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Molecular Characterization of rhlR and fliC Genes in Biofilm-Forming, Multidrug-Resistant *Pseudomonas Aeruginosa* Isolated from Pediatric Clinical Specimens

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Abstract

Background: Quorum sensing (QS) and motility are vital for biofilm development and pathogenicity of *Pseudomonas aeruginosa*. The *rhlR* gene regulates the synthesis of rhamnolipids and biofilm maturity, whereas the *fliC* gene codes for flagellin, which is indispensable for cell adhesion to surfaces. In this project, we will investigate (a) the frequency of *rhlR* and *fliC* genes in pediatric isolates using PCR; (b) genetic diversity by DNA sequencing; (c) susceptibility to antimicrobials; and (d) correlations between gene load, biofilm development, and antibiotic resistance

Methodology: This study analyzed 100 isolates of *P. aeruginosa* from pediatric patients (6 months to 15 years old) with different infections in three Iraqi cities. The TCP test was used to quantify biofilm formation. *rhlR* and *fliC* were detected in 30 selected strains using PCR and subsequently sequenced (three amplicons for each gene). The antibiotic susceptibility test was performed against nine antipseudomonal antibiotics.

Results: In total, 70% of strains were found to produce moderate-to-strong biofilm. *rhlR* was identified in 43.3% and *fliC* in 33.3% of strains. Sequence analysis showed that *rhlR* had an identity value of 96.3-97.7%, with 18 reliable SNPs and a stop-gained mutation in codon 49. In contrast, there was a high variability in the *fliC* gene, showing an identity percentage of 31.6-49.4% within a small conserved core region of 79 bp. However, BLASTn analysis showed 99% identity with known sequences for the entire gene length (493 bp). Antibiotic resistance was highest with Aztreonam (35%) and Imipenem (28%). Spearman correlation indicated that the presence of *rhlR* and *fliC* positively correlated with antibiotic resistance severity (rho = 0.525, p = 0.0085).

Conclusion: *rhlR* and *fliC* are present in a considerable number of pediatric strains, where the *fliC* gene is highly variable. Moreover, the positive correlation between gene burden and resistance severity supports the development of new quorum-sensing-based therapies.

التوصيف الجزيئي لجينات rhlR و fliC في بكتيريا الزائفة الزنجارية المقاومة للأدوية المتعددة والمكونة للأغشية الحيوية، والمعزولة من عينات سريرية للأطفال.

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الخلاصة

المقدمة

وجود جيني *rhlR* و*fliC* شائع في عدد ملحوظ من العزلات البكتيرية لدى الأطفال، مع ملاحظة أن جين *fliC* يُظهر تباينًا جينيًا مرتفعًا. علاوة على ذلك، فإن الارتباط الإيجابي بين عدد الجينات الضراوية وشدة المقاومة يدعم التوجه نحو تطوير علاجات جديدة قائمة على تثبيط نظام استشعار النصاب (quorum sensing).

العينات وطرق العمل

تم الحصول على 100 عزلة من بكتيريا الزائفة الزنجارية من أطفال أعمارهم تتراوح بين 6 أشهر و15 عامًا، يعانون من حروق وجروح والتهابات الأذن والتهابات المسالك البولية، في بغداد والرمادي والفلوجة بالعراق، خلال الفترة من أغسطس إلى ديسمبر 2025. وتم تحديد العزلات بناءً على شكلها المستعمري وصبغة غرام، بالإضافة إلى اختبارات أخرى، بما في ذلك اختبارات كيميائية حيوية مثل الأوكسيداز والكاتالاز والإندول وMR–VP والسترات، ونموها عند درجة حرارة 42 درجة مئوية.

