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Original Article

Comparative Analysis of *Pseudomonas aeruginosa* Isolated from Different Clinical Sources Using Whole Genome Sequencing and Bioinformatics

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Abstract

Introduction: This study aimed to evaluate the antibiotic resistance patterns, virulence characteristics, and genetic and epidemiological relationships of clinical isolates of *Pseudomonas aeruginosa* obtained from various sources in hospitals and clinics in the city of Baqubah, Iraq.

Materials and Methods: *P. aeruginosa* isolates (n = 80) were tested for antimicrobial susceptibility. Eight isolates, selected for diversity, underwent Whole Genome Sequencing (WGS). Analyses included the identification of Antibiotic Resistance Genes (ARGs), antimicrobial Resistance (AMR) Mechanism, Multi-Locus Sequence Typing (MLST), and Virulence Factors (VFs). A biofilm production assay was performed, and results were correlated with resistance patterns.

Results: High resistance rates were observed for the isolates, with the majority being multi-drug resistant: MDR 42.5%, XDR 27.5%, and PDR 10%. The highest resistance rates were recorded against Piperacillin (97.5%) and Polymyxin B (97.5%). Statistically significant resistance was also documented against Cefepime (70%, p = 0.0003) and Meropenem (36.3%, p = 0.013). 100% of the isolates were biofilm producers (68.75% strong, 31.25% moderate). Statistical analysis revealed a significant correlation between resistance patterns (MDR/XDR/PDR) and the capacity for strong biofilm formation (X squared = 18, p = 0.0004). Using MLST and WGS, the spread of high-risk clones was confirmed: ST-235 (in PA4 and PA18) and ST-244 (in PA7 and PA40). A substantial accumulation of ARGs was identified. Isolate PA 56 (ST-654) carried the most dangerous combination: bla_NDM-1, bla_KPC-2 (carbapenemases), and rmtF (high-level aminoglycoside resistance). The core set of essential VFs (such as the Type III Secretion System (TTSS), Exotoxin A, and Quorum Sensing) was conserved across all eight isolates.

Conclusions: Findings confirm widespread Multidrug-Resistant *P. aeruginosa* in Iraqi hospitals, driven by high-risk international clones and acquired *carbapenemase* genes. The combination of Mobile Genetic Elements (MGEs) in resistance transfer and strong biofilm formation presents a major therapeutic and epidemiological challenge, necessitating strict genomic surveillance and infection control. However, these findings are based on a local, small-scale comparison.

Keywords: *Pseudomonas aeruginosa*, Whole Genome Sequencing, Comparative Genomics, Antimicrobial Resistance, Virulence Factors

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Introduction

The rise of antimicrobial resistance among bacterial pathogens is a major global health concern, with multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) bacteria posing substantial challenges to infection control and treatment. *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen, is particularly worrisome because of its association with severe hospital-acquired infections, including pneumonia, urinary tract infections, and bloodstream infections, especially in immunocompromised patients.[1] The adaptability of *P. aeruginosa* enables it to develop resistance to multiple classes of antibiotics, resulting in high rates of MDR, XDR, and even PDR strains in clinical settings.[2] Resistance mechanisms in *P. aeruginosa* include efflux pump overexpression, biofilm formation, enzymatic degradation, and genetic mutations, all of which allow it to persist despite antimicrobial therapy.[3] *P. aeruginosa* exhibits a nonclonal epidemic population structure, with 1,392 sequence types (STs) identified globally. The ten major high-risk clones (ST235, ST111, ST233, ST244, ST357, ST308, ST175, ST277, ST654, and ST298)[4] are widely disseminated in hospital environments.[5] The success of these high-risk clones likely reflects the acquisition and accumulation of antimicrobial resistance genes (ARGs) carried on accessory genetic elements (AGEs). AGEs, including integrative and conjugative elements (ICEs), plasmids, and integrons, play a pivotal role in the accumulation and dissemination of ARGs within *P. aeruginosa* populations.[6]

Although polymerase chain reaction (PCR)-based techniques have traditionally been employed to detect resistance genes, these methods are limited by their reliance on known targets and cannot detect novel or unexpected resistance determinants.[7] In contrast, high-throughput whole-genome sequencing (WGS), combined with bioinformatics pipelines, offers a powerful tool for the rapid and comprehensive identification of resistance genes in bacterial genomes.[8] WGS is one of the latest approaches to study resistance mechanisms in organisms. It provides extensive information on the genes present within a pathogen, as this technique can process multiple DNA sequences in parallel with high throughput at a reduced cost and time.[9] WGS has proven indispensable in elucidating the genetic composition, resistance factors, and evolutionary adaptations of *P. aeruginosa*.[10] The study of molecular epidemiology and genetic diversity in multidrug-resistant *P. aeruginosa* that harbor genes conferring resistance to a wide range of antibiotics and virulence factors is key to understanding its role in the dissemination of such resistance determinants among clinical and environmental isolates.[11] This study aimed to evaluate the antibiotic resistance patterns, virulence characteristics, and genetic and epidemiological relationships of clinical isolates of *Pseudomonas aeruginosa* obtained from various sources in hospitals and clinics in the city of Baqubah, Iraq.

