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Estimate of cold plasma on antibiotic resistance and biofilm formation in Staphylococcus aureus isolated from clinical cases

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Abstract:

Background: *Staphylococcus aureus* is a commensal and opportunistic bacterium and the leading cause of human bacterial infections worldwide. *S. aureus* exhibits significant resistance to antibiotics, particularly methicillin-resistant Staphylococcus aureus (MRSA) strains. This resistance is a growing global concern, as *S. aureus* can rapidly adapt to various antibiotic treatments, making these bacteria difficult to treat. The ability to form biofilms enhances this resistance, allowing *S. aureus* to persist in infections and become resistant to antibiotics. Therefore, alternative treatment options to antibiotics are needed to control antibiotic resistance and biofilm formation, and cold plasma may be an alternative. Objective: This study aimed to evaluate the effect of cold plasma on *S. aureus* antibiotic resistance and its ability to form biofilms. Methodology: 100 clinical samples were collected, isolated, and identified. *S. aureus* isolates were exposed to cold plasma for different time periods (3, 6, and 9 minutes). Changes in susceptibility to several antibiotics were assessed using the disk diffusion method. A biofilm formation assay was also performed using crystal violet staining. Results: The results demonstrated the clear effectiveness of cold plasma against *S. aureus* antibiotic resistance and ability to form biofilms. The effectiveness of the tested antibiotics was increased, while 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 these bacteria. Conclusion: The results demonstrated the potential of using cold plasma technology as an alternative to antibiotics to treat diseases associated with *S. aureus*.

Keywords: *S. aureus*, cold plasma, antibiotic resistance, biofilms, clinical isolates, biosterilization

Introduction:

Staphylococcus aureus is a Gram-positive, non-motile, non-spore-forming, oxidase-negative, hemolytic, catalase-positive, and coagulase-positive bacterium. It is a facultative anaerobe that obtains energy for growth through aerobic respiration or fermentation (Tigabu & Getaneh, 2021). *S. aureus* is also characterized as a commensal and opportunistic bacterium

(Yamazaki et al., 2024). *S. aureus* plays a significant role in causing infections in both hospitals and the community, ranging from minor infections to life-threatening infections (Alkan et al., 2023; Abdulrazzaq et al., 2025). It is a major cause of human bacterial infections worldwide, the most common cause of skin and soft tissue infections, and can cause various other infections, including pneumonia and osteomyelitis, as well as life-threatening infections such as sepsis and infective endocarditis (Yamazaki et al., 2024; Abbood and Hateet, 2025).

Various virulence factors contribute to these infections, including surface proteins, enzymes, toxins, and others. These factors play an important role in helping *S. aureus* invade, colonize, and survive in the host to cause staphylococcal diseases (Pal et al., 2020). Cold plasma has recently emerged as a modern technology that is highly efficient in inactivating microbes and their toxins, including bacteria, fungi, and viruses, with minimal adverse effects and environmental friendliness compared to chemical disinfectants (Subrahmanyam et al., 2024; Salman and Ahmed, 2025). Cold plasma is a partially ionized, active gas (Smith et al., 2024) with a relatively low temperature of 30–50°C (Von Woedtke et al., 2019). It contains reactive oxygen and nitrogen species, which directly kill bacteria, modify virulence factors, and enhance innate immune responses (Smith et al., 2024). 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 (Akpor et al., 2024). These species interact either with the cell membrane or with intracellular functions (Tanaka et al., 2020).

It induces 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 signaling pathways. Furthermore, oxidative stress induced by cold plasma can lead to significant changes in RNA and DNA within the nucleus (Jeong et al., 2024). Cold plasma technology relies on the production of reactive species at low temperatures, making it an alternative to antimicrobial therapy while minimizing thermal damage to biological tissues (Karthik et al., 2024; Mumtaz et al., 2023). Its applications are primarily in biomedicine and therapy (Karthik et al., 2024). It is widely used in oral medicine, tissue regeneration, wound healing, and cancer treatment (Thomas et al., 2025). Cold plasma selectively targets cancer cells, inducing apoptosis and inhibiting tumor growth while sparing healthy cells, making it an attractive option for treating localized cancer with minimal side effects (Thomas et al., 2024).