النتائج

اجمالا وُجد أن 70% من السلالات تُنتج غشاءً حيويًا متوسطًا إلى قوي. تم تحديد جين *rhlR* في 43.3% من السلالات وجين *fliC* في 33.3% منها. أظهر تحليل التسلسل أن جين *rhlR* يتمتع بنسبة تطابق تتراوح بين 96.3% و97.7%، مع 18 طفرة نيوكليوتيدية مفردة موثوقة وطفرة توقف في الكودون 49. في المقابل، لوحظ تباين كبير في جين fliC، حيث بلغت نسبة التطابق فيه 31.6% إلى 49.4% ضمن منطقة أساسية محفوظة صغيرة بطول 79 زوجًا قاعديًا. مع ذلك، أظهر تحليل BLASTn تطابقًا بنسبة 99% مع التسلسلات المعروفة لكامل طول الجين (493 زوجًا قاعديًا). كانت مقاومة المضادات الحيوية أعلى ما يمكن مع أزتريونام (35%) واميبنم (28%). أشارت علاقة ارتباط سبيرمان إلى أن وجود *rhlR* و fliC يرتبط بشكل إيجابي بشدة مقاومة المضادات الحيوية (rho = 0.525، p = 0.0085)

الاستنتاج

يتواجد الجينان *rhlR* و *fliC* في عدد كبير من سلالات البكتيريا لدى الأطفال، حيث يتسم جين *fliC* بتنوع كبير. علاوة على ذلك، فإن العلاقة الإيجابية بين عبء الجين وشدة المقاومة تدعم تطوير علاجات جديدة تعتمد على استشعار النصاب.

1. Introduction

P. aeruginosa remains one of the most prevalent pathogens of nosocomial infections in pediatric populations, especially in individuals suffering from cystic fibrosis, burns, immunosuppressed conditions, and critical care unit admissions (Schwartz *et al.*, 2024) .Its ability to form biofilms and develop multidrug resistance makes it difficult for clinicians to treat infections, often leading to treatment failure, prolonged hospitalization, and fatalities (Qin et al., 2022;.(Haidar *et al.*, 2024) Biofilm formation by *P. aeruginosa* is a complicated multi-step process that requires precise regulation of several genes encoding proteins responsible for adhesion, matrix production, and maturation. Quorum sensing (QS) is a communicative mechanism of cells that enables coordinated gene expression with respect to the density of the bacterial population (Azimi *et al.*, 2020). *P. aeruginosa* possesses a hierarchical QS circuit involving three interconnected QS circuits: Las (lasI/lasR), Rhl (rhlI/rhlR), and PQS (pqsABCDE/pqsR). Generally, the Rhl system works under the influence of the Las system; nevertheless, during phosphate starvation conditions, the Rhl system acts independently (Soto-Aceves *et al.*, 2021); (Simanek *et al.*, 2023). The *rhlR* gene codes for RhlR, a LuxR-type transcriptional activator that, after interaction with its autoinducer N-butyryl-homoserine lactone (C4-HSL), triggers the expression of genes coding for the biosynthesis of rhamnolipids, elastases, pyocyanin, and biofilm formation (Lim *et al.*, 2022);(Ghosh, Das and Mallik, 2019). *rhlR* acts as a post-transcriptional activator of the Psl polysaccharide synthesis, collaborating with the Las system in promoting the development of the biofilm structure (Carey *et al.*, 2024)). The main products of the regulation by *rhlR* gene transcription include the biosurfactants known as rhamnolipids, which perform significant functions in biofilm stability, swarming motility, and mushroom-like microcolonies formation (Boyle *et al.*, 2015). The presence of the *rhlR* gene in the *Pseudomonas aeruginosa* isolates, accounting for approximately 90%, has been positively associated with biofilm formation and multidrug-resistance of the bacteria, being found in more than 94% of the MDR strains (Hemmati *et al.*, 2024). The *fliC* gene codes for flagellin, which is a primary component of the bacterial flagellum, important for motility and adherence to surfaces (Haiko and Westerlund-Wikström, 2013) .During early stages of biofilm development, flagella enable free-floating bacteria to swim to surfaces and overcome repulsive force, thus enabling movement from reversible to irreversible adhesion (Bouteiller *et al.*, 2021) .Importantly, after the biofilm structure is formed, the expression of *fliC* is usually reduced because flagella are no longer needed for movement within the established biofilm community (Suriyanarayanan *et al.*, 2016). However, *fliC* is not an absolute requirement for biofilm formation as some bacteria use alternate mechanisms for biofilm formation that do not involve flagella (Sriramulu *et al.*, 2005).In addition, flagellin acts as an effective immunogen and binds Toll-like receptor 5 (TLR5), triggering innate immunity reactions that lead to inflammation and damage of tissues during infection (Zhang and Stallforth, 2024).The frequency of fliC-positive clinical strains is about 60-85% (Płókarz *et al.*, 2022);(Ghadaksaz *et al.*, 2015). In spite of their clear involvement in the pathogenesis of *P. aeruginosa* infection, insufficient data have been provided concerning the genetics of *rhlR* and *fliC* in pediatric isolates in Iraq. Children are more vulnerable to infections caused by biofilms due to immunological immaturity and the high incidence of the use of catheters and other medical equipment. Also, antibiotic-resistant strains pose an additional threat to treatment success, and the exploration of genetic mechanisms behind QS and motility can help discover new drug targets. The objectives of this research are to investigate the occurrence of *rhlR* and *fliC* genes in pediatric clinical isolates using PCR; analyze genetic diversity using DNA sequencing techniques, evaluate antibiotic sensitivity; and investigate correlations between gene presence and antibiotic resistance.

