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Prevalence of *pslA* and *pslB* Biofilm-Related Genes in *Pseudomonas aeruginosa* Isolates from ICU Patients: A Cross-Sectional PCR-Based Study

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ABSTRACT

Background and Aim: Biofilm formation is a key factor in persistence and antibiotic resistance of *Pseudomonas (P.) aeruginosa*, especially in intensive care units (ICU). This study aimed to explore how common the *pslA* and *pslB* genes are in multidrug-resistant (MDR) strains of *P. aeruginosa* isolated from ICU patients in Tehran and how these genes relate to biofilm development.

Materials and Methods: For this cross-sectional study, 112 *P. aeruginosa* isolates were collected from patients admitted to the ICUs of 1000-bed tertiary care of Milad Hospital, Tehran, Iran. The isolates were identified using standard microbiological techniques. To assess biofilm formation, crystal violet staining was conducted using 96-well microtiter plate. DNA was extracted using a commercial kit, and polymerase chain reaction (PCR) was carried out to detect the *pslA*/*B* genes.

Results: Of 112 samples, 92 (82.1%) isolates were biofilm producers, among them the vast majority of biofilm-producing strains were tested positive for both *pslA* (91, 98.9%) and *pslB* (92, 100%) genes. One *pslA*-negative isolate from a wound specimen retained weak biofilm-forming capacity. These genes showed a strong association with biofilm development (P<0.05).

Conclusion: The high prevalence of *pslA*/*B* genes in biofilm-forming MDR isolates suggests their significant role in enhancing biofilm formation and antibiotic resistance. This highlights the need to understand biofilm-related genes in managing ICU infections, though methodological limitations warrant further validation.

Keywords: Biofilm, ICU, Multidrug Resistance, *Pseudomonas aeruginosa*, *pslA*, *pslB*

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1. Introduction

Pseudomonas (P.) aeruginosa is a significant opportunistic Gram-negative bacterium, responsible for the serious life-threatening hospital-acquired infections. These infections predominantly affect immunocompromised patients and those in intensive care units (ICUs) **(1)**. This pathogen causes both acute and chronic human infections and poses a serious threat to public health. *Pseudomonas aeruginosa* possesses both intrinsic and acquired antibiotic resistance mechanisms, along with biofilm formation, making it a particularly challenging pathogen **(2)**.

Biofilm-associated infections pose a significant challenge for eradication, as biofilms provide protection against antimicrobial agents and host defense mechanisms **(3)**. Exopolysaccharides within the biofilm matrix play a vital role in adhesion, stability, and structural integrity **(4)**. Due to its biofilm-forming capacity, *P. aeruginosa* exhibits tolerance towards a wide variety of antimicrobials, making its eradication difficult **(5, 6)**. Consequently, characterizing novel biofilm-related targets is urgently needed.

The composition of biofilm matrices differs significantly between microbial species. *Pseudomonas aeruginosa* produces three main exopolysaccharides— Psl, Pel, and alginate (Alg) — which work synergistically to support biofilm development and stability **(7)**. Psl, a neutral polysaccharide consisting of repeating pentamers of D-glucose, D-mannose, and L-rhamnose, exhibits a helical distribution around the bacterial cell surface **(8)**. It plays a key role in surface attachment during initial biofilm formation **(9)**; it also acts as a signaling molecule that regulates exopolysaccharide production by stimulating c-di-GMP synthesis, thereby promoting the formation of stronger, more robust biofilms **(10, 11)**.

The Psl operon harbours 15 genes, with 11 (pslACDEFGHIJKL) essential for Psl synthesis **(12)**. The biosynthetic machinery involves an inner membrane-associated multiprotein complex coupled to Psl export. PslA exhibits similarity to WbaP, suggesting it may provide an assembly site for oligosaccharide repeat units onto an isoprenoid lipid carrier **(13)**. PslB, a paralogue of *P. aeruginosa* WbpW, functions as a bifunctional enzyme with phosphomannose isomerase (PMI) and GDP-D-mannose pyrophosphorylase (GMP) activities. It participates in producing sugar nucleotide precursors **(14)**. Collectively, these genes are indispensable for Psl production and play a key role in early biofilm development by facilitating cell-to-cell and cell-to-surface adhesion, while also contributing to the structural integrity of mature biofilms **(15)**.

