

An Emerging Uropathogenic Threat: High-Virulence, Stress-Adapted, and Drug-Resistant *Pseudomonas aeruginosa* from Clinical UTI Iraqi Isolates

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DOI : <https://doi.org/10.61796/jmgcb.v3i8.1886>



Sections Info

Article history:

Submitted: May 09, 2026

Final Revised: June 14, 2026

Accepted: July 19, 2026

Published: August 05, 2026

Keywords:

Pseudomonas aeruginosa

Virulence

Tolerance

Antibiotic resistance

ABSTRACT

Objective: *Pseudomonas aeruginosa* is one of the most important urinary tract infection-causative pathogens that is greatly associated with Urinary Tract Infections (UTI) because of its great ability to adaptation at different harsh environments due to the presence of a set of genes that contribute to the expression of virulence factors and the development of antibiotic resistance. The present study aimed to phenotypically characterize the multidimensional phenotypic profile of *Pseudomonas aeruginosa* isolated from UTI Iraqi patients. **Methods:** Attachment and biofilm formation, blood hemolysis, urease, lipase, gelatinase, protease, and siderophores producing all were evaluated as virulence factors. Isolates' tolerance to harsh conditions, including heat, cold, salt, acid, and alkaline, was estimated. Antibiotic susceptibility test have been performed to assess isolates resistance against piperacillin, ceftazidime, cefepime, azteronam, imipenem, meropenem, amikacin, gentamicin, tobramycin, levofloxacin and colistin. **Results:** Results explain that the majority of isolates had a concurrent presence of virulence traits and the ability for antibiotic resistance and tolerance to harsh conditions. **Novelty:** which indicate that *Pseudomonas aeruginosa* represents an emerging uropthological threat characterized by high adaptive flexibility and virulence, which further complicates therapeutic options and underscores the need for continuous surveillance and more stringent preventive and therapeutic strategies to limit their spread in clinical settings.

INTRODUCTION

Pseudomonas aeruginosa bacteria have exceptional metabolic adaptability, and a wide range of virulence factors are responsible for its clinical success [1]. Clinical isolates from regional hotspots like Iraq have started to display high virulence characteristics, in contrast to typical uropathogens [2]. These strains evade the host's innate immune response by using a variety of secretion systems, biofilms, and siderophores³. Additionally, the urinary tract offers a harsh environment with nutritional constraints and osmotic oscillations [2]. Consequently, stress-adapted strains have evolved specialized mechanisms to thrive under these physiological pressures, making them harder to eradicate than standard laboratory strains [3]. The escalating threat of Multidrug Resistant (MDR) and Extensively Drug Resistant (XDR) *P. aeruginosa* is now a critical public health concern in Iraq [4]. The selection of isolates with complex resistance genes has been influenced by the usage of aminoglycosides and carbapenems [5]. The convergence of high virulence with drug resistance creates a perfect storm where the pathogen is not only more aggressive in causing tissue damage but also effectively untreatable with conventional antibiotic regimens [6]. There is a dearth of thorough information about the molecular and phenotypic traits of *P. aeruginosa* isolates that were

particularly obtained from UTI patients in the Iraqi clinical setting, despite the clinical relevance of these infections [7]. Selective pressures, such as exposure to sub-lethal disinfectant doses, temperature variations, and osmotic shifts, are constantly applied to bacterial populations [8]. Recent evidence suggests a robust cross resistance phenomenon where the genetic and phenotypic pathways that allow *P. aeruginosa* to tolerate severe environmental stresses such as thermal, salinity, and pH variations, also reinforce its intrinsic and acquired antibiotic resistance mechanisms, making these emerging uropathogenic strains exceptionally resilient within the hospital ecosystem [9]. There is a dearth of thorough information about the molecular and phenotypic traits of *P. aeruginosa* isolates that were particularly obtained from UTI patients in the Iraqi clinical setting, despite the clinical relevance of these infections [10]. The majority of current research concentrates on generic antibiotic sensitivity without examining the interaction between virulence and stress adaptation [11]. Thus, the purpose of this work is to describe the drug-resistance patterns, stress-response capacities, and virulence profiles of *P. aeruginosa* isolates collected from Iraqi hospitals [12].

A complex network of extracellular enzymes and adaptive survival mechanisms controls *P. aeruginosa*'s pathogenicity in the urinary system [13]. The Adhesion and Biofilm Formation Ability, which promotes long term persistence and defense against host immune clearance, is essential to its colonization approach [14]. In addition, a number of exoenzymes, including urease, lipase, gelatinase, and protease, are produced [14]. These enzymes work together to break down host tissues and change the local microenvironment to promote bacterial growth [15]. Additionally, the clinical isolates' capacity for siderophore synthesis and blood hemolysis highlights their aggressive nutrient acquisition tactics, especially for iron scavenging in the human host's iron-limited environment [16]. These newly discovered uropathogenic strains show exceptional environmental stress tolerance in addition to biochemical virulence [17]. Significant heat, cold, and salinity tolerance as well as a strong ability to tolerate acid-alkaline stress are characteristics of their resilience [18]. Antibiotic resistance profiles seen in clinical settings are further complicated by this physiological adaptability, which not only helps them survive the changing conditions of the urinary tract but also adds to their persistence in hospital settings [18]. Thus, by characterizing the virulence profiles, stress response capacities, and drug resistance patterns of *P. aeruginosa* isolates collected from Iraqi hospitals, this study aims to offer crucial insights that could guide improved diagnostic procedures and focused treatment approaches.

RESEARCH METHOD

1. Adhesion and Biofilm Formation Ability

Assessment of isolates capacity for biofilm formation ability have been equipped using Crystal violet Microtiter Plate Assay (CVMPA), in which, bacterial suspension McFarland 0.5 had been prepared from each isolate then diluted to 1:100 broth nutrient broth media, then, 200 μ L of the suspension was added to the plate wells (three replicates)

followed by incubation at 37 C° for 42 hours [19]. By gently washing with Phosphate Buffer Saline (PBS) to remove unattached cells, 100 µL Crystal violet stain was added to each well leaving wells without bacteria as negative control then the plate was incubated for 10-15 minute followed by reading with ELISA reader at 580 nm. Mean plus Standard deviation for each replicate was assessed for all isolates. Optical Density Cutoff (ODC) was calculated according to the following formula: $ODC = \text{Mean} + (3 \times SD)$.