Materials and Methods

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller–Hinton agar. The results were interpreted as *susceptible*, *intermediate*, or *resistant* according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI M100, 33rd Edition, 2023).[12] The antibiotics tested included piperacillin (100 µg), ticarcillin (75 µg), piperacillin–tazobactam (100/10 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg), polymyxin B (MIC method), tobramycin (10 µg), ciprofloxacin (5 µg), and levofloxacin (5 µg). The results were verified using the standard reference strain *P. aeruginosa* ATCC 27853.

Quantitative Biofilm Formation Assay

Quantitative assessment of biofilm formation by *P. aeruginosa* was performed as previously described.[13] Briefly, all isolates were cultured overnight in brain–heart infusion broth at 37 °C and adjusted to a turbidity equivalent to 0.5 McFarland standard. Each isolate was inoculated into Mueller–Hinton broth supplemented with 1% glucose and mixed thoroughly. A 200 µl aliquot of each bacterial suspension was transferred in duplicate into sterile 96-well U-bottom polystyrene microplates. Plates were covered and incubated aerobically at 37 °C for 24 hours. Following incubation, planktonic cells were removed by rinsing the wells twice with deionized water, and residual fluid was discarded by tapping the plates on filter paper. The plates were then dried and fixed at 65 °C for 1 hour. The adherent bacterial cells were fixed with 200 µl of absolute methanol (99%) for 20 minutes at room temperature and subsequently stained with 200 µl of 0.1% crystal violet for 15 minutes. Excess stain was removed, and the plates were air-dried. To solubilize the bound dye, 33% glacial acetic acid was added to each well. The optical density (OD) was measured at 630 nm using an ELISA microplate reader to quantify biofilm formation. Biofilm assay was run in triplicates (with controls); results were averaged from replicates. The biofilm-forming capacity of each isolate was classified based on OD values according to the criteria presented in Table 1.

Table 1. Classification of Bacterial Adherence and the biofilm-forming capacity by Microtiter Plate method

Mean OD630	Biofilm intense
OD ≤ ODc*	Non-biofilm producer
ODc < OD ≤ 2ODc	Weak
2ODc < OD ≤ 4ODc	Moderate
OD > 4ODc	Strong

*Cut off value (ODc) = Mean OD of negative controls + 3 (Standard Deviation of control).

DNA Extraction and Sequencing

Eight *P. aeruginosa* isolates were available for WGS. DNA was extracted using the High Pure Template Preparation Kit (Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions. The quality of the extracted DNA was assessed using a Nanodrop spectrophotometer. The eight isolates underwent library preparation and paired-end sequencing on an Illumina MiSeq sequencer.[14] Briefly, sequencing libraries were prepared using the Illumina TruSeq Nano DNA kit (Illumina, Inc., San Diego, CA, USA), followed by paired-end sequencing on an Illumina MiSeq sequencer. Library layout (paired), genomic source, and Illumina sequencing parameters were recorded for subsequent analysis.

Bioinformatics Data Analysis

The pipeline WGSBAC (v2.0.0) was used to analyses the data from isolates of *P. aeruginosa* that were sequenced for this investigation.[14] In essence, WGSBAC used FastQC (v0.11.5) to control the quality of the raw sequencing data, and the coverage was determined.[15] Shovel (v1.0.4) was constructed using SPAdes (v3.14.0).[16] QUAST (v5.0.2) was used to inspect the quality of the assembly.[17] The databases MiniKraken (v2) and Kraken 2 (v2.0.7 beta) were used to categorise readings and assemblies and check for contamination.[18] The NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP), which uses AMR Finder

Plus, ResFinder, and CARD for antimicrobial resistance profiling and determination of AMR genes, annotated the assembled genome sequence.[19] The virulence characteristics were discovered using the VFDB database.[20] The GC-Profile tool was used to identify the genomic sequence's areas with aberrant G + C contents.[21] To check for the existence of plasmids and plasmid replicons, PlasmidFinder and Platon were employed.[22]

Analysis of Sequences

Sequence types (STs) were determined using staramr version 0.7.2 (https://github.com/phac-nml/staramr; accessed October 1, 2025).[23] *Pseudomonas aeruginosa* serotypes were predicted using the *P. aeruginosa* Serotyper (PAst) 1.0 tool within the Center for Genomic Epidemiology (CGE) platform.[24]

Sequence Annotation and Comparison

Genome sequences were annotated using the Rapid Annotation using Subsystem Technology (RAST) platform,[25] combined with BLASTP/BLASTX/BLASTN,[26] the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and RefSeq.[27] Resistance genes, mobile genetic elements (MGEs), and other genomic features were identified using CARD,[28] ResFinder,[29] ISfinder,[30] INTEGRALL,[31] and the Tn Number Registry.[32] Virulence factors were detected using the VFDB database.[20] Multiple and pairwise sequence comparisons were performed using BLASTN. Gene organization diagrams were created using Inkscape 1.0 (https://inkscape.org/en/).

Statistical Analysis

Dendrograms were generated using the UPGMA algorithm with Jaccard similarity coefficients. Dendrogram construction was performed in RStudio using the dendextend package.[33] Network plots were created using the network package in RStudio.[34,35] Stacked charts were generated via the ggplot2 package using the Chart Builder tool in RStudio. Intersections of gene sets across multiple samples were visualized using UpSet plots generated with the UpSetR package,[36] which provides a scalable alternative to traditional Venn diagrams for identifying shared gene subsets.