2. Materials and Methods

2.2. Study Subjects

In total, 100 isolates of *Pseudomonas aeruginosa* from children aged 6 months to 15 years suffering from burns, wounds, ear infections, and UTIs were obtained from Baghdad, Ramadi, and Fallujah, Iraq, during the period from August to December 2025. The identification of isolates was done based on their colonial morphology and Gram staining, along with other tests, including biochemical tests such as oxidase, catalase, indole, MR-VP, citrate, and growth at 42°C.

2.2. Biofilm Quantification

To assess biofilm production, we used the tissue culture plate (TCP) method. In brief, the isolates were grown in brain heart infusion (BHI) medium containing 1% glucose in 96-well microtiter plates and stained with crystal violet, with the OD recorded at 490nm. The OD value of each isolate was compared to previously defined cutoff values for determining whether it produced no, weak, moderate, or strong biofilms.

2.3. Antimicrobial Susceptibility Testing

Isolation profile was done by Kirby-Bauer disc diffusion technique in Mueller-Hinton medium according to CLSI guideline 2025. The following nine antibiotics tested were given: amikacin (30 µg), aztreonam (30 µg), ceftazidime (30 µg), cefepime (30 µg), ciprofloxacin (10 µg), imipenem (10 µg), levofloxacin (5 µg), meropenem (10 µg), and piperacillin-tazobactam (100/10 µg). The zone sizes of bacteria for the different antibiotics were determined after a 24-hour incubation period at 37°C.

2.4. DNA Extraction and PCR Amplification

Genomic DNA was extracted from 30 selected isolates using a commercial kit (Promega, USA). PCR was performed to detect *rhlR* (371 bp product) and *fliC* (385 bp product) using gene-specific primers Table1. The PCR reaction mixture (20 µl) contained 12.5 µl master mix, 1 µl each forward and reverse primer (10 pmol/µl), 1.5 µl DNA template, and 4 µl nuclease-free water. Thermal cycling conditions were: initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec; final extension at 72°C for 7 min. PCR products were visualized by agarose gel electrophoresis (1.5% gel, 80V for 1 hour) with ethidium bromide staining.

Table1: Primer Sequences and Expected PCR Product Sizes for *Rhir* And *Flic* Genes

Name of Gene	Primer sequence (5′- 3)	Product size (bp)	Manufacturing company (Origin)
rhlR	GAAATGGTGGTCTGGAGCGA F ---------- R TGGAAGTTCACCGTGCTCTC	371	Microgene (Korea)
fliC	F TCGACATGAAGGGCAACGAA ---------- R GTGTTGTCGAAGCGGTTC	385	Microgene (Korea)

