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Characterization of *Pseudomonas aeruginosa* Isolates from Clinical Specimens: Biofilm-Forming Ability and Association with Antibiotic Resistance

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ABSTRACT

Pseudomonas aeruginosa is a versatile pathogen known for its ability to cause infections and survive in various environments. Biofilm formation plays a critical role in its virulence in many types of bacteria spatially *P. aeruginosa*. This study investigated different *P. aeruginosa* isolates collected from clinical specimens, focusing on their identification, characterization, antibiotic resistance profiles, multi-drug resistance patterns, and biofilm formation abilities. Among the isolates, twenty were as *P. aeruginosa* based on standard biochemical tests. Antibiotic susceptibility testing revealed varying levels of resistance, including multidrug resistance (MDR). Biofilm formation was assessed, showing diverse production levels without significant differences between non-multidrug-resistant (non-MDR) and multidrug-resistant isolates. PCR analysis detected biofilm genes (PslA and GacA) in all isolates, with a few lacking the Pelf gene. Associations between biofilm formation patterns, drug resistance, and biofilm genes were explored, revealing no strong correlations. These findings highlight the prevalence of biofilm formation in *P. aeruginosa* isolates regardless of their multidrug resistance status. The presence of specific biofilm genes may not strongly correlate with biofilm formation patterns or drug resistance.

Introduction

Pseudomonas aeruginosa is a rod-shaped, heterotrophic, motile, Gram-negative bacterium that is 0.5–1.0 μm in width and about 1–5 μm in length. It is a facultative aerobe that uses nitrate as the terminal electron acceptor to develop through both anaerobic and aerobic respiration. As a prototroph, *P. aeruginosa* can thrive on a minimal salt growth medium using only one source of carbon and energy. It can use more than 100 organic molecules as a source of carbon and/or energy. *P. aeruginosa* can withstand temperatures in the range of 4–42°C, however it thrives best at 37°C (Diggle and Whiteley, 2020). *P. aeruginosa* is widely distributed in aquatic environments and is commonly associated with healthcare-associated infections (HAIs) (Whistler *et al.*, 2019; Fiscarelli *et al.*, 2021). It has long been used as a model organism to study bacterial life and disease due to its ability to cause recurrent infections in immunocompromised individuals, particularly those with cystic fibrosis (Services, 2017). As a result of its increasing incidence of hospital-acquired infections and its ability to develop resistance to various classes of antibiotics, this widespread bacterial pathogen is now considered a global

public health risk (Moradali *et al.*, 2017).

Healthcare experts around the world are very concerned about antimicrobial resistance (AMR) because there are limited treatment options available for infections caused by multidrug-resistant (MDR) microorganisms (Gajdács and Albericio, 2019; Zhen *et al.*, 2020). The problem of drug-resistance infections has become genuine and concerning due to the "superbugs"—microorganisms that are resistant to the majority of known antimicrobials—spreading quickly around the world. The World Health Organization (WHO) has recognized AMR as one of the top three greatest dangers to public health. After cardiovascular disorders, antimicrobial-resistant infections are the third most common cause of death (Antimicrobial Resistance Collaborators, 2022). According to a significant study released in January 2022, 1.27 million deaths were expected to be related to antimicrobial-resistant illnesses in 2019 alone, while drug-resistant infections were somehow responsible for approximately 5 million deaths. By 2050, this number is predicted to rise to 10,000,000 annually, far exceeding cancer-related mortality (O'Neill, 2014).

In the United States, these pathogens cause an estimated 35,000 fatalities annually, and infect over 2.8 million people every year (Services, 2019). In the European Union, AMR pathogen infections result in 33,000 deaths annually, and an annual economic loss of 1.5 billion dollars. If no action is taken, AMR mortality and morbidity are predicted to surpass all acute and chronic illnesses, including heart disease and cancer, by 2050, resulting in an estimated 10 million annual deaths (O'Neill, 2016). The majority of Middle Eastern and North African (MENA) countries have high rates of MDR *P. aeruginosa*, likely due to comparable populations and antibiotic prescription cultures. Isolates from critical care units in Saudi Arabia, Libya, Egypt, Lebanon, and Syria exhibited high levels of resistance to aminoglycosides, carbapenems, and cephalosporins (Al-Orphaly et al., 2021).

The National Institutes of Health estimates that up to 80% of human microbial diseases are caused by biofilms (Karatan and Watnick, 2009; Joo and Otto, 2012; Jamal et al., 2018) including periodontitis, endocarditis, rhinosinusitis, meningitis, cystic fibrosis, kidney infections, osteomyelitis, prosthesis, non-healing chronic wounds, and implantable device-related infections (Veerachamy et al., 2014;

Buttner et al., 2015; Chao et al., 2015). Biofilms pose serious therapy challenges because of their diagnosis and dearth of relevant biomarkers. In addition, biofilms can be difficult to eradicate in clinical settings due of their strong resistance to antibiotics (Paharik and Horswill, 2016). A large number of biofilm infections related to host tissue are chronic. Examples of these include periodontitis, endocarditis, chronic prostatitis, chronic rhinosinusitis, chronic otitis media, and chronic lung infections in people with cystic fibrosis (Burmolle et al., 2010).