Results

Antibiotic Susceptibility Testing

Table 2 shows the results of the antimicrobial susceptibility test rates for 80 *P. aeruginosa* isolates obtained in our study. *P. aeruginosa* isolates were categorized by resistance patterns: Most were multidrug-resistant (MDR; 34 isolates, 42.5%), followed by XDR (22 isolates, 27.5%) and PDR (8 isolates, 10%). Only 16 isolates (20%) of the total showed resistance to the majority of antibiotics.

Biofilm Production Assay

All 80 *P. aeruginosa* isolates (100%) were biofilm producers. Most (68.75%) showed a strong capacity, with the remainder (31.25%) being moderate. The relationship between biofilm production and antibiotic resistance is detailed in Table 3.

Table 2. Distribution of Antibiotic Resistance rates of *P. aeruginosa* Isolates (n = 80)

Antibiotic		Antimicrobial susceptibility patterns	
Antimicrobial class	**Agent included generic name**	**Resistance**	**Sensitive**
Penicillin	Piperacillin	78 (97.5%)	2 (2.5%)
		X-squared = 72.2, df = 1, $P < 2.2$	
	Ticarcillin	72 (90%)	8 (10%)
		X-squared = 51.2, df = 1, $p = 8.34$	
B-lactam combination agents	Piperacillin-tazobactam	26 (32.5%)	54 (67.5%)
		X-squared = 9.8, df = 1, $P = 0.0017$*	
Cephems	Ceftazidime	62 (77.5%)	18 (22.5%)
		X-squared = 24.2, df = 1, $P = 8.68$	
	Cefepime	56 (70%)	24 (30%)
		X-squared = 12.8, df = 1, $P = 0.0003$*	
Monobactams	Aztreonam	26 (32.5%)	54 (67.5%)
		X-squared = 9.8, df = 1, $P = 0.0017$*	
Carbapenems	Imipenem	17 (21.3%)	63 (78.8%)
		X-squared = 26.45, df = 1, $P = 2.70$	
	Meropenem	29 (36.3%)	51 (63.8%)
		X-squared = 6.05, df = 1, $P = 0.013$*	
Lipopeptides	Polymyxin B	78 (97.5%)	2 (2.5%)
		X-squared = 64.8, df = 1, $P = 8.29$	
Aminoglycosides	Tobramycin	38 (47.5%)	42 (52.5%)
		X-squared = 0.2, df = 1, $P = 0.6547$	
Fluoroquinolones	Ciprofloxacin	27 (33.8%)	53 (66.3%)
		X-squared = 8.45, df = 1, $P = 0.0036$*	
	Levofloxacin	28 (35%)	52 (65%)
		X-squared = 7.2, df = 1, $P = 0.0072$*	

x2: Chi-Square Test, *df*: Degree of freedom, *P* = p-value, * Significant difference

Table 3. Patterns of MDR, XDR, and PDR of *P. aeruginosa* Isolated and their Relation to Biofilm Formation

Antimicrobial patterns	Isolates	Isolate number				
		Biofilm formation isolates	Strong biofilm formation	Moderate biofilm formation	Weak biofilm formation	Non biofilm producer
PDR	8	8 (100%)	3	5	0	0
XDR	22	22 (100%)	16	6	0	0
MDR	34	34 (100%)	25	9	0	0
Sensitive	16	16 (100%)	11	5	0	0
Total*	80	100%	55	25	0	0
X-squared = 18, df = 3, *P = 0.0004						

Selected Isolates for Whole Genome Sequencing

Eight isolates were selected for further analysis based on the variety of outcomes observed across the following categories: source, socio-demographics, antibiotic resistance, biofilm formation, sample collection site, and isolation date (Table 4).

Table 4. Criteria Used to Select *P. aeruginosa* (PA) Isolates for WGS

Character	*PA1*	*PA4*	*PA7*	*PA18*	*PA40*	*PA54*	*PA56*	*PA58*
Source	Burns	Blood	Burns	Burns	Urine	Burns	Urine	Burns
Gender	Female	Male	Female	Female	Female	Female	Male	Male
Age	23	38	60	12	37	50	44	5
Resistance categories	8/12 (XDR)	10/12 (XDR)	8/12 (MDR)	9/12 (XDR)	2/12 (SDR)	9/12 (XDR)	12/12 (PDR)	12/12 (PDR)
Biofilm	Strong	Strong	Strong	Strong	Moderate	Strong	Strong	Moderate
Site of sample collection	Baquba Teaching Hospital (burns Wards)	Baquba Teaching Hospital (educational laboratories)	Baquba Teaching Hospital (burns Wards)	Baquba Teaching Hospital (burns Wards)	Consultation clinic (labs)	Baquba Teaching Hospital (burns Wards)	Consultation clinic (labs)	Baquba Teaching Hospital (burns Wards)
Date of isolation	7/11/2023	8/11/2023	13/11/2023	4/12/2023	26/12/2023	11/2/2024	11/2/2024	21/2/2024

Whole Genome Sequencing, Annotation and Assembly

Following Whole-Genome Sequencing (WGS), the eight *P. aeruginosa* genomes were annotated using the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) with PAO1 as the reference. Genome attributes and annotation statistics, compared to other reference genomes, are detailed in Table 5.

