Diyala Journal of Medicine

ORIGINAL RESEARCH
Published: 25 April 2026
DOI: 10.26505/djm.v30i1.1416

Investigation the Role of Quorum Sensing Genes in Biofilm Formation and Antibiotic Resistance Among Clinical Isolates of *Pseudomonas Aeruginosa*

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Received: 19 April 2025
Accepted: 29 June 2025
Published: 25 April 2026

Abstract

Background: Biofilm development in P. aeruginosa is a complicated mechanism controlled by several genetic factors, among which quorum sensing (QS) plays a pivotal role.

Objectives: Investigating the Impact of Quorum-sensing Systems genes (*rhlI*, *rhlR*, *lasI*, and *lasR*) on biofilm-mediated antibiotic resistance in *Pseudomonas aeruginosa.*

Patients and Methods: A total of 30 Pseudomonas aeruginosa isolates were collected from four major referral hospitals in Baghdad, including Al-Imamian Al-Kadhimiyain Medical City and the Iraq Medical City complex (comprising Baghdad Teaching Hospital, Burns Hospital, and Ghazy Al-Hariri Hospital for Surgical Specialties). Antibiotic susceptibility testing and minimum inhibitory concentration (MIC) determination were evaluated. Quantitative microtiter plate assays were used to study the biofilm formation ability. Conventional PCR was used to detect quorum-sensing genes using specific primer pairs after DNA extraction.

Results: Of the 30 *Pseudomonas aeruginosa* isolates,43% were identified as extensively drug-resistant (XDR), while 17% were classified as multidrug-resistant (MDR). The highest level of antibiotic resistance was observed against levofloxacin (60%). Formation of Biofilms was detected in 93.33% of the isolates, with varying strength. The occurrence of quorum-sensing genes among the isolates was as follows: *lasI* (96.67%), *lasR* (76.67%), *rhlI* (93.33%), and *rhlR* (63.33%).

Conclusion: The quorum-sensing genes *lasI* and *rhlI* were consistently noticed in all isolates that formed moderate to strong biofilms. Additionally, a correlation was observed between the existence of *lasR* and *rhlR* genes and resistance to fluoroquinolones and aminoglycosides.

Keywords: *Pseudomonas aeruginosa*, Quorum-sensing genes, Biofilm, *rhlI*, *lasI*.

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Introduction

Pseudomonas aeruginosa is a rod-shaped, Gram-negative bacterium that causes chronic and serious infections in immunocompromised and cystic fibrosis patients. It is a highly intractable pathogen that is resistant to most antimicrobial agents, making treatment and containment challenging (1). A critical factor contributing to its pathogenicity is its ability to form Biofilm-structured populations of bacteria enclosed within a self-generated extracellular matrix (2). These biofilms not only improve the bacterium's resistance to antimicrobial agents but also shield it from host immune responses, leading to persistent and challenging infections (3). Biofilm development in *P. aeruginosa* is a complicated mechanism controlled by several genetic and environmental factors, of which quorum sensing (QS) plays a pivotal role (4). *Pseudomonas aeruginosa* exhibits multidrug resistance via diverse mechanisms: overexpression of efflux pumps, reduced outer membrane permeability, enzymatic degradation (e.g., β-lactamases, aminoglycoside-modifying enzymes), target site mutations, biofilm formation, and horizontal gene transfer. These adaptations enable survival in hostile environments and complicate the treatment of infections in clinical settings (5). Quorum Sensing, a unique system that enables bacterial cells to communicate with other bacterial cells, is regulated by a gene system that modulates its expression, which subsequently affects many virulence factors and genes that enhance biofilm (6). The two quorum-sensing systems in *P. aeruginosa* are *Rhl* and *Las* that have N-(3-oxododecanoyl)-L-homoserine lactone (3O-C12 HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) (7). Once a threshold concentration is reached, specific signaling molecules that are independently created by *LasI* and *RhlI* bind to their corresponding receptors (*LasR* and *RhlR*) (8). The *Rhl* system contributes to different stages of biofilm formation, including microcolony formation. It also regulates several virulence factors in *Pseudomonas aeruginosa*, such as rhamnolipid, hydrogen cyanide, elastase, and pyocyanin (9). The Las system, on the other hand, contributes to other sides of biofilm formation, particularly biofilm maturation (10). Additionally, quorum sensing (QS) influences the control of antibiotic-modifying enzymes and drug efflux pumps, which may contribute to the development of antibiotic resistance in *P. aeruginosa*. (5) This research aims to examine the impact of quorum-sensing systems (*rhlI*, *rhlR*, *lasI*, and *lasR*) on biofilm-mediated antibiotic resistance in *Pseudomonas aeruginosa*.