The ability of bacteria to form biofilms is a widespread phenomenon that enhances their survival under harsh conditions, such as exposure to antibiotics or nutrient deprivation (Costerton et al., 1995; Mah et al., 2001). One important characteristic of biofilms is that they can protect bacteria from the host immune system and antimicrobial agents, contributing to the development of chronic infections (Hall-Stoodley et al., 2009). The complex communities of biofilm-producing bacteria are composed of individual cells attached to a surface and embedded in an extracellular polysaccharide matrix (Whittaker et al., 1996). There are five stages in the biofilm growth establishment; Single bacterial attachment (both reversible and

irreversible), bacterial aggregation, microcolony formation, maturation, and dispersion/detachment (Sauer *et al.*, 2002; Klausen *et al.*, 2003; Gu *et al.*, 2013; Speziale and Geoghegan, 2015; Juhlin *et al.*, 2017).

P. aeruginosa is a well-established model for studying biofilm formation, owing to its known ability to form biofilms (Ghafoor *et al.*, 2011). In the lungs of cystic fibrosis patients, the elastic biofilm of *P. aeruginosa* plays a vital role in its survival, competition, and dominance in the polymicrobial environment (Oluyombo *et al.*, 2019).

Biofilm compositions are different according to its own strains. The extracellular polymeric substances (EPS), which consist mainly of polysaccharides, extracellular DNA (eDNA), proteins, and lipids, are the major components of the *P. aeruginosa* biofilm matrix (Stempel *et al.*, 2013). Most *P. aeruginosa* strains can synthesize three exopolysaccharides, namely pellicle (Pel), polysaccharide synthesis locus (Psl), and Alginate, which play crucial roles in the biofilm formation as matrix components (Høiby *et al.*, 1974; Govan and Deretic, 1996; Friedman and Kolter, 2004; Matsukawa and Greenberg, 2004; Overhage *et al.*, 2005; Ma *et al.*, 2006).

The Psl polysaccharide is a key element gene at early stage of biofilm

formation when cells explore surfaces for adhesion (Overhage *et al.*, 2005). It is anchored around cells in a helical arrangement initiating biofilm formation by enhancing cell migration, cell-cell interaction and cell-surface adhesion whereas in mature biofilms it is located to the periphery of mushroom shaped macrocolonies (Ma *et al.*, 2009; Zhao *et al.*, 2013). Psl can exist as a fiber-like matrix requiring type 4 pili-mediated migration of cells (Wang *et al.*, 2013). It protects cells against phagocytosis and oxidative stress during infection (Mishra *et al.*, 2012). Recent studies suggested that Psl can provide an instant protective role against anti-biofilm agents and a broad spectrum of antibiotics particularly at early stage of biofilm development (Zegans *et al.*, 2012; Billings *et al.*, 2013).

The chemical composition and biological role of Pel, a crucial structural element of the biofilm stalk that connects eDNA, have been revealed by recent investigations. (Jennings *et al.*, 2015). Pel has been shown to protect bacteria against specific aminoglycoside antibiotics (Colvin *et al.*, 2011). Pel is a cationic polysaccharide, containing acetylgalactosamine and acetylglucosamine sugars, that has been shown to aid in cell-cell adherence, surface attachment, DNA crosslinking, and protection against aminoglycosides

(Colvin *et al.*, 2011; Jennings *et al.*, 2015). The global activator (GacA/S) are two-components global regulatory system that has been demonstrated to be an essential virulence factor for *P. aeruginosa* pathogenesis independently in animal, plant, nematode and insect models of infection (Rahme *et al.*, 1995; Mahajan-Miklos *et al.*, 1999; Tan *et al.*, 1999 ; Jander *et al.*, 2000). GacA plays a significant part in biofilm formation where it can regulate an alternative pathway essential for achieving optimal biofilm formation (Parkins *et al.*, 2001), which has been demonstrated to regulate positively the production of several virulence factors, specifically N-butyryl-L-homoserine lactone, pyocyanin, cyanide and lipase in *P. aeruginosa* (Reimann *et al.*, 1997). The relationship between the ability to form biofilms and multidrug resistance (MDR) in pathogenic bacteria has been extensively researched (Karballei Mirzahosseini *et al.*, 2020; Senobar Tahaei *et al.*, 2021).

The current study aims to investigate the prevalence of biofilm formation and the frequency of biofilm-related genes, as well as their associations with antibiotic resistance patterns, in twenty *P. aeruginosa* isolates obtained from clinical samples in Egypt.

Materials and Methods:

Sample Collection:

This study was conducted during the period from June 2020 to December 2022. A total of 35 specimens were collected from El Kasr Alainy Hospital–Cairo University, Cairo Egypt. The samples were obtained from various sources, including urine, blood culture, pus, sputum, and wounds. This study received approval from the Institutional Review Board, Faculty of Medicine, Tanta University, Tanta, Egypt with the approval code 36178/12/22.

Bacterial isolates identification and culture conditions:

Thirty -five samples were collected from different sources, sample -collected by swabs were placed directly in nutrient broth transport medium, then transferred to labs of bacteriology. Each sample was streaked on plates contain Nutrient Agar medium which is general-purpose medium for bacterial isolation.

For the isolation and purification of *P. aeruginosa*, Cetrimide agar medium (Oxoid, UK), a selective and differential medium, was used following the method described by Bachoon *et al.*, (2008). The presence of a green or bluish color on media was interpreted as an indication of pyocyanin production, while the presence of a yellow pigment alone indicated pyoverdine or

fluorescein production. Isolates showing the appropriate pigmentation were selected as *P. aeruginosa* for further analysis. The selected colonies were sub-cultured on MacConkey agar medium to identify non-lactose-fermenting colonies exhibiting colorless or pale coloring. Subsequently, these colonies were sub-cultured on Simmons citrate agar to assess citrate utilization. Additional tests including Gram staining, colony morphology, and standard biochemical tests were conducted, such as the oxidase test using 1% tetramethyl-p-phenylenediamine dihydrochloride and gelatinase utilization, following the protocols described by Engelkirk *et al.*, (2008). All tests were read after incubating the cultures for 18 to 24 hours at 35°C.