Table 5. Genome Features of Eight *P. aeruginosa* Strains

Characteristic	Genome quality	Contigs	Genome length	GC content (%)	Contig N50	Contig L50	CDS	tRNA	rRNA	Repeated regions
P. aeruginosa 1	Good	81	6,841,731 bp	65.82	271,449	9	6,341	59	3	53
P. aeruginosa 4	Good	190	6,912,033 bp	65.90	203,116	12	6,411	61	3	0
P. aeruginosa 7	Poor	234	10,716,087 bp	56.20	212,509	15	9,822	127	6	108
P. aeruginosa 18	Good	186	6,914,534 bp	65.90	212,525	11	6,411	61	3	0
P. aeruginosa 40	Good	109	6,529,484 bp	66.23	279,465	7	6,003	59	3	58
P. aeruginosa 54	Good	124	6,679,419 bp	65.98	232,288	9	6,193	59	3	39
P. aeruginosa 56	Good	162	7,068,220 bp	65.87	186,694	11	6,633	61	3	80
P. aeruginosa 58	Good	129	7,233,123 bp	65.57	225,897	12	6,735	62	3	7

Comparative Genome Analysis

MLST assessed the genetic diversity of eight *P. aeruginosa* strains using seven housekeeping genes. Substantial variation was found, revealing the following Sequence Types (STs): PA1 (ST 1194), PA4 (ST 235), PA7 (ST 244), PA18 (ST 235), PA40 (ST 244), PA54 (ST 316), PA56 (ST 654), and PA58 (ST 664). Serotyping classified the strains into O-types: PA1 (O7), PA4 (O11), PA7 (O5), PA18 (O11), PA40 (O5), PA54 (O11), PA56 (O4), and PA58 (O5). Full epidemiological details are in Table 6. WGS of *P. aeruginosa* isolates revealed a widespread multidrug resistance profile (Supplementary 1). All eight isolates were resistant to at least one aminoglycoside, with four isolates harboring more than five resistance genes. Isolate PA56 contained the highest number of resistance genes. All isolates carried at least two β-lactam resistance genes. Additionally, two amphenicol-resistance genes were detected: *catB7* was present in all isolates, and *catA3* was identified in one. The *fosA* gene, conferring fosfomycin resistance, was detected in all isolates. The *crpP* gene was found in five isolates, and *qnrD1* in one, both encoding quinolone resistance. Resistance determinants to tetracyclines (*tet(B)* and *tet(G)*), folate pathway antagonists (*sul1* and *dfrA1*), aminocyclitols (*aadA2b* and *aadA6*), and rifamycin (*ARR-2*) were also identified. Acquired disinfectant resistance genes, including *qacE*, *qacL*, and *qacH*, were present in five, two, and one isolate, respectively.

Supplementary 2 summarizes the AMR genes and corresponding mechanisms annotated in the genomes of the eight isolates. Although all isolates carry a high number of resistance genes, the specific genes and mechanisms vary between them. An UpSet plot effectively visualizes the relationships and overlaps among the eight datasets (Figure 1). The largest overlap comprises 94 elements shared across all isolates, indicating a strong core commonality, while smaller intersections highlight subset-specific gene patterns. Supplementary 3 summarizes the virulence factor (VF) classes and corresponding genes identified in the genomes. All isolates shared a core set of virulence factor genes, although some genes varied among isolates. UpSet plot analysis revealed a conserved core of 204 genes across all eight isolates (Figure 2). Smaller intersections included three genes shared by seven isolates (excluding PA58) and two genes present in only three or four isolates, suggesting limited gene variability or conditional expression differences.

WGS also revealed significant diversity of insertion sequence (IS) and transposon (Tn) elements (Supplementary 4). The number of IS elements per isolate varied widely. The most frequently observed IS types were ISPa6, ISPsy24, ISAs31, and ISPa32. Several isolates carried unique IS elements: PA1 harbored ISPsy20, PA4 contained ISAs33, PA18 had ISPa26, PA45 carried ISPsy29, PA56 had IS1071 and ISPa1635, PA58 contained seven unique IS elements, and PA7 harbored 15 unique IS elements. The 52 identified transposons included 42 unit transposons and 10 composite transposons, with Tn6061 being the most common type. Certain isolates carried unique Tn elements; for example, PA56 had two copies of Tn4661, while PA58 contained Tn5563 and other elements. PA1 and PA7 also possessed unique transposons. Isolates 4, 7, 18, and 56 each contained an integrative conjugative element, and a Col3M plasmid was detected only in PA7.

A high number of antibiotic resistance genes (ARGs) were located on mobile genetic elements (MGEs) in the *P. aeruginosa* isolates (Supplementary-4). The ARGs conferred resistance to multiple antibiotic classes, including aminoglycosides (e.g., *aph(3')-VIb, ant(2'')-Ia, aph(6)-Id, aph(3'')-Ib, aac(2')-Ia, aph(3')-VI*), β-lactams (e.g., *blaOXA-2, blaPER-1, blaOXA-392, blaVIM-4, blaCMY-16*), fosfomycin (*fosA*), quinolones (*crpP* and *qnrD1*), tetracyclines (*tet(A)* and *tet(G)*), amphenicol (*catA3* and *catB7*), folate pathway antagonists (*sul1*), and quaternary ammonium compounds (*qacE*, *qacL*).

