PERIÓDICO TCHÊ QUÍMICA
ARTIGO ORIGINAL

FORMAÇÃO DE BIOFILME, RESISTÊNCIA A ANTIBIÓTICOS E DETECÇÃO MOLECULAR DO GENE *rhlI* EM ISOLADOS CLÍNICOS DE *Pseudomonas aeruginosa*

BIOFILM FORMATION, ANTIBIOTIC RESISTANCE, AND MOLECULAR DETECTION OF THE *rhlI* GENE IN CLINICAL ISOLATES OF *Pseudomonas aeruginosa*

تكوُّن الأغشية الحيوية، ومقاومة المضادات الحيوية، والكشف الجزيئي عن جين *rhlI* في العزلات السريرية لبكتيريا *Pseudomonas aeruginosa*

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RESUMO

Introdução: *Pseudomonas aeruginosa* é um patógeno de origem hospitalar frequentemente envolvido em diversas infecções associadas à assistência à saúde, especialmente em pacientes imunocomprometidos. Sua capacidade de formar biofilme e desenvolver resistência a agentes antimicrobianos desempenha papel fundamental nas falhas terapêuticas. **Objetivo**: O presente estudo teve como objetivo avaliar fenotipicamente a formação de biofilme, identificar o padrão de suscetibilidade a antibióticos e detectar o gene *rhlI* em isolados clínicos de P. aeruginosa. **Métodos**: Trinta e cinco amostras clínicas foram obtidas de diversas fontes, incluindo urina, swabs de feridas, swabs auriculares e amostras de queimaduras. A identificação dos isolados bacterianos foi realizada com base em métodos microbiológicos padrão e confirmada pelo sistema VITEK-2. O teste de difusão em disco de Kirby-Bauer foi empregado para determinar a suscetibilidade antimicrobiana. O ensaio em placa de cultura de tecidos (TCP) foi realizado para avaliar a capacidade de formação de biofilme, e a reação em cadeia da polimerase (PCR) foi utilizada para detectar a presença do gene *rhlI*. A análise estatística utilizou o teste de Fisher ($p < 0,05$). **Resultados**: Os isolados apresentaram resistência à ticarcilina, ceftriaxona e ciprofloxacina (100% cada). Novobiocina (96%), cloranfenicol (93%) e trimetoprima também apresentaram altas taxas de resistência (90%). Por outro lado, imipenem (9%) e amicacina (5%) apresentaram taxas de resistência mais baixas, indicando que esses antibióticos foram comparativamente mais eficazes. A análise do biofilme revelou que 60% dos isolados eram fortes produtores de biofilme, enquanto 40% apresentaram formação moderada de biofilme. Os testes de PCR revelaram que os isolados (100%) possuíam o gene *rhlI*. **Conclusões**: Os isolados clínicos de *P. aeruginosa* demonstraram elevada resistência antimicrobiana, alta capacidade de formação de biofilme e presença universal do gene *rhlI*. Esses resultados ressaltam a necessidade de associar abordagens fenotípicas e moleculares para melhor compreender e controlar as infecções causadas por esse microrganismo.

Palavras-chave: *Detecção de quorum, Pseudomonas aeruginosa, Fatores de virulência, Infecções Associadas à Assistência à Saúde, Reação em cadeia da polimerase, Resistência a drogas.*

ABSTRACT

Background: *Pseudomonas aeruginosa* is a hospital-acquired pathogen that commonly causes healthcare-associated infections, particularly in immunocompromised patients. Its ability to form biofilms and develop resistance to antimicrobial agents plays a key role in treatment failure. **Purpose**: The study aimed to assess the phenotypic biofilm formation, identify the pattern of antibiotic susceptibility, and identify the *rhlI* gene in clinical isolates of P. aeruginosa. **Methods**: Thirty-five clinical samples were obtained through various sources, including urine, wound swabs, ear swabs, and burn samples. Bacterial isolates were identified using standard microbiological methods and examined with the VITEK-2 system. Kirby-Bauer disk diffusion testing was done to determine antimicrobial susceptibility. A tissue culture plate (TCP) assay was conducted to assess biofilm-forming ability, and polymerase chain reaction (PCR) was performed to detect the *rhlI* gene. The statistical analysis used Fisher's test ($p < 0.05$). **Results**: The isolates were resistant to ticarcillin, ceftriaxone, and ciprofloxacin (100% each). Novobiocin (96%), chloramphenicol (93%), and trimethoprim also had high resistance rates (90%). On the

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contrary, imipenem (9%) and amikacin (5%) showed lower resistance rates, indicating that these antibiotics were comparatively more effective. The analysis of biofilm revealed that 60% of the isolates were strong biofilm producers, while 40% formed moderate biofilms. PCR tests revealed that all isolates (100%) carried the *rhlI* gene. **Conclusions**: *P. aeruginosa* clinical isolates displayed high antimicrobial resistance and biofilm-forming capacity, and the *rhlI* gene was found in all isolates. These results highlight the need to consider a combination of both phenotypic and molecular approaches to gain more insight and manage infection by this organism.