Pseudomonas aeruginosa that resists multiple antibiotics is a growing problem in hospitals, especially when it forms biofilms that make treatment harder. Because of this, it is worth looking into certain genes tied to biofilm formation like *pslA* and *pslB* that show up in patient samples, particularly in ICUs, where infection risks are higher. In this study, we aimed to find out how common these genes are in resistant *P. aeruginosa* strains and whether they are connected to the bacterial ability to form biofilms.

2. Materials and Methods

2.1 Study Design and Ethical Considerations

We conducted a descriptive cross-sectional study to characterize biofilm formation in *P. aeruginosa* isolates. These isolates were obtained from patients admitted to the ICUs of 1000-bed Milad Hospital, a tertiary care facility in Tehran, Iran, between January 2023 and October 2024. The Research Ethics Committee of Iran University of Medical Sciences, Tehran, Iran, approved all aspects of the study protocol under ethical code IR.IUMS.REC.1401.1039.

2.2 Sample Collection and Isolates Confirmation

A total of 112 *P. aeruginosa* isolates were obtained from clinical samples collected from ICU patients, including urine, blood, wounds, tracheal aspirate/fluid, soft tissue specimens, and sputum. Initial culturing was performed on blood agar and MacConkey agar (Merck, Germany) plates in the hospital laboratories. Isolates were then transfer to the Department of Microbiology, Iran University of Medical Sciences, for further analysis. Identification as *P. aeruginosa* was confirmed using a series of microbiological and biochemical tests: Kligler Iron Agar (KIA), catalase, oxidase, oxidative-fermentative (OF) metabolism, motility, and growth on Mueller-Hinton agar at 42°C. The standard strain *P. aeruginosa* ATCC 27853 served as a positive control to validate the accuracy and ensure reliable identification. For long-term preservation, the isolates were stored in Tryptic Soy Broth (TSB) with 20% glycerol at -80°C **(16-18)**.

2.3 Antibacterial Susceptibility Tests

Antibiotic susceptibility was assessed using Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2024) guidelines **(19)**. Antibiotic disks (Oxoid Ltd., Basingstoke, UK) included piperacillin/tazobactam (100/10 µg), imipenem (10 µg), Meropenem (10 µg), Amikacin (30 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), and

Aztreonam (30 µg). Following incubation at 37°C for 16-18 hours, zones of inhibition were measured and interpreted using CLSI breakpoints. Colistin susceptibility was assessed by broth microdilution in accordance with CLSI guidelines, with resistance defined as a minimum inhibitory concentration (MIC) ≥ 4 µg/mL. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 served as reference strains. Multidrug-resistant (MDR) and extensively drug-resistant (XDR) classification was performed according to the standardized international definitions proposed by Magiorakos et al **(20)**. MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial and XDR was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories.

2.4 Biofilm Formation Assay

Biofilm-forming capacity of *P. aeruginosa* strains was quantitatively assessed using crystal violet (CV) microtiter plate assay according to the established protocols **(21-24)**. Bacterial strains were cultured in microplate wells at a 1:100 dilution in TSB from a 0.5 McFarland standard suspension. Following 24 hr incubation at 37°C, planktonic cells and media were removed by gently washing the wells three times with distilled water. Adherent cells (biofilm) were then stained with 0.1% (w/v) CV solution for 15 min at room temperature. Excess CV was discarded, and wells were washed three times with distilled water before air-drying at room temperature. Bound dye was solubilized by adding 33% glacial acetic acid (Merck, Germany) to each well. After 15 min, the solution was transferred to a new optically clear microplate, and absorbance was measured at 570 nm wavelength using an ELISA reader. Based on the established criteria **(21)**, strains were categorized as non, weak, moderate, or strong biofilm producers. All assays were performed in duplicate with average absorbance values used for analysis. The sterile TSB served as the negative control.

2.5 DNA Extraction and PCR Amplification

Chromosomal DNA was extracted from all *P. aeruginosa* isolates using a commercial kit (Favorgen, Taiwan) according to the manufacturer's protocol. DNA purity (A_{260}/A_{280} = 1.8–2.0) and concentration were assessed using a Nanodrop spectrophotometer. Conventional polymerase chain reaction (PCR) amplified *pslA* and *pslB* genes, using specific primers synthesized by Metabione, Germany **(25)**: [*pslA* (F-GTTCTGCCTGCTGTTGTTCA) and (R-GGTTGCGTACCAGGTATTCG)] and [*pslB* (F-GCTTCAAGATCAAGCGCATC) and (R-ACCTCGATCATCACCAGGTC)]. Each PCR reaction contained: 12.5 µL master mix (Amplicon, Denmark), 0.4 µM of each primer (forward and reverse), 2 µL template DNA, and water to reach 25 µL final volume. Amplification was performed in a thermal cycler (Eppendorf, Germany) under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 54°C and 72°C for 90 sec, and a final extension at 72°C for 5 min. The amplified products were electrophoresed on 1.5% agarose gel in tris–borate-EDTA (TBE) buffer at 90 v for 45 min, stained with DNA green viewer. Specific bands were visualized using gel documentation system to confirm the presence and size **(12, 26-28)**.

