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Prevalence of the *pilS2* gene in *Pseudomonas aeruginosa* isolates from various infections and its relationship with bacterial pathogenicity

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Abstract

Pseudomonas aeruginosa, a prevalent opportunistic bacterium, leads to several diseases., like wounds, sputum, throat, urine, and blood infections. This research aimed to assess the presence of the *pilS2* gene and its correlation with the *P. aeruginosa* plasmid *pKLC102* and the *PAPI-1* pathogenicity island in *P. aeruginosa* isolates. Patient samples were obtained from individuals with various infections, including wounds, sputum, throat, urine, and blood cultures. DNA extraction from these samples utilized a DNA extraction kit for subsequent PCR analysis. PCR analysis involved the use of two primer pairs, *SojR/4541F* and *intF/sojR*, to detect the presence of *PAPI-1*. Additionally, the detection of *pKLC102* was accomplished using the primer pair *cp44F/cp44R*. Forty-three isolates of *P. aeruginosa* were collected from urine (16), wounds (12), sputum (7), throats (2), and blood (6). According to the higher resistance of samples to the different antibiotic discs, we selected 15 samples: wound (4), urine (6), sputum (4), and blood (1). PCR results from 15 samples revealed that the *pilS2 gene* was present in 7 strains, accounting for 46% of the samples. Among the isolates, 8 showed integration of *PAPI-1-attB*, constituting 53.3%, while the circular form of *PAPI-1* was identified in 6 isolates, representing 40%. Furthermore, *pKLC102* tested positive in 5 strains, totaling 33%. The qRT-PCR analysis showed significant decreases in gene expression for three genes in the disease groups. *Pils2* gene expression decreased by 0.045-fold (P-value=0.037), *Cp44* gene expression decreased by 0.032-fold (P-value=0.013), and *Soj/4541* gene expression decreased by 0.059-fold (P-value=0.041) compared to controls. We found the majority of the virulence may be related to the presence of *pils2* genes, plasmid *PKLC102*, and *Soj/4541* genes of *PAPI-1*, which showed a significant down-regulation of gene expression in the disease group of tested *Pseudomonas aeruginosa* isolates that showed higher antibiotic resistance levels relative to the control group using qRT-PCR analysis.

Keywords: *PilS2* Gene, *Pseudomonas aeruginosa*, Bacterial Pathogenicity, Gene Prevalence, Gene expression.

1. Introduction

Pseudomonas aeruginosa commonly causes chronic bronchial infections in individuals with cystic fibrosis, making it the predominant bacterial pathogen in such cases. (Heidary et al., 2016) This pathogen can cause several diseases, including nosocomial pneumonia, urinary tract infections, conjunctivitis, and keratitis. (Tarashi et al., 2017) *Pseudomonas aeruginosa's* pathogenesis is attributed to its ability to produce a diverse array of extracellular virulence factors, such as pili, hemolysins, pyocyanin, proteases, alginate, lipopolysaccharide, and the Type III secretion system. Additionally, *P. aeruginosa* produces effector proteins like ExoU, ExoT, ExoY, and ExoS, which play crucial roles in its pathogenesis.(Harrison et al., 2010).

Pseudomonas aeruginosa strains have shown an ability to bind to different receptors and create biofilms using Type IV pili. These pili are divided into two main categories: IVa (T4aP) and IVb (T4bP). T4bP is more diverse than T4aP, and among them, Tad pili, a specific group within T4bP, has been observed in various environmental species. (Carter et al., 2010), While T4aP and Tad genes exist in all strains of *Pseudomonas aeruginosa*, the *pilS2* gene, responsible for the primary component of Type IVb pili, is exclusively found in strains containing associated elements like the *P. aeruginosa* plasmid *pKLC102* or the *PAPI-1 pathogenicity island*. (Carter et al., 2010; Würdemann & Tümmler, 2007). Carter et al. found that *PAPI-1* can transfer between different *Pseudomonas aeruginosa* strains once it's removed from the donor's chromosome. They also noted that *PAPI-1* enters recipient *P. aeruginosa* through conjugation, facilitated by a Type IV pilus encoded by a ten-gene cluster within *PAPI-1.*

In the genome of *Pseudomonas aeruginosa*, there are two genes encoding tRNALys: PA0976.1 and PA4541.1. One of these sites is occupied by *pKLC102*, a 103,532 bp integrative and conjugative element. (Klockgether et al., 2004) However, *PAPI-1* has the capability to integrate into the attB sites found at both PA0976.1 and PA4541.1 positions. (Lu et al., 2015) Besides its integrated state, *PAPI-1* can also be detected as an extrachromosomal circular entity in certain bacterial communities. (Qiu et al., 2006)

The *pilS2* gene in *PAPI-1* encodes a significant pilin subunit, which is a 176-amino-acid protein with a conserved *PilS* superfamily domain. Horizontal gene transfer is recognized as a key evolutionary process that enhances the pathogenicity of many bacterial diseases. (Johnston et al., 2023).