***Keywords**: Quorum sensing, Pseudomonas aeruginosa, Virulence factors, Healthcare-Associated Infections, Polymerase Chain Reaction, Drug Resistance.*

الملخص

الخلفية: تُعدّ بكتيريا الزائفة الزنجارية من الممرضات الانتهازية المرتبطة بطيف واسع من العدوى المرتبطة بالرعاية الصحية، ولا سيما لدى المرضى ذوي المناعة الضعيفة. وتسهم قدرتها على تكوين الأغشية الحيوية وتطوير مقاومة مضادات الميكروبات إسهاماً كبيراً في فشل العلاج. **الهدف:** هدفت هذه الدراسة إلى تقييم تكوّن الأغشية الحيوية ظاهريًا، وتحديد أنماط الحساسية للمضادات الحيوية، والكشف عن وجود جين *rhlI* في العزلات السريرية لبكتيريا الزائفة الزنجارية. **طرائق العمل:** جُمعت 35 عينة سريرية من مصادر مختلفة، شملت البول والجروح ومسحات الأذن والحروق. تم التعرف على العزلات البكتيرية باستخدام الطرق الميكروبيولوجية القياسية، وأُكِّد التشخيص باستخدام جهاز VITEK-2 كما أُجري اختبار الحساسية للمضادات الحيوية باستخدام طريقة انتشار الأقراص (Kirby–Bauer). وتم تقييم تكوّن الأغشية الحيوية باستخدام اختبار صفيحة زراعة الأنسجة (TCP)، في حين تم الكشف عن جين *rhlI* باستخدام تفاعل البوليميراز المتسلسل.استخدم التحليل الإحصائي اختبار فيشر ($p < 0.05$) **النتائج:** أظهرت العزلات معدلات مقاومة مرتفعة لكلٍّ من التيكارسيلين (100%)، والسيفترياكسون (100%)، والسيبروفلوكساسين (100%). كما سُجّلت معدلات مقاومة عالية للنوفوبيوسين (96%)، والكلورامفينيكول (93%)، والتريميثوبريم (90%). في المقابل، لوحظت معدلات مقاومة منخفضة للإيميبينيم (9%) والأميكاسين (5%)، مما يشير إلى فعاليتهما النسبية. وكشف تحليل تكوّن الأغشية الحيوية أن 60% من العزلات كانت منتجة قوية، و40% منتجة متوسطة. كما أظهرت نتائج PCR أن جميع العزلات (100%) تحمل جين *rhlI*. **الاستنتاجات:** أظهرت العزلات السريرية لـ *Pseudomonas aeruginosa* مستويات عالية من مقاومة المضادات الحيوية وقدرة على تكوين الأغشية الحيوية، مع وجود شامل لجين *rhlI*. وتؤكد هذه النتائج أهمية دمج الطرائق الظاهرية والجزيئية لفهمٍ أفضل وإدارةٍ أكثر فاعلية للعدوى التي تسببها هذه البكتيريا.

الكلمات المفتاحية: استشعار النصاب, *الزائفة الزنجارية، عوامل الضراوة، العدوى المرتبطة بالرعاية الصحية، تفاعل البوليميراز المتسلسل، مقاومة الادوية.*

1. INTRODUCTION:

Pseudomonas aeruginosa is a well-known opportunistic pathogen belonging to the family Pseudomonadaceae and is often associated with colonization in both humans and animals (Mohamed *et al*., 2022). It is Gram-negative, non-fermentative, rod-shaped, and non-sporing, with a diameter of 0.5-0.8 µm and a length of 1.5 -3.0 µm. The organism is very common in natural habitats such as soil and water, and can also infect human and animal hosts. It is mostly aerobic, though it can grow under anaerobic conditions in the presence of nitrate. It moves using a polar flagellum (Spagnolo *et al*., 2021).

The optimal temperature for this bacterium's growth is 37 °C, although it can grow up to 42 °C. In addition, *P. aeruginosa* is oxidase-positive, lactose-non-fermenting, and produces pigments, including pyocyanin, fluorescein, pyorubin, and pyomelanin; these pigments play an important role in its identification (Zavascki *et al*., 2010).