Breast, lung, and skin cancers have been successfully treated using cold plasma (Shakya et al., 2022). Cold plasma is also used to sterilize dental and surgical equipment used in hospitals (Mohammed & Al-Marjani, 2024). It is also used to treat dental diseases and whiten teeth, as it is highly effective in inhibiting bacteria, sterilizing oral diseases, and is painless before cavity preparation, root canal treatment, and treating disturbed tissues (Adnan Hammudi et al., 2023). Therefore, the study aims to find an environmentally friendly method with minimal side effects to control *Staphylococcus aureus* instead of antibiotics.

Materials and Methods:

Bacterial Isolates:

100 clinical samples were collected from various sources, including burns, wounds, ear, tonsils, and urine, from patients aged 1–60 years at Al-Diwaniyah Teaching Hospital, Afak General Hospital, and the Specialized Burns Center 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 (Jaffar et al., 2016), as shown in Table (1).

Detection of Biofilm Formation:

The ability of *S. aureus* 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
***S. aureus* detection**	**F**	ACGGTCTTGCTGTCACTTATAG	**519 bp**	(Young *et al.*, 2022)
	R	CACTGGTGTTCCTCCATATCTC		
lasR	**F**	AAGTGGAAAATTGGAGTGGAG	**130 bp**	(Young *et al.*, 2022)
	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		

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 (Shaheed, 2023) + Image (Figures 2, 3).

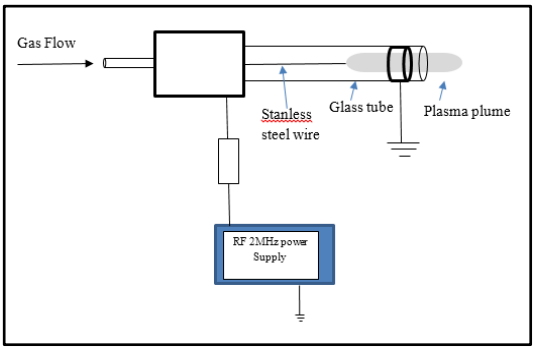


Figure (1) Cold plasma device (Shaheed (2023)

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 (Hudzicki, 2009). 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 liters per minute. The nozzle of the device was directed directly at the center of the plate, maintaining a distance of 1 cm between the nozzle and the plate surface (Shaheed, 2023). Different time 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 of time, the plates were left at room temperature for 10 minutes to allow the plasma-treated surface to stabilize 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.

Statistical analysis:

The study results were statistically analyzed to determine significant differences. The Chi-square test and the LSD test were used, and significant differences were determined at the 5% level using the Statistical Package for Social Sciences (SPSS) version (27).

Ethical Approval:

This study was approved by the local Publication Ethics Committee at Al-Qadisiyah University, Iraq, according to document number 216 dated June 1, 2025.

Results:

The phenotypic and biochemical results showed that 15 (15%) isolates were *S. aureus*, distributed among different clinical cases, 60% (6 isolates) from the skin, 40% (4 isolates) from the urine, 20% (2 isolates) from the nose, 20% (2 isolates) from the sputum, and 10% (1 isolate) from the eyes. Five isolates were molecularly identified using PCR, as the results of the primer amplification targeting the 16S rRNA gene showed that all bacterial isolates contained 100% of this gene with a product size of 519 bp, (Figure 3).