As far as we know, no previous studies have been conducted specifically on the prevalence of the *pilS2* gene in *Pseudomonas aeruginosa* isolates and its connection with the bacterial pathogenicity island (*PAPI-1*) and its association with the *P. aeruginosa plasmid pKLC102* in Egypt.

This study aimed to examine the frequency of occurrence of the *pilS2* gene and its association with the pathogenicity island of the *P. aeruginosa* plasmid *pKLC102* and *PAPI-1* in *P. aeruginosa* strains. obtained from various types of infections, including urine, sputum, throat, blood, and wound infections.

2. Material and methods

2.1. Study design, data, and specimens collection

Samples were obtained from patients diagnosed with urinary tract infections, wounds, chest infections, and cystic fibrosis. Sputum samples underwent macroscopic examination and were cultured on blood agar, MacConkey agar, and cetrimide agar. Throat swabs, wound swabs, and urine, Additionally, blood culture samples were obtained and subjected to culture on MacConkey agar, blood agar, and cetrimide agar for further analysis. (Huus et al., 2016)

The colonies collected from positive cultures were examined to identify the biochemical characteristics of *P. aeruginosa*. This included examining characteristics such as gram staining, pigment production, colony morphology, oxidase production capability, and Microbact biochemical kites (Oxoid - Product Detail, n.d.).

2.2. Antimicrobial susceptibility testing

The antimicrobial susceptibility testing utilized the Kirby-Bauer disk diffusion method under the recommendations from the Clinical and Laboratory Standards Institute. This method was employed to assess the susceptibilities of all isolates to various antibiotics by using amikacin (30 mg). ceftazidime (30 mg), meropenem (10), imipenem (10 mg), gentamicin (10 mg), piperacillin-tazobactam (10 mg), cefoperazone-sulbactam (10 mg), chloramphenicol (10 mg) Ampicillin/Sulbactam (20 mg), Tigecycline (15 mg), Levofloxacin (5 mg), Amoxycillin/Clavulanic Acid (30 mg), and aztreonam (30 mg) (Pang et al., 2019).

2.3. Microtiter Dish Biofilm Formation Assay

Biofilms, composed of bacteria, adhere to various surfaces in medical, industrial, and natural settings, constituting a prevalent growth mode for microorganisms. Mature biofilms showcase distinct traits, with bacteria ensconced in an extracellular matrix for structural support and defense. Microbial growth within biofilms adopts a specific arrangement, featuring macrocolonies encircled by fluid-filled channels. Notably, bacteria thriving in biofilms demonstrate resilience against diverse antimicrobial agents, including essential medications. (O'Toole, 2011).

Important for the study of the earliest stages of biofilm formation, the microtiter dish assay has been utilized primarily in the investigation of bacterial biofilms. (Mah et al., 2003). The procedure has successfully identified many components crucial for biofilm formation, including flagella, pili, adhesins, and enzymes related to cyclic-di-GMP binding and metabolism. (Toutain et al., 2007). Moreover, genes are implicated in extracellular polysaccharide synthesis (Kuchma et al., 2005).

The microtiter dish assay facilitates biofilm growth on the walls or base of the dish. Its efficient throughput lends itself well to genetic screening and the evaluation of biofilm formation across various strains under diverse growth conditions. Different versions of this assay have been employed to analyze the initial stages of biofilm formation in various microorganisms, such as *Pseudomonas aeruginosa.*

This method involves exploring biofilm growth in the model organism, *Pseudomonas aeruginosa*. Crystal violet

(C.V) is used to measure the amount of biofilm., although additional colorimetric and metabolic stains have been employed in microtiter plate assays for quantification. The assay uses a microtiter plate with numerous wells, each containing a diluted microbial culture. The amount of biofilm development was assessed by employing a violet dye crystal.
(CV), which was dissolved in acetic acid and quantified with a plate reader PR4100(BIO-RAD

The ideal parameters for biofilm formation, such as growth medium, temperature, and incubation time, were empirically identified for *Pseudomonas aeruginosa*. For each strain or condition, many replicates were performed along with positive and negative controls. The assay was adaptable to different microbial species, with motile microorganisms often sticking to the well walls or bottoms and non-motile germs adhering to the well bottoms.