Most P. aeruginosa strains possess virulence factors on their surface, enabling them to attach, colonize, and invade host tissues. The virulence factors stimulate cytokine secretion from injured tissues, which facilitates invasion and toxin secretion (Liao *et al*., 2022). This bacterium can invade numerous organs and suppress the immune system, leading to infections that cannot be fully cured. The main virulence factors contributing to bacterial colonization are the Lipopolysaccharide (LPS), flagella, and type IV pili (Saleh and Motib, 2023). In addition, other extracellular virulence factors include exotoxin A, alkaline proteases, elastases, pyocyanin, biofilm formation, and quorum sensing (Vadakkan *et al*., 2024). Biofilm formation has been identified as a major virulence factor in many bacteria. It entails the creation of a well-organized community of microbes, deposited within a thick protective network. The major components of this matrix are extracellular polymeric substances (EPS), which include exopolysaccharides, proteins, nucleic acids, and other secreted molecules (Mann and Wozniak, 2012). Though biofilms were earlier viewed as simple aggregates of bacterial cells, it is now known to have a multifaceted, highly organized architecture. They are structured microcolonies linked together through pathways that facilitate the flow of water, nutrients, and waste products. This structural organization creates a heterogeneous microenvironment with nutrient and oxygen gradients (Hall-Stoodley *et al*., 2004).

P. aeruginosa uses intrinsic, acquired, or adaptive resistance as its defense mechanism against antibiotics. In the intrinsic phase, the outer membrane is impermeable, efflux pumps are

expressed to expel antibiotics from cells, and enzymes that degrade antibiotics are produced. A horizontal shift of resistance genes or mutational changes might achieve the acquired phase (Artono *et al*., 2025).

Pseudomonas aeruginosa shows resistance to a number of the most important antibiotic classes, including β-lactam antibiotics, aminoglycosides, and fluoroquinolones. Resistance to beta-lactams is primarily mediated by several mechanisms, including mutations in PBPs (penicillin-binding proteins) that limit drug binding, decreased permeability of the bacterial outer membrane, enhanced antibiotic efflux, and enzymatic degradation. Gene transfer also facilitates the acquisition of β-lactamase enzymes from other bacterial species, further contributing to resistance (Glen and Lamont, 2024). *P. aeruginosa* has been examined for innate causes that contribute to biofilm formation. *P. aeruginosa* DNA microarray analysis detected that a small fraction of the genome (approximately 1%) shows differential expression during biofilm growth, with about 0.5% of the genes being repressed and 0.5% of the genes being activated (Whiteley *et al*., 2001). *Pseudomonas aeruginosa* isolates that carry the lasR and rhlR genes are believed to be the most critical factors that control the expression of virulence factors and biofilm formation. The lasR gene, especially, is positively regulated in biofilm formation, proteolytic, pyocyanin, and rhamnolipid biosynthesis. Similarly, the quorum-sensing regulator rhlR enhances protease and rhamnolipid synthesis (Elnegery *et al*., 2021).

Despite the established role of quorum-sensing systems in regulating virulence and biofilm formation in *Pseudomonas aeruginosa*, there is a lack of local data on the coexistence of antimicrobial resistance with biofilm-forming ability and the presence of the *rhlI* gene in clinical isolates. The combined assessment of these factors will provide a more holistic understanding of this organism's pathogenic behavior in the clinical setting. To this end, the study will explore trends in antibiotic resistance, biofilm-forming capacity, and *rhlI* gene expression in clinical isolates of *Pseudomonas aeruginosa* using a hybrid methodology that combines phenotypic and molecular methods.

2. MATERIALS AND METHODS:

2.1. *Study design and sample collection:*

The 35 samples were collected from various clinical sources, including burn swabs, urine, stool, wound swabs, ear swabs, throat swabs, and blood samples. All specimens were handled according to standard microbiological procedures (Wilson, 1996). The sample size was determined based on the available clinical isolates at the time of the study, and all samples were collected from patients with a confirmed or suspected bacterial infection.

2.2. *Isolation and Identification*

Clinical specimens were transported to the laboratory in transport media, inoculated onto a MacConkey's agar plate, and incubated at 37 °C for 18-24 hours. The primary diagnosis of isolates was made using different media and biochemical tests, as described by Figueroa-Bossi *et al*.(2022). The final diagnoses of the isolates were confirmed biochemically using the VITEK 2 automated system (GN ID card).

2.2.1. GN-ID Identification through VITEK 2 Compact System

According to the manufacturer's instructions, the isolates were diagnosed using the VITEK-2 Compact system (bioMérieux, France) with GN ID cards.