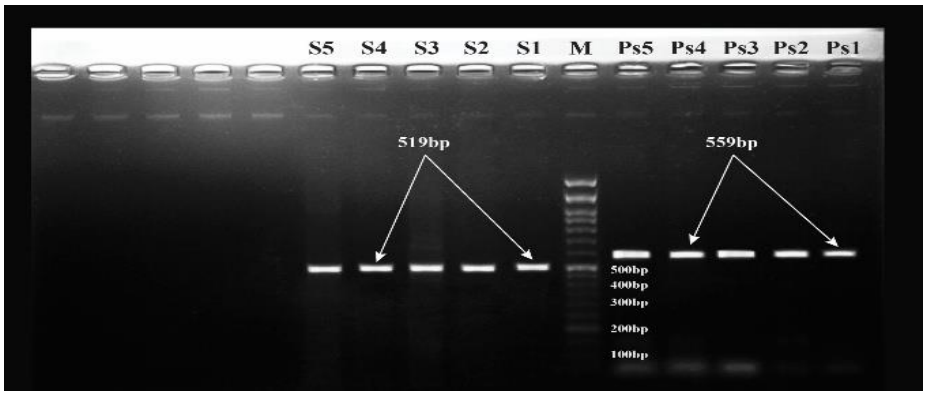


Figure (3) Primer amplification

Antibiotic susceptibility testing:

The results of the disk diffusion antibiotic susceptibility testing showed that the isolates varied in their resistance to the tested antibiotics. The highest resistance rate was 67% to the antibiotic. PCR results also showed that the isolates possessed the blaZ gene, which is responsible for resistance to β-lactam antibiotics, at 80% (Figure 4).

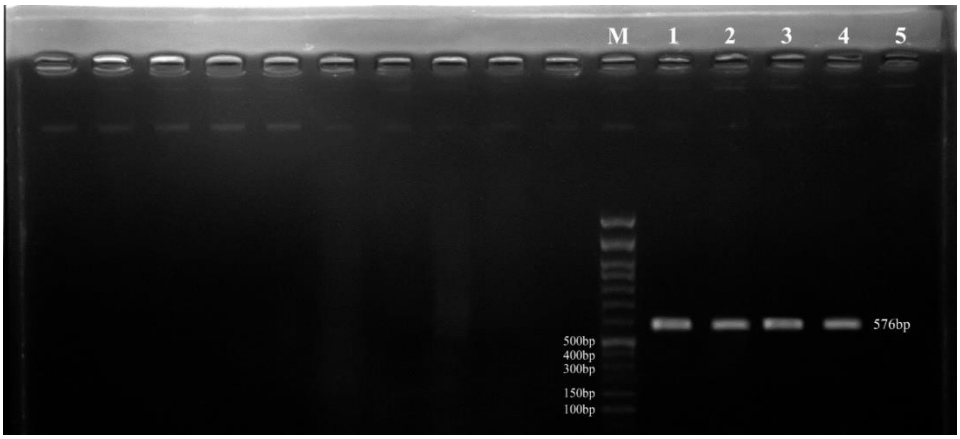


Figure (4): Agarose gel electrophoresis showing the PCR analysis of the blaZ gene in *S. aureus*. M represents the marker ladder with a size of 100-1500 bp, holes (1, 2, 3, 4) show the isolates positive for the blaZ gene with a PCR result of 576 bp, while hole (5) shows the isolate negative for this gene. Electrophoresis conditions: agarose gel (1.5%), potential difference (100 V), current (80 A), time (1 h).

Detection of Biofilm Formation:

Phytochemical detection of biofilm production was performed using the microtiter plate method. Results showed that these isolates were 100% biofilm-forming. Molecular detection revealed the icaD gene, responsible for biofilm formation in *S. aureus*. Results showed that all isolates were biofilm-producing and possessed this gene at 100% expression.

