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Molecular and Genetic Factors Contributing to *Pseudomonas aeruginosa* Virulence in Urinary Tract Infections

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen, and the incidence of complicated UTIs caused by *P. aeruginosa* has been increasing; however, very little is known with respect to virulence factors encoded by locally collected *P. aeruginosa* isolates. To the best of our knowledge, there is no published data to characterize these virulence factors in the south of Iraq; thus, it was aimed to determine the molecular and genetic basis of increased pathogenesis by *P. aeruginosa* from UTIs and to identify *P. aeruginosa* virulent clonal isolates causing UTIs in the southern part of Iraq. A total of 430 suspected UTI patients were enrolled from four general hospitals in Nasiriyah City, Iraq; 108 non-duplicate isolates of *P. aeruginosa* were recovered. Virulence genes (lasB, toxA, exoS, exoU, algD, lasR, and rhlR) were detected by polymerase chain reaction (PCR), biofilm formation was evaluated, and the relationship between virulence factors, including MDR, was investigated. The results indicated high frequencies of several VFs, vigorous biofilm production, and clear associations between the carriage of these genes and MDR, highlighting enrichment in exoU-positive strains. There was considerable genetic clustering in the isolates.

Keywords: *P. aeruginosa*, urinary tract infections, virulence genes, biofilm formation, molecular characterization

1. Introduction

Urinary tract infections (UTIs) continue to represent one of the most common bacterial infections in both community and hospital settings [1-2]. Although E. coli continues to be the predominant uropathogen, nonfermenting gram-negative bacilli are responsible for difficult and nosocomial UTIs [3]. *P. aeruginosa* is one of these, and it is intrinsically resistant to several classes of antimicrobial agents and closely correlated with catheter-related infections, prolonged hospitalization, and morbidity [4-5]. The pathogen is able to cause UTI from *P. aeruginosa*; it establishes a chronic disease in the urinary tract and evades host immune responses [6].

The virulence of *P. aeruginosa* pathogenesis is largely controlled by the expression of multiple virulence factors, all believed to be linked to specific genes [7]. LasB promotes tissue damage and immune response modulation, whereas exotoxin A (toxA) inhibits host protein synthesis, leading to cellular

damage [8]. Acute infection and host cell invasion are regulated by the type III secretion system (T3SS) effectors exoS/exoU, which have disparate cytotoxic vs. invasive capabilities and connect to disease severity [9-10]. In addition, the biofilm-related gene algD is also crucial for alginate production, which facilitates susceptible bacteria adhering to uroepithelium and indwelling medical devices [11].

The production of these virulence factors is highly controlled by the quorum-sensing systems (QSS), which are regulated predominantly by the transcriptional regulators lasR and rhlR [12]. Such regulators regulate population density-dependent gene expression and, as such, link production of virulence factors to the presence within the urinary tract [13-14]. Multiple reports have demonstrated that the hyperactivation of QSS correlates directly with high levels of biofilm, antimicrobial tolerance, and chronic infection [15-17]. Nevertheless, data about its prevalence and coexistence with other genes in the urinary *P. aeruginosa* isolates are limited from many regions, especially regarding its implication in uti-rPDR. Therefore, the specific molecular characterisation of these virulence genes is required for a better understanding of the pathogenesis of UTIs caused by *P. aeruginosa* and also the treatment and management of infections.

Notably, though the *P. aeruginosa* species is considered clinically significant in UTI, the available molecular information on the prevalence of virulence-associated genes and their association with biofilm formation, multidrug resistance, and genotyping is scarce from different regions, including the southern part of Iraq [18]. The absence of region-specific molecular epidemiological data for *P. aeruginosa* prevents successful infection control measures and restrains the selection of appropriate therapeutic interventions to treat *P. aeruginosa* UTIs.

This study aimed to explore molecular and genetic determinants of *P. aeruginosa* virulence in UTIs. The objectives of this study were to (i) establish the prevalence of important virulence-related genes among clinical urinary isolates of *P. aeruginosa*; (ii) evaluate the correlation between carriage of virulence genes, biofilm-forming ability, and MDR; and (iii) document relatedness between genotypes using profiles from putative virulence factors.