2.3. Antimicrobial susceptibility testing

The Kirby-Bauer method was followed as described by Peterson *et al*. (1980) to determine antibiotic susceptibility to 8 antibiotics (ticarcillin, ceftriaxone, imipenem, novobiocin, amikacin, ciprofloxacin, trimethoprim, and chloramphenicol). A bacterial suspension was prepared by picking one to two well-isolated colonies from the original culture and placing them in a test tube containing 4 mL of normal saline to yield a suspension of moderate turbidity, as measured by DENSICHEK (0.5–0.63). A portion of the bacterial suspension was transferred and uniformly distributed on Mueller-Hinton agar using a sterile cotton swab, then incubated for 10 minutes. Next, the antibiotic discs were inoculated onto the agar surface with sterile forceps and pressed firmly to ensure adequate contact. The plates were then inverted and incubated at 37 °C for 18-24 hours. According to the Clinical and Laboratory Standards Institute (CLSI) standards, the diameter of the inhibition zone around antimicrobial discs was measured in millimeters (mm) using a metric ruler (Weinstein and Lewis, 2020). The sensitivity of the individual isolates was later established by comparing the observed inhibition zones with the standard criteria in the literature and categorizing them into three groups: susceptible, intermediate, and

resistant to the antimicrobial agent in question. All findings were read according to CLSI (2025) recommendations for standardized breakpoint values.

2.4. Biofilm assay

Each experiment was carried out three times, and the mean optical density (OD) was calculated. Negative control wells containing sterile broth have been added, and the cut-off optical density (ODc) was set at the average OD of the negative control plus 3 standard deviations. Table 1 summarizes the classification of biofilm formation depending on the OD values.

2.4.1. Tissue culture plate method (TCP)

The most commonly used method of biofilm assessment is the tissue culture plate (TCP) biofilm detection methodology, as identified by Christensen *et al*. (1985), and it is considered the standard method for assessing biofilm formation.

2.5. DNA Extraction

Genomic DNA was extracted using a commercially available extraction kit (Promega Genomic DNA Kit). A *Pseudomonas aeruginosa* suspension was prepared by inoculating Luria broth with the bacteria and incubating at 37 °C for 24 hr. DNA isolation was performed using a Promega kit. The entire process involved taking 1 ml of suspension and centrifuging it at 14000 rpm for 1 min. The supernatant is then discarded to obtain a lysate. 200 µL of GT solution is added for precipitation, and the precipitated cells are resuspended by vigorous shaking and incubated at room temperature for 5 minutes. Next, 200 µL of GB solution is added to the tube, mixed vigorously for 5 seconds, and incubated at 60 °C for at least 10 minutes. After lysis, 200 µL of absolute ethanol is added to the tube, and the mixture is immediately shaken vigorously for 10 seconds. The GD column tube was placed in a collection tube. The components were transferred to the GD column tube and centrifuged for 2 minutes. The collection tubes containing the flux were discarded.

400 µl of W1 was added to the GD column tube, then centrifuged for 30 seconds. The flux was discarded, and the GD column tube was returned to the 2 ml collection container. tube. 600 µL of wash buffer was added to the GD column tube, and the mixture was centrifuged for 30 seconds. The flow-through in the collection tube was discarded, and the GD column tube was placed back in the 2ml collection tube

tube and centrifuged again for 3 min. to dry the column matrix. Finally, the dried GD column tube was placed in a clean 1.5 ml centrifuge tube, and 100 µL elution buffer was added; the mixture was left for 3 min. Then, the DNA was centrifuged for 30 seconds to elute the target DNA solution, which was stored at -20 °C.

2.6. PCR amplification of rhlI

The *rhlI* gene, involved in biofilm formation in *Pseudomonas aeruginosa*, was detected by conventional PCR. The primers were designed by Alpha DNA (Canada) according to Edward *et al*. (2023) with the following sequences: The amplification of a 245 bp fragment was carried out using the forward primer (5′-CTCTCTGAATCGCTGGAAGG-3) and the reverse primer (5′-GCGAAGACTTCCTTGAGCAG-3). PCR amplification of the *rhlI* gene was conducted using the thermal cycling conditions listed in Table 2. The PCR products were subsequently amplified and subjected to electrophoresis on a 1% agarose gel to verify the expected fragment size. 4 µL of 10 mg/mL ethidium bromide was used to stain the gel, and 80 V was applied for 90 minutes. The bands of DNA were visualized using a UV transilluminator (Cleaver, UK) and recorded with a gel documentation system (Cleaver, UK). A 100 bp fragment was added to the dish to determine the size of the amplified fragments. PCR was performed in a 25 µL master mix containing primers and template DNA, with a total volume of 25 µL, under standard PCR conditions. The same reaction was used for the negative control, but without a DNA template.