2. Heat Tolerance

Evaluation of *Pseudomonas aeruginosa* isolates for heat tolerant have been outfitted by prepare five tubes containing a standardized bacterial suspensions adjusted to 0.5 McFarland of each isolate, the first four tubes were left at 55 C° in water bath for 5, 10, 15 and 20 minutes subsequently, while the remaining fifth tube was the negative control [20]. After cooling with ice for 2-3 minutes, 100 m\ L of each tube have been cultured on nutrient agar media and incubated for 24 - to 24 hours at 37 C°. Each of CFU\ mL then, colony forming unit (CFU\ mL) and survival percentage were calculated according the following formula;

$$CFU\ mL = \text{number of colonies} \backslash \text{volume of isolates} \times 1 \backslash \text{dilution factor}$$

$$\text{Survival \%} = CFU\ mL \text{ after heating} \backslash CFU\ mL \text{ before heating} \times 100\%$$

3. Cold Tolerance

The ability of isolates to tolerate low temperature was assessed using a cold stress assay using five tubes containing a standardized bacterial suspensions adjusted to 0.5 McFarland of each isolate, four tubes were exposed to low temperature by placing in refrigerator maintained at 4° for sequential time periods of 6, 12, 24 and 48 hours, whereas the fifth tube was kept without cold exposure and served as a control for comparison. Then, the tubes were removed and gradually returned to room temperature [19]. Subsequently, 100 m\ L of each tube have been cultured on nutrient agar media and incubated for 24 - to 24 hours at 37 C°. Each of CFU\ mL then, colony forming unit (CFU\ mL) and survival percentage were calculated according the following formula;

$$CFU\ mL = \text{number of colonies} \backslash \text{volume of isolates} \times 1 \backslash \text{dilution factor}$$

$$\text{Survival \%} = CFU\ mL \text{ after heating} \backslash CFU\ mL \text{ before heating} \times 100\%$$

4. Acid-Alkaline Stress Tolerance

By using acid-alkaline stress assay, capacity of isolates for tolerate hydrogen ion concentration (PH) have been evaluated via toke five tubes containing a standardized bacterial suspensions adjusted to 0.5 McFarland of each isolate. PH of four tubes was adjusted to different levels using appropriate buffer solution, representing low PH condition (PH 5 and PH 6) and high PH condition (PH 8 and PH 9) whereas the fifth tube was maintained at neutral PH (PH 7) and served as a control for comparison. Then, the tubes were removed and gradually returned to room temperature [21]. Subsequently, 100 m\ L of each tube have been cultured on nutrient agar media and incubated for 24 - to 24 hours at 37 C°. Then, after detection of bacterial growth in each tube, isolates were classified into Acid-tolerant (growth at PH (5-6) only), Alkali-tolerant (growth al alkaline PH (8-9), Amphoteric (growth at both acidic and alkaline PH) and PH-sensitive (Growth only at PH 7).

5. Salinity Tolerance

By an osmotic stress assay, the ability of isolates to tolerate salinity was assessed by five tubes containing a standardized bacterial suspensions adjusted to 0.5 McFarland of each isolate. The sodium chloride (NaCl) concentration in four tubes was adjusted to different levels to represent increasing salinity conditions, namely 2% (2 gram of NaCl dissolved in 100 mL solution), 4%, 6% and 8% (w/v), whereas the fifth tube was maintained at a normal saline concentration (0.85% NaCl) and served as a control for comparison. Then, the tubes were removed and gradually returned to room temperature. Subsequently, 100 mL of each tube have been cultured on nutrient agar media and incubated for 24 – to 24 hours at 37 C°. Then, after detection of bacterial growth in each tube, the number as well as percentage of survival isolates were be recorded [22], [23].

6. Blood Hemolysis Ability

The ability of isolates to hemolyse blood was investigated using a blood agar hemolysis assay. For each isolate, a standardized bacterial suspension adjusted to 0.5 McFarland was prepared. The isolates were then inoculated onto blood agar plates containing 5% blood using the streaking inoculation method, and the plates were incubated at 37C° for 24 C° hours. Following incubation, the plates were examined visually for the presence of hemolytic activity surrounding the bacterial colonies. Based on their appearance of the zone around each colony, the types of hemolysis were complete hemolysis (β -hemolysis) which was recorded when a clear transparent zone was observed, partially hemolysis (α -hemolysis) which was recorded when a greenish, non-transparent zone was observed and Gamma hemolysis (γ -hemolysis) which was detected by the absence of any visible change in the surrounding medium [24] .

7. Urease Production

The ability of isolates to produce urease was investigated using the standard urease test. For each isolate, a standardized bacterial suspension adjusted to 0.5 McFarland was prepared. The isolates were then inoculated onto urea agar or into urea broth containing urea and a pH indicator, using the streaking method, followed by incubation at 37 °C for 18–24 hours. After the incubation period, the media were examined visually for any color change resulting from urea hydrolysis and the subsequent release of ammonia, which leads to an increase in pH. A positive urease reaction was recorded when the medium changed in color from yellow or orange to pink or fuchsia, whereas the absence of any color change was considered indicative of a negative urease reaction. These observations were used to evaluate the urease-producing capability of the isolates [25].

8. Lipase Production

The ability of isolates to produce lipase was investigated using the standard lipase assay. For each isolate, a standardized bacterial suspension adjusted to 0.5 McFarland was prepared. The isolates were then inoculated onto a selective medium for lipase detection, tributyrin agar containing an appropriate lipid substrate, using the streaking method, and incubated at 37 °C for 18–24 hours. After the incubation period, the plates were examined visually for lipase activity, as indicated by the presence of a clear zone or distinct halo surrounding the bacterial colonies due to lipid hydrolysis in the medium.

The appearance of this zone was considered a positive result for lipase production, whereas the absence of any visible change in the surrounding medium was regarded as a negative result [26].

