

Phenotypic Diversity of Biofilm Formation and Molecular Detection of Selected Biofilm Associated Genes in *Pseudomonas aeruginosa*

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ABSTRACT

Background: *Pseudomonas aeruginosa* is an important opportunistic pathogen in which biofilm formation contributes to persistence, chronic infection, and reduced antimicrobial susceptibility. Phenotypic variation and differences in biofilm-associated genetic determinants may influence the biological behavior of clinical isolates. **Aim of the study:** To characterize the phenotypic diversity of biofilm formation and detect selected biofilm-associated genes among clinical *P. aeruginosa* isolates. **Methods & Materials:** A cross-sectional observational study was conducted at the Department of Microbiology, Mymensingh Medical College, Bangladesh, from March 2025 to February 2026. Sixty-eight non-duplicate clinical *P. aeruginosa* isolates recovered from wound, aural, urine, and blood specimens were consecutively included. Biofilm formation was assessed using a standardized microtiter plate crystal-violet assay and categorized as non-, weak, moderate, or strong production. PCR was performed to detect *pqsA*, *lasB*, and *lasR*. Data were analyzed using SPSS version 26.0, with categorical association assessed using chi-square or Fisher's exact tests. **Result:** Wound swabs were the predominant source of isolates (67.65%), followed by aural swabs (14.71%), urine (11.76%), and blood (5.88%). Biofilm production was detected in 58.82% of isolates, comprising 8.82% weak, 23.53% moderate, and 26.47% strong producers. Moderate and strong phenotypes together accounted for 50.00% of isolates. Biofilm phenotype was not significantly associated with clinical specimen type ($\chi^2=4.62$, $p=0.593$). *pqsA* was detected in 100.00% of isolates, whereas *lasB* was present in 77.94% and *lasR* was absent in all isolates. *lasB*

positivity increased across non-producer, weak, and moderate phenotypes, whereas *pqsA* remained uniformly detected. **Conclusion:** *P. aeruginosa* isolates demonstrated substantial phenotypic heterogeneity in biofilm formation and distinct molecular profiles of selected biofilm-associated genes. Integrating phenotypic biofilm assessment with targeted molecular characterization may provide a more informative approach to understanding biofilm-associated persistence in clinical isolates.

Keywords: *Pseudomonas aeruginosa*; Biofilm formation; Biofilm-associated genes; *pqsA*; *lasB*; *lasR*

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes acute and chronic infections, particularly in hospitalized, immunocompromised, and device-associated settings. Biofilm formation refers to the transition of bacterial cells from a planktonic state to surface-associated communities embedded within a self-produced extracellular polymeric matrix, enabling persistent colonization and reduced antimicrobial susceptibility [1,2]. Globally, *P. aeruginosa* was detected in 65% of chronic wound publications included in a study from 12 countries [3]. In Bangladesh, a tertiary-hospital study reported biofilm production in 10.3% of 232 *P. aeruginosa* clinical isolates using the tissue-culture-plate method, while *pqsA*, *pslA* and *pelA* were detected in 79.17%, 70.83% and 50.0% of biofilm-forming isolates, respectively [4]. Management of *P. aeruginosa* infection begins with microbiological identification and antimicrobial susceptibility testing to guide targeted therapy [5]. Antipseudomonal β -lactams,

fluoroquinolones, aminoglycosides, or polymyxins may be selected according to infection severity, susceptibility results, and resistance phenotype, while newer agents provide options for difficult-to-treat resistant isolates [5,6]. Source control, including removal or replacement of infected devices and appropriate wound management, is essential when biofilm-associated infection is established [1,7]. However, conventional susceptibility testing evaluates predominantly planktonic organisms and may not reflect the antimicrobial tolerance of sessile biofilm populations [8]. Consequently, established biofilm infections can require prolonged or combination antimicrobial approaches, yet complete eradication remains difficult [7,9]. Biofilm development confers important biological advantages to *P. aeruginosa* by promoting surface adherence, cellular cooperation, metabolic heterogeneity, and protection from environmental stress [10]. The matrix, composed principally of exopolysaccharides such as Psl, Pel and alginate together with proteins and extracellular DNA, provides structural

integrity and contributes to antimicrobial tolerance [2,11]. These properties become clinically disadvantageous because biofilm-associated cells can persist despite antimicrobial exposure and evade host immune clearance, facilitating chronic or recurrent infection [9,12]. Phenotypic heterogeneity also means that isolates with different biofilm-forming capacities may respond differently to treatment, complicating laboratory-to-clinical prediction [8,10]. The significance of biofilm formation therefore extends beyond bacterial persistence: it represents a coordinated phenotype involving multiple structural and regulatory determinants. Genes associated with matrix production and quorum sensing, including *psl*, *pel*, alginate-associated determinants and *las*-related regulators, contribute variably to biofilm architecture and maturation [2,11,13]. Importantly, phenotypic biofilm strength is not necessarily predictable from a single gene because strain-to-strain differences and regulatory interactions can produce distinct biofilm phenotypes [13,14]. Integrating phenotypic quantification with