2.7. Statistical analysis

Statistical analysis was performed using SPSS version 30 (IBM, USA). Categories were defined as means and percentages. Relationships between categories were analyzed using Fisher's exact test for small samples, and significance was determined at $p < 0.05$. Biofilm formation categories and the extent of antibiotic resistance were used to group the data in the current study. The rhlI gene was detected in all 15 isolates, with a prevalence of 100%. The confidence interval (CI) was calculated using the Clopper-Person method.

3. RESULTS

3.1. Results

3.1.1. Isolation and identification of *P. aeruginosa*

A total of 35 clinical specimens were collected from different sources. Among these, 25 samples showed bacterial growth, while 10 did

not. Based on cultural and biochemical characteristics, 20 isolates were initially suspected to be Pseudomonas species. Based on VITEK-2 confirmation, 15 isolates were identified as *Pseudomonas aeruginosa*. These isolates formed non-lactose-fermenting colonies on MacConkey agar, produced pyocyanin pigment, were highly positive in oxidase and motility tests, and were strongly positive in the K/K reaction on triple sugar iron (TSI) medium (Brook and Frazier, 1995). On MacConkey agar, 15 isolates were identified as lactose non-fermenters, whereas 10 isolates were identified as other bacterial species. The identification was finally conducted by placing the samples in the automated VITEK-2 Compact system with GN-ID cards, which contain 47 biochemical tests and a single negative control well. The VITEK-2 system confirmed all suspected isolates, with identification confidence levels very good to excellent and probability values between 96% and 99%. Identification of isolates by sample source showed that the most frequent sources of *P. aeruginosa* were wound and burn specimens, followed by urine and ear swab samples.

3.1.2. Antibiotic susceptibility test

The antibiotic susceptibility of the 15 *Pseudomonas aeruginosa* clinical isolates was evaluated using the Kirby–Bauer disk diffusion method according to CLSI (2025) guidelines. Eight antibiotics representing various antimicrobial classes were evaluated. Table 3 shows the antimicrobial susceptibility profiles of the isolates. Notably, ticarcillin, ceftriaxone, and ciprofloxacin showed full resistance (100%), whereas relatively low rates of resistance were observed with imipenem (9%) and amikacin (5%). These results are illustrated graphically in Figure 1.

3.1.3. Biofilm formation in *P. aeruginosa* isolates.

All *Pseudomonas aeruginosa* isolates formed biofilms, as determined using the tissue culture plate (TCP) method. These isolates, 9 (60%) were strong biofilm producers, and 6 (40%) showed moderate biofilm-forming ability. None of the isolates was classified as weak or non-biofilm formers.

3.1.4. Biofilm formation and antimicrobial resistance

The results of the statistical analysis, according to Fisher's test, showed highly significant differences (P-Value = 0.004) between the biofilm formation categories and the extent of antibiotic resistance. The results showed that all isolates had a strong ability to form biofilms; 9 were completely resistant to antibiotics (100%), and none were sensitive. This indicates a strong positive correlation between biofilm formation and the severity of antibiotic resistance, as shown in Table 5. Among isolates with moderate biofilm-forming capacity, 6 (66.7%) were resistant, and 3 (33.3%) were non-resistant. This indicates that biofilm formation intensity decreases with decreasing resistance.

3.1.5. *rhlI* Gene Detection

The *rhlI* gene was screened for using a predictive polymerase chain reaction (PCR) in all 15 *Pseudomonas aeruginosa* isolates. These outcomes revealed that no negative isolates were detected (100% positive), and each produced a clear 245 bp amplification band, as shown in Figure 2. The negative control used showed no band. The *rhlI* gene was detected in all 15 isolates, with a prevalence of 100%. The confidence interval (CI) calculated using the Clopper-Pearson method ranged from 78.2% to 100%, as shown in Table 6.

3.2. DISCUSSION

The obvious differences in resistance patterns suggest the presence of multiple resistance mechanisms underlying the isolates. The findings of this study are consistent with the prior literature on multidrug resistance in *Pseudomonas aeruginosa* (Bassetti *et al*., 2018; El Zowalaty *et al*., 2015). A combination of factors, such as reduced membrane permeability, activation of excretory pump systems, and the formation of antibiotic-inactivating enzymes, can explain this resistance. The high levels of resistance detected in this research have critical clinical implications, as they could limit the number of available treatment options and underscore the importance of ongoing antimicrobial surveillance.