Antimicrobial susceptibility evaluation of the selected *P. aeruginosa* isolates

According to the Clinical Laboratory Standards Institute (CLSI, 2020), Mueller-Hinton agar (bioMérieux, France) was used for evaluating the antimicrobial susceptibility of isolates using the Kirby-Bauer disc diffusion method. A total of seven antibiotics from four distinct families (Oxoid, UK) were examined. The aminoglycoside family included Gentamicin (CN; 10 µg), Amikacin (AK; 30 µg), and Tobramycin (TOB; 10

µg). The fluoroquinolone family consisted of Ciprofloxacin (CIP; 5 µg), while the carbapenem family included Meropenem (MEM; 10 µg) and Imipenem (IPM; 10 µg. Piperacillin-Tazobactam (TPZ) (a broad-spectrum antibiotic) belongs to the β-lactam family, specifically the penicillin and β-lactamase inhibitor combination was also utilized. The isolates were categorized as multidrug-resistant (MDR) if they were found to be resistant to at least one agent in at least three antibiotic families, following the criteria outlined by Magiorakos *et al.*, (2012).

Quantification of biofilm formation in the selected *P. aeruginosa* isolates using a microtiter-plate-based assay

Using a microtiter-plate-based technique, the ability of the corresponding *P. aeruginosa* isolates to generate biofilm was investigated—described by STEPANOVIĆ *et al.*, (2007). Briefly, *P. aeruginosa* cultures were inoculated overnight into 5 mL of Trypticase Soy Broth (TSB; Oxoid, UK). The following day, 180 µL of TSB and 20 µL of *P. aeruginosa* suspension (set at 10⁶ CFU/mL) were inoculated into 96-well flat-bottomed microtiter plates, reaching a final volume of 200 µL. After that, the plates were incubated for 24 hours at 37°C. After the incubation period, the supernatants were carefully removed, and the wells were

washed three times with phosphate-buffered saline (PBS) with a pH of 7.3 to remove any planktonic cells. Adherent biofilms were fixed using 99% methanol (**Sigma-Aldrich, USA**) for 15 minutes, followed by the removal of the solutions and air-drying of the plate. To visualize and quantify the biofilms, 200 μ L of 0.1% crystal violet (**CV; Oxoid, UK**) was added to each well and allowed to incubate for fifteen minutes at room temperature. The wells were then rinsed with water and allowed to dry. The contents of the wells were resuspended in 200 μ L of 95% ethanol, and the absorbance was measured at 570 nm (OD_{570}) using a microtiter plate reader (**Sunrise, Tecan, Austria**).

All experiments were performed in triplicate. The cut-off value for determining biofilm formation was determined using the negative control sample (negative control (media only without bacteria)). The cut-off value was calculated by taking the mean optical density (OD) of the negative control. This cut-off value served as a threshold to classify the isolates into different categories based on their biofilm production levels. Based on the optical density values obtained, the isolates were categorized into four groups: non-biofilm producers ($OD < OD_c$), weak biofilm producers ($OD_c < OD < 2 \times OD_c$), moderate biofilm producers

($2 \times OD_c < OD < 4 \times OD_c$), and strong biofilm producers ($4 \times OD_c < OD$).

Detection of biofilm genes in the selected *P. aeruginosa* Isolate

The 20 selected bacterial isolates were subjected to detection of biofilm genes. A Specific primer for the selected biofilm genes (PelF, PslA, and GacA) were designed using Primer3 Plus software. The process of designing specific primers typically involves two stages. First, the primers flanking regions of interest are generated either software tools NCBI (The National Center for Biotechnology Information) then they are searched against an appropriate nucleotide sequence database using tools Primer3 Plus software to examine the potential targets. The primers' specificity was confirmed using the National Centre for Biotechnology Information (NCBI) server. Sequence of primers, amplicon sizes, and thermal conditions are mentioned in (**Table 1**). Genomic DNA extraction from bacterial cultures was performed using the Qiagen DNeasy kit (**Qiagen, Germany**). PCR amplification was carried out using a thermal cycler apparatus (**Applied Biosystem, USA**). A total reaction volume of 25 μ l was prepared, containing 150 ng of extracted DNA, 1 μ l of each forward and reverse primer, and 12.5 μ l of Taq PCR Master Mix (**Sigma Aldrich, USA**). The final

volume was adjusted to 25µl with nuclease-free water. PCR conditions were as follows: denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute. This was followed by a final extension at 72°C for 10 minutes. The amplified PCR products were evaluated using 1.5% agarose gel electrophoresis. The gel was stained with Midori green for 45 minutes. Visualization and imaging of the gel were performed using a gel documentation system (**Biometra, Germany**).

Statistical Analysis

Descriptive statistical analyses, including means \pm SD (standard deviation) and percentages, were performed using Microsoft Excel 2023 (**Microsoft Corp., USA**). Further

statistical analyses were conducted using the Statistical Package for the Social Sciences (**SPSS v. 26, USA**). A two-way ANOVA (Analysis of Variance) was performed to compare the measurements of OD₅₇₀ readings for biofilm production between non-MDR and MDR bacterial groups and different biofilm production patterns (weak, moderate, and strong). The Chi-square test was employed to determine if there was a significant association between the resistance profile of the bacteria and their biofilm formation capacity, as well as the presence of selected genes among the provided isolates.