Patients and Methods

Study design: This cross-sectional study was conducted from November 2024 to February 2025. A total of 30 *Pseudomonas aeruginosa* isolates were obtained from wound samples collected at four major referral hospitals in Baghdad: Al-Imamian Al-Kadhimiyain Medical City and Iraq Medical City, which include Baghdad Teaching Hospital, Burns Hospital, and Ghazy Al-Hariri Hospital for Surgical Specialties. These institutions serve as primary referral centers for patients from Baghdad, its surrounding suburbs, and other governorates.

Bacterial growth and identification: The bacterial isolates were initially identified based on colony morphology, including shape, size, odor, color, and pigment production. A single colony was selected using a sterile loop to establish a pure subculture on nutrient agar (11). Further identification was performed using the VITEK® 2 system (BioMérieux, France), which was also employed for antibiotic susceptibility testing and minimum inhibitory concentration (MIC) determination, following the manufacturer's instructions (Table 1).

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Table 1. AST-N222 card used for *Pseudomonas aeruginosa*, which contained the following antibiotics.

Antibiotic	MIC	Antibiotic	MIC
Amikacin	<=2	Piperacillin	<=4
Cefepime	<=1	Piperacillin/ tazobactam	<=4
Ceftazidime	<=1	Levofloxacin	<=1
Ciprofloxacin	<=0.25	Meropenem	<=0.25
Colistin	<=0.5	Imipenem	<=1
Gentamicin	<=1		

Detection of biofilm formation: Quantitative microtiter plate tests for biofilm production with slight modifications were accomplished as explained by Müsken et al (12). Equal volumes (100 µL) of a 0.5 McFarland bacterial suspension and LB broth with 20% glucose were added to 96-well plates, then incubated at 37°C for one day. The wells were washed with PBS, fixed with methanol (200 µL, 10 min), stained with 0.4% crystal violet for 15 minutes, and then rinsed and dried. Biofilm quantification was done by adding 250 µL of 33% glacial acetic acid and measuring absorbance at 595 nm. Appropriate positive and negative controls were included. The biofilm-forming capacity of the isolates was classified into four categories depending on optical density (OD) measurements: non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤ 2×ODc), moderate biofilm producers (2×ODc < OD ≤ 4×ODc), and strong biofilm producers (OD > 4×ODc). All experiments were conducted in triplicate, with *Pseudomonas aeruginosa* strain (Js 6234), obtained from the Medical Research Center, College of Medicine, Al-Nahrain University, serving as the positive control.

Molecular detection of quorum-sensing genes (*rhlI*, *rhlR*, *lasI*, and *lasR*): The diagnosed *P. aeruginosa* isolates were used to extract genomic DNA from a fresh overnight culture using the Geneaid Presto™ Mini gDNA Bacteria Kit (Taiwan). After that, DNA templates were used to target *lasI*, *lasR*, *rhlI*, and *rhlR* with specific primer sequences, as listed in Table 2. The PCR products were visualized by electrophoresis on a 1.5% agarose gel (70V, 60min) with ethidium bromide (0.3 µg/ml), and their sizes were estimated using a DNA ladder (100-1000 bp) (Promega USA).

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Table 2. AST-N222 card used for *Pseudomonas aeruginosa*, which contained the following antibiotics.

Gene	Primer (5′ 3′)	Annealing (Tem. /time)	Amplicon size (pb)	Reference
rhlI	F- TTC ATC CTC CTT TAG TCT TCCC	55 °C/1 min	155	13
	R- TTC CAG CGA TTC AGA GAG C			
rhlR	F-TGC ATT TTA TCG ATC AGG GC	55 °C/55 s	133	13
	R- CAC TTC CTT TTC CAG GAC G			
lasI	F- CGT GCT CAA GTG TTC AAG G	56 °C/1 min	295	14
	R- TAC AGT CGG AAA AGC CCA G			
lasR	F- AAG TGG AAA ATT GGA GTG GAG	56 °C/50 s	130	14
	R- GTA GTT GCC GAC GAC GAT GAAG			

Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 24 (Chicago, USA). Numerical data were expressed as mean ± standard deviation, while categorical variables were summarized using frequencies and percentages. The chi-square test was employed to assess associations between categorical variables. A p-value of less than 0.05 was considered statistically significant.