The results showed that biofilm formation was a prevalent property of clinical isolates of *P. aeruginosa*, which can improve their survival and persistence in the clinical setting by increasing resistance. Biofilm development is one of the fundamental adaptive strategies by which bacteria can adhere to surfaces and grow within a self-assembled extracellular polymeric matrix of polysaccharides, proteins, and extracellular DNA. The matrix also provides resistance to environmental stresses and antimicrobial agents, which enhances bacterial resistance to antibiotics (Hall-Stoodley *et al*., 2004; Mann and Wozniak, 2012; Kunwar *et al*., 2021). The biofilm-forming capacity of the isolates was described in Table 4**,** and 60% and 40% were strong and moderate producers, respectively. This difference aligns with previous studies reporting heterogeneity in *P. aeruginosa* biofilm production among clinical

isolates (Chimi *et al*., 2024). Biofilm formation is a highly controlled, dynamic process that generally undergoes phases of initial adhesion, microcolony development, maturation, and eventual dissemination, thereby supporting colonization of new environments (Hall-Stoodley *et al*., 2004). The high rates of antibiotic resistance could be partly explained by the majority of strong biofilm-forming isolates in the present study, which form biofilms that serve as physical barriers, restricting antibiotic penetration and helping bacteria survive. These findings raise the possibility of a relationship between biofilm-forming capacity and antimicrobial resistance in the investigated isolates.

Ratajczak *et al.* (2021) mentioned that the majority of strains (73.6%) were strong biofilm producers, 17.0% were moderate, and 9.4% were weak biofilm producers. Both multiple antibiotic resistance and biofilm formation may complicate the treatment of *P. aeruginosa* infections. The present study showed that all *P. aeruginosa* strains isolated from clinical specimens formed biofilms in vitro. However, they differed in the degree of biofilm formation. Most strains showed strong biofilm-forming capacity (74.0%), while significantly fewer strains exhibited moderate (16.4%) and weak (9.6%) biofilm-forming potential. The study demonstrated that all *Pseudomonas aeruginosa* strains isolated from various clinical samples formed biofilms under laboratory conditions; however, they differed in the extent of biofilm formation. A 2018 study by da Costa Lima et al. estimated biofilm-forming ability and found that 77.5% of clinical *P. aeruginosa* strains were capable of forming biofilm. (Da Silva Carvalho & Perez, 2019) found that 86.5% of *P. aeruginosa* strains studied were classified as strong or moderate biofilm producers.

This observation indicates a high prevalence of this quorum-sensing gene among the clinical isolates studied. The *rhlI* gene is another important component of the quorum-sensing system in P. *aeruginosa*, which controls the synthesis of signaling molecules involved in biofilm formation and the expression of several virulence factors. Quorum-sensing systems, such as *rhlI*-mediated responses, allow bacterial communication and are critical for biofilm formation and maintenance (De Kievit *et al*., 2001; Elnegery *et al*., 2021). The prevalence of the *rhlI* gene across all isolates is also consistent with earlier studies highlighting its role in biofilm formation and pathogenicity. Disruption of *rhlI*-mediated signaling was shown to affect biofilm development and decrease bacterial virulence, highlighting the importance of this functional process (Favre-Bonté *et al*., 2003).

Nevertheless, the presence of the *rhlI* gene may not always guarantee active quorum-sensing activity or direct participation in biofilm formation regulation. Further studies are needed to determine the functional role of gene expression levels in clinical conditions. Thus, its presence in detecting possible participation; further molecular research is required to clarify its specific role in biofilm formation and antimicrobial resistance.

4. CONCLUSIONS

The study demonstrates that biofilm-forming capability, antimicrobial resistance, and the ubiquitous presence of the *rhlI* gene occur concomitantly in clinical isolates of *Pseudomonas aeruginosa*. These results indicate the demand for routine screening of biofilm-producing bacteria and quorum-sensing-related genes in hospital labs. Future studies should focus on the quantitative analysis of the *rhlI* gene expression under different environmental and antimicrobial stress conditions.

5. DECLARATIONS

5.1. Study Limitation

The sample size used is relatively small, which might limit the scope of the findings. Also, an antimicrobial susceptibility test by disk diffusion was performed without confirmation by minimum inhibitory concentration (MIC). Bonferroni or other multiple-comparison correction was not applied to the eight antibiotic susceptibility comparisons. It was also restricted to *rhlI* gene detection, with no evaluation of its expression level or functional activity. In addition, the analysis did not include other genes associated with quorum sensing and biofilm.

5.2. Acknowledgments

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5.3. Funding Sources

This research received no external funding. The authors funded this research.

5.4. Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this manuscript.

5.5. Data Availability

All data presented in this study are available in the manuscript tables and figures. Raw data are available upon request from the corresponding author.