2.4. DNA extraction and PCR analysis

The isolated *P. aeruginosa* colonies were then transferred to 5 mL of Luria-Bertani medium and incubated at 37°C overnight after the samples were cultivated on cetrimide agar. It was possible to identify the pathogenicity island of *P. aeruginosa* PAPI-1 through a PCR assay employing two primer pairs: *SojR/4541F* and *intF/sojR*. The detection of the *P. aeruginosa plasmid pKLC102* was achieved using the *cp44F/cp44R* primer pair in a PCR assay. Primer sequences utilized for genetic profiling of Clinical strains of *P. aeruginosa* and the *pilS2* gene identification are presented in Table 1. After amplification, the products undergo electrophoresis on a 1% agarose gel stained with a safe dye. A clear and well-defined band observed during DNA amplification indicated a positive outcome.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

The Maxima SYBR Green/ROX qPCR Master Mix (2X) by Thermo Scientific (LOTT 01043850, REF K02) was employed for quantitative comparative (RT PCR), utilizing the Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument with SDS Software. This system illuminates samples with specific-wavelength light beams to measure the fluorescence emitted by the excited fluorophore. The SYBR Green method employs a double-stranded DNA dye during the PCR reaction, which emits fluorescence upon binding to newly synthesized double-stranded DNA.

PCR typically involves a series of temperature adjustments repeated 25 to 50 times. These cycles generally comprise three stages: initial denaturation around 95°C to separate the nucleic acid double strand; annealing at 50-60°C for primer binding to the single-strand DNA template; and extension at 68-72°C to facilitate DNA polymerization by the DNA Taq polymerase.

Temperature and timing for each cycle depend on various factors, including the DNA synthesis enzyme, concentration of divalent ions and deoxyribonucleotides (dNTPs), and primer annealing temperature. The primer sequences for target and reference genes are detailed in Table 1.

Table 1. Primer sequence used in the genetic characterization of clinical isolates of *Pseudomonas aeruginosa*. *PilS2* and *cp44* primers were used in regular PCR genotyping. The primers for qRT-PCR are *pils2*, *cp44*, *sojR/4541F*, *intF/sojR*, and *pbp-2*.

Gene/genomic islands	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Size of PCR product (bp)	Tm (°C)	References	Gene function
pilS2	GCGGTTTCGTTTCCATCGAG	GGTCACTTCTCCGGTGATCG	428	60	(Bahramian et al., 2019)	Twitching and Swimming Motility
pKLC102						
cp44	GGGTCCGCAAAACTTTCCGC	GCTTGAGGTTGGGCCAATCG	272	61	(Klockgether et al., 2007)	vector for gene cloning and expression.
PAPI-1						
SojR/4541F	CGAGCACAGAAATGTCCTGA	GACAAGACCAGCCACAACCT	1,600	58	(Qiu et al., 2006)	plasmid partitioning and maintaining PAPI-1 in the bacterium
intF/sojR	AGCTACATCGAGGCCGACTA	CGAGCACAGAAATGTCCTGA	1,600	58	(Qiu et al., 2006)	excision of PAPI-1 from its chromosomal location
pbp-2	CCGCCACTACCCGCTGAAG	TGCCGTGCAACTCGCTCTC		60	(Savli et al., 2003)	cell wall synthesis and integrity

2. Data analysis

Data were analyzed using SPSS statistical software, version 22 (IBM SPSS Statistics, USA), and Microsoft Excel 2016 (Microsoft Corporation, USA). Qualitative data were described using frequencies and percentages, while quantitative data were characterized by the median and interquartile range. The normality of the data was assessed using the Shapiro-Wilk test. The Fisher exact test was utilized to examine the association between two qualitative variables. The Mann-Whitney U test was conducted to determine differences between two quantitative variables. Additionally, the Kruskal-Wallis test was used to detect differences among more than two quantitative variables. A p-value of ≤ 0.05 was considered statistically significant. Relative gene expression was calculated using the fold change method using $2^{-\Delta\Delta Ct}$ method (also known as the Livak method).