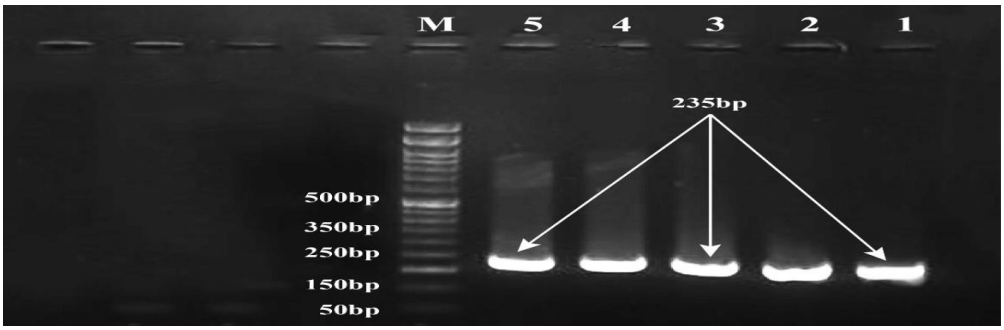


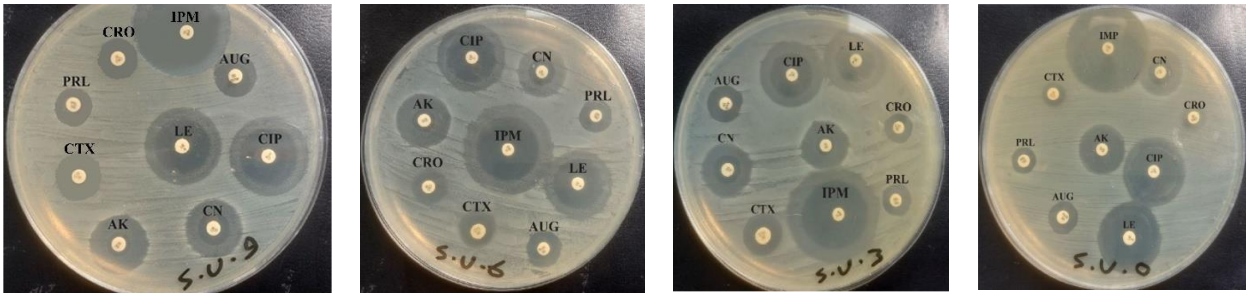
Figure (5)Agarose gel electrophoresis showing the PCR analysis of icaD gene in *S. aureus*. M represents the marker ladder with a size of 100-1500 bp, and the wells (S1, S2, S3, S4, S5) show the isolates positive for icaD gene with a PCR product of 235 bp. Electrophoresis conditions: agarose gel (1.5%), voltage (100 V), current (80 A), time (1 h).

The effect of cold plasma on antibiotic resistance:

The results showed that the resistance of bacterial isolates to antibiotics decreased 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, as shown in Table (2) and Figure (6).

Table (3): Diameters of inhibition of antibiotics for *S. aureus* after exposure to cold plasma

antibiotics	Skin infections				Nasal infections				Urinary tract infections				LSD
	0	3	6	9	0	3	6	9	0	3	6	9	
Piperacillin	15Aa	16Ab	16Ab	16Ab	17Ac	18Ad	18Ad	19Ae	13Af	16Ab	16Ab	16Ab	0.51
Augmentin	18Ba	18Ba	21Bb	21Bb	21Bb	22Bbc	23Bcd	24Bd	16Be	18Ba	18Ba	20Bb	1.04
Ceftriaxone	12Ca	19Bb	18Cb	19Cb	19Cb	18Ab	24Bc	26Cd	14Ce	18Bb	18Bb	18Cb	1.11
Cefotaxime	18Ba	19Ba	Bb 22	Bb 22	13Dc	23Cb	23Bb	23Bb	12Dc	21Cb	bC 21	21Bb	1.34
Imipenem	40Da	40Ca	46Db	47Dc	40Ea	45Db	45Cb	45Db	44Eb	45Db	Db 44	45Db	1.22
Gentamicin	20Ea	20Da	Ba 20	Ca 20	25Fb	24Eb	26Dbc	27Cc	24Fb	25Eb	Eb 25	25Eb	1.3
Amikacin	22Fa	24Eb	bE 25	bE 26	30Gc	34Fd	33Ed	34Ed	25Gb	25Eb	Fe 27	27Fe	1.18
Ciprofloxacin	13Ga	12Fb	Fc 13	13Fc	30Gd	34Fe	33Ef	34Ee	34He	35Fg	Gg 35	35Gg	0.96
Levofloxacin	16Ha	18Bb	19Cb	Cb 19	30Gc	33Gd	33Ed	34Ed	33Jd	33Gd	Ge 35	35Ge	1.01
LSD	0.91	1.12	1.21	1.2	0.56	0.82	1.01	1.06	0.84	0.71	1.21	0.132	