5.6. Author Contribution

The author confirms sole responsibility for the following: study conception and design, sample processing, laboratory analysis, data interpretation, and manuscript preparation.

Code	English	Português
CD	Conception and Design	Concepção e Design
DC	Data Collection	Coleta de Dados
DAI	Data Analysis and Interpretation	Análise e Interpretação de Dados
MW	Manuscript Writing	Escrita do Manuscrito
CR	Critical Review	Revisão Crítica
FA	Final Approval	Aprovação Final

5.7. AI and Computational Tools Declaration

Tool: Grammarly (Grammarly Inc.), QuillBot (QuillBot Inc.), Turnitin (Turnitin LLC). Purpose: Grammarly was used for grammar and language editing. QuillBot was used for limited paraphrasing in the introduction section. Turnitin was used for plagiarism detection and similarity checking.

Extent: The use of AI tools was limited to language improvement and minor text refinement (less than 10% of the manuscript).

Human Verification: All outputs generated or modified using AI tools were carefully reviewed, validated, and substantially revised by the authors. No AI tools were used for data analysis, statistical testing, results interpretation, or scientific decision-making.

5.8. Research Integrity Declaration

The author confirms that this research was conducted in accordance with accepted standards of scientific integrity. The data presented in this study are original, have not been fabricated or manipulated, and accurately reflect the research findings. The manuscript has not been published previously and is not under consideration for publication elsewhere.

5.9. Originality & Plagiarism Statement

The authors declare that this is an original manuscript and have attached the Turnitin similarity index, which was less than 20%.

5.10. Open Access License

6. STUDIES INVOLVING HUMAN AND ANIMAL SUBJECTS

6.1. Ethics Committee Approval

Ethical approval for this study was obtained from the ethics committee at the University of Alkafeel, College of Medical and Health Technologies, with reference number HMT 05/2024 on April 8/2024

The requirement for informed consent was waived due to the study's design. All data were anonymized and handled in accordance with corporate confidentiality and data governance policies.

6.2. INFORMED CONSENT

Informed consent was waived because the study was conducted on anonymized clinical isolates collected as part of routine diagnostic procedures, without direct patient intervention.

7. REFERENCES:

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Table 1. *Classification of biofilm formation based on OD values (Christensen et al., 1985)*

Mean of OD value at 630 nm'	Biofilm formation
<0.120	Non
0.120-0.240	Moderate
>0.240	High

Table 2. *PCR program of (rhlI) primer that applies in the thermocycler*

Gene	Initial denaturation	Denaturation	Annealing	Extension	No. of cycles	Final extension
rhlI	94 °C for 3 min	94 °C for 30 sec	55.5 °C 30 sec	72 °C for 1 min	30	72 °C for 5 min

Table 3. *Antimicrobial susceptibility profile of Pseudomonas aeruginosa isolates (n=15)*

Antibiotic	Abbreviate	Concentration (µg/disk)	Resistance No. (%)	Intermediated No. (%)	Sensitive No. (%)
Ticarcillin	TIC	30	100%	0%	0%
Ceftriaxone	CRO	30	100 %	0 %	0%
Imipenem	IMP	10	9%	11 %	80%
Novobiocin	NOV	30	96 %	4 %	0%
Amikacin	AK	10	5%	0%	95%
Ciprofloxacin	CIP	10	100 %	0 %	0%
Trimethoprim	TMP	10	90%	7%	3%
Chloramphenicol	CHL	30	93 %	7 %	0%

Table 4. *Distribution of biofilm formation among P. aeruginosa isolates*

Biofilm category	Number of isolates	Percentage (%)
Strong	9	60%
Moderate	6	40%
Weak	0	0%

Table 5. *Association between biofilm formation and antimicrobial resistance among Pseudomonas aeruginosa isolates (n = 15)*

Biofilm Category	Resistant N (%)	Non Resistant N (%)	Total	P-Value
Strong	9 (100%)	0	9	
Moderate	4 (66.7%)	2 (33.3%)	6	
Total	13	2	15	0.004*

**P-Value calculated by using Fisher's exact test*

Table 6. *rhlI gene prevalence among clinical isolates*

Total number	Prevalence%	Interval coincident Clopper-Pearson95%
15	100%	%(78.2-100)