Table (1): PCR primers, amplicon sizes, and thermal conditions for the three biofilm genes

Target primer	Primer (5' to 3')	Amplicon Sizes	PCR Thermal Conditions	Reference
PslA	F: GTTCTGCCTGCTGTTGTTCA R: GTTGCGTACCAGGTATTTCG	230 bp	{95 °C (20 Sec), 55 °C (15 Sec), 72 °C (20 Sec)} ×35	Original
PelF	F: CCTTGCTGTGGAGAAAGTCC R: TGCACATCGAGGAACACTTC	207 bp	{95 °C (20 Sec), 56 °C (15 Sec), 72 °C (20 Sec)} ×35	Original
GacA	F: GTGATTAAGGTGCTGGTGGTC R: CGGTAGCGATAGGTATTACCG	560 bp	{95 °C (20 Sec), 55 °C (15 Sec), 72 °C (20 Sec)} ×35	Original

Results

Identification and characterization of *P. aeruginosa* isolated from various clinical specimens:

Twenty out of 35 bacterial isolates capable of growing on cetrimide agar and producing yellow-green or yellow-brown, fluorescent pigments were selected as *P. aeruginosa*. These isolates were collected from various specimens, including five from urine, three from blood culture, seven from pus, two from sputum, and three from superficial wounds. The twenty isolates exhibited gram-negative rod morphology with opaque colonies and a grape-like odor on cetrimide agar medium. Using oxidase test strips containing a chromogenic substrate, all twenty isolates exhibited a color change indicating the presence of the oxidase enzyme. The catalase test of the twenty isolates showed the presence of the catalase enzyme, as evidenced by the presence of oxygen bubbles. The twenty isolates could grow on MacConkey agar. However, they appeared as colorless or pale colonies, indicating non-lactose fermentation. The isolates were cultured on a citrate agar medium to determine their ability to utilize citrate as a sole source of carbon. All the isolates were able to grow on the medium, and the medium's color changed from green to blue due to the production of alkaline products,

indicating positive citrate utilization. Gelatinase activity was also observed in all isolates when cultured on a gelatin agar medium. Based on the results of these tests, all twenty isolates were confirmed to be *P. aeruginosa*.

Antibiotic resistance profile, multi-drug resistance patterns, and biofilm formation assay of the selected *P. aeruginosa* isolates

The disc diffusion method was used to assess the resistance of *P. aeruginosa* isolates to various specific antibiotics. Most of the isolates (75%, n=15) were found to be resistant to Piperacillin-Tazobactam (TPZ), while 70% (n=14) showed resistance to Tobramycin (TOB) and 60% (n=12) to Gentamicin (CN). Resistance to Amikacin (AK) was observed in 35% (n=7) of the isolates, while 40% (n=8) were resistant to both Meropenem (MEM) and Ciprofloxacin (CIP). In addition, 20% (n=4) of the isolates were resistant to Imipenem (IPM). Only two isolates (no. 6 and no. 17) exhibited resistance to all tested antibiotics. Based on these results, the isolates were categorized into 9 (45%) non-multidrug-resistant (non-MDR) and 11 (55%) multidrug-resistant (MDR) isolates (**Fig. 1, A and B**).

The ability of twenty *P. aeruginosa* isolates to form biofilms was assessed using a microtiter-plate-based

method, which revealed that 40% (n=8) were weak biofilm producers, 35% (n=7) were moderate, and 25% (n=5) were strong. The intensity of biofilm formation was significantly different between weak, moderate, and strong biofilm-forming isolates ($P < 0.05$), as determined by optical density measurements at 570 nm. However, there was no significant difference in biofilm formation between non-MDR and MDR groups ($P > 0.05$). Among non-MDR isolates, 62.5% (5 out of 8) had weak biofilm formation ability, 42.8% (3 out of 7) had moderate ability, and 20% (1 out of 5) had strong ability. In contrast, only 37.5% (3 out of 8) of MDR isolates had weak ability, 57.2% (4 out of 7) had moderate ability, and 80% (4 out of 5) had strong ability (**Fig. 1 A,C**). These findings suggest that biofilm formation is prevalent among *P. aeruginosa* isolates, regardless of their MDR status. The results also indicate that biofilm-formation ability varies among isolates, with some exhibiting stronger biofilm-forming ability than others.

Prevalence of biofilm-encoding genes in *P. aeruginosa* isolates and their association with antibiotic resistance.

The Polymerase chain reaction (PCR) revealed that a large proportion of the selected *P. aeruginosa* isolates carried all three biofilm genes, with no

significant difference in prevalence between all isolates. The presence of PslA and GacA genes was observed in all screened isolates, while the pelF gene was absent in only three isolates (no. 8, no. 14, and no. 15) that were moderate, strong, and moderate biofilm-forming bacteria, respectively (**Fig. 1, A**). The PCR products of PslA, PelF and GacA genes were detected at 230 bp, 207 bp, and 560 bp, respectively (**Fig. 2**). This finding suggested that there was no significant difference in the prevalence of biofilm genes between non-MDR and MDR isolates.

Association analysis of PsIA, PeIF, and GacA genes with biofilm formation patterns and drug resistance in *P. aeruginosa* Isolates.

