Augmentin	0Ba	0Ba	0Ba	0Ba	15Ab	25Bc	25Bc	25Bc	20Bd	25Bc	25Bc	25Ac	1.18
Ceftriaxone	0Ba	0Ba	0Ba	0Ba	8Cb	0Ca	14Cc	15Cc	10Ad	11Cd	14Cc	15Bc	1.24
Cefotaxime	0Ba	0Ba	0Ba	15Ab	15Ab	0Ca	16Dbc	16Cbc	14Cb	14Db	15Cb	17Cc	1.40
Imipenem	30Ca	34Cb	33Cb	35Cb	24Dc	27Dd	25Bc	27Dd	23De	24Be	27Dd	27Dd	1.56
Gentamicin	0Ba	0Ba	0Ba	0Ba	0Ea	0Ca	0Ea	0Ea	13Cb	15Dc	15Cc	16Cc	1.12
Amikacin	13Da	13Da	14Dab	15Ab	10Fc	12Ea	12Fa	13Fa	10Ac	14Db	15Cb	15Cb	1.32
Ciprofloxacin	0Ba	0Ba	8Ea	0Ba	15Ab	25Bc	25Bc	28Dd	20Be	24Bc	24Bc	25Ac	1.16
Levofloxacin	0Ba	0Ba	0Ba	0Ba	15Ab	25Bc	27Gd	27Dd	20Be	24Bc	25Bc	25Ac	1.32
LSD	0.42	0.45	0.52	0.5	0.86	1.16	1.36	1.21	1.41	1.28	1.13	1.45	

Results of exposing bacterial isolates to cold plasma showed a clear decrease in the optical density associated with biofilm formation, indicating that cold plasma inhibits biofilm

formation in a manner directly proportional to the increase in exposure duration. Significant differences ($P < 0.05$) were observed between the control group (0 minutes) and the treated isolates (3, 6, and 9 minutes). The control group (without exposure) had the highest value in biofilm formation, while the isolates treated with plasma for 9 minutes showed a loss of their ability to form biofilms. Biofilm formation was classified as strong (>0.24), medium (0.12-0.24), and none (<0.12) (22). Optical density values of isolates treated with cold plasma for different durations (3, 6, 9) minutes were compared with a control (no exposure) (Table 3).

Table (3) biofilm optical density values of *P. aeruginosa* isolates from different regions and with different exposure periods

Isolation site	Exposure time (mins)			
	0	3	6	9
Ear infections	0.58 Aa (strong)	0.09 Ab (None)	0.08 Ab (None)	0.07 Ab (None)
Tonsillitis infections	0.55 Aa (strong)	0.11 Ab (None)	0.09 Ab (None)	0.06 Ab (None)
Wound infections	0.55 Ba (strong)	0.09 Ab (None)	0.07 Ab (None)	0.07 Ab (None)
LSD	0.061			

Capital letters indicate a comparison between rates vertically, and lowercase letters indicate a comparison between rates horizontally; Different letters between any two rates (vertically or horizontally) indicate a significant difference between the rates

Discussion

Of the 100 samples collected, 9 (9%) *P. aeruginosa* isolates were found, which agrees with (22, 25). This is lower than the findings of Ding et al. (26), which were 15.4% and 19.4%. This may be attributed to differences in sample size, study population, length of hospital stay, exposure of patients to high-risk medical devices, and prescription of antibiotics without antibiotic susceptibility testing (27). Inconsistencies in the prevalence of *P. aeruginosa* between studies may also be due to variations in clinical samples, hospitals, populations examined, geographic areas, and healthcare practices (28).

According to our results, the *aac(6')-Ib* gene responsible for aminoglycoside resistance was detected in *P. aeruginosa*, and the presence of this gene was detected in all isolates at a rate of 100%. Our results were consistent with the results of the study by Abednezhad et al. (29), while the percentage was lower in the study, which was 74% (30). However, the *gyrA* gene responsible for resistance to fluoroquinolones was detected in *P. aeruginosa*. The results showed the presence of this gene in three isolates at a rate of 60%. This percentage is close to the study by Rieuwpassa et al. (31) and Rehman et al. (32), which were 54.5% and 75%, respectively. The percentage was higher in the study of Al-Ramlawee (33) and Abed & Kareem (34) that reached 95.6% and 100%, respectively.