9. Gelatinase Production

The ability of isolates to produce gelatinase was investigated using the standard gelatin hydrolysis assay. For each isolate, a standardized bacterial suspension adjusted to 0.5 McFarland was prepared. The isolates were then inoculated into gelatin agar medium using the stab inoculation method, followed by incubation at 37 °C for 18–24 hours (and up to 48 hours when required). After the incubation period, the inoculated media were examined for evidence of gelatin hydrolysis. For gelatin agar, the presence of a clear zone surrounding the bacterial growth was considered indicative of gelatinase production [27]

10. Protease Production

The ability of isolates to produce protease was investigated using a standard protease activity assay. For each isolate, a standardized bacterial suspension adjusted to 0.5 McFarland was prepared. The isolates were then inoculated onto a protease detection medium, such as skim milk agar, containing protein substrates suitable for proteolytic activity, using the streaking method, followed by incubation at 37 °C for 18–24 hours. After the incubation period, the plates were examined visually for evidence of proteolytic activity. Protease production was indicated by the presence of a clear zone (halo) surrounding the bacterial colonies as a result of protein hydrolysis in the medium. The appearance of this zone was recorded as a positive protease reaction, whereas the absence of any visible clearing in the surrounding medium was considered a negative result [28].

11. Assessment of Siderophore production

The ability of bacterial isolates to produce siderophores was evaluated using the chrome azule S (CAZ) assay. Bacterial cultures were incubated under standard conditions. The development of a color change surrounding bacterial colonies was considered indicative of siderophore production. The extent of color change was used as a semi-quantitative measure of siderophore activity [29]

12. Resistance to Antibiotics

Antibiotics resistance was assessed using Kirby-Bauer disk diffusion method in which, all isolates were cultured on Mueller-Hinton agar, then, several types of antibiotic discs including piperacillin, tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, colistin, tobramycin, ciprofloxacin and levofloxacin have been distributed onto medium using sterile forceps in appropriate distances followed by incubation at 37 °C for 16 – 18 hours. sensitivity of isolate had assessed by the formation of clear hallow around the antibiotic discs, and according to Clinical and Laboratory Standard Institute (CLSI) isolates have been classified into Sensitive (S), Intermediate (I), and Resistant (R) [30]. [31].

Statistical Analysis

Data were analyzed using appropriate statistical software. Quantitative data were expressed as mean + standard deviation. Comparasions between study groups were

performed using analysis of variance (ANOVA) or corresponding non-parametric tests. Correlation analyses were conducted to evaluate associations between bacterial iron-related activities and hematological parameters. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Results

1. Adhesion and Biofilm Formation Ability

According to their ability for adhesion and biofilm formation, isolates were classified into four classes as seen in table (1), fig.1. the present work showed that 42.1% of isolates were strong adherent, 32.8 moderat adherent, 21.1 weak adherent and 4.2 were non adherent.

Table 1. Classification of *Pseudomonas aeuroginosa* isolates according to Adhesion ability.

Adhesion Category	OD range	Number of isolates	Percentage %
Non-adherent	$\leq$ ODC	5	4.2
Weak-Adherent	$\leq 2 \times$ ODC	25	21.1
Moderate-Adherent	$\leq 4 \times$ ODC	39	32.8
Strong-Adherent	$> 4 \times$ ODC	50	42.1
Total		119	

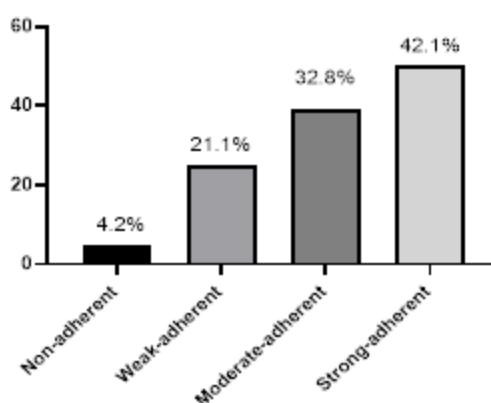


Figure 1. *Pseudomonas aeuroginosa* adhesion ability.

2. Thermal Tolerance

As shown in table 2, fig.2, *Pseudomonas aeuroginosa* isolates survival percentage were 67%, 21%, 9% and 2% under thermal exposure stress, 55 C⁰ for 5, 10, 15 and 20 minutes respectively.

Table 2. Distribution of isolates regarding thermal tolerance.

Exposure time (min.)	CFU\mL (mean $\pm$ SD)	Survival %
0	$1 \times 10^8 \pm 2.3$	100%
5	$1 \times 10^7 \pm 11.5$	67%
10	$1 \times 10^5 \pm 5.8$	21%
15	$1 \times 10^3 \pm 8.4$	9%
20	$1 \times 10^2 \pm 1.9$	2%

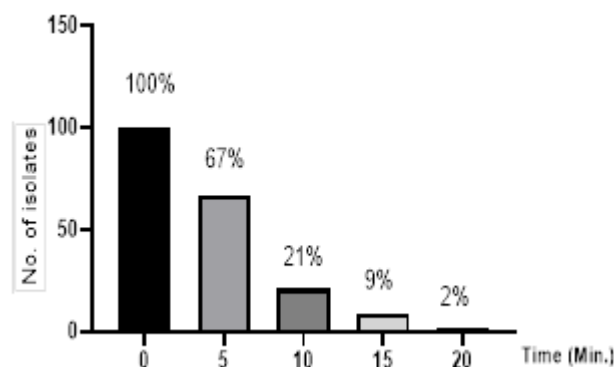


Figure 2. *Pseudomonas aeruginosa* survival at 55 C.

3. Cold Tolerance

As shown in table 3, fig.3, *Pseudomonas aeruginosa* isolates survival percentage were 23%, 8%, 2% and 0.7% under cold exposure stress, 4 C⁰ for 5, 10, 15 and 20 minutes respectively.

Table 3. Distribution of isolates regarding cold tolerance.

