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Evaluation of the Antibiofilm Efficacy of Ammonium Chloride (NH_4Cl) Against Gram-Negative Bacterial Isolates

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ABSTRACT

Background: *The bacterial Biofilms, which are communities of bacteria shielded by an Extracellular Polymeric Substance (EPS) matrix, contribute to over 65% of clinical infections and exhibit resistance levels up to 1,000 times higher than free-floating (planktonic) bacteria, so lead to increased antimicrobial resistance.* ***Method:*** *The study evaluated the antibiofilm efficacy of Ammonium Chloride (NH_4Cl) against 111 Gram-negative bacterial isolates. The bacteria were detected by vitek-2-compact system, then The biofilm was identified by the tissue culture plate method, after that tested the activity of NH_4Cl at two concentrations (0.5, 1mg/mL).* ***Result:*** *From 111 Gram-negative bacterial isolates in current study,20 isolate was not growth while 91 was detected as Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae and Acinetobacter baumanii at different ratio. Then detect the biofilm production for 91 isolate, 38 have the ability to form biofilm . The Ammonium Chloride established a strong, dose-dependent inhibitory effect on the bacterial biofilm formation. The biofilm creation was reduced to (71.1% and 100% eradication by 0.5, 1 mg/ml) concentration, respectively.* ***Conclusion:*** *The concentration of 1 mg/mL from Ammonium Chloride efficiently founding as the Minimum Biofilm Inhibitory Concentration (MBIC) for the isolates tested.*

Keywords: Ammonium Chloride, Minimum biofilm inhibitory concentration, Gram-negative bacteria.

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INTRODUCTION

Every year, millions of people are impacted by the growth of biofilms, which are responsible for over 50 % of infectious infections. The persistence of bacterial infections in clinical and industrial environments is largely attributed to the ability of microorganisms to organize into complex, sessile communities known as biofilms. Biofilms are collections of microbial cells that are encased in a matrix that they have generated and are affixed to a surface or to one another. The matrix creates a unique microenvironment by combining microbial biopolymers such as proteins, exopolysaccharides, and extracellular DNA. A biofilm helps to build antibiotic resistance by forming a protective barrier around bacteria, therefore preventing the entry of drugs, and so enabling the transmission of resistance genes between bacterial cells within the biofilm community before becoming resistant. The formation of biofilms

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is the underlying source of the problem, and it has been unequivocally revealed that bacteria may develop resistance against antibiotic (Ehud Banin *et al.,* 2017). A biofilm is a highly structured aggregation of bacterial cells embedded within a self-produced matrix of Extracellular Polymeric Substances (EPS), including polysaccharides, proteins, and extracellular DNA (Flemming HC *et al* .,2023). Because of their many uses, including bioremediation, power generation, bio-hydrogen production, and commercial chemical synthesis, biofilms are not always dangerous (Haggag, 2010).

Amongst the numerous chemical approaches under examination, inorganic salts and ammonium-based compounds must increase care for their capability to adapt the microenvironment of the bacterial cell surface. Ammonium Chloride (NH_4Cl), readily available and constant inorganic salt, has established possible in changing the ionic strength and osmotic pressure of growth media. Operating the chemical environment throughout the first attachment phase, NH_4Cl may avoid the rescindable adhesion of bacteria to surfaces, by halting the biofilm maturing procedure before the defensive EPS matrix is completely recognized (Kula N *et al.,* 2022). This mode of growth provides a significant evolutionary advantage, as the EPS matrix acts as a robust physical barrier that limits the penetration of conventional antibiotics and shields the bacteria from the host's immune response, such as phagocytosis (Thi MTT. *et al.*,2020)

The persistent nature of Gram-negative pathogens, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, is fundamentally driven by their transition into structured biofilm communities. The resulting Extracellular Polymeric Substance (EPS) matrix is no longer viewed merely as a static barrier, but as a dynamic biochemical shield. Emerging research highlights the role of Outer Membrane Vesicles (OMVs) embedded within the matrix. These vesicles serve as decoys for antimicrobial peptides and carry antibiotic-degrading enzymes, such as beta-lactamases, providing collective resistance to the entire community. Furthermore, the biofilm environment facilitates a high rate of Horizontal Gene Transfer (HGT), allowing for the rapid dissemination of multi-drug resistance (MDR) genes. As these sessile populations mature, they create metabolic gradients that foster "persister cells," which remain dormant and highly tolerant to even the most potent last-resort carbapenems, leading to chronic, recurrent clinical infections(Omwenga and Awuor , 2024).