2.6 Statistical Analysis

Statistical analyses were performed using chi-squared tests in SPSS version 23.0 (IBM SPSS Inc., Chicago, IL, USA). The results were presented as descriptive statistics, primarily using percentages. Statistical significance was defined as $P<0.05$.

3. Results

3.1 Sample Collection and Demographics

A total of 112 non-duplicate *P. aeruginosa* isolates were obtained, primarily from urine specimens (38, 33.9%), followed by tracheal aspirate/fluid (35, 31.2%), wound and soft tissue specimens (31, 27.6%), blood (6, 5.3%), and sputum (2, 1.7%). The patients' age ranged from 7 to 89 years old (59.7 ± 28.27), which 68 (60.7%) were from male patients, while 44 (39.2%) from female.

3.2 Antimicrobial Susceptibility Profile

Antimicrobial susceptibility testing revealed high resistance rates among isolates: ceftazidime (73.2%), ciprofloxacin (68.7%), meropenem (66.9%), aztreonam (60.7%), imipenem (58.9%), amikacin (57.1%), piperacillin/tazobactam (34.8%), and colistin sulfate (4.4%). The highest resistance rates to the tested antibiotics occurred among patients aged 35-59 years. Table 1 details antibiotic resistance rates and their correlation with isolation sources. Colistin-resistant strains originated exclusively from tracheal aspirate and wound specimens. Ceftazidime demonstrated the lowest anti-pseudomonal activity, while colistin showed the highest activity regardless of patient age or specimen type. No pan-resistant isolates were identified, while 91 isolates (81.2%) were MDR, among which 58 (51.7% of total) were XDR.

3.3 Biofilm Production

Overall, 92 *P. aeruginosa* isolates (82.1%) were biofilm producers while 20 (17.8%) were non- producer. Among biofilm producers, 13/92 (14.1%) were strong producers, and 62/92 (67.4%) and 17/92 (18.5%) were moderate and weak biofilm producers, respectively. The isolates from tracheal aspirate/fluid exhibited significantly stronger biofilm formation than other sources. Within

this subgroup, weak, moderate, and strong biofilm producers consisted of 16.1%, 54.8%, and 29.0%, respectively. Table 2 details biofilm formation patterns across all specimen types.

3.4 Frequency of biofilm-Associated Genes

All 29 urine isolates were positive for biofilm-forming genes (*pslA*/*pslB*). Among the 26 wound/soft tissue isolates, *pslB* was present in 100%, while *pslA* was absent in only one isolate. This *pslA*-negative wound isolate formed weak biofilms. Table 2 shows gene distribution across other specimen types. No significant association emerged between fluoroquinolones/ aminoglycoside susceptibility (excluding amikacin), *pslA*/*pslB* presence ($P \geq 0.05$). Conversely, carbapenem-, monobactam-, and amikacin-resistant strains showed high prevalence of biofilm regulator genes ($P = 0.003$). Notably, some isolates harboring these genes exhibited reduced biofilm formation, though this inverse relationship was not statistically significant ($P \geq 0.05$).

Table 1. Antimicrobial resistance for 112 non-duplicated isolates of *P. aeruginosa* strains isolated over study period.

Antibiotic	Urine (38)	Tracheal aspirate/fluid (35)	Wound and soft tissue (31)	Blood (6)	Sputum (2)	Total
AZT	19 (50)	25 (71.4)	19 (61.3)	4 (66.6)	1 (50)	68 (60.7)
CAZ	28 (73.7)	31 (88.6)	18 (58.0)	3 (50)	2 (100)	82 (73.2)
AMK	21 (55.2)	23 (65.7)	19 (61.3)	1 (16.6)	0 (0)	64 (57.1)
CIP	25 (65.8)	26 (74.2)	20 (64.5)	5 (83.3)	1 (50)	77 (68.7)
CS*	0 (0)	4 (11.4)	1 (3.2)	0 (0)	0 (0)	5 (4.4)
IPM	20 (52.6)	22 (62.8)	19 (61.3)	4 (66.6)	1 (50)	66 (58.9)
MRN	23 (60.5)	28 (80.0)	19 (61.3)	3 (50)	2 (100)	75 (66.9)
PTZ	10 (26.3)	14 (40.0)	13 (41.9)	1 (16.6)	1 (50)	39 (34.8)

AZT: Aztreonam, **CAZ**: ceftazidime, **AMK**: amikacin, **CIP**: ciprofloxacin, **CS**: colistin sulfate, **IPM**: Imipenem, **MRN**: meropenem, and **PTZ**: piperacillin/tazobactam; * colistin was reported descriptively, based on MIC thresholds, without applying susceptibility labels per CLSI M100 (2024).