2.5. DNA Sequencing and Analysis

Positive PCR products for *rhlR* (three isolates: H260120-015_K10_s46_of.ab1, H260120-015_M10_s47_of.ab1, H260120-015_I10_s45_of.ab1) and *fliC* (three isolates: H260120-015_O10_s48_FF.ab1, H260120-015_C12_s50_FF.ab1, H260120-015_A12_s49_FF.ab1) were purified and sequenced using an ABI 3730XL Genetic Analyzer (Microgene, Korea). Sequence analysis was performed using BioEdit software and BLASTn against the NCBI database. Multiple sequence alignment identified SNPs and INDELs. Pairwise genetic similarity was calculated, and mutations were classified as synonymous, non-synonymous, or stop-gained after conceptual translation. For *fliC*, due to

length variations, only a shared core region (79 bp) was used for alignment, with BLASTn confirmation using the full-length sequence (approximately 493 bp).

2.6. Statistical Analysis

Statistical analysis was conducted in R software. The differences in inhibition zone values between resistant and sensitive isolates were checked using Mann-Whitney U test with Benjamini-Hochberg correction. Spearman's rank correlation was used to determine correlation between gene count (presence of *algD, pslA, rhlR, fliC)* and resistance score. Linear regression analysis was used to investigate the impact of gene count on resistance score. The resistance scores were then analyzed using Kruskal-Wallis test followed by Dunn's post hoc test for gene count groups (0–1, 2, 3–4 genes). P-value < 0.05 was considered statistically significant.

2.7. Ethical Approval

for this study was acquired from the Research Ethics Committee, College of Applied Sciences, University of Fallujah. Clinical specimens were collected from pediatric patients admitted to hospitals in Baghdad, Ramadi, and Fallujah. All samples were completely anonymized prior to analysis to protect patient confidentiality. The study was conducted in accordance with ethical guidelines for handling clinical specimens, and no personal identifying information was recorded.

3. Results

3.1. Biofilm Formation and Antimicrobial Resistance Profile

The TCP method demonstrated that among the 100 clinical isolates, 70% were biofilm Antimicrobial susceptibility testing showed variable resistance among the pediatric isolates. Aztreonam exhibited the highest resistance (35%), followed by Meropenem (21%), Levofloxacin (27%), Imipenem (28%), Ciprofloxacin (21%), Ceftazidime (22%), Amikacin (20%), Cefepime (25%), and Piperacillin/tazobactam (15%). The overall mean resistance rate was 23.9%.

Mann–Whitney U analysis showed significant differences in inhibition zone diameters between resistant and susceptible isolates for all tested antibiotics (adjusted p-values ranged from 0.00000143 to 0.0000972).

3.2. Prevalence of *rhlR* and *fliC* Genes

PCR analysis of 30 selected isolates revealed that 13 isolates (43.3%) carried the *rhlR* gene, while 10 isolates (33.3%) carried the *fliC* gene, as shown in Fig.1. and Fig.2.