Table 6. Multi-locus Sequence Typing and Serotyping of Eight *P. aeruginosa* Isolates

Gene	*P. aeruginosa* 1	*P. aeruginosa* 4	*P. aeruginosa* 7	*P. aeruginosa* 18	*P. aeruginosa* 40	*P. aeruginosa* 54	*P. aeruginosa* 56	*P. aeruginosa* 58
acsA	28	38	17	38	17	13	17	9
aroE	24	11	5	11	5	8	5	5
guaA	10	3	12	3	12	9	26	11
mutL	5	13	3	13	3	3	3	3
nuoD	1	1	14	1	14	1	4	4
ppsA	6	2	4	2	4	6	4	40
trpE	2	4	7	4	7	9	26	18
ST	1194	235	244	235	244	316	654	664
O type	O7	O11	O5	O11	O5	O11	O4	O5

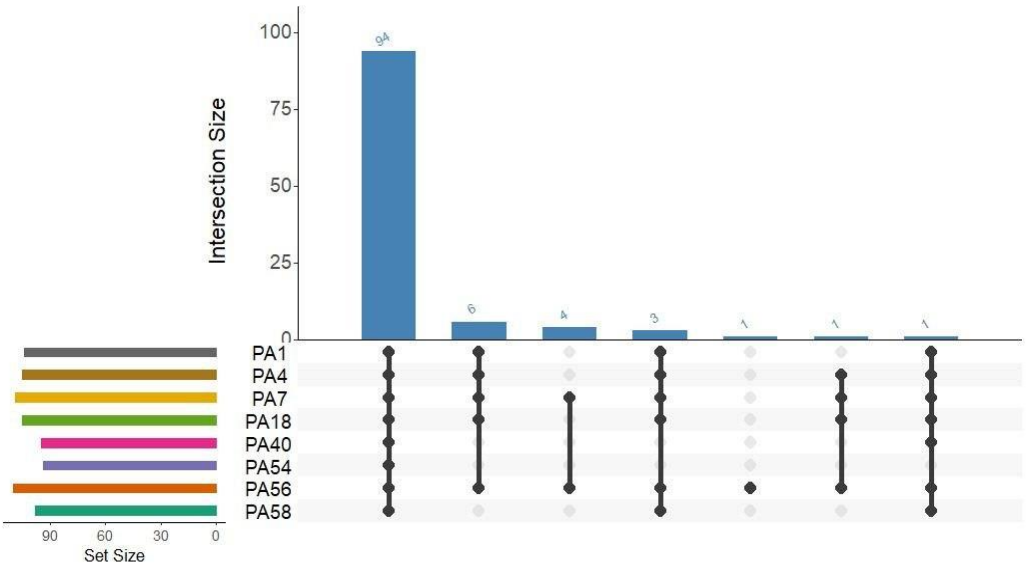

Figure 1. Eight *P. aeruginosa* (PA) Gene Sets (PA1, PA4, PA7, PA18, PA40, PA54, PA56, PA58) Were Compared for Shared and Unique AMR Mechanism Gene Content. UpSetR uses a matrix to display the data: Rows show the size of the individual gene sets, and columns show the size of the intersections between the sets.

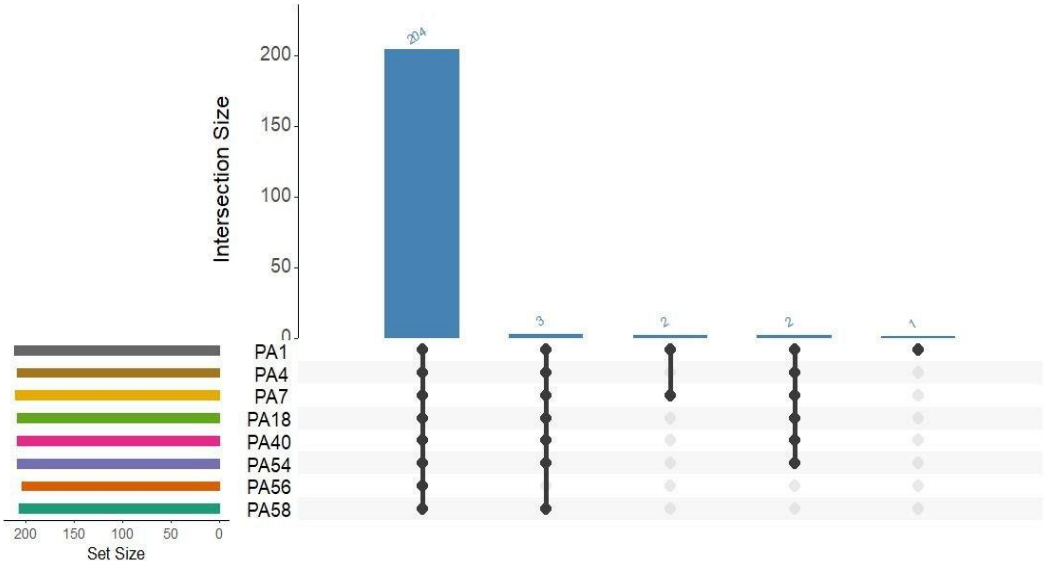

Figure 2. Eight *P. aeruginosa* (PA) Gene Sets (PA1, PA4, PA7, PA18, PA40, PA54, PA56, PA58) Were Compared for Shared and Unique Virulence Factor Gene Content. UpSetR uses a matrix to display the data: Rows show the size of the individual gene sets, and columns show the size of the intersections between the sets.