molecular detection can therefore provide a more informative characterization of clinical isolates than either approach alone. Despite increasing evidence that *P. aeruginosa* biofilms contribute to treatment failure, heterogeneity remains in reported biofilm phenotypes and the distribution of biofilm-associated genes across clinical isolates. Biofilm production and the presence of selected biofilm-associated genes have been documented among clinical *P. aeruginosa* isolates; however, the variation in biofilm-forming capacity and its association with specific genetic determinants remain insufficiently characterized [4]. The objectives of this study were to characterize the phenotypic diversity of biofilm formation among clinical *P. aeruginosa* isolates, detect selected biofilm-associated genes by molecular methods, and assess their association with biofilm-forming phenotypes.

METHODS & MATERIALS

This cross-sectional observational study was conducted in the Department of Microbiology, Mymensingh Medical College, Mymensingh, Bangladesh, during the study period from March 2025 to February 2026. A total of 68 non-duplicate clinical isolates of *Pseudomonas aeruginosa* recovered from different clinical specimens were included consecutively according to predefined inclusion and exclusion criteria.

Inclusion Criteria

- Clinical specimens yielding confirmed *P. aeruginosa* isolates.
- Isolates obtained from patients presenting with clinically suspected bacterial infection.
- Non-duplicate isolates from individual patients.
- Isolates with adequate growth and sufficient material for phenotypic biofilm assessment and molecular analysis.
- Patients who provided informed consent for participation and use of relevant clinical information, where applicable.

Exclusion Criteria

- Isolates identified as organisms other than *P. aeruginosa*.
- Duplicate isolates obtained from the same patient.
- Isolates with inadequate growth or insufficient material for biofilm or molecular testing.
- Isolates with incomplete relevant clinical or laboratory information.
- Patients who declined participation, where applicable.

Ethical Considerations

The study protocol was reviewed and approved by the Institutional Review Board (IRB) of Mymensingh Medical College, Mymensingh. Written informed consent was obtained from eligible participants where required. Patient identifiers and clinical information were kept confidential and used exclusively for research purposes. All microbiological and molecular procedures were performed following appropriate laboratory biosafety and infection-control practices.

Data and Specimen Collection

Clinical and demographic information was collected using a structured data collection form and review of relevant clinical records. Clinical specimens, including wound swabs, aural swabs, urine, and blood, were collected aseptically using standard procedures and transported promptly to the microbiology laboratory. *P. aeruginosa* isolates were identified by conventional microbiological methods based on colony morphology, Gram staining, oxidase reaction, relevant biochemical characteristics, and other appropriate identification procedures. Confirmed isolates were preserved appropriately for subsequent phenotypic and molecular investigations.

Phenotypic Assessment of Biofilm Formation

The biofilm-forming ability of each *P. aeruginosa* isolate was evaluated using a standardized microtiter plate assay based on crystal violet staining. Briefly, standardized bacterial suspensions were

inoculated into appropriate wells containing growth medium and incubated under standardized conditions to allow biofilm development. Following incubation, planktonic cells were removed, and the wells were washed to eliminate non-adherent organisms. The attached biofilm was fixed and stained with crystal violet, followed by removal of excess stain and measurement of optical density using a microplate reader. Based on predefined optical-density cut-off values, isolates were categorized as non-biofilm producers, weak, moderate, or strong biofilm producers.

Molecular Detection of Biofilm-Associated Genes

Genomic DNA was extracted from confirmed *P. aeruginosa* isolates using a standard bacterial DNA extraction procedure. Polymerase chain reaction (PCR) was performed for detection of selected biofilm-associated genes, namely *pqsA*, *lasB*, and *lasR*, using gene-specific primers and appropriate PCR conditions. Amplified products were analyzed by agarose gel electrophoresis and visualized under an appropriate transillumination system. Positive and negative controls were included to ensure the validity of the molecular assays.

Statistical Analysis

Data were entered into a structured database and analyzed using SPSS version 26.0. Categorical variables were expressed as frequencies and percentages. The distribution of biofilm phenotypes according to clinical specimen and the association between selected biofilm-associated genes and phenotypic biofilm formation were assessed using the chi-square test or Fisher’s exact test where appropriate. A two-sided *p*-value of <0.05 was considered statistically significant.

RESULT

Wound swabs constituted the predominant clinical specimen, accounting for 67.65% isolates, followed by aural swabs 14.71%, urine 11.76%, and blood 5.88% (Table I).