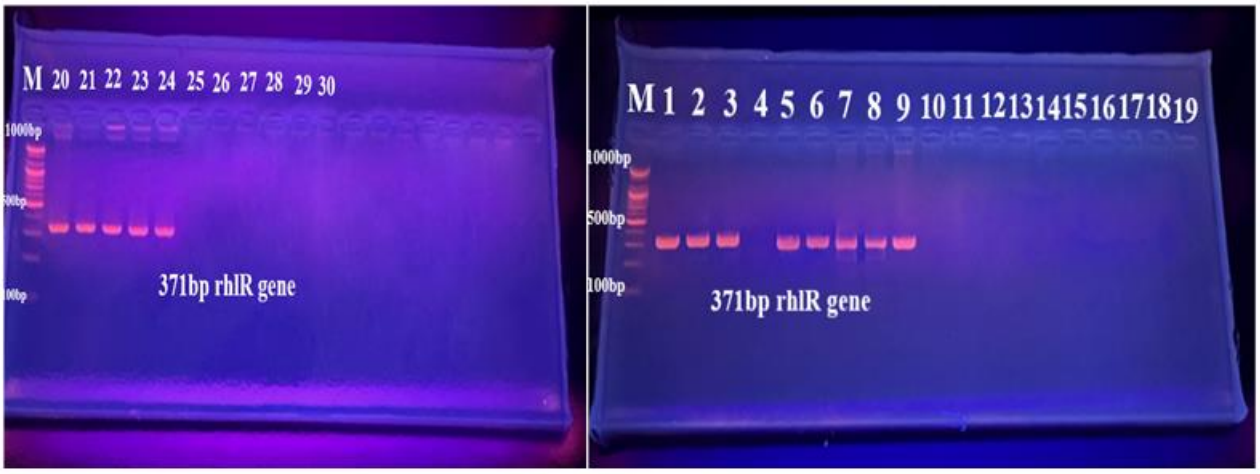

Figure1: Agarose gel electrophoresis of PCR-amplified *rhlR* gene. PCR products were separated on an agarose gel, demonstrating successful amplification of the *rhlR* gene with the expected amplicon size of 371 bp. M: 100 bp DNA ladder; lanes 1–30: clinical isolates analyzed, with positive amplification indicated by the presence of a distinct 371 bp band.

Figure2: Agarose Gel Electrophoresis of PCR-Amplified *Flic* Gene in *Pseudomonas Aeruginosa* Isolates. PCR products were separated on an agarose gel, demonstrating successful amplification of the *fliC* gene with the expected amplicon size of 385 bp. M: 100 bp DNA ladder; lanes 1–38: clinical *P. aeruginosa* isolates analyzed. The presence of a distinct 385 bp band indicates a positive result for the *fliC* gene.

3.3. Correlation Between Gene Count and Resistance Severity

Spearman's rank correlation analysis showed a statistically significant positive correlation between the total number of biofilm-associated genes (algD, pslA, rhlR, and fliC) and antibiotic resistance score (rho = 0.525, p = 0.0085). Linear regression analysis revealed a significant association between gene count and resistance severity (β = 0.684, p = 0.0175), accounting for 23.1% of the variation. Results from a Kruskal–Wallis analysis indicated significant differences in resistance score among isolates grouped according to gene count (p = 0.0313). Dunn's test posts hoc showed that the resistance scores of isolates that carried 3–4 genes were significantly higher than those that contained 0–1 genes (adjusted p = 0.0462), as shown in Fig.3.

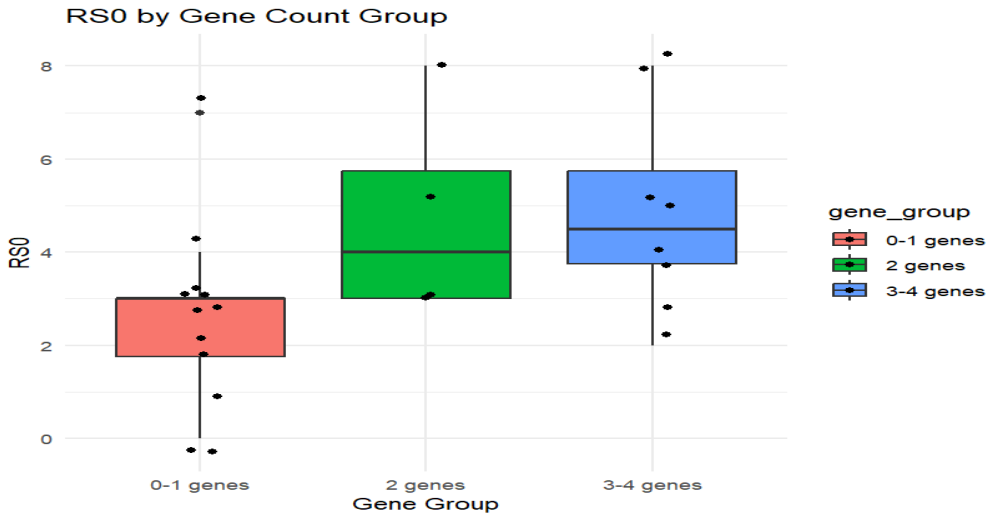

Figure3: **Association Between Gene Count and Resistance Severity Among Resistant Isolates**.