Exposure time (min.)	CFU\mL (mean $\pm$ SD)	Survival %
0	$1 \times 10^8 \pm 4.8$	100%
5	$1 \times 10^4 \pm 23.5$	23%
10	$1 \times 10^3 \pm 11.6$	8%
15	$1 \times 10^2 \pm 6.8$	2%
20	$1 \times 10^1 \pm 1.1$	0.7%

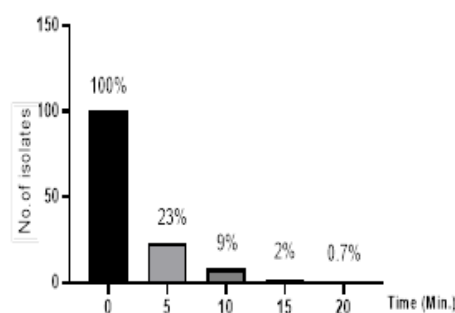


Figure 3. *Pseudomonas aeruginosa* survival at 4 C.

4. Acid-Alkaline Tolerance

As shown in table 4, fig.4, *Pseudomonas aeruginosa* isolates survival percentage which were tolerant for both acidic and alkaline stress (Amphoteric) were 65%, while acid-tolerant isolate percentage were 34% and alkaline-tolerant isolate were 29% and the percentage of PH-sensitive isolates were 7%.

Table 4. Classification of isolates regarding cold tolerance.

Acidity Profile	Operational detention	Survival %
acid-tolerant	Growth at acidic pH (5, 6) only	34%
alkaline-tolerant	Growth at alkaline pH (8, 9) only	29%
Amphoteric	Growth at both acidic and alkaline pH (5, 6, 7, 8, 9)	65%
PH-sensitive	No growth at both extremes (only at pH 7)	7%

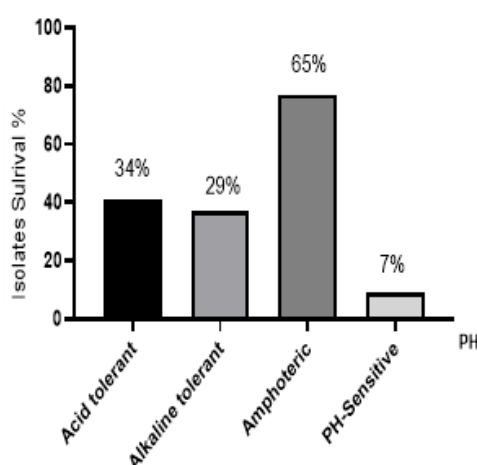


Figure 4. *Pseudomonas aeruginosa* Acid-Alkaline Tolerance.

5. Salinity Tolerance

According to table 5, fig.5, 77% of *Pseudomonas aeruginosa* isolates was tolerant to 2% NaCl, and 72% was tolerant to 4% NaCl, 4% of isolates were tolerant to 6% NaCl and no growth of any isolates were detected at % NaCl.

Table 5. Distribution of isolates regarding salinity tolerance.

NaCl (%)	No. of Survival isolates	%
2	92	77%
4	89	72%
6	5	4%
8	Zero	0.0%
Total	119	

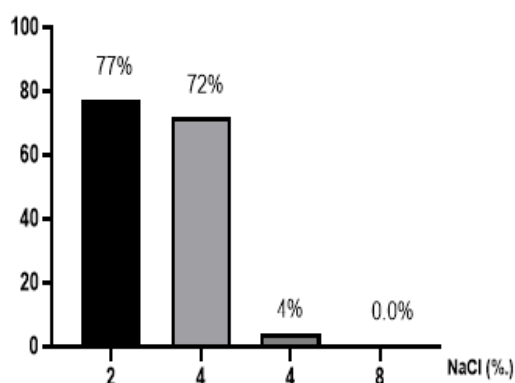


Figure 5. *Pseudomonas aeruginosa* Salinity tolerance.

6. Blood hemolysis

Depending on their ability to blood hemolysis, 31% of isolates were a-hemolysis, 48.7% were b-hemolysis and 19.3% were non-hemolysis as shown in table 6, fig 6.

Table 6. Classification of isolates regarding hemolysis activity.

Hemolysis type	Description	No. of Survival isolates	%
a- Hemolysis	Partial RBC lysis with a greenish halo around colonies	38	31.9
B- Hemolysis	Complete RBC lysis with a clear zone around colonies	58	48.7
Y-Hemolysis	No RBC lysis and no changes in media	23	19.3
Total		119	

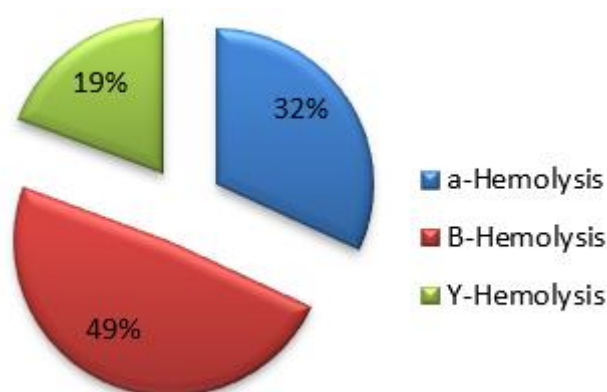


Figure 6. *Pseudomonas aeruginosa* Hemolysis Activity.

7. Urease Producing Capacity

Depending on their ability to produce urease enzyme, 74.8% of isolates were urease producing while 25.2% were urease negative isolates as shown in table 7, fig 7.

Table 7. Classification of isolates according to urease production.

Urease Production	No. of Isolates	Percentage %
Positive	89	74.8
Negative	30	25.2
Total	119	100

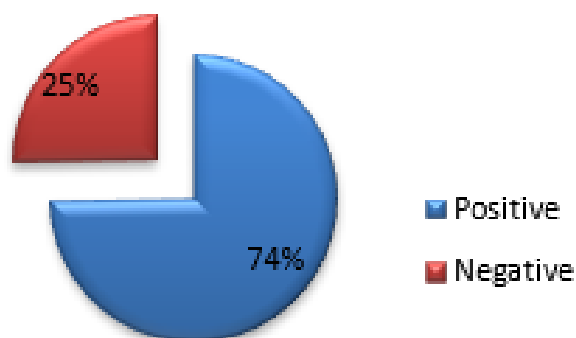


Figure 7. *Pseudomonas aeruginosa* Urease production.