Table 2. The biofilm formation and presence of biofilm-forming genes in *P. aeruginosa* based on the specimen type.

Source	Mean of OD at 600 (nm)	Urine (38)			Tracheal aspirate/fluid (35)	
		Strong	Moderate	Weak	*pslA*	*pslB*
Urine (29)	0.17±1.09	0 (0)	26 (89.6)	3 (10.3)	29 (100)	29(100)
Tracheal aspirate/fluid (31)	0.25±0.95	9 (29.0)	17 (54.8)	5 (16.1)	31 (100)	31(100)
Wound/soft tissue (26)	0.21±0.83	4 (15.4)	16 (61.5)	6 (23.1)	25 (96.1)	26(100)
Blood (4)	0.19±1.14	0 (0)	1 (25)	3 (75)	4 (100)	4(100)
Sputum (2)	0.20±0.29	0 (0)	2 (100)	0 (0)	2 (100)	2(100)
Total (92)		13(14.1)	62(67.4)	17(18.5)	91(98.9)	92(100)

4. Discussion

Infections with *P. aeruginosa* are difficult to eradicate because of their ability to form biofilms. The biofilms are not only less susceptible to the host cell immune responses, but also have a high tolerance to antibiotics than the planktonic cells **(29)**. The present study focused on *pslA* and *pslB* genes that were found in *P. aeruginosa* isolates from ICU patients. These genes are important parts of the Psl polysaccharide synthesis pathway. Our investigation of 112 ICU-associated *P. aeruginosa* isolates from Tehran revealed elevated incidence of the biofilm-associated genes *pslA* and *pslB*. These rates are analogous to

those documented in contemporary literature. In Iran, Farshchi et al **(30)** identified *pslA* in 76.7% of the clinical isolates **(30)**, whereas other research in the Tehran region showed *pslA* in 83–89% of *P. aeruginosa* strains **(31)**. Similarly, *pslB* has been identified in approximately 86% of the hospital isolates **(32)**.

These results highlight that Psl exopolysaccharide locus is extensively preserved among clinical *P. aeruginosa*, particularly in critical-care environments. The identification of an isolate that formed weak biofilms despite lacking *pslA* gene is a very interesting finding that deserves full investigation. This paradoxical case challenges the conventional understanding of Psl-dependent biofilm formation and suggests the existence of alternative compensatory mechanism (e.g. Pel, and Alg) in clinical strains of *P. aeruginosa* **(33)**.

The occurrence of *pslA* in our MDR isolates reflects prior research **(34)**. Similarly, we found *pslB* in the vast majority of isolates, in line with a recent 2024 study reporting *pslB* in 86% of clinical strains **(32)**. These comparisons show that our results are in line with the growing trend that ICU-derived *P. aeruginosa* isolates in Iran and nearby areas mostly have *psl* operon genes. It was important that our isolates had *pslA* and *pslB* because they were linked to multidrug resistance. Most of the MDR isolates we have in our collection contained these biofilm genes. This is similar to what reported by Abdulhaq et al **(34)**, where they found *pslA* in all biofilm-producing isolates and 90% of MDR strains. It is also similar to what a recent study from Sonqor hospitals found, where they showed *pslB* carriage highly correlated with an MDR/high-biofilm phenotype **(32)**. The correlation between resistance and high *psl* prevalence raises the possibility that biofilm development plays a role in these ICU infections. However, other scientists have pointed out that susceptible strains can also include biofilm genes, which may indicate intricate control of biofilm morphologies **(35)**. Yet, most of the current studies suggest that biofilm-forming *P. aeruginosa* frequently coexists with multidrug resistance. The remarkable co-occurrence of *psl* genes and antibiotic resistance phenotypes in our research reinforces the notion that robust biofilm producers present a therapeutic challenge.