3.4. Sequence Analysis of *rhlR* Gene

Sequence analysis of *rhlR* from three selected isolates identified 24 variable nucleotide positions, including 18 confirmed SNPs after excluding INDEL regions. Pairwise nucleotide identity ranged from 96.26% to 97.67%. The highest identity was observed between isolates K10 and M10 (97.67%), whereas the lowest was observed between K10 and I10 (96.26%). A stop-gain mutation was detected at codon 49 in isolate K10, resulting in substitution of glycine with a stop codon, as shown in Fig.4.

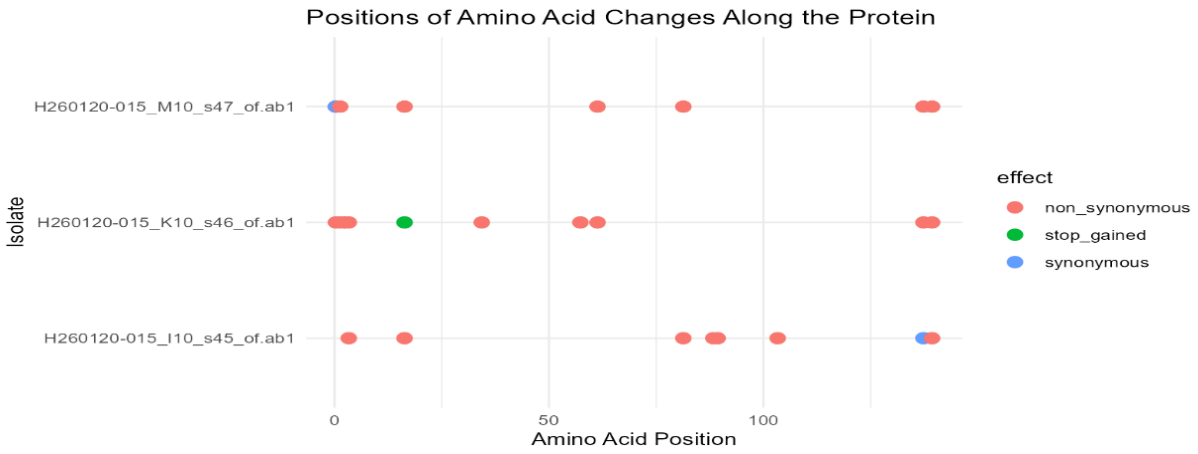

Figure4: Positions of Amino Acid Changes Along the *Rhir* Protein

4.4. Sequence Analysis of *fliC* Gene

Sequence analysis of the *fliC* gene showed variable sequence lengths among the three isolates. The common alienable region was 79 nucleotides. Within this region, 62 polymorphic sites were identified. Pairwise nucleotide identity ranged from 31.6% to 49.4%. BLASTn analysis of the approximately 493 bp amplicon from isolate O10 showed 99% identity with *Pseudomonas aeruginosa* strain IP40a (GenBank accession: KX709966.1), as shown in Fig.5.