8. Lipase Producing Capacity

Depending on their ability to produce lipase enzyme, 57% of isolates were lipase producing while 42% were lipase negative isolates as clarified in table 8, fig 8.

Table 8. Classification of isolates according to lipase production.

Lipase Production	No. of Isolates	Percentage %
Positive	68	57.1
Negative	51	42.9
Total	119	100

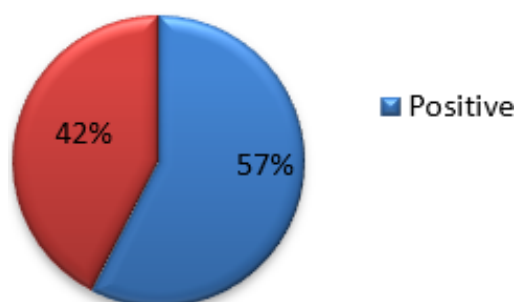


Figure 8. *Pseudomonas aeruginosa* lipase production.

9. Gelatinase Producing Capacity

Depending on their ability to produce gelatinase enzyme, 55% of isolates were gelatinase-producing, while 53 were gelatinase negative classified as in table 9, fig 9.

Table 9. Classification of isolates according to gelatinase production.

Gelatinase Production	No. of Isolates	Percentage %
Positive	66	55.46
Negative	53	44.54
Total	119	100

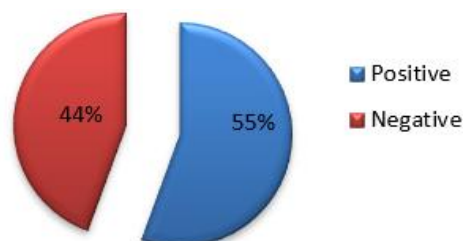


Figure 9. *Pseudomonas aeruginosa* gelatinase production.

10. Protease Producing Capacity

Depending on their ability to produce protease enzyme, 61% of isolates were protease positive while 46 were protease negative, as shown in Table 10, fig 10.

Table 10. Classification of isolates according to protease production.

Protease Production	No. of Isolates	Percentage %
Positive	73	61.3
Negative	46	38.7
Total	119	100

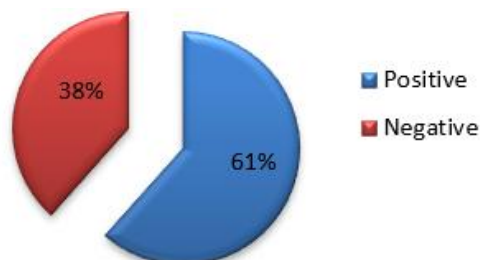


Figure 10. *Pseudomonas aeruginosa* proteas production.

11. Siderophore Producing Capacity

Depending on their ability to produce siderophore, 63% of isolates were siderophore-producing while 44% were negative, as shown in Table 11, fig 11.

Table 11. Classification of isolates according to protease production.

Siderophore Production	No. of Isolates	Percentage %
Positive	75	63.0
Negative	44	37
Total	119	100

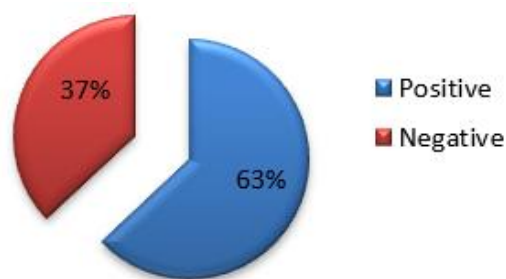


Table 11. *Pseudomonas aeruginosa* siderophore production.

12. Antibiotic Resistance

Using Antibiotic Susceptibility test, antibiotic resistance profile of isolates have explained at table 12, fig.12 which revealed a high prevalence of resistance among isolates since the resistance rates exceeding 51% were observed against piperacillin, 45% against ceftazidime, 52% against cefepime, 50% against aztreonam, 43% against imipenem, 42% against meropenem, 34% against amikacin, 38% against gentamicin, 37% against tobramycin, 48% against levofloxacin and 16% against colistin.

Table 12. Antibiotic resistance profile of *Pseudomonas aeruginosa*.

Antibiotic	Class	S (%)	I (%)	R (%)
Piperacillin	Antipseudomonal penicillin	41 (34%)	17 (14%)	61 (51%)
Ceftazidime	3rd generation cephalosporin	45 (37%)	20 (16%)	54 (45%)
Cefepime	4th generation cephalosporin	48 (40%)	18 (15%)	63 (52%)
Aztreonam	Monobactam	43 (36%)	16 (13%)	60 (50%)
Imipenem	Carbapenem	54 (45%)	13 (10%)	52 (43%)
Meropenem	Carbapenem	56 (47%)	12 (10%)	51 (42%)
Amikacin	Aminoglycoside	70 (58%)	15 (12%)	34 (28%)
Gentamicin	Aminoglycoside	57 (47%)	16 (13%)	46 (38%)
Tobramycin	Fluoroquinolone	60 (50%)	14 (11%)	45 (37%)
Levofloxacin	Fluoroquinolone	44 (37%)	17 (14%)	58 (48%)
Colistin	Polymyxin	100 (100%)	0 (0%)	19 (16%)

S; sensitive, R; resistant, I; intermediate

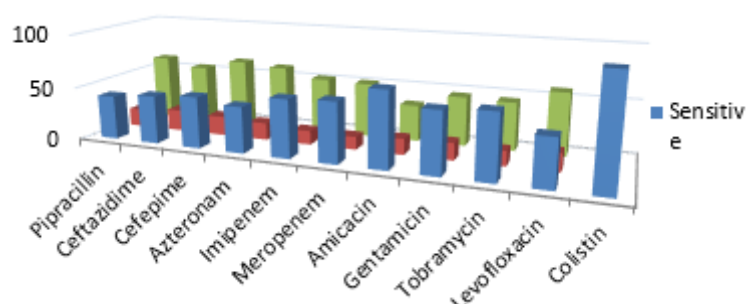


Figure 12. Antibiotic resistance profile of *Pseudomonas aeruginosa*.