3. Results

From 43 *P. aeruginosa* isolates in total, samples were collected from urine (16), wounds (12), sputum (7), throats (2), and blood (6). All samples were cultured on blood agar, MacConkey agar media, and cetrimide agar. The cetrimide agar test is a biochemical test performed to identify

Pseudomonas aeruginosa from other microorganisms (Figure 1).

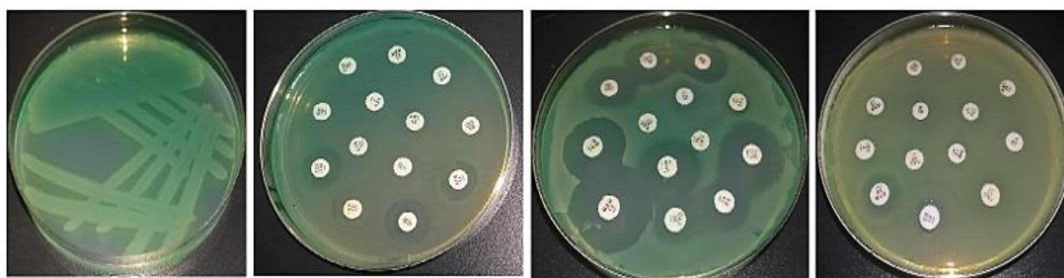


Figure 1. Growth of *P. aeruginosa* on Citrimide Agar Plates and Test for Antibiotic Sensitivity and Resistance in *P. aeruginosa* isolates.

3.1.Antibiotic susceptibility testing

Table 2 and Figure 2 show the patterns of antibiotic susceptibility of *P. aeruginosa* isolated from clinical specimens. The highest resistance was to Chloramphenicol (100%), Ampicillin/Sulbactam (100%), and Amoxicillin/Clavulanic Acid (88.3%). On the other hand, the lowest resistance was to Aztreonam (27.9%), meropenem (25.5%), and imipenem (23.2%) in clinical samples. Moreover, there was severe antibiotic resistance among the 43 isolates of *P. aeruginosa* collected from patients.

Table 2. Patterns of antibiotic resistance in isolates of *Pseudomonas aeruginosa*.

Antibiotic	Number of resistant wound samples./Total Wound samples	Number of resistant urine samples./Total urine samples	Number of resistant sputum samples./Total sputum samples	Number of resistant throat samples./Total throat samples	Number of resistant blood samples./Total blood samples	Total Resistant Sample/Total Sample	Resistant Sample/Total. Sample (%)
Aztreonam	3/12	5/16	2/7	0/2	2/6	12/43	27.9
Meropenem	2/12	5/16	4/7	0/2	0/6	11/43	25.5
Amikacin	3/12	6/16	1/7	1/2	2/6	13/43	30.2
Piperacillin/Tazobactam	4/12	6/16	4/7	0/2	2/6	16/43	37.2
Imipenem	2/12	4/16	3/7	0/2	1/6	10/43	23.2
Gentamycin	3/12	7/16	4/7	0/2	4/6	18/43	41.8
Ceftazidime	7/12	12/16	5/7	0/2	3/6	27/43	65.1
Ampicillin/Sulbactam	12/12	16/16	7/7	2/2	6/6	43/43	100
Chloramphenicol	12/12	16/16	7/7	2/2	6/6	43/43	100
Levofloxacin	4/12	8/16	4/7	0/2	2/6	18/43	44.1
Amoxicillin/Clavulanic Acid	10/12	13/16	7/7	2/2	6/6	38/43	88.3
Tigecycline	7/12	12/16	5/7	1/2	3/6	28/43	65.1
Cefoperazone/sulbactam	5/12	8/16	5/7	2/2	4/6	24/43	55.8