These bacteria isolated from tonsillitis infections after exposure to cold plasma showed sensitivity to the antibiotics Piperacillin, Ciprofloxacin, and Levofloxacin, after previously being resistant to them. The diameter of inhibition for these antibiotics before exposure was 15 mm; after exposure, the diameters were 20 mm, 28 mm, and 27 mm, respectively, at 9 minutes. However, the other antibiotics showed increased efficacy with increasing exposure time. In wound infections, post-exposure results showed increased diameters of inhibition for Ceftriaxone and Cefotaxime, and increased efficacy for Augmentin and Imipenem. It is worth noting that these bacteria were resistant to Piperacillin, Gentamicin, Amikacin, Ciprofloxacin, and Levofloxacin before exposure. After exposure, the bacteria became sensitive to it, with the inhibition diameters before exposure being 10 mm, 13 mm, 10 mm, 20 mm, and 20 mm, respectively. After exposure, the inhibition diameters became 24 mm, 16 mm, 15 mm, 25 mm, and 25 mm, respectively. Cold plasma treatment improves the effectiveness of antibiotics against *P. aeruginosa*, and in some cases, the bacteria regain their sensitivity to antibiotics. The results of this study agree with Paldrychová et al. (35), which showed that cold plasma treatment increased the effectiveness of relatively low concentrations of antibiotics. It is also agreed with Maybin et al. (36), who showed that plasma treatment enhanced the sensitivity of *P. aeruginosa* to antibiotics. Also, the results of our study were similar to Brun et al. (37), which showed that plasma had similar efficacy to antibiotics, but showed a faster killing rate.

The statistical analysis showed significant differences at the 5% significance level between exposure times and isolates based on their source. The source of the isolate plays an important role in its ability to form biofilms. This may be because bacteria in the aforementioned isolation site encounter a volatile surface environment

containing chemical and physical inhibitory factors. These factors combined prevent bacterial anchoring and biofilm. Therefore, exhibit a higher response to cold plasma treatment. The underlying mechanism of cold plasma's anti-biofilm effect may involve a combination of direct damage to bacterial cells, disruption of the extracellular matrix, and changes in the expression of genes related to biofilm formation and virulence, leading to biofilm destruction (19).

Cold plasma affects the essential components of biofilms. Cold plasma inactivates EPS by oxidising lipids, modifying and degrading proteins, and breaking the chemical bonds of carbohydrates, ultimately leading to EPS destruction (38). This biochemical change in EPS is attributed to oxidative processes caused by ROS and RNS molecules. By inactivating EPS, the adhesion of the biofilm to the mounting surface decreases, which in turn disrupts or even disintegrates the three-dimensional structure of the biofilm (39). Cold plasma can convert bacteria from a biofilm to a planktonic state. It can also kill bacteria in combination with other types of disinfectants that have a weak effect on biofilms but an excellent effect on bacteria (40, 41).

Conclusions

The study concludes that clear effectiveness of cold plasma against antibiotic resistance and the biofilm-forming ability of *P. aeruginosa*. The effectiveness of the tested antibiotics was increased, and the bacteria lost their ability to form biofilms by 100%. The results support the idea of using cold plasma technology as an alternative to antibiotics to eliminate pathogenic and antibiotic-resistant bacteria, and thus treat some diseases associated with this bacterium. The results demonstrated the potential of using cold plasma technology as an alternative to antibiotics to treat diseases associated with this bacterium.

List of Abbreviations

°C: degrees Celsius
ROS: Reactive Oxygen Species
RNS: Reactive Nitrogen Species
DNA: Deoxyribonucleic Acid
PCR: Polymerase Chain Reaction
16S rRNA: 16S ribosomal ribonucleic acid
NCBI: National Centre for Biotechnology Information
GenBank: Genetic Sequence Database
CLSI: Clinical Laboratory Standards Institute
β-lactamase: beta-lactamase
bla OXA-10: beta-lactamase gene-oxacillinase-10
aac(6')-Ib: Aminoglycoside 6'-N-acetyltransferase type Ib
gyrA: DNA gyrase subunit A gene
lasR: Las quorum-sensing Regulator
h: Hour
V: Volt
A: Ampere
kV: Kilovolt
kHz: Kilohertz
M: Marker ladder
bp: Base pair
LSD: Least Significant Difference
cm: Centimetre
mm: Millimetre
EPS: Exopolysaccharides

Declarations

Ethics approval and consent to participate

This study was approved by the local publication ethics committee of the Department of Biology, Faculty of Education, Al-Qadisiyah University, Iraq, under approval reference number 216 dated September 19, 2024.

Consent for publication

The authors agree to publication in this journal.

Availability of data and materials

All relevant data are within the paper and its supporting information files.

Competing interests

The authors have declared that no competing interest exists.

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The study is part of a master's degree, but there are no funds for this project.

Authors contributions

FZS: Methodology and Writing-Original Draft Preparation; Software, Data Curation.
A-SMKA: Supervision, Investigation, Writing-Review and Editing.

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