Discussion

The data confirms a strong link between high phenotypic resistance rates (Table 2) and complex genotypic mechanisms (Supplementary 1 & 2). The prevalence of MDR, XDR, and PDR resistance is caused by a two-part defensive strategy: Accumulation of high-risk acquired genes and a broad, shared intrinsic defense system present in all strains. β-Lactam Resistance: Resistance in this class is a critical threat, with its complexity explained by the interplay of enzymatic and structural factors. The high resistance observed for piperacillin-tazobactam (32.5%, p = 0.0017), cefepime (70%, p = 0.0003), and meropenem (36.3%, p = 0.013) is statistically robust. This confirms a true, non-random spread of mechanisms like ESBLs and carbapenemases within the sampled population. The near-complete resistance to Piperacillin (97.5%) is largely attributed to the *blaPAO* gene (found in all isolates) and acquired *blaPER-1* (in 3 isolates). The dramatic drop in resistance when tazobactam is added precisely confirms that the dominant mechanism here is Inactivation Enzymes (Supplementary 1) which are susceptible to the inhibitor. Phenotypic resistance is primarily explained by the acquisition of *blaVIM-4* (in PA4, PA18) and the severe combination of *blaNDM-1* and *blaKPC-2* (in PA56), highlighting critical treatment challenges.[37] This enzymatic resistance is universally potentiated by the intrinsic mechanism of protein-modulating permeability (41 genes, Supplementary 1). This high gene count includes elements like *OprD* loss, confirming that reduced outer membrane permeability is a constitutive resistance factor that works synergistically with carbapenemases. Aminoglycoside and Quinolone Resistance: Resistance in these classes is determined by a combination of target protection and efflux mechanisms: Aminoglycosides (Tobramycin 47.5%) resistance is explained by the widespread dissemination of Aminoglycoside Modifying Enzymes (AMEs) such as aph(3')-IIb(found in all isolates). Consistent with previous findings, aph(3′)-IIb can be found in virtually all *P. aeruginosa* isolates.[38] The acquisition of the *rmtF* gene in PA56 is the most critical finding. This gene, listed under Inactivation/Target Protection Enzymes in Supplementary 1, confers high-level resistance to all aminoglycosides, thereby surpassing the moderate resistance resulting from conventional AMEs. The presence of these genes results in exceptionally high-level resistance to all parenterally administered aminoglycosides currently used in clinical settings.[39] According to the findings resistance to fluoroquinolones was 33.8%-35%. Resistance results from target protection genes such as *crpP* (in 5 isolates) and the dual-acting gene *aac(6')Ib-cr* (in PA56, PA58), which reduces ciprofloxacin effectiveness, ensuring a baseline level of resistance against multiple drug classes, including quinolones and tetracyclines with *tet(B)* detected in PA7 and *tet(G)* detected in PA56 and PA58. Crucially, the fundamental role belongs to the Efflux Pump systems (22–26 genes in all isolates). These pumps (such as MexXY-OMP and MexAB-OprM) provide broad intrinsic resistance that elevates the resistance level to Quinolones, even in the absence of *qnr* genes.

The extreme phenotypic resistance to polymyxin B (97.5%) is explained by genomic mechanisms, not acquired genes (*mcr* is absent). Resistance stems from regulated chromosomal mechanisms: mutations in genes controlling lipopolysaccharide (LPS) modification. The presence of protein altering cell wall charge genes (*GdpD* and *PgsA*) in all isolates confirms the mechanism: modifying the cell's

surface charge to reduce antibiotic binding. The presence of disinfectant resistance genes (*qacE*, *qacL*, and *qacH*), which encode efflux pumps, poses a severe threat. This undermines hospital cleaning protocols, promoting the persistence and spread of MDR pathogens.