This study presents an in-depth molecular understanding of the virulence properties of *P. aeruginosa* isolates leading to UTIs in Nasiriyah City, Iraq. By linking virulence gene profiling, biofilm, MDR linkage, and genetic clonal complex, the results provide informative regional data to the published data. Results enhance the knowledge about the pathogenicity of *P. aeruginosa* in

UTIs and could help to improve clinical management, antimicrobial stewardship, and infection control policies in such healthcare facilities.

2. Materials and Methods

2.1 Study Design and Sample Collection

This was a hospital-based descriptive cross-sectional study performed in Nasiriyah City, Iraq, from 12 July to 24 November 2025. Four hundred and thirty urine samples were collected from patients with a clinical suspicion of UTI being referred to 4 major general hospitals in the city. The midstream urine samples were aspirated aseptically and processed according to the standard microbiological techniques. One hundred eight (25.1%) samples out of the whole sample set were *P. aeruginosa* positive. Out of the identified *P. aeruginosa* isolates, 43 (39.8%) were obtained from male patients, while 65 (60.2%) were female, indicating a higher proportion in females than males. The age of patients with proven infection ranged from 25 to 70. The research considered the non-duplicate isolates, and one isolate per patient was used to avoid duplication. The multicentre nature of the sampling helped conduct a representative sample and thus enhanced the generalizability of these results in relation to regional healthcare services.

2.2 Identification of *P. aeruginosa*

The isolates were inoculated on MacConkey agar and cetrimide agar and incubated in the presence of a 24 h plate. It was found on Gram staining to be a Gram-negative bacillus. Further, catalase and oxidase tests were done. If these tests were positive, bacteria were identified by SIM for motility, indole, and H_2S. Remaining biochemical tests, i.e., TSI, MR-VP, OF (oxidase fermentation), urease broth, the citrate test, lysine and ornithine decarboxylase, and growth at 42ºC for identification of *P. aeruginosa*, were also carried out. All the strains were stored in Luria Bertani (LB) of 20% glycerol at -20°C and restored from colonies picked in 50 ml of LB medium for 24 h at 37°C under shaking. The medium (1.5 ml) was removed after incubation, transferred into a new test tube, and centrifuged at 12000 rpm for 1 min at 4°C; repeat this process another two or three times, and the supernatant was discarded when the precipitate of bacteria ceased to be removed. The bacterial chromosomal genome was extracted using a DNA purification kit (MBST Inc., Iran) and stored at -20 °C in a helix for further experiments.

2.3 Antibiotic susceptibility

The *P. aeruginosa* isolates obtained from urine samples were subjected to an antibiotic susceptibility test by the Kirby-Bauer method as described in CLSI guidelines (Table 1). Briefly, overnight incubated bacterial cultures were standardised to 0.5 McFarland turbidity and then swab-inoculated on Mueller-Hinton agar plates. Pseudomonas test strain was inoculated on the surface of each tube (Mueller-Hinton broth) plate, and then antibiotic discs corresponding to the quality control of urine isolates were placed. Further, the plates were aerobically incubated at 37°C for approximately 18–24 h. Interpretation of the zone of inhibition was performed according to CLSI, and the data is expressed as susceptible or resistant based on Clinical and Laboratory Standards Institute breakpoints. MDR was defined as described by [19] as not susceptible to at least one drug in three or more antimicrobial categories

Table 1. Antimicrobial Susceptibility Profile of *P. aeruginosa* UTI Isolates (n= 108)

Antibiotic	Susceptible n (%)	Resistant n (%)
Piperacillin–Tazobactam	60 (55.6)	48 (44.4)
Ceftazidime	54 (50.0)	54 (50.0)
Cefepime	57 (52.8)	51 (47.2)
Imipenem	46 (42.6)	62 (57.4)
Meropenem	49 (45.4)	59 (54.6)
Aztreonam	53 (49.1)	55 (50.9)
Amikacin	66 (61.1)	42 (38.9)
Gentamicin	56 (51.9)	52 (48.1)
Ciprofloxacin	40 (37.0)	68 (63.0)
Levofloxacin	45 (41.7)	63 (58.3)
Colistin	102 (94.4)	6 (5.6)
Fosfomycin	31 (28.7)	77 (71.3)