Results

Antibiotic resistance and biofilm-forming ability of *pseudomonas aeruginosa*: Among the 30 *P. aeruginosa* isolates, the highest resistance was to Levofloxacin (60%), Ciprofloxacin (57%), and Imipenem (53%). Moderate resistance was seen for Piperacillin and Ceftazidime (50%), Gentamicin (47%), and Amikacin (43%). Lower rates were noted for Cefepime (40%), Meropenem and Piperacillin-Tazobactam (37%), with the lowest for Colistin (23%) (Figure 1). Based on the OD_{595} cutoff (0.123), most of the 30 *P. aeruginosa* isolates were biofilm producers: 13.33% strong, 33.33% moderate, 46.67% weak, and 6.67% non-producers. (Figure 2).

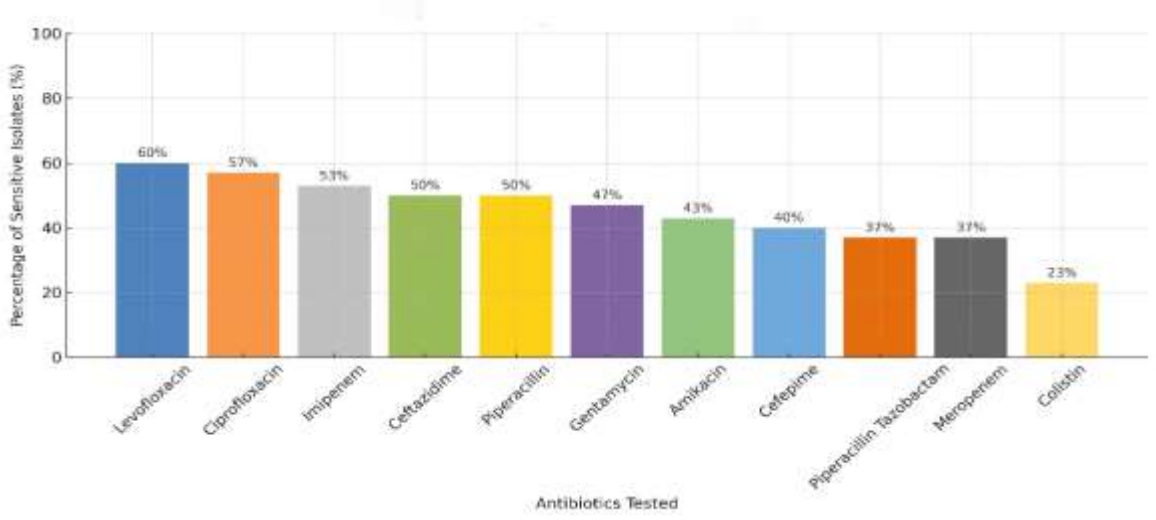

Figure 1. Antibiotic resistance rate of P. aeruginosa Isolates.

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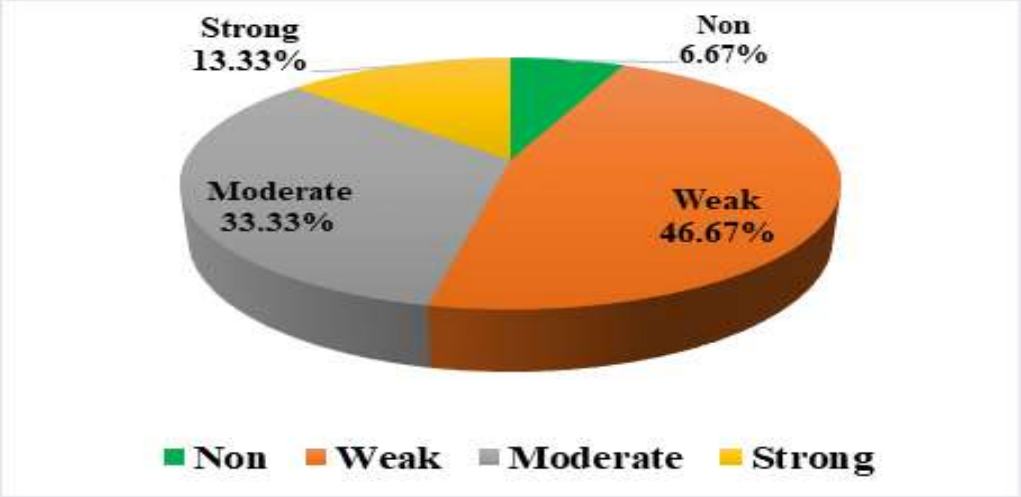

Figure 2. Distribution of Pseudomonas aeruginosa isolates based on biofilm production.