Hospital infection control relies on Quaternary Ammonium Compounds (QACs) for surface disinfection.[40] The *qac* genes encode efflux pumps that confer multidrug and antiseptic resistance.[41] These pumps actively expel QAC from the bacterial cell, preventing lethal internal biocide concentration. Routine QAC use becomes ineffective against *qac* positive strains. Disinfection acts as selective pressure, favoring the survival and persistence of biocide-resistant pathogens in the clinical environment. The critical concern is that *qac* genes are often located on mobile genetic elements (like plasmids) that also carry antibiotic resistance genes. This is called co-selection. QAC resistance is frequently linked to antibiotic resistance.[42] In MDR bacteria, qac genes are associated with β-lactam resistance[43] in *S. aureus*, *E. coli*, and *A. baumannii*, reducing β-lactam susceptibility. *Qac* genes are also linked to resistance against aminoglycosides, chloramphenicol, tetracycline, erythromycin, and sulfamethoxazole.[44] When QAC disinfectants are used, they select for qac-carrying bacteria. Since antibiotic resistance genes are linked to qac, disinfectant use inadvertently drives the selection and horizontal spread of MDR strains, even without antibiotic pressure. Figure 1 uses an UpSetR plot to illustrate the genetic distribution of AMR mechanisms across eight *P. aeruginosa* isolates, analyzing the core and accessory resistance. The rows show the total number of AMR genes per isolate (resistance content). Differences (e.g., PA1 vs. PA58) indicate variability in resistance size. Isolates with more AMR genes are typically MDR and pose a higher clinical concern. This allows for tentative grouping (low, high, or pan-resistant resistance). The columns show the sizes of the intersections between isolate groups. The large intersection across all eight isolates reveals the core set of genes responsible for intrinsic or widely disseminated resistance in the species, defining the minimum inherent resistance capability. Partial intersections (e.g., PA1, PA4, PA7) point to acquired resistance genes. These may have spread via Horizontal Gene Transfer (HGT) or be shared among related clones, contributing to the MDR phenotype. Intersections for single isolates (genes not shared) highlight recent genetic mutations or acquisitions unique to that isolate's profile, key to understanding resistance evolution in specific niches.

Table 3 reveals a highly significant association between the AMR profile (MDR, XDR, PDR) and biofilm formation (p = 0.0004). 100% (80/80) of *P. aeruginosa* isolates were biofilm producers (Strong: n = 55; Moderate: n = 25). The absence of non-producers confirms biofilm formation as a predominant, conserved virulence trait. This capability provides a fundamental, non-specific defense against stresses (e.g., antibiotics), independent of acquired mechanisms. Biofilm formation complicates clearance and contributes to chronic, treatment-resistant infections.[45] While all categories produced biofilm (100%)., MDR (73.5%) and XDR (72.7%) isolates showed the highest propensity for Strong biofilm formation. This suggests a synergistic relationship, as a robust biofilm provides structural defense (the EPS matrix acts as a crucial barrier) intrinsically linked to the highest drug resistance levels, complementing biochemical resistance from acquired genes. The high prevalence of Strong biofilm formers even in Sensitive isolates (68.75%) emphasizes that biofilm status is a primary virulence determinant, independent of the current resistance genotype. Its combination with MDR and XDR traits significantly compounds the clinical challenge.

The MLST and O-typing data define the evolutionary and epidemiological relationships among the eight *P. aeruginosa* isolates in Table 6. The prevalent ST-235 clone (PA4, PA18) is associated with serotype O11. The O11 serotype is a pattern commonly linked to carbapenem-resistant isolates. Previous reports have linked ST235 clones and O11 serotype to multiple drug resistance,[46] global dissemination potential,[46] and Middle Eastern outbreaks.[47] The ST-244 clone (PA7, PA40) is linked to serotype O5. Isolate PA58 (ST-664) is also linked to serotype O5. Interestingly, isolate PA54 (ST-316) is associated with the O11 serotype (like ST-235), despite having a vastly different sequence type. This discrepancy indicates that serotyping alone may be insufficient for determining true genetic relatedness. MLST (which analyzes seven housekeeping genes) offers higher accuracy in defining true epidemiological strains. MLST/O-typing strongly supports prior resistance findings: Identifying ST-235 (linked to blaVIM-4) confirms that resistance spread is not random, but occurs via specific, robust clonal lineages capable of clinical establishment. Identifying two clones (ST-235 and ST-244) is crucial for epidemiological investigations to curb their spread. The prevalence of ST-235 and ST-244 in this dataset aligns with global reports of these sequence types being associated with high-risk clones exhibiting multidrug resistance and virulence.[48] ST-654, though less prevalent, is highly significant: it carries the most dangerous gene combination (*NDM*-1, *KPC*-2, *rmtF*). This shows that less common (non-clonal) *P. aeruginosa* strains can acquire a lethal genetic makeup, posing a formidable clinical threat despite rarity.

Supplementary 3 compares key virulence factors (VFs) across eight *P. aeruginosa* strains. The majority of crucial VF classes are highly conserved and present in all isolates, supporting a robust pathogenic foundation linked to their MDR/XDR/PDR and biofilm capabilities. Genes for Flagella (*fla*, *flg*, *fli*, *mot*, and *pil*) and Type IV Pili are essential for initial colonization, microcolony formation, and spreading