2.4 DNA Extraction and PCR Amplification

The chromosomal DNA of these confirmed *P. aeruginosa* isolates was extracted using a ready-to-use commercial kit for bacterial genomic DNA extraction as instructed by the manufacturer. In brief, a colony from an overnight culture on cetrimide agar was suspended in lysis buffer, lysed with proteinase K, and purified with spin-column chromatography. The purity and concentration of the DNA were measured spectrophotometrically (A 260/A /A 280 ratio; ~1.8–2.0), and the integrity of the same was checked by electrophoresing on a 1% agarose

gel. Seven of the virulence-associated genes (lasB, toxA, exoS, exoU, algD, lasR, and rhlR) were amplified by PCR in 25 µL reactions under the following conditions: 12.5 µL PCR Master Mix Taq DNA polymerase, including taq buffer and dNTPs (Promega), and 0.5 µM targeting primers, each with forward and reverse sequences, are shown in Table 2. As a control for all reactions, nuclease-free water was added instead of template DNA, and a further volume of nuclease-free water was added up to the total reaction volume. The amplifications were carried out in a thermal cycler with an initial denaturation of 5 min at 95°C, followed by 30 cycles of denaturation for 0.3 min at 95°C and gene-specific annealing for 0.3 min in each case, extension at 72°C not exceeding 90 s, and a subsequent efficiency test to a final extension of 7 min at 72°C. The products were resolved on a 1.5% agarose gel with ethidium bromide (final resolution) and compared to a 100-base-pair ladder under UV illumination. Positive (*P. aeruginosa* ATCC 27853) and negative control (nuclease-free water) strains were included after every run.

Table 2. Primer sequences used for amplification of virulence-associated genes in *P. aeruginosa*

Target gene	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temp (°C)	Reference
lasB	F: GGAATGAACGAAGCGTTCTC R: GGTCCAGTAGTAGCGGTTGG	300	58	[20]
toxA	F: GGTAACCAGCTCAGCCACAT R: TGATGTCCAGGTCATGCTTC	352	56	[20]
exoS	F: CTTGAAGGGACTCGACAAGG R: TTCAGGTCCGCGTAGTGAAT	504	57	[20]
exoU	F: GCTAAGGCTTGGCGGAAT	204	58	[21]

Target gene	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temp (°C)	Reference
	A R: : AGATCACACCCAGCGGTAAC			
algD	F: CTACATCGAGACCGTCTGCC R: CATCAACGAACCGAGCATC	593	58	[22]
lasR	F: AAGTGGAAAATTGGAGTGGAG R: GTAGTTGCCGACGACGATGAAG	130	56	[23]
rhlR	F: TGCATTTTATCGATCAGGGC R: CACTTCCTTTTCCAGGACG	133	55	[23]

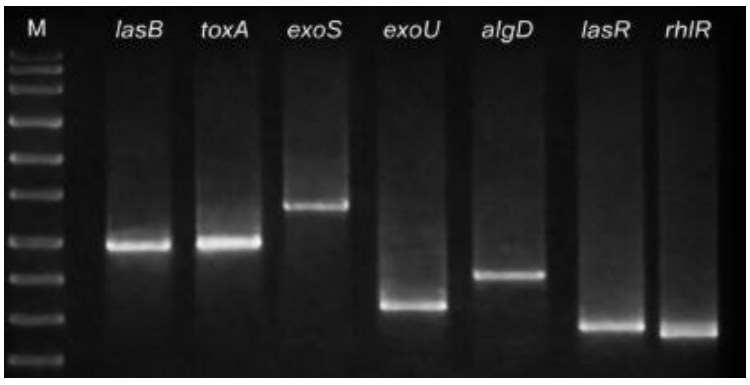

Figure 1. The result of virulence factors on *P. aeruginosa* was demonstrated using agarose gel electrophoresis (1.5%): Lane M: 100 bp ladder. Lane 1 has lasB (300 bp); Lane 2 has toxA (352 bp); Lane 3 has exoS (504 bp); Lane 4 has exoU (204 bp); Lane 5 has algD (593 bp); Lane 6 has lasR (130 bp); and Lane 7

has rhlR (133 bp). The bands that matched the expected molecular weight indicated that target genes were amplified successfully.