Molecular detection of quorum-sensing genes in *pseudomonas aeruginosa*: Detection rates of Quorum Sensing Genes under study in P. aeruginosa were 29/30 (96.67%) for lasI (295 bp), 23/30 (76.67%) for *lasR* (130 bp), 28/30 (93.33%) for *rhlI* (155 bp), and 19/30 (63.33%) for rhlR (133 bp) (as illustrated in Figures 3,4,5, and 6, respectively).

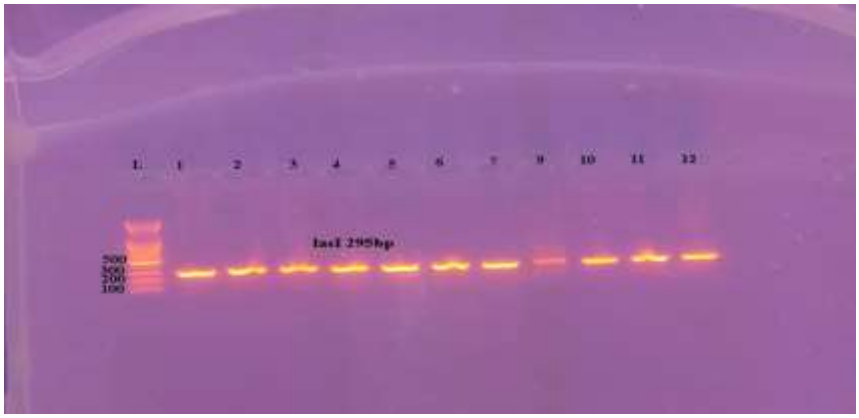

Figure 3. Gel electrophoresis (1.5 % agarose,70 Volt/cm for 60 min) amplification of the *lasI* gene, after staining with ethidium bromide. (L), 100bp ladder lanes (1-12), PCR product at 295bp.

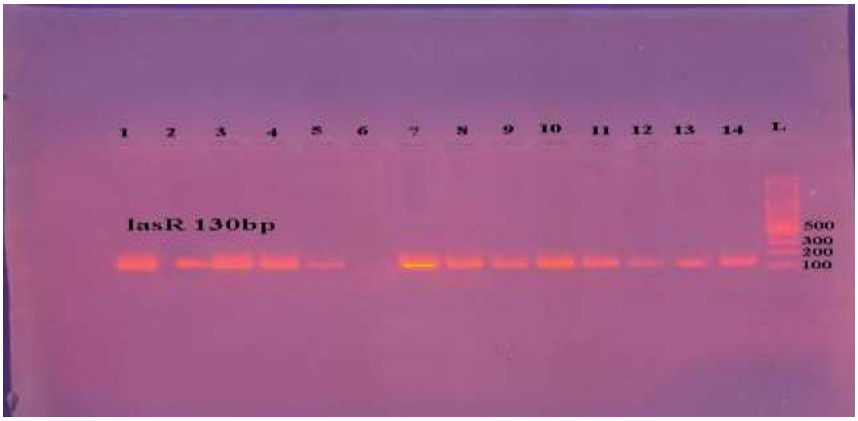

Figure 4. Gel electrophoresis (1.5 % agarose,70 Volt/cm for 60 min), amplification of the *lasR* gene, after staining with ethidium bromide (L), 100bp ladder lanes (1-14), PCR product at 130bp.

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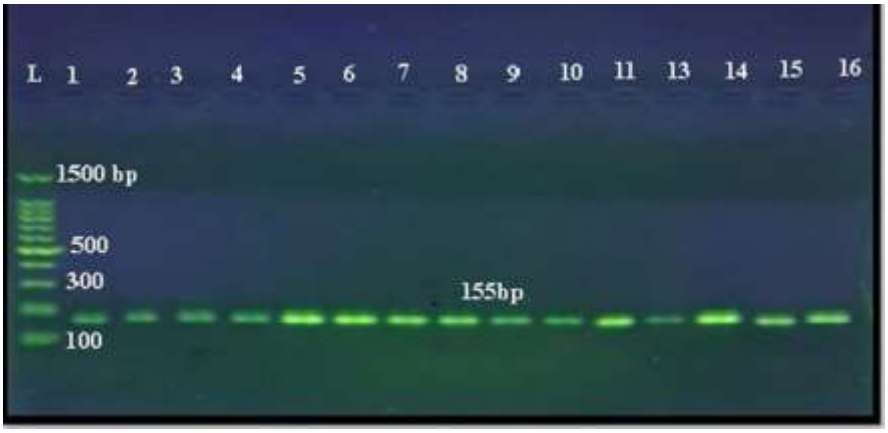

Figure 5. Gel electrophoresis (1.5 % agarose,70 Volt/cm for 60 min.), Amplification of the *rhII* gene, staining with Red Safe™ Nucleic Acid Staining. (L) 100bp ladder lanes (1-16) PCR product at 155bp.