Discussion

- **Adhesion and Biofilm Formation**

The results of the present study demonstrate a high prevalence of biofilm-forming ability among *P. aeruginosa* isolates, with 74.9% of the isolates exhibiting moderate to strong adherence (42.1% strong and 32.8% moderate).

As Comparison with Global Trends, the high percentage of strong biofilm producers (42.1%) in this study is consistent with findings El-sayed *et al.*, (2021), who reported similar trends in clinical isolates [33]. However, it contrasts with studies on environmental isolates, where biofilm production is often weaker. This suggests that clinical strains of *P. aeruginosa* have evolved robust mechanisms to survive the stresses of the host environment and medical interventions [33].

However, *P. aeruginosa*'s capacity to build biofilms is a sophisticated survival strategy that has biological and clinical significance [34]. The majority of our isolates exhibit strong adhesion, which is associated with the synthesis of extracellular polymeric substances (EPS) [35]. Antibiotic resistance biofilms function as a physical barrier that lessens the entry of antibiotics such as beta-lactams and aminoglycosides [34], [35]. Because only 4.2% of isolates were non-adherent, the Correlation with Virulence revealed that biofilm formation is a fundamental virulence determinant for this disease rather than merely a bonus characteristic [35]. Catheter-associated urinary tract infections (CAUTIs) and ventilator-associated pneumonia (VAP), where surface attachment represents the initial stage of infection, are probably caused by these strong adherent strains [35].

- **Survival Under Thermal Stress**

The results shown in Table 2 and Figure 2 show that when *P. aeruginosa* isolates are subjected to a continuous heat stress of 55°C, their vitality decreases over time [36]. After 20 minutes of exposure, the survival rate decreased dramatically from 67% at 5 minutes to just 2% [36]. The Mechanism of Thermal Inactivation Several deadly biological processes take place at 55°C, which is significantly higher than *P. aeruginosa*'s ideal development temperature (usually 37°C), Protein Denaturation when vital enzymes and structural proteins unfurl due to heat, metabolic function is lost [37]. Ribosomal Damage, the 30S and 50S ribosomal subunits (which we discussed in the antibiotic section) are sensitive to heat; their degradation stops protein synthesis entirely [38].

Survival Under Salinity (Osmotic) Stress: *P. aeruginosa*'s capacity to control osmotic pressure is demonstrated by its survival in a range of sodium chloride (NaCl) concentrations [38]. The Osmotic Adaptation Mechanism: The bacterial cell faces two main dangers when exposed to high salt concentrations: dehydration and ion toxicity [39]. To survive, the isolates probably use the following strategies. The Limits of tolerance of results show a sharp drop at very high concentrations above 10% NaCl, it indicates that breaking point of the cell's homeostatic mechanisms [40]. While plasmolysis at extreme salinity, the cytoplasmic membrane shrinks away from the cell wall (plasmolysis), leading to permanent structural damage [39], [40].

- **Acid-alkaline pH tolerance**

The outcomes shown in Table 4 and Figure 4 demonstrate the *P. aeruginosa* isolates' exceptional metabolic flexibility. The most startling discovery is that 65% of the isolates had an amphoteric character, which means they could withstand both alkaline and acidic stress [40]. Only 7% of the isolates were found to be pH sensitive, underscoring the ruggedness of these clinical strains. The Molecular Basis of pH tolerance and the majority (65%) to tolerate both extremes suggests a very efficient. In addition of producing Acid shock Proteins (ASPs), which shield cellular DNA and proteins from denaturation, *P. aeruginosa* probably uses proton pumps to actively remove ions from the cytoplasm in order to survive acidic environments [41]. Alkaline tolerance (29%) meaning Anti porters, which carry protons into the cell to neutralize the alkaline interior and keep the internal pH within a functioning range, are usually necessary for survival in high pH situations [42]. Also, the Clinical Significance as Amphoteric Advantage, high percentage of amphoteric isolates (65%) has direct clinical implications in Iraq Survival in [42]. The body fluids clinical environments vary greatly in pH as in the urinary tract can be acidic, while infected wounds or certain parts of the gastrointestinal tract can become alkaline. An amphoteric isolate can transition between these environments without losing viability [43]. On the another hand, the Resistance to Disinfectants, many cleaning agents used in hospitals are either highly acidic or highly alkaline [43]. The fact that only 7% are sensitive means that standard chemical disinfection might fail against 93% of these isolates. The Correlation with Virulence and Biofilm, there is often a link between pH tolerance and Biofilm Formation which discussed above [44]. The biofilm matrix (EPS) acts as a buffer zone, protecting the bacteria from rapid pH shifts. While the strong Adherent isolates (42.1%) are likely the ones that make up the 65% amphoteric group, as the biofilm provides the physical stability needed to survive pH stress [44] .

- **Salinity Tolerance and Osmoadaptation**

The phenotypic assessment of osmotic stress tolerance revealed that the clinical *Pseudomonas aeruginosa* isolates possess a robust capacity to survive under elevated hypersaline conditions (Table-5, Fig.5). Specifically 77% of the isolates demonstrated viability at 2% NaCl, and a remarkably high proportion 72% retained tolerance at 4% NaCl. At higher osmotic thresholds, however, a significant decrease in survival was noted; only 4% of the isolates could withstand 6% NaCl and all isolates completely stopped growing at 8% NaCl .The remarkable physiological adaptability of these clinical strains in comparison to their typical laboratory counterparts is highlighted by the great viability retention at (2% and 4% NaCl) [45].The human urinary tract is a highly hostile environment characterized by drastic fluctuations in osmolarity and urea concentrations, driven by host hydration and renal metabolic activity [45].

Crucially, the high tolerance to 4% NaCl observed in nearly three-quarters of our isolates (72%) signals an emerging "stress-adapted" phenotype within the regional clinical ecosystem [44]. In Iraqi healthcare settings, bacterial populations are continuously subjected to selective environmental pressures, including exposure to sub-lethal concentrations of local disinfectants, saline flushes, and ambient desiccation on medical surfaces [46]. Consequently, the high halotolerance documented in this study strongly

correlates with the multidrug-resistant (MDR) and extensively drug-resistant (XDR) profiles of these isolates, confirming that environmental stress adaptation actively drives clinical persistence and limits the efficacy of conventional antipseudomonal therapy [46].