2.5 Biofilm

Biofilm formation was quantified using the typical 96-well microtiter plate method. Briefly, overnight cultures of each *P. aeruginosa* in tryptic soy broth (TSB) were incubated at 37°C, inoculated to an optical density equivalent to ~0.5 McFarland units, and subsequently diluted 1:100 in fresh TSB. The above culture was added to sterile, flat-bottom 96-well polystyrene microplates (200 µL/well in triplicate) and incubated statically for 24 h at 37°C, after which the planktonic cell suspension was removed, and the wells were thoroughly washed three times with PBS to eliminate non-adherent cells. Biofilms were fixed with methanol (15 min), air dried, and stained with 0.1% crystal violet (15 min) after allowing them to adhere. Unbound dye was aspirated, the wells were washed with dH_2O, and bound dye was solubilised in 95% ethanol (or 33% acetic acid). Biofilm biomass was expressed as the optical density measured at 570 nm (OD570) by a microplate reader. Biofilm formation was rated as Weak, Moderate, and Strong using OD cut-off values compared to the negative control.

2.6 Virulence-Based Clustering

Genetic relatedness of *P. aeruginosa* isolates with UTIs was assessed based on the virulence gene profiles (binary data), represented as the presence of each virulence-associated gene in all the isolates. A hierarchical cluster analysis, using the unweighted pair group method with the arithmetic average agglomeration option, was carried out to determine isolate similarity based on their virulence gene combination profiles. The resulting dendrogram was constructed for clustering analysis and genetic diversity of the isolates.

2.7 Ethics Approval

This study was also ethically approved by the Scientific Research Ethics Committee, College of Science, University of Thi-Qar, Iraq. All participating subjects gave written informed consent in advance of the intervention. All patient data were anonymous, and procedures were in accordance with ethical standards of the Committee on Human Experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2008.

2.8 Statistical Analysis

Data were analyzed using SPSS V19. Associations between virulence genes, AMR profile, and clinical data were tested using chi-square tests ($p < 0.05$ was considered to be significant).

3. Results

3.1 Prevalence of Virulence Genes

Table 3 shows the distribution of virulence factors among the confirmed 108 *P. aeruginosa* urinary isolates. The most prevalent gene was lasB, found in 90 (83.3%) isolates. This indicated that these strains may have high potential to induce tissue damage by producing elastase. Distribution of quorum-sensing regulators in lasR89 (78.7%); rhlR79 (73.1%). It is therefore evident that quorum-sensing networks are also well spread out among the strains. The presence of the gene exotoxin A (toxA) was detected in 80 isolates (74.1%), indicating the cytotoxicity capability of isolates. Regarding exoSS effectors, the gene for exoS was identified in 76 (70.4%) isolates and that of exoU in 32 (29.6%) isolates, demonstrating an absolute prevalence of invasive rather than highly cytotoxic phenotypes. Furthermore, the biofilm-associated gene algD was detected in 71 (65.7%) isolates, suggestive of high biofilm-formative potential among urinary *P. aeruginosa* strains.

Table 3. Prevalence of virulence-associated genes in *P. aeruginosa* urinary Isolates

Virulence Gene	Positive Isolates (n)	Prevalence (%)
lasB	90	83.3
toxA	80	74.1
exoS	76	70.4
exoU	32	29.6
algD	71	65.7
lasR	85	78.7
rhlR	79	73.1

3.2 T3SS Effector Distribution

The prevalence of the exoS gene was much higher than that of exoU, and this suggests an increased proportion of invasive compared with highly cytotoxic phenotypes in UTI isolates. However, the prevalence of antimicrobial resistance was significantly greater in exoU strains.