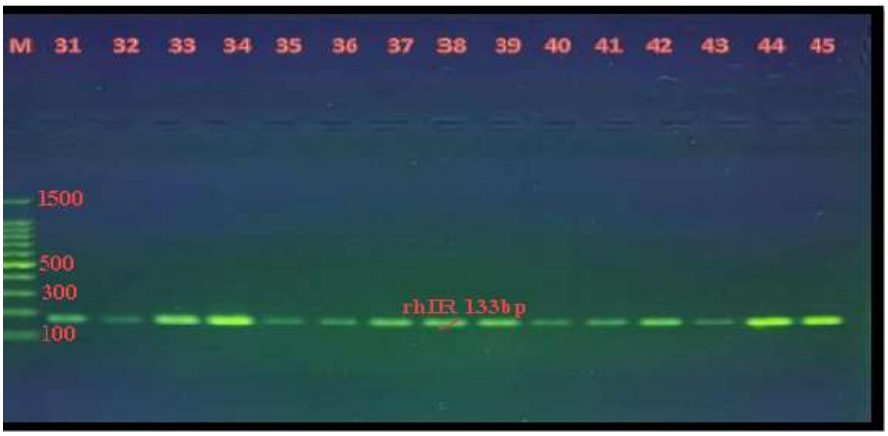

Figure 6. Gel electrophoresis (1.5 % agarose,70 Volt/cm for 60 min.), Amplification of the *rhIR* gene, staining with Red Safe™ Nucleic Acid Staining. (L) 100bp ladder lanes (1-16) PCR product at 133bp.

Association between quorum sensing genes (*LasI*, *LasR*, *RhlI*, *RhlR*) and antibiotic resistance in different antibiotic categories: The association between quorum-sensing genes (*LasI*, *LasR*, *RhlI*, and *RhlR*) and four antibiotic classes, Quinolones, Aminoglycosides, β-lactams, and Polymyxins, was analyzed in Figure 7. The figure presents the percentage of isolates that were sensitive or resistant to each antibiotic group, stratified by the presence (Positive) or absence (Negative) of quorum-sensing genes. For Quinolone groups, isolates positive for *LasI* showed 100% positivity among sensitive strains, while resistant strains showed 94.4% positivity, with no significant association (p-value = 0.600). Those that were positive for *LasR* in sensitive strains showed 66.7% positivity and 83.3% in resistant strains, with no significant association p-value: (0.266). The results exhibit a consistent pattern in *RhlI* in which the sensitive strains showed 83.3% positivity, while the resistant strains showed 100% positivity, with no significant association p-value (0.152). While that was positive for *RhlR* in sensitive strains showed 50% positivity and 66.7% in resistant strains, with no significant association p-value: (0.296). The data follows a similar trend in other antibiotic classes tested (Aminoglycoside, β-Lactam, Polymyxins) in which analysis revealed no statistically significant associations (all p-values > 0.05) between the existence /absence of quorum sensing genes and Sensitive / Resistance patterns (Figure 1).

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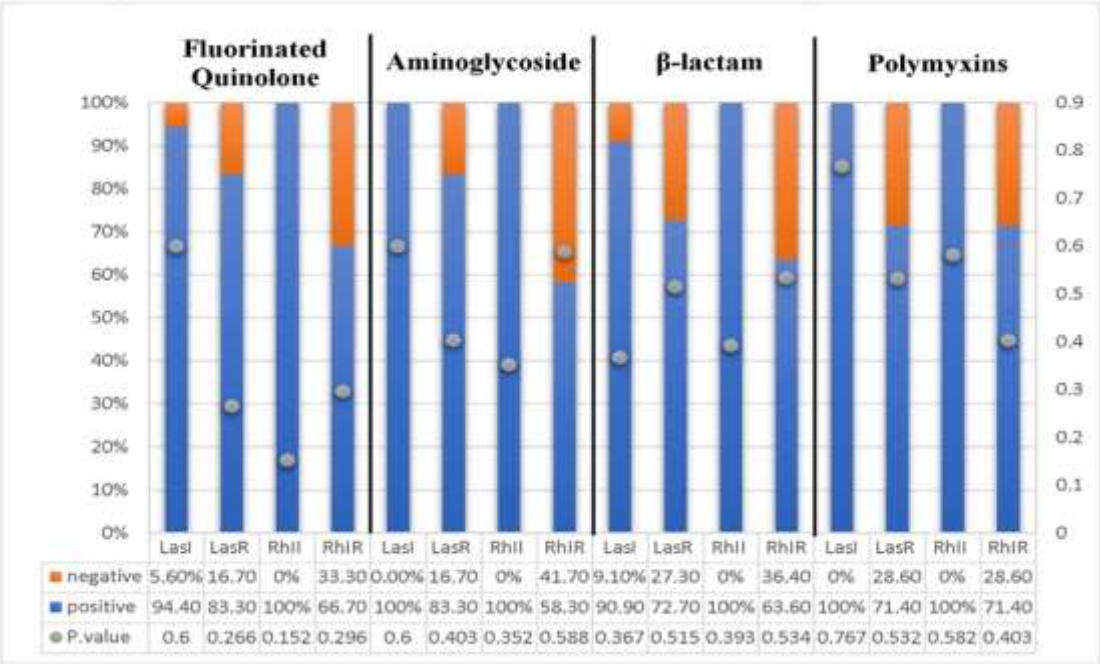