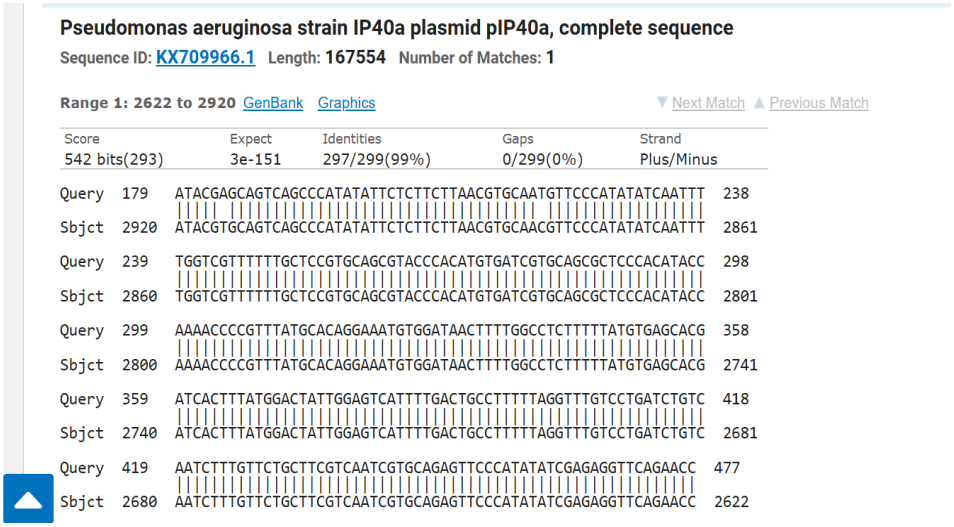

Figure5: Blast Alignment of The Flic Gene Fragment (~493 Bp) Showing 99% Identity with *Pseudomonas Aeruginosa* Reference Sequence (KX709966.1).

4. Discussion

The moderate/strong biofilm production in the analysis reported in this study suggests that biofilm formation is a common characteristic in pediatric clinical isolates of *Pseudomonas aeruginosa* infection. This corresponds to previous studies indicating biofilm formation as a key factor for persistence and treatment failure in clinical settings(Haidar *et al.*, 2024); (Qin *et al.*, 2022)Similar to reports from Vietnam and Iraq(Nga, Le Thi Thanh Thuy and Lam, 2025);(Alkhulaifi and Mohammed, 2023)) in which high resistance to fluoroquinolones, more specifically ciprofloxacin and levofloxacin, was observed. This is possibly due to mutated gyrA and parC genes in tandem with efflux pump overexpression (Yang et al., 2025). The observed carbapenem resistance in the isolates at this point is similar to the findings for pediatric ICU isolates (Nga, Le Thi Thanh Thuy and Lam, 2025), (Yin *et al.*, 2025) reflecting an increase in the therapeutic challenge**.** *rhlR* prevalence (43.3%) was lower than the prevalence documented by (Hemmati *et al.*, 2024). This difference may reflect differences in the source of infection, as the present study was predominantly performed on urinary isolates. The lower frequency of *fliC* compared with prior works(Płókarz *et al.*, 2022); (Ghadaksaz *et al.*, 2015);(Eidaroos *et al.*, 2024) may be a result of adaptation toward reduced motility in biofilm maturation. It has been reported that suppression of flagellar synthesis in biofilm development has been reported previously (Suriyanarayanan *et al.*, 2016). The association between biofilm-associated gene burden and resistance severity implies that the accumulation of virulence determinants contributes to multidrug resistance. This could occur through shared regulatory pathways such as quorum sensing, which can influence efflux pump expression and stress adaptation (Azimi *et al.*, 2020); (Carey *et al.*, 2024)High conservation of *rhlR* indicates the functional importance of this regulator. The stop-gain mutation detected in one isolate may affect quorum sensing activity, although compensatory regulation by LasR or PqsR pathways may maintain virulence (Soto-Aceves *et al.*, 2021); (Simanek *et al.*, 2023). When identifying *rhlR* and *fliC* in pediatric isolates, they have increased clinical importance due to their potential as virulence determinants. Given there is no requirement for quorum sensing regulators like *rhlR* in bacterial survival alone, they may represent candidates for therapeutic target(s) as they regulate virulence (Lim *et al.*, 2022); (Ghosh, Das and Mallik, 2019).The reported association of gene burden and resistance severity further supports the development of anti-biofilm therapeutics for the management of multidrug-resistant infections in pediatric patients**.**

Conclusion

This study demonstrates that *rhlR* and *fliC* are present in 43.3% and 33.3% of pediatric *P. aeruginosa* isolates, respectively. DNA sequencing revealed that *rhlR* is highly conserved (96.3–97.7% identity) with a stop-gained mutation in one isolate, while *fliC* exhibits extreme sequence variability, consistent with immune evasion. Antimicrobial resistance rates are alarmingly high, particularly for fluoroquinolones and carbapenems. A significant positive correlation exists between gene count and resistance severity, suggesting that biofilm-associated gene burden may serve as a marker for multidrug resistance. These findings support the development of QS-targeted therapies and highlight the need for routine molecular surveillance in pediatric healthcare settings.

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