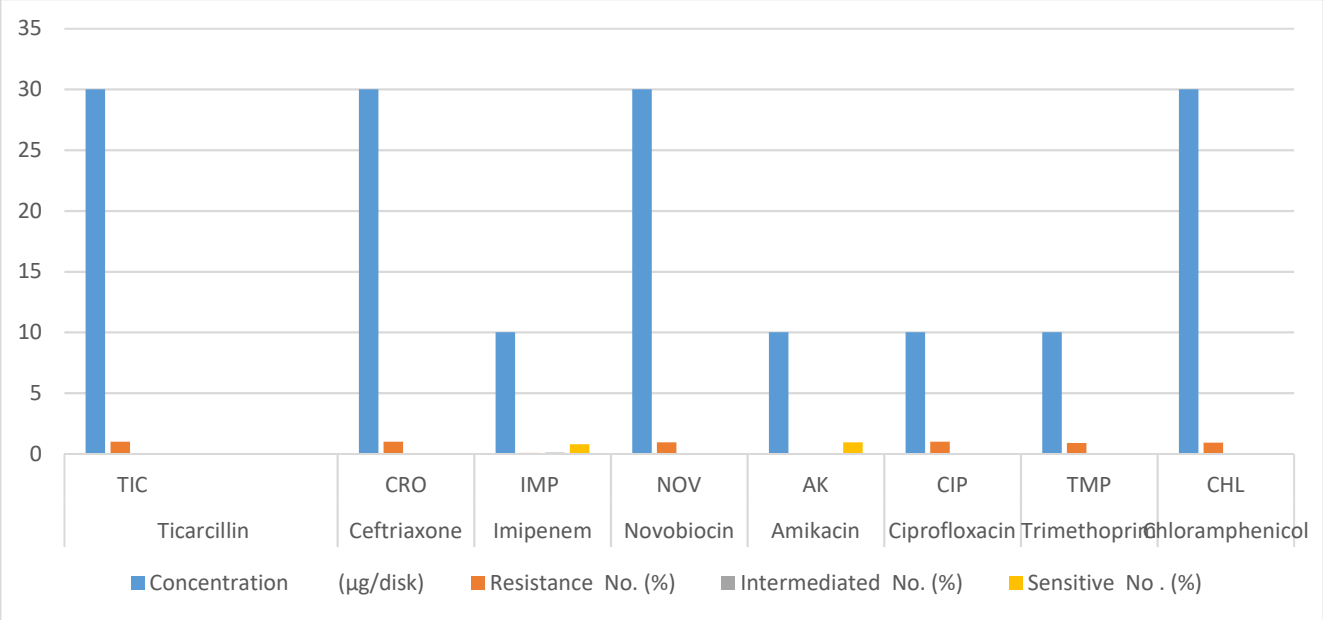

Figure 1. *Antimicrobial susceptibility profile of P. aeruginosa isolates*

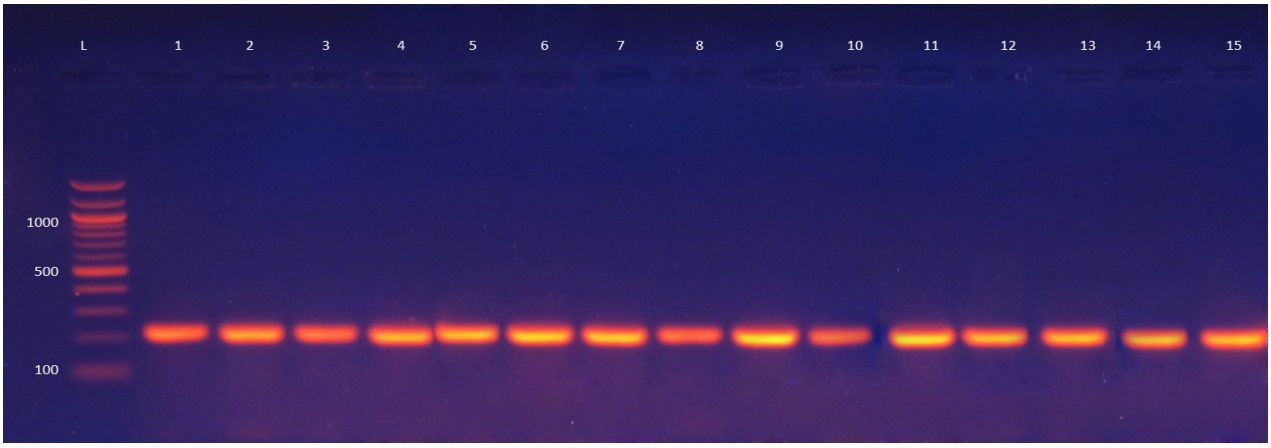

Figure 2. *PCR amplification products of P. aeruginosa isolates amplified with rhlI gene primers, yielding a 245 bp product. Lane (L), DNA molecular size marker (100-bp ladder). Lanes (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15) show positive results for the rhlI gene.*