Figure 7. Correlation between quorum-sensing genes and antibiotic resistance across different antibiotic categories.

Correlation between quorum-sensing genes and biofilm formation: Concerning the correlation between quorum-sensing genes (*LasI*, *LasR*, *RhlI*, and *RhlR*) and biofilm formation in *Pseudomonas aeruginosa*. The results indicate that the majority of biofilm-forming isolates, regardless of their biofilm strength (weak, moderate, or strong), were positive for these quorum-sensing genes. Notably, *LasI* and *RhlI* genes were detected in all moderate and strong biofilm producers. For the *LasR* and *RhlR* genes, their presence showed greater inconsistency, particularly among moderate biofilm producers, where some isolates tested negative. However, weak and moderate biofilm producers more frequently carried these genes compared to non-biofilm formers. Statistical analysis shows no significant link between the strength of these quorum-sensing genes and biofilm production ($p > 0.05$ for all genes) Table (3).

Table 3. Correlation between quorum-sensing genes and Biofilm formation in *Pseudomonas aeruginosa*.

		LasI		*LasR*		*RhlI*		*RhlR*	
		Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Biofilm formation	None	0	2	1	1	0	2	1	1
		0%	100%	50%	50%	0. %	100%	50%	50%
	Weak	1	14	2	13	1	14	5	10
		6.70%	93.30%	13.30%	86.70%	6.70%	93.30%	33.30%	66.70%
	Moderate	0	9	2	7	1	8	4	5
		0 %	100 %	22.20%	77.80%	11.10%	88.90%	44.40%	55.60%
	Strong	0	4	2	2	0	4	2	2
		0 %	100%	50%	50%	0%	100%	50%	50%
p-value		0.793		0.358		0.870		0.896	

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Discussion

Quorum sensing is a vital communication mechanism in bacteria that enables them to coordinate gene expression and behavior based on population density; quorum sensing plays a crucial role in bacterial survival and adaptability, allowing them to act as a collective rather than as individual cells. Understanding this process has significant implications in medicine and biotechnology (15). The rising resistance of *P. aeruginosa* is linked to its congenital and acquired resistance mechanisms. In this study, the highest resistance was observed for Levofloxacin and Ciprofloxacin, which agrees with the study in Baghdad, Iraq, by Wadi and Ali, who recorded the highest resistance towards Levofloxacin and Ciprofloxacin, and the lowest resistance to Colistin (16). The notably low colistin resistance rate among isolates is concerning, as Colistin is often considered a last-resort antibiotic for MDR and XDR infections. The emergence of Colistin resistance underscores the urgency for stringent antibiotic stewardship and the development of novel therapeutic strategies. Resistance to imipenem was consistent with the findings in Kufa and Baghdad (17, 18) but higher than that reported in Erbil city, Iraq (19). Meropenem and Cefepime resistance rates matched those reported by Ali et al. (20). Piperacillin resistance was in line with Al-Aboudi and Al-Jwaid (21) but lower than Khudair and Mahmood (22). Ceftazidime resistance matched reports from Baghdad (23) and Iran (24). Gentamicin and Amikacin resistance rates were consistent with previous Iraqi studies (21,25,26). Piperacillin/Tazobactam resistance was aligned with the Poland data (27). The observed increase in antibiotic resistance in *Pseudomonas aeruginosa*, particularly in our country, can be attributed to several key factors, such as unregulated antibiotic use, self-medication, lack of susceptibility testing, and weak infection control in some Iraqi healthcare settings.