3.3 Biofilm-Associated Genes

Out of 108 *P. aeruginosa* isolates, 48 (44.4%) were strong biofilm producers, while 34 (31.5%) and 26 (24.1%) belonged to moderate and weak biofilm producer categories, respectively. There is an algD gene in 71 (65.7%) isolates of the 108 *P. aeruginosa* isolates confirmed to have strong biofilm formation. Among the strong biofilm producers, algD and lasR were present at the highest rate (90.3% and 87.1%, respectively), with a gradual decrease in their prevalence among moderate to weak biofilm formers compared to controls (Table 4).

Table 4. Biofilm formation capacity in relation to virulence gene expression

Biofilm Strength	Isolates (n)	*algD* Positive (%)	*lasR* Positive (%)
Strong	48	90.3	87.1
Moderate	34	63.6	68.2
Weak	26	29.4	41.2
Total	108	—	—

3.4 Association Between Virulence Genes and MDR

A significant association of the virulence gene carrying and MDR was observed in the 108 clinical *P. aeruginosa* isolates (Table 5). The positive rates of lasB, toxA, exoS, exoU, and algD of MDR isolates were higher than those of non-MDR isolates ($p < 0.05$), as high as the positive value of the exoU gene. These results indicate that virulence and resistance features are closely linked in *P. aeruginosa* urinary infection.

Table 5. Association of Virulence Genes With MDR in *P. aeruginosa* (n = 108)

Virulence Gene	MDR (%)	Non-MDR (%)	*p*-value
lasB (n = 90)	64 (71.1)	26 (28.9)	0.018
toxA (n = 80)	55 (68.8)	25 (31.2)	0.026
exoS (n = 76)	45 (59.2)	31 (40.8)	0.041

Virulence Gene	MDR (%)	Non-MDR (%)	*p*-value
exoU (n = 32)	27 (84.4)	5 (15.6)	0.003
algD (n = 71)	52 (73.2)	19 (26.8)	0.012

3.5 Virulence Profile Similarity

Unweighted pair group method with arithmetic mean clustering (UPGMA) analysis was performed to determine the genetic relationship among the *P. aeruginosa* urinary isolates based on virulence gene profiles (binary data), where virulence-associated genes were represented as binary (present/absent). The dendrogram demonstrated that the isolates fell into 4 clusters (A–D), suggesting a substantial genetic diversity among them. Although the isolates in each cluster were highly related to each other, there was evidence of discrete clustering by the virulence gene content. This clustering implies that closely related *P. aeruginosa* genotypes that may circulate in this population (Figure 2) differ distinctly with respect to their food web and the arsenal of virulence factors at their disposal.