In recent healthcare, biofilm-associated infections are accountable for over 65% of all bacterial infections, mainly those involving catheters, medical implants, and chronic wounds. The inherent resistance of biofilms frequently mentioned as being 100 to 1,000 times greater than of planktonic cells has reduced many traditional antimicrobial procedures ineffective (Zafer MM *et al.,*2024). This mounting disaster of antimicrobial resistance (AMR) has required the examination of alternate chemical agents that goal the biofilm lifecycle rather than just the metabolic pathways of separate cells. Current research aims to measure the inhibitory ability of NH_4Cl in contradiction of a varied set of bacterial isolates, providing a statistical framework for its effectiveness as an antibiofilm agent.

MATERIALS AND METHODS

Bacterial Isolates

This study included 111 clinical bacterial isolate from patients with urinary tract infection from AL-Sader teaching hospital in Al-Najaf province, Then All the growth bacterial isolates have been identified by using standard biochemical tests and confirmed by automated systems (VITEK-2,

bioMérieux, France) according to CLSI guidelines (Abd Al-Hassan *et al.*, 2024).

Biofilm formation

Initial screening was performed to identify biofilm-producing strains using Tissue culture plate Method (Han A, Lee SY. 2023)

Ammonium Chloride as antibiofilm

The bacterial isolates were divided into three groups: a control group (untreated) and two experimental groups treated with NH_4Cl at concentrations of 0.5 mg/mL and 1 mg/mL, respectively, After an incubation period (typically 24 hours at 37°C), the biofilms were stained with crystal violet, and the optical density (OD) was measured to categorize the isolates as biofilm-positive or biofilm-negative.

Statistical analysis

The paired statistical comparisons were used to decide the decrease ratio and the significance of the results (P-values), ensuring the dependability of the experiential inhibitory effects.

RESULTS

Bacterial identification

The current study result of bacterial isolate identification by Vitek-2-compact system indicate the percent of bacteria as shown in Table 1.

Table 1. Distribution of identified bacterial species among clinical isolates (n = 91).

Bacterial isolate	Number
Escherichia coli	29
Proteus mirabilis	11
Pseudomonas aeruginosa	20
Klebsiella pneumoniae	21
Acinetobacter baumanii	10

Biofilm formation

This study demonstrates the use of two concentrations of ammonium chloride on biofilm-forming bacterial isolates to determine whether it has an inhibitory effect on bacterial biofilm formation.

The result of current study revealed that 41.7% (n=38) of the 91 isolates were primarily able to biofilms formation detected by microtiter plate method as shown in **Figure1**

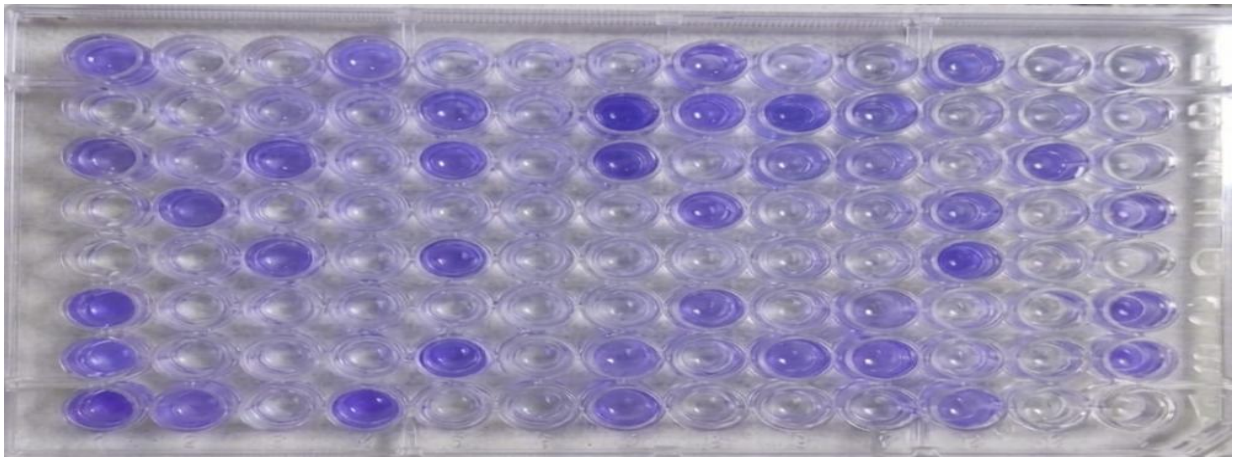

Figure 1: Biofilm production in bacterial isolate.