This study found that most of the *P. aeruginosa* isolates (93.33%) exhibited some level of biofilm production, with varying degrees of strength ranging between strong (4 isolates), moderate (9 isolates), and weak (15 isolates) biofilm producers. These findings are consistent with several studies conducted in Baghdad, Iraq (28,29, 30). Our findings regarding the detection of quorum-sensing genes in *Pseudomonas aeruginosa* align with previous reports, though variations exist due to differences in sample size, location, and clinical sources. For comparison, Al Bayatia in Baghdad reported frequencies of 96–100% for all QS genes (29). Lima et al. (13) found 97.5% for *lasI* and 100% for *lasR*, *rhlI*, and *rhlR*. Al-Kilabi et al. (31) Reported lower rates in otitis media isolates (lasI: 87%, *lasR*: 80.6%, *rhlI*: 80.6%, *rhlR*: 90.3%). Ghanem et al. (Egypt) noted 81.6%, 80%, 69.6%, and 94.4%, respectively (32). Similarly, Elnegery et al. recorded *lasR* at 94% and *rhlR* at 90% (33). Another study of 120 isolates found *lasI*: 89.1%, *lasR*: 78.3%, *rhlI*: 81.6%, and *rhlR*: 90.8% (7). Our findings show that QS genes are prevalent among biofilm-forming *P. aeruginosa* isolates. Notably, *lasI* and *rhlI* were detected in all moderate and strong biofilm producers, indicating a strong association with biofilm development. Despite *lasI* and *rhlI* being found in all moderate and strong biofilm producers, statistical analysis did not confirm a significant correlation, suggesting other regulatory elements may modulate biofilm strength ($p > 0.05$). These results align with previous studies, such as a study by Hemmati et al, who reported high *rhlR* and *lasI* prevalence in strong biofilm producers, and *rhlI/rhlR* in moderate ones (7). Al Bayatia (Baghdad) found all QS genes present in strong biofilm producers (29). While Elnegery et al. (Egypt) reported the universal presence of *lasR* and *rhlR* in such isolates. (33). Some studies suggest QS gene mutations affect biofilm architecture rather than initiation. Overall, while QS genes are common

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in biofilm-forming isolates, their presence alone may not predict biofilm strength, pointing to additional regulatory or environmental factors in biofilm maturation. (34,35) The results of this study suggest a potential association between quorum-sensing genes, particularly *LasR* and *RhlR*, and antibiotic resistance. Resistance to *fluoroquinolones* and *aminoglycosides* is more prevalent in isolates positive for these genes, indicating a possible link. Although gene presence suggests potential involvement, gene expression studies are required to confirm functional roles in biofilm formation. In contrast, β-lactam-sensitive isolates predominantly express quorum-sensing genes, while resistance patterns vary. For polymyxins, resistance is less frequent, and most sensitive isolates are positive for quorum-sensing genes, though without strong statistical significance. These findings highlight a possible role of quorum sensing in antibiotic resistance, particularly for *fluoroquinolones* and *aminoglycosides*. These findings align with existing research, indicating a complex interplay between QS systems and antibiotic resistance in *P. aeruginosa*. For instance, a study by Sikdar and Elias demonstrated that mutations in QS genes (*lasI* and *rhlI*) can influence susceptibility to various antibiotics, including fluoroquinolones. The researcher found that deficient mutants in QS genes exhibited altered resistance profiles, suggesting that QS activity modulates antibiotic susceptibility (36) Moreover, the role of QS in heteroresistance, especially in the fluoroquinolones group, was shown in a study by Lu Y et al, which observed that the downregulation of QS genes (*lasI* and *rhlI*) was associated with increased heteroresistance in many antibacterial therapies (37). However, the relationship between QS and antibiotic resistance is multifaceted. Some studies have noted that when the quorum-sensing gene is unexpressed in some bacterial strains, these bacteria can increase antibiotic tolerance. (7,32). Surprisingly, in this study, β-lactam-sensitive isolates predominantly express QS genes with varying sensitive patterns, which contradicts many studies that mentioned QS has been implicated in regulating β-lactamase production, leading to β-lactam resistance (38, 39). The predominance of QS genes in β-lactam-sensitive isolates suggests that additional factors, such as specific regulatory pathways or environmental conditions, may influence this dynamic, which may add a new perspective to this complex. Regarding polymyxins, the finding in the current study reported that resistance is less frequent and that most sensitive isolates are positive for QS genes, though without strong statistical significance, which aligns with the notion that QS may not play a central role in mediating polymyxin resistance. This is consistent with studies indicating that polymyxin resistance in *P. aeruginosa* is primarily associated with modifications to the lipopolysaccharide structure and efflux pump activity rather than QS mechanisms (40,41).