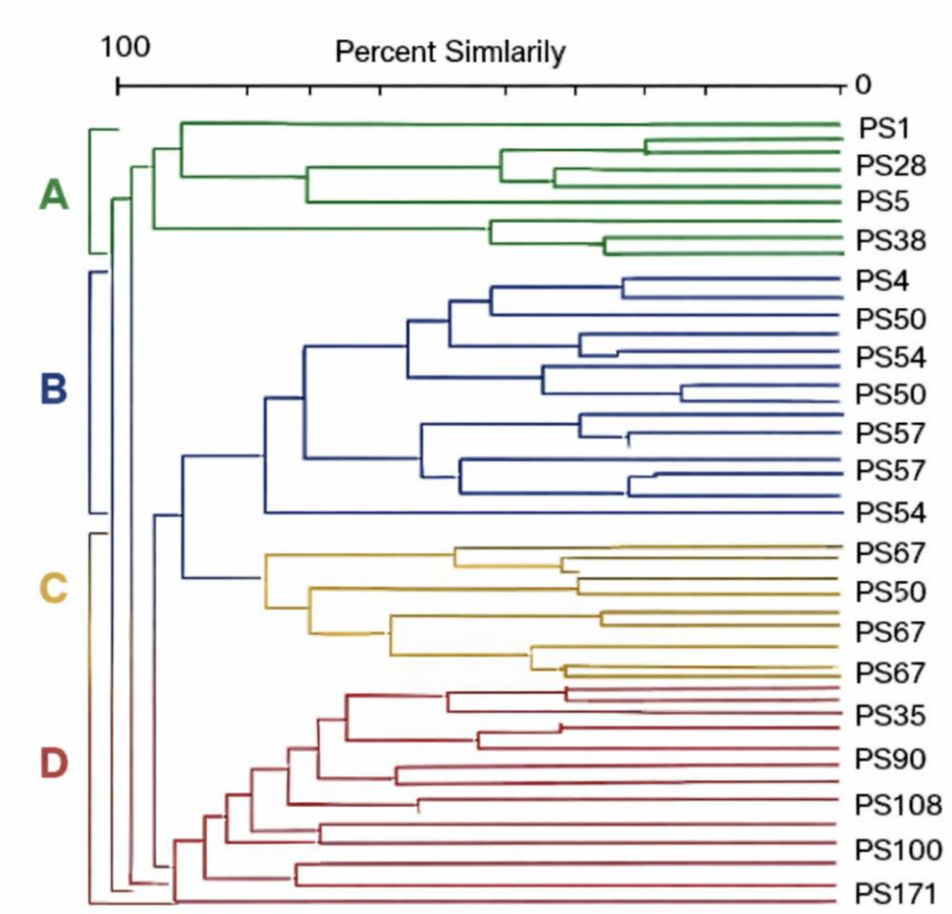

Figure 2. Dendrogram illustrating the genetic relatedness among *P. aeruginosa* urinary isolates based on virulence gene profiles.

4. Discussion

This study studied the molecular characteristics of P. aeruginosa isolates from UTI cases in southern Iraq, showing a high rate of major virulence genes, strong biofilm formation, and marked associations between virulence genes and MDR strains. Our findings underscore the convergent evolution of pathogenicity and resistance in this opportunist, an emerging motif among global clinical isolates.

The sensitivity of P. aeruginosa isolated based on antimicrobial profile demonstrates a high MDR profile, where there was a noteworthy non-susceptibility to ciprofloxacin (63.0%), imipenem (57.4%), and ceftazidime (50%), whilst colistin remains quite effective among the tested antibiotics, with a susceptibility rate of 94.4%. This is in consonance with recent regional reviews from Iraq [24], Iran [25], and Egypt [26], where MDR was estimated to be between 61% and 65% for both hospital-induced and catheter-associated UTIs. The carbapenem resistance in European ICUs is lower (40%–48%), but European ICUs are experiencing rising rates of colistin heteroresistance, which needs attention. The activity of colistin being retained vindicates the use of colistin as a last-resort option, but caution might be warranted in view of the resistance trend reported in Lebanon [27]. These findings highlight the need for prudent antimicrobial use, recommending antibiotic stewardship programmes and timely diagnosis to limit the spread of MDR P. aeruginosa within hospitals in Iraq.

A high prevalence (83.3%) of lasB and QS regulators lasR (78.7%) and rhlR (73.1%) encoding sequences was detected here, similar to previously reported studies that emphasised the significance of QS in P. aeruginosa uropathogenesis. Reports have shown 76% and 71% for the lasR and rhlR of urinary isolates, respectively, in a study [28], where their expression has been linked to persistence in chronic infections. A study from Iran [29] found that the positive rate of lasB was 85.3% in P. aeruginosa wound isolates, suggesting that its involvement has remained at different sites of infection. The predominance of exoS (70.4%) over exoU (29.6%) in our study group indicates this trend among the invasive T3SS phenotype as found in Baghdad [30], Egypt [31], and Mexico [32], such that community-acquired, catheter-related UTIs were linked with exoS strains. Remarkably, although there were fewer exoU$^+$ strains, they were of higher association with MDR ($p = 0.003$), as reported by [33], which demonstrated that exoU P. aeruginosa were more resistant to carbapenems and fluoroquinolones by combining the virulence factors with resistance genes in plasmids and transposon-like elements in Chinese ICU patients

The fact that algD was detected in 65.7% of the strains and is associated with biofilm formation (90.3% among strong producers) emphasises the role of alginates in biofilm structural stability. Our application of the quantitative, reproducible microtiter plate assay further strengthens this observation compared to previous qualitative assays. A study by [34] determined that algD was present in 78.6 % of urinary P. aeruginosa with a comparable biofilm profile and showed by RT-qPCR that optical density values were directly correlated to the extent of algD expression [35]. Conversely, the low incidence of algD (52%), but high rates of pelA and pslA expression, were reported by [36], suggesting that biofilm factors are closely related to geographical area.