Activity of NH_4CL as antibiofilm

The current study show the ability of 41.7% (n=38) from the 91 isolates to biofilms formation. The 0.5 mg/mL concentration of NH_4Cl lowered the numeral of biofilm-positive isolates to 11 (28.9%), indicating a 71.1% reduction ($P < 0.0001$). Increasing the concentration to 1 mg/mL led to complete eradication (0% positive, 100% reduction) as shown in **Figure 2,3 and Table (2).**

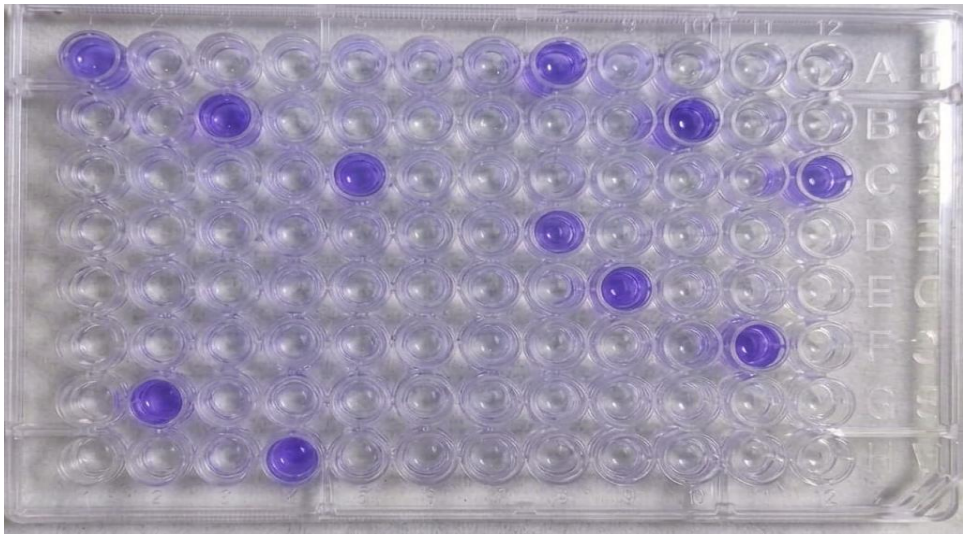

Figure 2 : NH_4Cl activity as antibiofilm with 0.5 mg/ml concentrations show 28.9% inhibition.

Figure 3 : NH_4Cl activity as antibiofilm with 1 mg/ml concentrations show 100% inhibition of biofilm.

Table 2. Effect of ammonium chloride (NH_4Cl) on biofilm formation among bacterial isolates (n = 38).

Experimental condition	Biofilm positive n (%)	Biofilm negative n (%)	Total
Control (untreated)	38 (41.7)	53 (58.2)	91
NH_4Cl (0.5 mg/mL)	11(28.9)	27 (71.1)	38
NH_4Cl (1 mg/mL)	0 (0.0)	38 (100.0)	38

The statistical evaluation of the current study result shows inhibitory effect of Ammonium Chloride (NH_4Cl) on the adhesive abilities of clinical isolates. As shown in **(Table 3)**, the addition of NH_4Cl at a concentration of 0.5 mg/mL may able to decreased the number of positive adherent isolates from 38 to 11, getting a reduction ratio of 71.1% ($P < 0.0001$). Extremely, increasing the concentration to 1 mg/mL lead to complete eradication of bacterial adhesion (100% reduction), where no positive isolates were detected after addition.

Table 3. Paired statistical comparison of biofilm formation before and after NH4Cl treatment.

Comparison	Positive before treatment	Positive after treatment	Reduction %	P-value
Control vs NH4Cl (0.5 mg/mL)	38	11	71.1	<0.0001
Control vs NH4Cl (1 mg/mL)	38	0	100.0	<0.0001
NH4Cl (0.5 mg/mL) vs NH4Cl (1 mg/mL)	11	0	100.0	<0.001

The study as shown in **(Table 4)** expose an effective inhibitory of Ammonium Chloride (NH_4Cl) on biofilm formation, categorized by a complete cessation of biofilm assembly at a concentration of 1 mg/mL. This alteration from moderate inhibition (71.1%) at 0.5 mg/mL to total inhibition at 1 mg/mL highlights an important concentration tolerance essential to interrupt the bacterial sessile lifestyle.