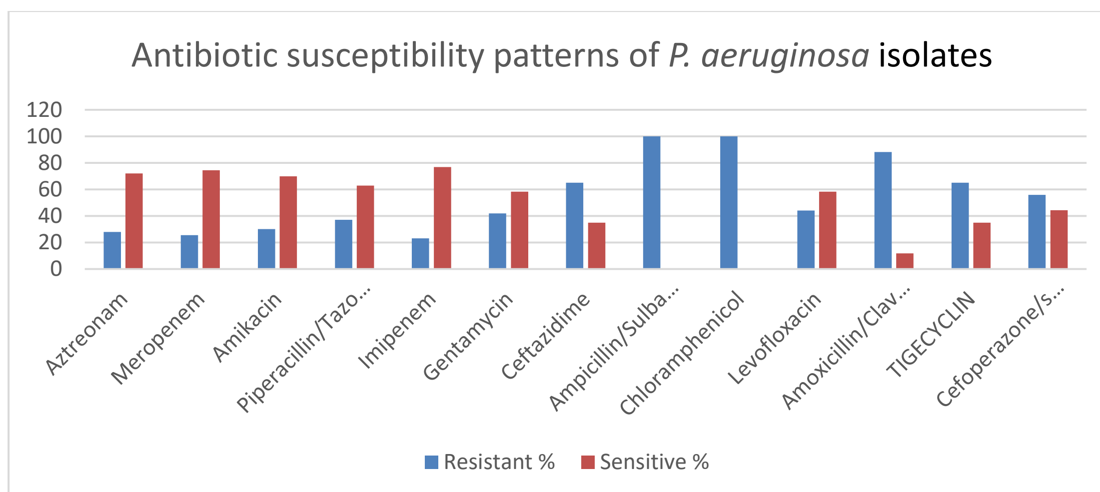


Figure 2. Antibiotic susceptibility patterns in *Pseudomonas aeruginosa* isolates.

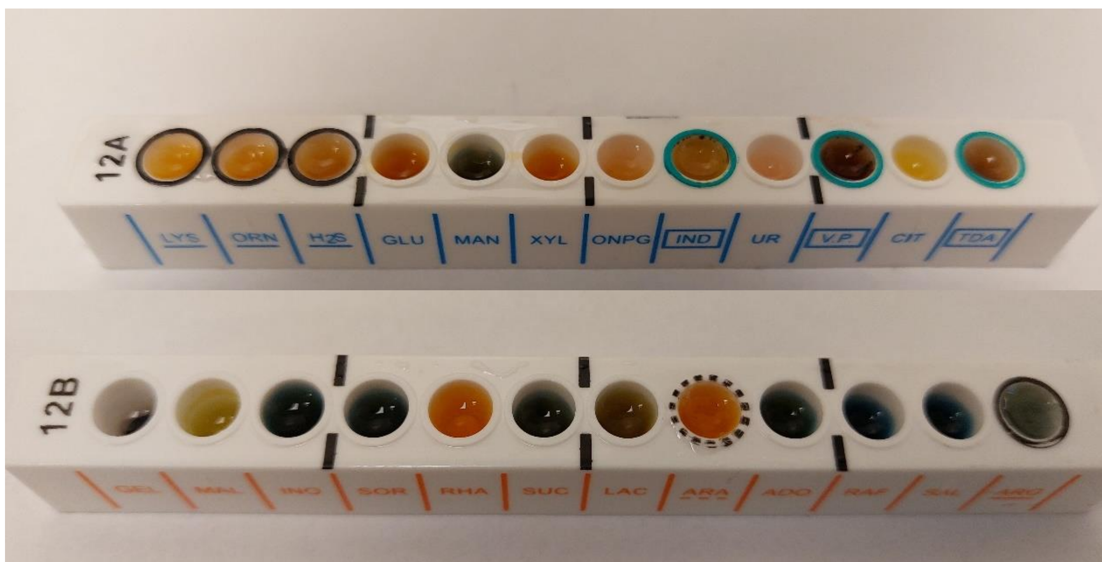
3.2. Biochemical Tests and Identification of *Pseudomonas aeruginosa*

The biochemical tests and identification of *Pseudomonas aeruginosa* by Microbact kits (Oxoid - Product Detail, n.d.) are shown in Table 3. *P. aeruginosa* is a gram-negative rods bacillus. It is motile, non-sporing, and has a single flagellum. It reacts with catalase, oxidase, nitrate, citrate, and gelatin. In contrast, it gives a negative reaction to Ornithine, H_2S, Indole, Urease, and Voges Proskauer (V.P.). Also, the fermentation was positive for malonate and negative for other fermentative tests. Finally, regarding the enzymatic reaction, it was positive for Arginine hydrolysis and negative for Lysine (Figure 3 and Table 3).

Table 3. The basic properties and identification of *Pseudomonas aeruginosa* isolates in the study.