These findings are consistent with previous reports of carrying algD in a proportion of biofilm-forming P. aeruginosa UTIs. Growth that is dependent on biofilm acts as a defence mechanism against antimicrobial and host defences, thus resulting in chronic and recurrent infections mainly among catheterised patients. Similar ranges of biofilm formation are reported in Indian [37], Portugal [38], and Egyptian [39] studies, emphasising the global significance of biofilm-mediated persistence in P. aeruginosa infections.

The relationship between the virulence genes and MDR (Table 4) suggested that dominant clones causing UTIs were disseminating with the ability to cause infection, in addition to carrying an antimicrobial resistance pattern. In a recent study, [41] found exoU and bla_VIM-2 co-carriage in ST235 P. aeruginosa in Europe, linking virulence to carbapenem resistance. In the same line, [42] observed aac(6')-Ib and qnr genes in lasB/algD/exoU isolates from Saudi Arabia, which made them resistant to aminoglycosides and quinolones. Our data are in agreement with this synergism, particularly for exoU strains, which might represent locally adapted high-risk lineages. With respect to clustering of virulence profiles, the UPGMA analysis generated in the current work was also able to cluster isolates in four major clusters based on gene combinations, a fact that mirrors what has been seen before in Tunis [43], where cluster-specific virulence profiles were associated with hospital wards. However, again, this reflects the identical vit rather than a genetic commonality.

The dominance of strong biofilm producers (44.4%) is also a considerable clinical concern, since the presence of biofilms may enhance antibiotic resistance by as much as 1000-fold [44]. This might be responsible for the high prevalence of recurrent UTIs and catheter failure in our setting. An updated meta-analysis by [45] also confirmed that P. aeruginosa biofilm-positive UTIs demanded longer therapy (2–3 times) and had a higher chance of relapse (threefold increase).

It is worth noting that a very significant relationship between genes associated with virulence and MDR existed in the present study. In this study, MDR isolates had a significantly higher prevalence of toxA, lasB, exoS, exoU, and algD genes, among which algD was more often detected in strains that were positive for exoU. These links are also well established by European, Asian, and Middle-Eastern investigations, in which co-selection of the virulence and resistance traits is believed to have been achieved under antimicrobial challenge in hospital environments. The presence of exoU, which is strongly positively associated with MDR isolates, is particularly concerning, as these strains are often linked to poor patient outcomes and limited therapeutic options.

Genetic relationship analysis with a similarity coefficient matrix of their virulence-gene profiles resulted in four major clusters demonstrating significant diversity between the isolates. Although lacking the sequence resolution of typing schemes based on sequence, virulence gene profiling can provide a valuable and meaningful perspective in directing epidemiological surveillance in poor-resource settings. This virulence-based clustering is similar to that reported in both studies from other developing countries, suggesting that several pathogenic lineages are present in hospital settings. The observed degree of genetic diversity may indicate diverse sources of infection, patient populations, or transmission dynamics in health care facilities.

The genetic similarity of this study was conducted on pathogenic gene profiles as binary data, not a sequence-based genotype approach. This is a useful indicator of pathogenicity but does not necessarily reflect true clonal relationships among the strains. Follow-up studies using sequencing are required to validate and expand upon these clustering patterns.

5. Conclusion

Our findings emphasised that *P. aeruginosa* urinary isolates from southern Iraq possess a burden of major virulence factor genes (lasB, exoS, and algD), have a high biofilm production percentage (44.4%), are associated with important virulent genotypes—particularly exoU—and are highly associated with MDR. Assignment of isolates into four categories by using virulence reflects the geographic differences in pathogenicity. These results demonstrate that the two dangers of increased virulence and AMR can be linked, and they add weight to calls for integrated monitoring strategies of resistance/virulence profiling. Utilizing anti-virulence strategies and antimicrobial stewardship might help to achieve an optimal management of *P. aeruginosa* UTI in this population. Prospective studies with accompanying transcriptomic analysis and larger

multicentre databases are needed to gain more understanding of virulence control during UTI.

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Conflict of Interest

The authors declare no conflict of interest.

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