Table 4. Magnitude of antibiofilm activity of NH4Cl according to concentration.

NH4Cl concentration	Remaining biofilm producers n (%)	Inhibited isolates n (%)	Relative efficacy rank
0.5 mg/mL	11(28.9)	27 (71.1)*	Moderate
1 mg/mL	0 (0.0)	38 (100.0)*	Highest

DISCUSSION

This study demonstrates the use of two concentrations of ammonium chloride on biofilm-forming bacterial isolates to determine whether it has an inhibitory effect on bacterial biofilm formation. The discussion of the results are as follows:

In the absence of NH_4Cl, 41.7% (n=38) of the isolates were biofilm-positive. This serves as the baseline for the natural pathogenic potential of the samples. at (0.5 mg/mL) concentration of NH_4Cl, a sharp decline in biofilm formation was observed, with only 28.9% (n=11) of isolates remaining positive. This indicates that even low concentrations of ammonium chloride can disrupt the initial stages of bacterial attachment. While at (1 mg/mL) of NH_4Cl concentration achieved 100% inhibition (0.0% positive). All 38 isolates were rendered biofilm negative; establishing 1 mg/mL as the Minimum Biofilm Inhibitory Concentration

(MBIC) for this study and is a"Highest" rank of antibiofilm activity. The results align with findings by (Sasinova, K., *et al.*, 2025) in their study on clinical isolates in Baghdad, which highlighted that chemical salts and ammonium-based compounds significantly reduce the expression of adhesion genes in multi-drug resistant bacteria like Pseudomonas aeruginosa. (Qu, Y .*et al.*,2024) explored how ammonium compounds disrupt the Quorum Sensing (QS) pathways, the chemical communication system bacteria use to coordinate biofilm building.

This dose-dependent modulation from 71.1% to 100% proposes that NH_4Cl exploits as an effective chemical stressor that expected interrupts the electrostatic interactions between the bacterial cell wall and the attachment surfaces. From a biological perspective, this total inhibition at 1 mg/mL indicates that the salt concentration reached a threshold capable of completely down-regulating the expression of key adhesion-related genes (such as *pel* and *psl*), which are essential for the initial stages of biofilm formation, these findings align with the study by (Almatroudi A. 2024), which emphasized that ammonium-based compounds serve as effective anti-adhesive agents against multi-drug resistant (MDR) bacteria in Baghdad's clinical settings. According to (Songtai Gao *et al.*, 2023), the presence of ammonium ions in the growth medium creates an osmotic and chemical stress environment that interferes with the production of Extracellular Polymeric Substances (EPS). (Khawla E. Alsamhary, 2025), established that NH_4Cl significantly decreases biofilm formation in Gram-negative bacteria like *Pseudomonas aeruginosa* by disrupting the constancy of the biofilm matrix and down-regulating the signaling pathways responsible for colony consolidation.

The observed ability of NH_4Cl is reliable with the proven understanding of exactly how ionic compounds manipulates bacterial adhesion. (Kilic, T. ,2025), highlights that the first stage of biofilm formation is greatly sensitive to the physicochemical properties of the environment, including surface charge and ionic strength. The establishment of NH_4Cl liable interferes with these initial docking forces, stopping the irreversible attachment of planktonic cells.

Besides, the total inhibition done in this study (100% at 1 mg/mL) side with recent research by (Vishal Ahuja *et al.*, 2024), which debates the "Minimum Biofilm Eradication Concentration" (MBEC) of inorganic salts. Their work proposes that ammonium ions can access the protecting matrix more successfully than larger antibiotic molecules, disturbing the hydrogen bonding within the Extracellular Polymeric Substances (EPS). This mechanism supports the "Highest" rank of effectiveness watched in our results, as the salt effectively destabilizes the structural integrity of the biofilm. The significant reduction in biofilm creators watched here also confirm with the findings of (El-Sawy ER *et al.*, 2024), who informed that nitrogen-based salts can prevent the bacteria from coordinating the maturation of the biofilm, leaving them in a more vulnerable, planktonic state.

CONCLUSIONS

1-The current study show the possibility of NH_4Cl as the antibiofilm inhibition activity concentration dependent on some isolate of gram negative bacteria

2-The study indicate the critical concentration threshold of NH_4CL was (1 mg/mL) .

Ethical approval

This study was approved by the Committee on Publication Ethics at Al-Sader Medical City in Najaf province.

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