d **c** **b** **a**

Figure (6) Increase in the diameters of inhibition of *S. aureus* antibiotics with increasing exposure time to cold plasma. Where: (a) Control. (b) At 3 minutes (c) At 6 minutes (d) At 9 minutes.

Effect of cold plasma on biofilm formation in *S. aureus:*

The results of exposing bacterial isolates to cold plasma showed a significant decrease in the optical density associated with biofilm formation, indicating that cold plasma inhibits biofilm formation in direct proportion to the increase in exposure time. 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), moderate (0.12-0.24), and non-forming (< 0.12) based on Sultan and Nabiel (2019). The optical density (OD) values of the isolates treated with cold plasma for different periods (3, 6, 9) minutes were compared with a control group (without exposure) (Table 4).

Table (4): Optical density values of *S. aureus* biofilm isolates from different regions and at different exposure times

Isolation site	Exposure time (mins)			
	0	3	6	9
Urinary tract infections	0.57 Aa (strong)	0.14 Ab (medium)	0.11 Ab (None)	0.08 Ab (None)
Skin infections	0.18 Ba (medium)	0.09 Ab (None)	0.08 Ab (None)	0.07 Ab (None)
Nasal infections	0.21 Ba (medium)	0.12 Ab (medium)	0.12 Ab (medium)	0.09 Ab (None)
LSD	0.053			

Discussion:

The 100 samples collected, 15 isolates (15%) were found to be *S. aureus*. Ding et al. (2016) explained that discrepancies in the prevalence of the bacteria between studies could be due to variations in clinical samples, hospitals, populations examined, geographic regions, and healthcare practices (Ding et al., 2016). The isolates in the current study were distributed

unequally across different clinical settings. Kemal et al. (2025) reported that this variation in the prevalence of *S. aureus* may be attributed to differences in sample size, study population, length of hospital stay, patient exposure to high-risk medical devices, and prescription of antibiotics without antibiotic susceptibility testing. The studied isolates demonstrated high resistance to β-lactam antibiotics, which is linked to their possession of the blaZ gene, which is responsible for resistance to β-lactam antibiotics.

Vivekanandan et al. (2025) stated that antibiotic resistance in bacteria poses a serious global health challenge, driven by molecular mechanisms such as gene mutations, efflux pumps, enzymatic degradation of antibiotics, target site modifications, and biofilm formation. Horizontal gene transfer (HGT) accelerates the spread of resistance genes across bacterial populations. These mechanisms contribute to the emergence of multidrug-resistant (MDR) strains, rendering conventional antibiotics ineffective. Biofilm formation is one of the most important mechanisms of antibiotic resistance in bacteria. The isolates in the current study demonstrated a high capacity to form biofilms, which is linked to their high 100% presence of the icaD gene, thus explaining their multiple antibiotic resistances.

After exposing the studied bacterial isolates to cold plasma, the results of the antibiotic susceptibility test for *S. aureus* showed a significant increase in the diameters of inhibition for most antibiotics. This confirms that cold plasma treatment improves the effectiveness of antibiotics against *S. aureus*, and in some cases, the bacteria regain their sensitivity to antibiotics. This is consistent with the findings of Guo et al. (2021), which showed that cold plasma treatment enhances the effects of various antibiotics in reducing *S. aureus*, and bacteria can regain their sensitivity to antibiotics.