Hemolytic Activity and Virulence: The examination of hemolytic activity in *P. aeruginosa* isolates reveals a significant pathogenic potential [47]. As shown in Table 6 and Figure-6, a majority of the isolates (79.7%) exhibited some form of hemolysis, with 48.7% showing complete Beta-hemolysis and 31% showing Alpha-hemolysis. Only 19.3% were non-hemolytic (Gamma hemolysis).1. The Power of Beta Hemolysis (48.7%) are high prevalence of beta-hemolysis is a direct marker for the production of potent extracellular virulence factors, most notably Phospholipase C (PLC) and Exotoxin-A [48]. The mechanism of these toxins is to degrade the phospholipids in human cell membranes and cause the complete rupture of Red Blood Cells (RBCs). In the context of a UTI (Urinary Tract Infection), this ability allows the bacteria to acquire essential nutrients like Iron from the host's blood cells, which is a limiting factor for bacterial growth in the urinary tract [48]. Tissue Damage: beta-hemolytic strains are often associated with more invasive infections and higher rates of tissue necrosis-2 [49]. 31% of isolates showing alpha-hemolysis indicate the production of hydrogen peroxide or other oxidative agents that chemically modify hemoglobin [49]. These strains nonetheless have the ability to disrupt host immunological responses and harm mucosal surfaces, although being less aggressive than beta-hemolysis [49]. The fact that nearly 80% of your Iraqi UTI isolates are hemolytic explains why *P. aeruginosa* often causes severe complicated UTIs in the region [42]. Because they can both target the bladder lining and endure the harsh, acidic, and salty environment of urine, they are considered an Emerging Uropathogenic threat [50]. The clinical Implications appear high percentage of beta hemolysis (48.7%) in contrast to the comparatively low proportion of non-hemolytic strains (19.3%) indicates that *P. aeruginosa* has evolved toward a high pathogenicity phenotype in Iraqi hospitals. Early diagnosis and aggressive treatment are crucial to prevent the infection from entering the circulation which is Urosepsis or spreading to the kidneys (Pyelonephritis) [50].

Urease virulence Factor in UTIs: The high prevalence (74.8%) of urease production is a major finding. In the context of urinary tract infections, urease is not just a metabolic enzyme, it is a weapon pH Alteration [51]. Urease catalyzes the hydrolysis of urea into ammonia and carbon dioxide. These stones can protect bacteria from both antibiotics and the host's immune system [51]. Link to "Stress-Adapted and Drug Resistant, the Urease production plays a key role Survival in Acidic Stress [51]. While *P. aeruginosa* is versatile, the ability to modulate its local environment's pH allows it to survive the naturally acidic fluctuations of human urine [52]. The Iraqi studies, Note that *P. aeruginosa* is traditionally considered urease variable, but the high percentage suggests that the specific strains circulating in Iraqi clinical settings may have evolved (or acquired via horizontal gene transfer) enhanced ureolytic capabilities to survive the specific pressures of the local patient population as high antibiotic use or specific comorbidities [53].

- **Lipase as a Tool for Tissue Invasion**

Lipases are exoenzymes that break down lipids (fats). Since human cell membranes are composed of a lipid bilayer, lipase production is a direct attack mechanism [52]. Membrane Destabilization of Lipases degrade the phospholipids of host cell membranes, leading to cell lysis and tissue necrosis [54]. About 57% of the *P. aeruginosa* isolates were found to have the ability to produce lipase. Lipase secretion is a significant virulence mechanism for tissue colonization in the setting of uropathogenicity [55]. These enzymes promote cellular damage and nutrient release by attacking the uroepithelium's lipid bilayers [55].

The presence of lipase synthesis in more than half of the isolates highlights the high-virulence nature of these Iraqi clinical strains when combined with the high urease activity seen in our investigation [56]. This enzyme profile contributes to the Stress-Adapted phenotype that challenges conventional urinary tract infection treatment methods by indicating an evolutionary adaptation toward aggressive tissue invasion and environmental persistence [57]. These *P. aeruginosa* isolates are high urease and lipase production predominance points to a complex, multifaceted attack strategy [57]. While many standard *P. aeruginosa* strains are primarily known for respiratory or skin infections, these Iraqi UTI isolates appear specifically Stress adapted for the harsh environment of the urinary tract [56], [57]

- **Gelatinase as a Path Maker for Infection**

While urease changes the environment and lipase attacks cell membranes, gelatinase focuses on the glue that holds tissues⁵⁵. Degradation of the Extracellular Matrix (ECM). The Gelatinase breaks down collagen and other proteins in the ECM⁵⁶. This liquefaction of host tissue allows the bacteria to spread from the initial site of infection (the bladder) into deeper tissues and the bloodstream (bacteremia) [58]. Biofilm Maturity Gelatinase is often involved in the remodeling of biofilms [58]. By breaking down certain proteins within the biofilm matrix, the bacteria can detach and disperse to start new infection sites when the current environment becomes too "stressed" or nutrient poor. Chronic Infection: High gelatinase production is often a hallmark of chronic, hard-to-treat UTIs [59]. In many studies, *P. aeruginosa* gelatinase production varies significantly in UTI [59]. This finding of 55% suggests that more than half of these Iraqi clinical isolates have the specific toolkit required for invasive rather than just superficial infection.

The study further identified that 55% of the isolates possessed gelatinase-producing capacity. Gelatinase, a specialized protease, plays a pivotal role in the High virulence profile of these uropathogens by degrading the collagenous components of the extracellular matrix [60]. Through altering biofilm architecture and breaking down host immune signaling proteins, the enzymatic activity not only makes it easier for *P. aeruginosa* to physically disseminate across host tissues but also helps the infection become stress adapted [60].