The findings of **Table (2)** provide insights into the associations of PsIA, PeIF, and GacA genes with biofilm formation patterns and drug resistance patterns in twenty of the *P. aeruginosa* isolates. The interrelationship between biofilm formation patterns and drug resistance patterns among these isolates was examined, as well. For PsIA and GacA, no statistical computations were performed as these variables remained constant across all twenty isolates. The analysis revealed no significant associations between PeIF and either biofilm formation patterns or drug resistance. The chi-square tests and

symmetric measures of association indicate weak or nonexistent relationships between these variables. These findings suggest that PsIA, PeIF, and GacA may not be strongly associated with the presence of biofilm patterns or drug resistance in the

observed *P. aeruginosa* isolates. Furthermore, the results of the chi-square test indicate that there is no statistically significant association between biofilm formation patterns and drug resistance of the same isolates.

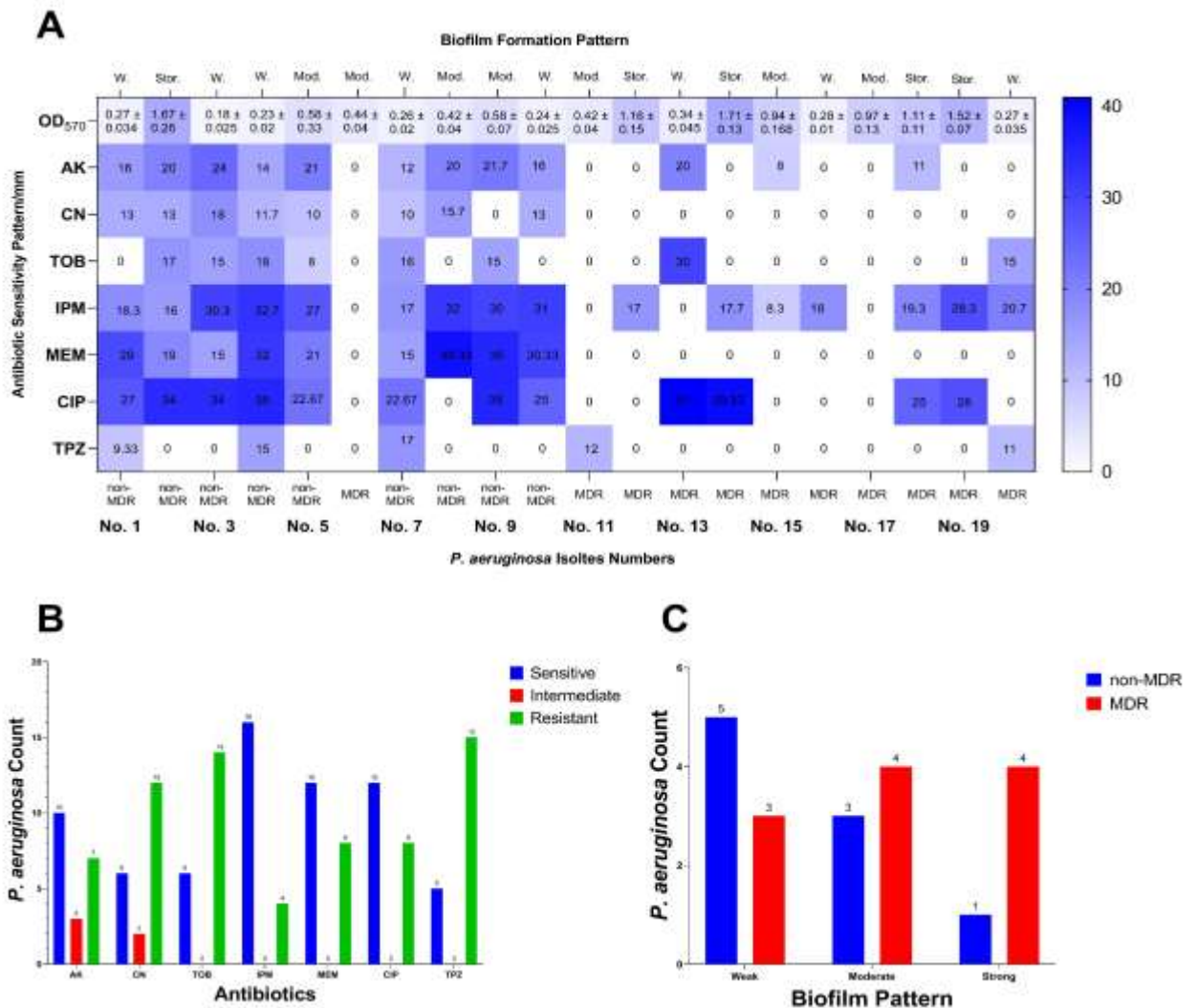


Fig. (1): Showing the relationship between biofilm formation capabilities and the corresponding drug resistance patterns among the twenty *P. aeruginosa* isolates. (A) Antibiotic resistance profiles and biofilm formation abilities of the isolates (No. 1 to No. 20), recognized by OD570 readings (mean ± standard deviation). The isolates are categorized based on their biofilm formation capabilities as weak (W.), moderate (Mod.), and strong (Stor.) biofilm producers. Additionally, the isolates are classified as non-multidrug-resistant (non-MDR) or multidrug-resistant (MDR) based on their resistance patterns. The color intensity in the figure represents the level of sensitivity to each antibiotic and the level of biofilm formation, with darker colors indicating higher sensitivity, or higher biofilm production, respectively. (B) Distribution of antibiotic resistance patterns among the twenty *P. aeruginosa* isolates. (C) Distribution of the biofilm formation patterns in relation to the drug resistance patterns of the twenty *P. aeruginosa* isolates.