Our results were also agreed with Brun et al. (2018), which reported that the effect of antibiotics was synergistically enhanced by cold plasma treatment. The results of our study were also agreed with Namini et al. (2019), whose results showed that cold plasma inhibited bacterial growth and inhibited bacterial growth. Our results also converged with the study of Nam et al. (2021), whose results showed that increasing the treatment time with cold plasma resulted in a decrease in the number of cultured microorganism colonies and an increase in the disinfected area.

According to the aforementioned results, cold plasma generates localized reactive oxygen and nitrogen species, which induces oxidative stress that damages bacterial cell membranes and DNA. It is believed that cell membrane damage or disruption results from the effect of oxidative stress on outer membrane proteins. This increases the susceptibility of bacterial cells to antimicrobials and facilitates their entry into the cell. Therefore, for the treatment of bacterial infections, particularly those caused by antibiotic-resistant pathogens, cold plasma represents a practical alternative or complement to conventional antibiotics (Maybin et al., 2023).

Gram-negative bacteria are generally more susceptible to cold plasma than Gram-positive bacteria. Gram-negative bacteria have a thinner outer membrane and peptidoglycan layer, and the proteins and LPS in the outer membrane are sensitive to

ROS molecules. Therefore, cold plasma damages the outer membrane, leading to damage to the cell wall and cell membrane. In contrast, Gram-positive bacteria are less susceptible to oxidative damage because they lack an outer membrane and are covered by a thick layer of peptidoglycan. As a result, cold plasma cannot easily damage their cell wall, but the active chemicals they produce can enter the cell and cause oxidative damage to intracellular components (Kang et al., 2021). The results shown in Table (3) showed a significant decrease in the ability of *S. aureus* to form a biofilm. Prolonged exposure time rendered the bacteria unable to form a biofilm. The results of our study were consistent with those of Liew et al. (2023) and Wang et al. (2020). The results showed that longer treatment led to a decrease in the bacteria's ability to survive. Our results were also consistent with a study (Dahle et al., 2024), which showed that increasing plasma exposure time and decreasing distance led to a reduction in the number of *S. aureus* cells in the biofilm.

Shaheed (2023) explained that direct bacterial cell injury, disruption of the extracellular matrix, and altered gene expression associated with biofilm formation may be the underlying mechanisms for the anti-biofilm effect of cold plasma, ultimately leading to the elimination of the biofilm. Cold plasma affects the essential components of the biofilm. Through lipid oxidation, protein modification and degradation, and the disruption of carbohydrate chemical bonds, the EPS is destroyed (Khosravi et al., 2021). This biochemical change in the EPS results from oxidation processes mediated by ROS and RNS molecules. When the EPS is disrupted, the adhesion of the biofilm to the immobilized surface decreases, ultimately leading to disruption of the three-dimensional biofilm structure or even disintegration (Soler-Arango et al., 2019).

This indicates that cold plasma can convert bacteria from a biofilm to a planktonic form. It can also kill bacteria in combination with other disinfectants that have a weak effect on biofilms but an excellent effect on bacteria (Zhang et al., 2023). The results of 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. Bacteria isolated from urinary tract infections showed the highest ability to form biofilms, while skin isolates showed the lowest ability to form biofilms. This may be due to the fact that bacteria in the isolation site are exposed to a volatile surface environment containing chemical and physical inhibitory factors. These factors combined prevent bacterial stabilization and biofilm formation, thus demonstrating a greater response to cold plasma treatment.

Conclusion:

The study found that cold plasma is effective in reducing *S. aureus* resistance to antibiotics and its ability to form biofilms. These results support the idea that cold plasma technology could be used to eliminate pathogenic bacteria instead of antibiotics, thereby treating some diseases associated with these bacteria.

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