According to the results for lipase (57%) and urease (74.8%), a highly aggressive uropathogenic threat is evident. These isolates make use of a complex enzymatic triad: lipase disrupts membranes, urease modifies ambient pH and gelatinase breaks down

structural tissue. The persistence and drug-resistant difficulties in treating *P. aeruginosa* UTIs in the Iraqi clinical population are probably explained by this combined enzymatic arsenal [59], [60]

- **Protease: The Master Degradar**

Protease producing capacity proteolytic activity was highly prevalent in the clinical *Pseudomonas aeruginosa* isolates, according to phenotypic screening Table-10, Fig.10. Specifically, 61% of the uropathogenic isolates were protease positive and had a clear zone on skim milk agar due to casein hydrolysis, whereas 39% of the isolates had a protease negative phenotype [58], [60]. These clinical strains have a strong pathogenic potential within the human host, as evidenced by the high frequency of protease production 61%. These enzymes have a variety of functions in the context of urinary tract infections UTIs, including promoting ascending colonization and impeding immune clearance [53], [55]. Also, interesting to note that the coexistence of protease positive (61%) and protease negative (39%) phenotypes within the same clinical pool illustrates *P. aeruginosa*'s diverse adaption methods in local healthcare environments [56]. Strong antibiotic pressures and protracted host immune responses are common in Iraq for the clinical strains of UTIs that are difficult or persistent [58]. Protease production is essential for acute tissue invasion, but during long term or biofilm mediated infections, some strains undergo metabolic rewiring or selective phenotypic switching to down regulate energy consuming extracellular enzymes [55], [57]. This enables them to avoid immune detection and continue as chronic reservoirs [59].

- **Siderophores: The Iron Scavenging Weapon**

In *P. aeruginosa* isolates, iron is absolutely necessary. The bacterium cannot carry out energy metabolism or DNA replication without it. The high proportion of producers 63% indicates that these isolates are well suited to the low nutrient environment of the human kidneys and bladder [60]. In biofilm regulation, one recognized cause of biofilm formation is iron signaling through siderophores [60]. In iron-limited conditions like a UTI, siderophore production ramps up, which in turn signals the bacteria to form a protective biofilm [60]. The infection becomes drug resistant as a result. About 63% of the *P. aeruginosa* isolates were found to be able to produce siderophores. This high prevalence is a hallmark of the Stress-Adapted phenotype mentioned in this research [59], [60]. The capacity to release high affinity iron chelators is crucial for bacterial persistence and pathogenicity in the iron restricted environment of the human urinary system [58], [60].

In addition to helping people survive under nutritional stress, siderophore synthesis serves as a crucial signaling mechanism for the formation of biofilms and the expression of other virulence factors, including proteases [60].

- **Antibiotic Resistance Profile**

A concerning trend of multidrug resistance (MDR) is revealed by the antibiotic susceptibility profiles of the *P. aeruginosa* isolates, as shown in Table-12 and Figure-12. Resistance to Beta Lactams and Carbapenems, the high resistance rates against Piperacillin (51%), Cefepime (52%), and Aztreonam (50%) are indicative of multiple resistance mechanisms, likely including the production of Extended Spectrum Beta-Lactamases

(ESBLs) and the upregulation of Efflux Pumps [7], [8], [60]. Resistance to Imipenem (43%) and Meropenem (42%) in the Carbapenem Challenge is very concerning. This resistance indicates the presence of carbapenemase-producing strains (MBLs) or the loss of OprD porins, which are the main entry points for these medications into the cell, since carbapenems are frequently the last line of defense [11], [13]. Aminoglycoside and Fluoroquinolone Resistance isolates also shown notable resistance to levofloxacin (48%) and aminoglycosides (gentamicin 38%, tobramycin 37%, and amikacin 34%). Aminoglycoside Modifying Enzymes (AMEs) or 16SrRNA methyl-transferases are typically responsible for *P. aeruginosa* resistance to aminoglycosides. The high resistance to Levofloxacin (48%) is often linked to mutations in the DNA gyrase (*gyrA*) or Topoisomerase IV (*parC*) genes, which we previously discussed in this study [14], [26]. Colistin is the Last Standing Pillar. In this study, Colistin remained the most effective agent with a resistance rate of only 16%. The mechanism of Colistin works by disrupting the bacterial outer membrane like a detergent. Correlation with Biofilm and Stress tolerance are Synergy with Biofilm [13]. The 42.1% strong biofilm producers identified likely account for the 42% resistance seen in Meropenem and Imipenem. Biofilms act as a physical barrier that prevents these antibiotics from reaching their targets [32]. Environmental Resilience are same physiological toughness that allowed these isolates to survive Thermal, Cold, and pH stress also contributes to their ability to survive high concentrations of antibiotics [44].

CONCLUSION

Fundamental Finding : This study underscores a critical evolutionary convergence where environmental stress adaptation and antibiotic selection pressures drive the emergence of hypervirulent, multidrug-resistant (MDR) *Pseudomonas aeruginosa* clinical uropathogens. The investigated isolates demonstrate a formidable, multifaceted virulence arsenal characterized by the strong biofilm mediated persistence and a high prevalence of extracellular enzymatic activities including potent tissue destructive proteases, lipases, gelatinase, ureases and hemolytic agents complemented by aggressive iron scavenging siderophores. Parallel to this aggressive phenotypic profile, these clinical strains display profound physiological plasticity, exhibiting robust cross-tolerance to severe thermal shocks, cold exposure, osmotic salinity fluctuations, and broad amphoteric pH shifts. Crucially, this environmental resilience directly mirrors an expansive antibiotic resistance profile that heavily compromises frontline anti-pseudomonal therapies, including anti-pseudomonal penicillins, cephalosporins, monobactams, fluoroquinolones, and core carbapenems, while leaving colistin as the only remaining therapeutic salvage option. **Implication :** Together, these findings reveal that the capacity to tolerate environmental and physiological stresses provides *P. aeruginosa* with a dual survival advantage, facilitating both long-term persistence within the hospital ecosystem and successful colonization of the hostile human urinary tract. **Limitation :** This study is limited to phenotypic characterization of *Pseudomonas aeruginosa* isolates from UTI patients and does not include molecular analysis of

resistance and virulence genes. The sample scope may also limit the generalizability of the findings to other clinical settings and geographic regions. **Future Research :** These insights highlight the urgent need to look beyond conventional antibiotic susceptibility testing and develop novel therapeutic strategies that actively target the pathogen's adaptive mechanisms and enzymatic virulence machinery to control this rising clinical threat.

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