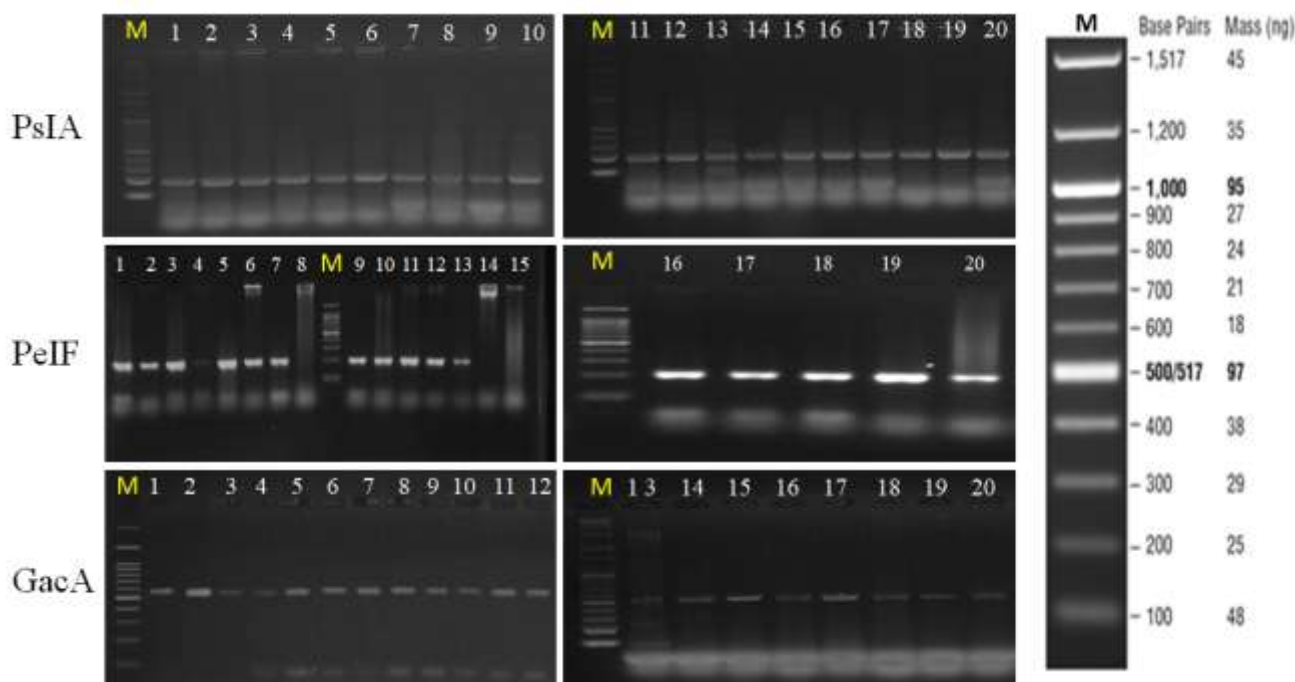


Fig. (2): Agarose gel electrophoresis analysis of PCR products obtained from the amplification of biofilm genes (PsIA, PeIF, and GacA) in selected *P. aeruginosa* isolates. Lanes 1-20 display the PCR products for each gene, with the corresponding amplicon size indicated. Lane M represents the 100bp DNA marker, with size markers indicated in base pairs (bp).

Table (2): Correlation analysis of PsIA, PeIF, and GacA with biofilm pattern and drug resistance, and the interrelationship between biofilm pattern and drug resistance pattern of the twenty *P. aeruginosa* isolates.

		PsIA		PeIF		GacA	
		+ve	-ve	+ve	-ve	+ve	-ve
Biofilm pattern	Weak (n)	8	0	8	0	8	0
	Moderate (n)	7	0	5	2	7	0
	Strong (n)	5	0	4	1	5	0
Pearson Chi-Square		^a 0		2.521		0	
Likelihood Ratio		0		3.529		0	
Linear-by-Linear Association		0		1.248		0	
<i>p</i> -Value		*NA		0.284		NA	
Drug Resistance Pattern	non-MDR (n)	9	0	8	1	9	0
	MDR (n)	11	0	9	2	11	0
Pearson Chi-Square		0		0.194		0	
Likelihood Ratio		0		0.198		0	
Linear-by-Linear Association		0		0.184		0	
<i>p</i> -Value		NA		0.660		NA	
		Drug Resistance Pattern					
		Non-MDR			MDR		
Biofilm Pattern	Weak (n)	5			3		
	Moderate (n)	3			4		
	Strong (n)	1			4		
Pearson Chi-Square		2.233					
Likelihood Ratio		2.376					
Linear-by-Linear Association		2.148					
<i>p</i> -Value		0.322					

a. No statistics are computed in case of no change between the tested parameters.
 *NA: *p*-Value not recognized in case no statistics are computed.
 The variable (n) represents the count of *P. aeruginosa* isolates in the corresponding parameters.

Discussion

The present study aimed to identify and characterize *P. aeruginosa* isolates collected from various clinical specimens. The findings focused on the antibiotic resistance profiles, biofilm formation ability, and the prevalence of biofilm-encoding genes in these isolates.