on host surfaces (for adherence and motility). The complete Type III Secretion System (TTSS) and components of the Type VI Secretion System (H-T6SS) are also present (as secretion systems). These systems inject toxins (exoS, exoT, exoY) directly into host cells, causing acute toxicity and mediating interbacterial competition.[47] Genes for Exotoxin A (toxA), Elastase (lasA, lasB), and Alkaline Protease (aprA, prpL) are also present, which cause significant tissue damage, protein degradation, immune evasion, and host cell death (as toxins and damage enzymes). Quorum sensing (QS) genes were also identified. The major las and rhl systems (lasI, lasR, rhlI, and rhlR) coordinate collective expression of virulence factors (elastase, toxins) in late infection stages and regulate biofilm maturation and stabilization.[47] The complete alginate biosynthesis genes (*alg*, *muc family*) are essential for mucoid phenotype formation, enhancing biofilm, and protecting bacteria from phagocytosis (Antiphagocytosis), which were present in all isolates. Pyochelin (pch) and pyoverdine (pvd) siderophores are necessary for Iron uptake, growth, and survival in the iron-limited host environment, which were identified in 8 strains. Figure 2 uses an UpSetR plot to analyze the genetic distribution of virulence factors (VFs) across eight *P. aeruginosa* isolates, identifying core and unique virulence genes. The rows show the total number of VF genes per isolate, reflecting variations in pathogenic potential. Isolates with higher VF content may be hypervirulent (e.g., more efficient at forming biofilms, adhering, or producing toxins), warranting investigation into specific VF types. The columns show the sizes of the intersections between VF gene sets. The large intersection defines the core VFs of *P. aeruginosa* (e.g., T3SS components, Quorum Sensing genes), essential to its identity as a pathogen. Partial intersections suggest VF groups shared among specific strains. These may correlate with ecological specialization (e.g., genes for vigorous biofilm formation in cystic fibrosis) or specific tissue tropism. Individual intersections highlight recently acquired or independently evolved VFs. These may explain observed variation in clinical symptoms or immune evasion specific to that isolate. Based on Supplementary 4, genetic analysis reveals that antibiotic resistance genes (ARGs) are carried on diverse mobile genetic elements (MGEs), primarily transposons (Tn), and insertion sequences (IS). Significant variation in MGE count reflects differing genomic plasticity. Strain PA7 recorded the maximum MGEs (39 elements), followed by PA4 and PA18 (34 each). This suggests that these critical strains are effective recipients for foreign genetic material. Strains PA54 (4 elements) and PA40 (5 elements) showed the least activity, mainly carrying common IS elements. The data reveal active transfer mechanisms for beta-lactam and carbapenem resistance; the critical Metallo-beta-lactamase (MBL) gene, *blaVIM*-4 (in PA4, PA18), is mobilized by the insertion sequence (IS) ISKpn4. This confirms that ISs are precise, highly effective agents for the rapid insertion of *blaVIM*-4 into new genomic sites. The composite transposon Tn1213 transfers the ESBL gene blaPER-1(in PA1, PA4, PA18). Other genes, like *blaOXA*-2 on Tn6196 and *Tn6082* (PA1) and *blaCMY*-16 (AmpC) on ISEc9 (PA7), confirm the diversity of mobilized resistance mechanisms. Aminoglycoside resistance involves diverse enzymes (APH, ANT, AAC); accordingly, the *aph(3')-VIb* gene (Amikacin/Gentamicin resistance) and blaPER-1 are co-transferred via Tn1213 or ISPssp2 in PA1, PA4, and PA18. This facilitates the co-evolution of multi-class resistance. The transposon Tn6082 is shared by three isolates (PA1, PA4, and PA56). It carries genes like *ant(2")-Ia* and *aph(6)-Id*, making it a critical vector for streptomycin and gentamicin resistance. PA7 acquired the *aac(2')-Ia* gene (Netilmicin resistance) on ISEch5, adding a unique resistance mechanism to that strain.

The analysis revealed transfer mechanisms for additional resistance classes and biocides showed that the *fosA* gene (Fosfomycin) (in 7/8 isolates) is carried by numerous ISs like (ISPa6, ISPa1, and ISPsy13). This IS-mediated spread indicates high flexibility, threatening remaining therapeutic options. For quinolone resistance (qnrD1); strain PA7 carries the *qnrD1gene* on the Col3M Plasmid, which is the most dangerous mechanism, enabling rapid transfer via conjugation. PA7 also carries *tet(B)* (Tetracycline) and *catA3* (Chloramphenicol) genes on ISEc39, confirming its status as a horizontal gene transfer (HGT) hotspot across multiple classes. Genes for biocide resistance (*qacE* and *qacL*) and sulfonamide resistance (*sul1* gene) were found linked on Tn6196 or IS26 (PA1, PA7). This linkage reinforces co-selection, where biocide use maintains antibiotic resistance in clinical settings.

Conclusion

This study provides compelling evidence of a significant public health threat. The high prevalence of drug resistance, particularly to last-resort antibiotics, coupled with strong pathogenic potential, underscores the urgent need for intervention. The findings highlight the importance of routine surveillance, genomic analysis, and targeted antimicrobial stewardship initiatives to monitor and control the spread of high-risk *P. aeruginosa* strains. The co-occurrence of resistance to both antibiotics and disinfectants presents a dual challenge for treatment and infection control in clinical settings. Overall, this work contributes to the growing recognition of PDR strains and emphasizes the need to understand the genomic basis of resistance to guide effective mitigation strategies. Of course, it should be noted that the small size of the sequenced cohort is a limitation that constrains the broad epidemiological generalization of the study's conclusions.

Authors' Contributions

Authors contributed equally to this study.

Ethical Approval

This research adhered to the principles outlined in the Helsinki Declaration and was approved by the Ethics Committee of the College of Education for Pure Sciences at the University of Diyala, Iraq (Ref No. CEPEC/03).

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Data Availability

The sequence data presented in this study has been deposited in GenBank as genome assemblies under submission accession number SUB15429138. The version described in this article is the first version.

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