The prevalence of MDR *P. aeruginosa* in the Middle East and North Africa (MENA) shows significant variation. In general, regarding clinical samples, Egypt exhibited the highest prevalence at 75.6%, whereas Morocco recorded the lowest prevalence at 0%. The prevalence of ICU samples in the MENA region shows significant variation, with Saudi Arabia at 61% and Syria at 54%. In contrast, lower rates are observed in Egypt (22.5%), Libya (36.4%), Lebanon (33.3%), and Morocco (28.5%). The prevalence of MDR *P. aeruginosa* isolates in Qatar was 5.9%, indicating a declining trend, with 95% of cases being hospital-acquired **(36, 37)**. The occurrence of MDR *P. aeruginosa* in ICU settings in Iran shows significant fluctuations. A comprehensive assessment **(38)** determined that the overall incidence of MDR *P. aeruginosa* in Iran stands at 58%, with the peak frequency observed in Tehran at 100% and the lowest recorded in Zahedan at 16%. Further investigation from Iran revealed varying rates: In Shiraz, 25.4% of clinical isolates exhibited MDR, while in an ICU in Tehran, 43% of *P. aeruginosa* isolates showed MDR characteristics **(18, 39, 40)**. In a recent investigation into *P. aeruginosa* in Tehran the resistance rates observed for *P. aeruginosa* isolates varied between 33% and 81% **(41)**.

Developing specific medications that disrupt Psl production or the stability of biofilms, such as enzymes, anti-biofilm agents, or inhibitors of signaling pathways, could improve the effectiveness of treatment **(42-44)**. Furthermore, examining the relationship between *pslA* and *pslB* expression and clinical outcomes could improve predictive tools and tailored treatment approaches. Exploring combination therapy that incorporates antibiotics alongside anti-biofilm agents or Psl inhibitors presents a promising approach to effectively tackle MDR *P. aeruginosa* infections.

The study limitations include single-center design, specific geographical focus, absence of gene expression analysis, and dependence on phenotypic biofilm assays. While these limitations are recognized, they do not diminish the valuable insights obtained; instead, they guide future research initiatives. Future studies should focus on clarifying the regulation of *pslA* and *pslB* gene expression and genetic typing in clinical isolates in relation to antibiotic and host environmental factors, utilizing transcriptome and proteomic approaches.

In this study, biofilm formation was assessed using crystal violet staining method, which provides a semi-quantitative, endpoint measurement and lacks specificity in differentiating between live and dead bacterial cells within the biofilm. To address these limitations, future investigations should incorporate complementary techniques such as confocal laser scanning microscopy (CLSM) to visualize biofilm structure in three dimensions and viability-based assays like XTT reduction or resazurin metabolism to specifically measure the metabolically active cells within biofilms. These combined approaches will provide a more comprehensive understanding of biofilm biology, facilitating the development of effective anti-biofilm therapeutic strategies.

5. Conclusion

This study demonstrates a critically high prevalence of biofilm-associated genes *pslA* (98.9%) and *pslB* (100%) among MDR *P. aeruginosa* isolates from ICU patients in Tehran, Iran. The near-ubiquitous coexistence of these genes with robust biofilm formation (observed in 82.1% of isolates) underscores their significant role in biofilm-mediated antibiotic resistance, particularly in carbapenem-, monobactam-, and amikacin-resistant strains. Notably, 81.2% of isolates were MDR, with ceftazidime resistance reaching 73.2%, highlighting the therapeutic challenge in ICU settings. The single *pslA*-negative isolate exhibiting weak biofilm formation suggests compensatory mechanisms may exist, warranting further investigation. These findings position *pslA* and *pslB* as potential biomarkers for the persistent infections and future targets for anti-biofilm therapies. Prioritizing research into Psl-disrupting agents could enhance treatment efficacy against MDR *P. aeruginosa* in critical care.

6. Declarations

6.1 Acknowledgment

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6.2 Ethical Considerations

All experiments were approved by the Ethics Committee guidelines of Iran University of Medical Sciences (Ethic codes: IR.IUMS.REC.1401.1039) at Tehran, Iran.

6.3 Authors' Contributions

All authors contributed to the study conception and design. MEA: methodology, experiments, and data collection. MEA and AZB: writing original draft. SR: design and implementation of molecular analysis. MR and PE: sample collection. AZB: study supervision, design the experimental scheme, carry out the overall planning, and improvement of the manuscript. All authors have read and approved the final manuscript.

6.4 Conflict of Interests

The authors declare no conflict of interest.

6.5 Financial Support and Sponsorship

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6.6 Using Artificial Intelligence Tools (AI Tools)

Not applicable.

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