Basic Characteristics	Properties (*Pseudomonas aeruginosa*)
Gram staining	Gram negative bacilli
Shape	Rods
Motility	Motile (Unipolar)
Spore	Non-Sporing
Flagella	Single Flagella
Catalase	Positive
Oxidase	Positive
Nitrate	Positive
Ornithine	Negative
H2S	Negative
Indole	Negative
Urease	Negative
V.P (Voges Proskauer)	Negative

Citrate	Positive
Gelatin	Positive
Fermentation of	
Glucose	Negative
Malonate	Positive
Inositol	Negative
Sorbitol	Negative
Rhamnose	Negative
Sucrose	Negative
Lactose	Negative
Arabinose	Negative
Adonitol	Negative
Enzymatic reaction	
Arginine hydrolysis	Positive
Lysine	Negative



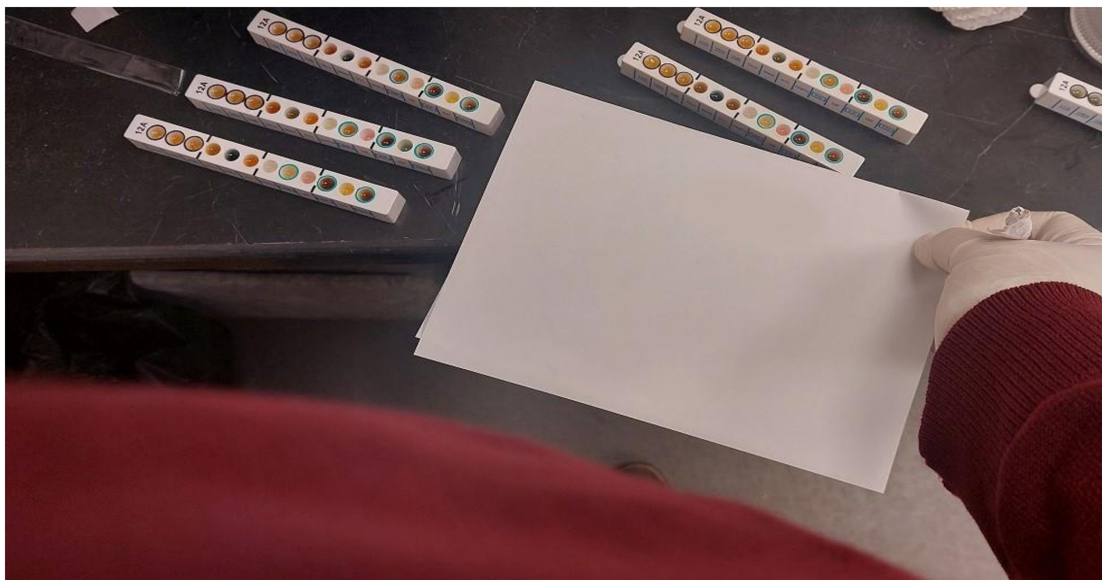


Figure 3. Results of simulated MICROBACT strip assay for identification *Pseudomonas aeruginosa* isolates.

3.3. Biofilm results

The qualitative assay showed the formation of *P. aeruginosa*, a motile organism, which formed a biofilm at the air-liquid interface and stained blue (Figure 4).

The Quantitative demonstrates that *P. aeruginosa* forms biofilms. The formation of strong biofilm was identified at 36 *h.* Biofilm moderately adhered on 48 h (Figure 5).