Conclusion

Our study concludes that the quorum-sensing genes *lasI* and *rhlI* were consistently found in all isolates that formed moderate to strong biofilms, indicating their possible role in biofilm development. Additionally, a correlation was observed between the presence of *lasR* and *rhlR* genes and resistance to fluoroquinolones and aminoglycosides, suggesting a link between quorum sensing and antimicrobial resistance. One of the key recommendations from our study is to investigate the interaction between quorum sensing (QS) and efflux pump expression, particularly regarding resistance against *fluoroquinolones* and *aminoglycosides*. Additionally, it is essential to examine the expression of QS-related genes—potentially through genomic sequencing and gene expression analysis—in the context of biofilm formation and antibiotic resistance.

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Source of funding: No source of funding.

Ethical clearance: Approval was sought and given by the Institutional Review Board (I.R.B.) in Al-Nahrain University College of Medicine." The ethics approval number is IRB/38/0380.

Conflict of interest: None.

Use of Artificial Intelligence (AI): The authors state they did not use any generative AI tools for creating or editing the manuscript's language.

Acknowledgments: The authors highly appreciate all patients enrolled in this study and the effort of all staff in Al-Imammian Al-Kadhimein Medical City during sample collection and processing.

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Diyala Journal of Medicine

ORIGINAL RESEARCH
Published: 25 April 2026
DOI: 10.26505/djm.v30i1.1416

التحري عن دور جينات الاستشعار النصابي في تكوين الأغشية الحيوية ومقاومة المضادات الحيوية بين العزلات السريرية لبكتيريا الزائفة الزنجارية (Pseudomonas aeruginosa)

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الملخص

الخلفية: تُعد عملية تكوين الأغشية الحيوية في بكتيريا الزائفة الزنجارية آلية معقدة تتحكم فيها عدة عوامل وراثية، ويُعد الاستشعار النصابي (Quorum Sensing) من أبرزها.

الأهداف: التحقيق في تأثير جينات نظام الاستشعار النصابي (*rhlI*، *rhlR*، *lasI*، و*lasR*) على مقاومة المضادات الحيوية المتواسطة بواسطة الأغشية الحيوية في بكتيريا الزائفة الزنجارية.

المرضى والطرق: تم جمع ٣٠ عزلة من بكتيريا الزائفة الزنجارية من أربعة مستشفيات إحالة رئيسية في بغداد، شملت مدينة الإمامين الكاظمين الطبية ومجمع مدينة الطب (الذي يضم مستشفى بغداد التعليمي، مستشفى الحروق، ومستشفى غازي الحريري للجراحات التخصصية). تم إجراء اختبارات الحساسية للمضادات الحيوية وتحديد التركيز المثبط الأدنى (MIC). استُخدم اختبار الأطباق الدقيقة الكمية لدراسة قدرة البكتيريا على تكوين الأغشية الحيوية. كما استُخدم تفاعل البلمرة المتسلسل (PCR) التقليدي للكشف عن جينات الاستشعار النصابي باستخدام بادئات نوعية بعد استخلاص الحمض النووي.

النتائج: من بين العزلات الثلاثين، تم تصنيف ٤٣٪ على أنها شديدة المقاومة للأدوية (XDR)، و١٧٪ كمقاومة لعدة أدوية (MDR). سُجلت أعلى نسبة مقاومة تجاه دواء الليفوفلوكساسين (٦٠٪). تم الكشف عن تكوين الأغشية الحيوية في ٩٣,٣٣٪ من العزلات، بدرجات متفاوتة من الشدة. نسب وجود جينات الاستشعار النصابي بين العزلات كانت كما يلي: (96.67٪) *lasI*، (76.67٪) *lasR*، *rhlI* (٩٣,٣٣٪)، و*rhlR* (٦٣,٣٣٪).

الاستنتاج: لوحظ وجود جيني *lasI* و*rhlI* بشكل دائم في جميع العزلات التي أظهرت تكويناً معتدلاً إلى قوياً للأغشية الحيوية. كما وُجد ارتباط بين وجود جيني *lasR* و*rhlR* ومقاومة الفلوروكينولونات والأمينوغليكوزيدات.

الكلمات المفتاحية: الزائفة الزنجارية، جينات الاستشعار النصابي ، الغشاء الحيوي، *rhlI*، *lasI*.

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تاريخ الاستلام: ١٩ نيسان ٢٠٢٥

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