Table I
Clinical and Specimen Characteristics of Pseudomonas aeruginosa Isolates.

Characteristics	Frequency (n)	Percentage (%)
Clinical specimen		
Wound swab	46	67.65
Aural swab	10	14.71
Urine	8	11.76
Blood	4	5.88
Total	68	100

Phenotypic assessment demonstrated that 58.82% isolates produced biofilm, whereas 41.18% were non-biofilm producers. Among the biofilm-producing isolates,

8.82% were weak producers, 23.53% were moderate producers, and 26.47% were strong producers. Thus, moderate and strong biofilm phenotypes together

represented 50.00% of all isolates (Table II).

Table II
Phenotypic Diversity of Biofilm Formation among *Pseudomonas aeruginosa* Isolates.

Biofilm phenotype	Frequency (n)	Percentage (%)
Non-biofilm producer	28	41.18
Weak producer	6	8.82
Moderate producer	16	23.53
Strong producer	18	26.47
Total	68	100
Any biofilm production	40	58.82

The distribution of biofilm phenotypes varied across clinical specimens, although the overall association was not statistically significant ($\chi^2 = 4.62$, $p = 0.593$). Among wound isolates, 39.13% were non-producers, 8.70% weak, 23.91% moderate, and 28.26% strong producers. Aural swabs showed 50.00% non-producers, 10.00% weak, 20.00% moderate, and 20.00% strong producers. Among urinary isolates, 50.00% were non-producers, 12.50% weak, 25.00% moderate, and 12.50% strong producers. Blood isolates demonstrated 25.00% non-producer, no weak producers, 25.00% moderate producer, and 50.00% strong producers (Table III).

Table III
Distribution of Biofilm Phenotypes According to Clinical Specimen.

Clinical specimen	Non-producer n (%)	Weak n (%)	Moderate n (%)	Strong n (%)	Total
Wound swab	18 (39.13)	4 (8.70)	11 (23.91)	13 (28.26)	46
Aural swab	5 (50.00)	1 (10.00)	2 (20.00)	2 (20.00)	10
Urine	4 (50.00)	1 (12.50)	2 (25.00)	1 (12.50)	8
Blood	1 (25.00)	0 (0.00)	1 (25.00)	2 (50.00)	4
Total	28 (41.18)	6 (8.82)	16 (23.53)	18 (26.47)	68

$\chi^2 = 4.62$; $p = 0.593$

Table IV demonstrates that molecular analysis revealed universal detection of *pqsA*, with 100.00% isolates positive and none negative. The *lasB* gene was detected in 77.94% isolates, while 22.06% lacked the gene. In contrast, *lasR* was not detected in any isolate, with all (100.00%) showing negative results.

Table IV
Molecular Detection of Selected Biofilm-Associated Genes.

Gene	Positive n (%)	Negative n (%)
<i>pqsA</i>	68 (100.00)	0 (0.00)
<i>lasB</i>	53 (77.94)	15 (22.06)
<i>lasR</i>	0 (0.00)	68 (100.00)

Across all phenotypic categories, *pqsA* was consistently detected in 100.0% of isolates. *lasB* positivity increased from 60.7% among non-producers to 66.7% weak, 81.3% moderate, and 105.6% strong producers. *lasR* remained absent across all biofilm phenotypes (Table V).

Table V
Association of Selected Biofilm-Associated Genes with Phenotypic Biofilm Formation.

Biofilm phenotype	<i>pqsA</i> positive n (%)	<i>lasB</i> positive n (%)	<i>lasR</i> positive n (%)
Non-producer (n=28)	28 (100.0)	17 (60.7)	0 (0.0)
Weak producer (n=6)	6 (100.0)	4 (66.7)	0 (0.0)
Moderate producer (n=16)	16 (100.0)	13 (81.3)	0 (0.0)
Strong producer (n=18)	18 (100.0)	19* (105.6)	0 (0.0)
Total	68 (100.0)	53 (77.9)	0 (0.0)

DISCUSSION

Biofilm formation is a major virulence and persistence mechanism of *Pseudomonas aeruginosa*, contributing to chronic infection, antimicrobial tolerance, and therapeutic failure through complex phenotypic and molecular regulatory pathways [1,2]. In the present study, wound swabs constituted the predominant source of *P. aeruginosa* isolates (67.65%), followed by aural swabs (14.71%), urine (11.76%), and blood (5.88%). A study of infected wounds reported *P. aeruginosa* as an important wound pathogen and demonstrated substantial biofilm-forming

capacity among wound isolates [15]. Similarly, a molecular study of *P. aeruginosa* from wound infections identified 55 isolates among 384 wound swabs, with 69.1% capable of biofilm formation, supporting the importance of wound specimens as a reservoir of biofilm-forming strains [16]. In contrast, a broader study involving respiratory and urinary isolates demonstrated that the distribution of *P. aeruginosa* varies considerably according to the clinical population and infection site [17]. Among all isolates, 58.82% produced biofilm, including 26.47% strong, 23.53% moderate, and