The results demonstrated that twenty out of the thirty-five bacterial isolates capable of growing on ceftrimide agar and producing characteristic fluorescent pigments which confirmed that they are *P. aeruginosa*. The isolates exhibited gram-negative rod morphology, opaque colonies, and a grape-like odor on ceftrimide agar medium. These observations align with the established characteristics of *P. aeruginosa* (Winn, 2006; Bachoon et al., 2008; Engelkirk et al., 2008).

The antibiotic resistance profile of the isolates revealed significant resistance to several commonly used antibiotics. Most notably, the high resistance rates to TPZ, TOB, and CN are of concern, as these antibiotics are frequently employed in the treatment of *P. aeruginosa* infections (Mancuso et al., 2021). Additionally, a considerable proportion of isolates displayed resistance to AK, MEM, and CIP. Notably, two isolates exhibited resistance to all tested antibiotics, highlighting the presence of

extensively drug-resistant strains. Based on these results, the isolates were categorized into 9 (45%) non-multidrug-resistant (non-MDR) and 11 (55%) multidrug-resistant (MDR) isolates.

These finding was consistent with previous study conducted by Al-Orphaly et al., (2021) who reported that Saudi Arabia had the highest resistance rates (100%) for TPZ among the three nations (Egypt, Libya, and Saudi Arabia), and Egypt having the second-highest resistance rates (36%) and no resistance data available for Libya. According to data from the European Antimicrobial Resistance Surveillance Network (EARS-Net), in 2020, 30.1% of *P. aeruginosa* isolates in the European Union/European Economic Area (EU/EEA) exhibited resistance to at least one of the antimicrobial groups under surveillance, with fluoroquinolones (19.6%) and piperacillin-tazobactam (18.8%) showing the highest resistance rates (Organization, 2022). On the other hand, our findings differ from the conclusions of other previous studies conducted by De Macedo and Santos (2005) and Galil et al., (2013) who identified IMP and AK as the most effective antimicrobial agents against *P. aeruginosa*, respectively.

Furthermore, Banar et al., (2016) found that more than 90% of their isolates were resistant to CN, AK, MEM,

and cefepime, indicating multidrug resistance (MDR). Similar results were reported by **alsadek mohamed (2020)** who observed MDR in 53% of *P. aeruginosa* isolates, with high levels of resistance observed for CN (62%), MEM (60%), and AK (56%). However, **Abdulhaq et al., (2020)** found the highest resistance levels in *P. aeruginosa* isolates against IMP (90.3%). These discrepancies in antimicrobial resistance results across published studies may be attributed to differences in the studied populations, geographical locations, or specific antibiotic usage patterns. Furthermore, the widespread misuse of antibiotics to treat nosocomial infections or mutations in the *P. aeruginosa* genome could contribute to the high prevalence of MDR strains. Therefore, it is crucial to establish appropriate treatment regimens for *P. aeruginosa* infections based on the geographical location of bacterial isolates (**El-sayed et al., 2021**).

Biofilm formation is recognized as a critical factor in the pathogenicity and antibiotic resistance of *P. aeruginosa*. The study revealed that 40% (n=8) were weak biofilm producers, 35% (n=7) were moderate, and 25% (n=5) were strong. In this study, the ability of the isolates to form biofilms was assessed, and the results showed that a substantial proportion of the isolates demonstrated biofilm-forming capabilities. Similar

findings were reported by **Mahmoud et al., (2021)** who showed that 70.4% of isolates demonstrated biofilm production, including 46.3% classified as moderate biofilm producers, 14.8% as strong biofilm producers, and 9.3% as weak biofilm producers, while 29.6% of isolates did not produce biofilm. Conversely, **Elhabibi and Ramzy (2017)** reported that all *P. aeruginosa* isolates exhibited biofilm production using the microtiter plate method with crystal violet (CV) assay. Among the 180 isolates tested obtained from Egyptian hospitals, 90% were classified as strong biofilm producers, 10% as moderate biofilm producers, and 8% as weak biofilm producers. Another study by **Abbas et al., (2012)** revealed that all isolates exhibited biofilm production using the microtiter plate method, with 80% classified as strong biofilm producers, 10% as moderate biofilm producers, and 10% as having low biofilm productivity.

Interestingly, the present study reported that the biofilm formation ability did not significantly differ between non-multidrug-resistant (non-MDR) and multidrug-resistant (MDR) isolates. This finding suggests that biofilm formation is prevalent among *P. aeruginosa* isolates, regardless of their antibiotic resistance status. In contrast to this finding, **Ghanbarzadeh et al., (2015)**

demonstrated a correlation between antibiotic resistance and biofilm development, with MDR *Pseudomonas* isolates exhibiting significantly higher biofilm production than non-MDR isolates. Additionally, several studies have shown that biofilm-producing colonies have a significantly higher tolerance to antibiotics, with the lowest inhibitory doses of several antibiotics increasing by 10-1000-fold in comparison to non-biofilm-producing colonies (**Donlan et al., 2002**). This difference could be attributed to several factors:

Sample Variability: Differences in the bacterial isolates used in the studies, such as their sources (e.g., clinical, environmental), geographical locations, or patient populations, may contribute to variations in biofilm production capabilities and MDR patterns. Microbial populations can vary widely, even within the same species, leading to differing biofilm formation and drug resistance characteristics (**Stewart et al., 2001**).

Environmental Factors: Environmental conditions and exposure to different antimicrobial agents can influence biofilm formation and the development of drug resistance (**Gebreyohannes et al., 2019**). Variations in the environmental factors in the different studies, such as antibiotic usage patterns or local antimicrobial resistance profiles, could contribute to the discrepancies observed.