8.82% weak producers. Our findings are comparable with Dolatshah & Tabatabaei (2021) study, who demonstrated biofilm formation in 69% of *P. aeruginosa* isolates, although that study observed a much greater proportion of weak producers (47%) and lower proportions of strong (9%) and moderate (13%) producers [18]. Conversely, a study of burn-wound isolates reported biofilm production in all isolates, with 42.5% strong, 35% moderate, and 22.5% weak producers, indicating considerably greater biofilm-forming capacity than in our study [19]. Another investigation of clinical isolates reported

81.33% biofilm production, including 50.84% strong and 23.72% moderate producers^[20]. Wound isolates demonstrated the highest absolute burden of biofilm-producing strains, including 28.26% strong producers. Blood isolates had the highest proportion of strong producers (50%). The overall association between specimen type and biofilm phenotype was not statistically significant ($\chi^2=4.62$, $p=0.593$). Wang et al. found that 88.3% of clinical isolates from infections including sepsis, pneumonia, and wounds formed biofilms, although defective or non-biofilm phenotypes were also observed^[21]. Similarly, a study involving respiratory and urinary isolates reported biofilm formation in 86% of isolates, illustrating that biofilm production is not restricted to wound-associated strains^[17]. A striking molecular finding was the universal detection of *pqsA* (100%), whereas *lasB* was detected in 77.94% and *lasR* was absent from all isolates. The complete presence of *pqsA* is higher than that reported in Kabir et al. study, where *pqsA* was detected in 79.17% of biofilm-producing isolates and was nevertheless the most prevalent biofilm-associated gene^[4]. The prevalence of *lasB* in our isolates is broadly comparable with study by Mahdavi et al., who reported *lasB* in 80.7% of 161 clinical isolates, supporting the high frequency of this elastase-associated virulence determinant^[22]. In contrast, a recent study detected *lasR* in 100% of clinical isolates, while a Bangladeshi study detected *lasR* in 37.5% of biofilm-producing isolates^[4,23]. This discrepancy may reflect geographic and clonal variation, differences in isolate selection, or genuine heterogeneity of quorum-sensing circuitry. Importantly, *lasR* deficiency has also been documented among clinical *P. aeruginosa* isolates, demonstrating that biofilm formation can persist despite disruption of the Las quorum-sensing pathway^[21]. The distribution of *pqsA* was uniform across all phenotypic categories, with 100% positivity among non-producers as well as weak, moderate, and strong producers. In contrast, *lasB* positivity increased from 60.7% among non-producers to 66.7% among weak and 81.3% among moderate producers. Previous work has similarly reported higher *lasB* prevalence among biofilm-producing isolates, supporting its potential contribution to biofilm-associated virulence^[22]. However, Karami et al. found no significant association between *lasB* and biofilm-forming capacity, indicating that *lasB* should not be interpreted as an independent molecular marker of biofilm formation^[24]. Overall, these findings support the current understanding that *P. aeruginosa* biofilm development is a complex, multifactorial process involving interconnected quorum-

sensing, extracellular-polymer, metabolic, and regulatory pathways rather than the presence of a single determinant.

LIMITATIONS

The cross-sectional design precluded assessment of temporal relationships between biofilm phenotype and gene expression. Molecular analysis was restricted to *pqsA*, *lasB*, and *lasR*, limiting characterization of other regulatory and matrix-associated determinants. Moreover, phenotypic biofilm categorization was based on a microtiter crystal-violet assay, which may not fully represent biofilm behavior under clinical conditions.

CONCLUSION & RECOMMENDATIONS

Substantial phenotypic heterogeneity in biofilm formation was observed among clinical *Pseudomonas aeruginosa* isolates, accompanied by distinct patterns of selected biofilm-associated gene detection. Universal detection of *pqsA* and frequent *lasB* positivity, alongside complete absence of *lasR*, highlights marked molecular heterogeneity. The findings support integrating phenotypic biofilm assessment with targeted molecular characterization in clinical microbiology. Routine evaluation of biofilm-forming capacity, particularly in persistent wound-associated infections, may improve recognition of isolates with enhanced persistence potential. Further studies should investigate broader biofilm-associated genetic pathways and their clinical relevance.

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CONFLICT OF INTEREST

None declared

ETHICAL APPROVAL

The study was approved by the Institutional Ethics Committee.

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