Sample Size and Selection Bias: Variations in sample size and selection criteria can affect the generalizability of the findings. If the sample sizes are small or not representative of the overall population, the results may not accurately reflect the true relationship between MDR and biofilm production.

Genetic Variability: *P. aeruginosa* is a genetically diverse species, and different strains may possess distinct virulence factors, biofilm-related genes, or mechanisms of antimicrobial resistance (**Heidari et al., 2022**).

The prevalence of biofilm-encoding genes (PslA, PelF, and GacA) was investigated in the *P. aeruginosa* isolates. Most of the isolates carried all three biofilm genes, indicating their potential for biofilm formation. Importantly, there was no significant difference in the prevalence of biofilm genes between non-multidrug-resistant (non-MDR) and multidrug-resistant (MDR) isolates, suggesting that the presence of biofilm genes is not strongly associated with antibiotic resistance. Previous studies have highlighted the importance of the Psl and Pel loci, particularly the PelA gene, as markers for biofilm formation in *P. aeruginosa* isolates (**Ghadaksaz et al., 2015**). In this study, the majority of the tested isolates possessed all three essential genes (PslD, AlgD, and PelF) for biofilm formation, and there was a

positive correlation between the presence of these genes and biofilm formation. However, no significant correlation was observed between biofilm formation and drug resistance.

Interestingly, three isolates were capable of producing biofilms despite lacking the PelF gene, suggesting the involvement of other genetic drivers or mechanisms in the development of the biofilm matrix. These findings indicate that the PelF gene may not play a significant role in the observed biofilm formation patterns or antibiotic resistance in the studied *P. aeruginosa* isolates. The association analysis between biofilm genes and biofilm formation patterns, as well as drug resistance, revealed that while PsIA and GacA genes were consistently present across all isolates, there were no significant associations between the presence of the PelF gene and either biofilm formation patterns or drug resistance. Similar to our findings, a study by **Eladawy *et al.*, (2021)** did not find significant correlations between antimicrobial resistance, biofilm development, and the presence of specific virulence factor genes in their investigation involving 190 isolates. These findings, combined with our results, highlight the complexity of biofilm formation in *P. aeruginosa* and suggest that the relationship between biofilm formation, antimicrobial resistance, and

the presence of specific virulence factors may vary among different *P. aeruginosa* isolates. Further studies are required to unravel the molecular mechanisms underlying biofilm development in *P. aeruginosa* and explore the implications for antimicrobial resistance and treatment strategies.

Conclusion

This study provides valuable insights into the identification, antibiotic resistance profiles, biofilm formation ability, and prevalence of biofilm-encoding genes in *P. aeruginosa* isolates. The high rates of antibiotic resistance and the prevalence of biofilm formation highlight the challenges in the management of *P. aeruginosa* infections. Understanding the complex relationship between biofilm formation, antibiotic resistance, and gene expression is crucial for developing effective treatment strategies against these infections. Further research is warranted to elucidate the underlying mechanisms and explore potential interventions to combat biofilm formation and reduce antibiotic resistance in *P. aeruginosa* isolates.

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توصيف عزلات بكتريا سيدوموناس ايرجينوزا من العينات السريرية: دراسة قدرتها على تشكيل الأغشية الحيوية والارتباط بمقاومة المضادات الحيوية

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بكتريا سيدوموناس ايرجينوزا تسبب العديد من الأمراض وذات قدرة على التسبب في العدوى والبقاء على قيد الحياة في بيئات مختلفة. يلعب تكوين الأغشية الحيوية دوراً حاسماً في ضراوتها تناولت هذه الدراسة عزلات مختلفة من سيدوموناس ايرجينوزا تم تجميعها من عينات سريرية، حيث تم التركيز على تحديدها وتوصيفها وملاحم مقاومة المضادات الحيوية وأنماط المقاومة للأدوية المتعددة وقدرتها على تكوين الأغشية الحيوية. تم عزل عشرين عزلة ومن بينها تم إختيار النوع الأكثر إنتاجاً للأغشية مخاطية بناءً على الاختبارات الكيموحيوية القياسية. كشف اختبار الحساسية للمضادات الحيوية عن مستويات متفاوتة من المقاومة، بما في ذلك المقاومة للأدوية المتعددة (MDR). تناولت الدراسة تقييم تكوين الأغشية الحيوية حيث أظهرت مستويات إنتاج متنوعة دون فروق ذات دلالة إحصائية بين العزلات غير المقاومة للأدوية المتعددة (غير المقاومة للأدوية المتعددة) والعزلات المقاومة للأدوية المتعددة. كشف تحليل PCR عن جينات الأغشية الحيوية (*GacA* و *PsIA*) في جميع العزلات، مع وجود عدد قليل منها يفتقر إلى جين *PelF*. تم استكشاف الارتباطات بين أنماط تكوين الأغشية الحيوية، ومقاومة الأدوية، وجينات الأغشية الحيوية حيث انه لم يظهر أي ارتباطات قوية. تسلط هذه النتائج الضوء على مدى انتشار تكوين الأغشية الحيوية في عزلات سيدوموناس ايرجينوزا بغض النظر عن حالة مقاومتها للأدوية المتعددة ولعل وجود جينات معينة من الأغشية الحيوية قد لا يرتبط بقوة بأنماط تكوين الأغشية الحيوية أو مقاومة الأدوية.