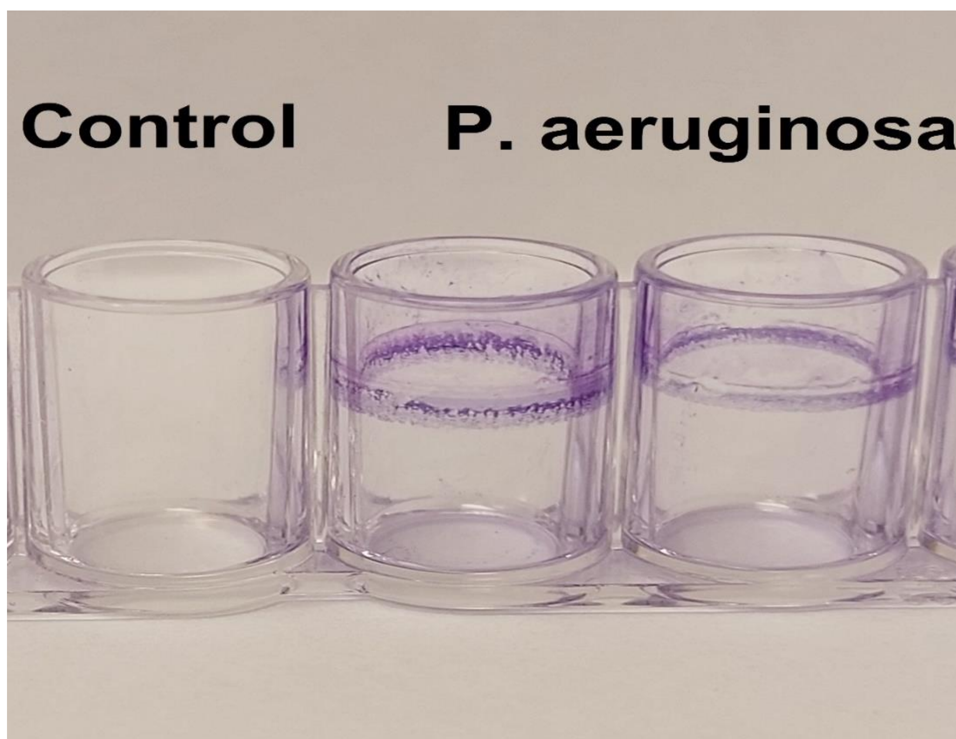


Figure 4. Biofilm formation assays conducted for *P. aeruginosa*: A side view of the well containing a biofilm of *P. aeruginosa*.

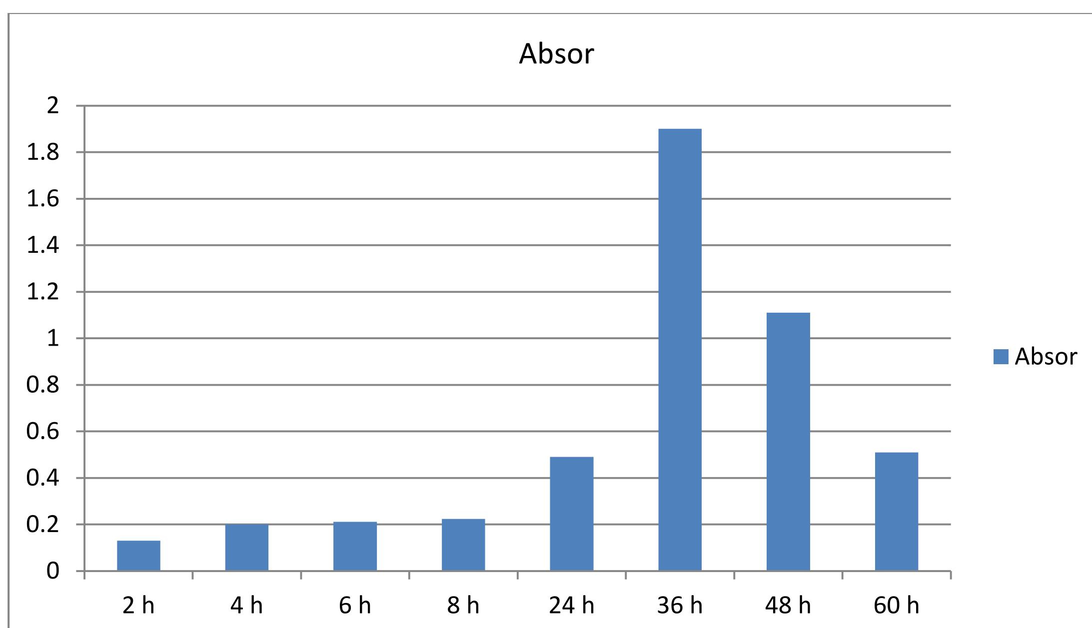


Figure 5. Biofilm formation by *P. aeruginosa.* Quantitative biofilm analysis for 60 hours in a microtiter plate.

3.4. Frequency of the *pilS2* and *Cp44* genes in *P. aeruginosa* isolates

Figure 1 shows the agarose gel electrophoresis of amplified *pils2* and *CP44* genes in *Pseudomonas aeruginosa* isolates. The 428 bands at lanes 3, 6, 7, 9, 13, 14, and 15 indicate the presence of the *pils2* gene, while the 272 bands at lines 2, 3, 7, 9, and 10 indicate the presence of the *CP44* gene.

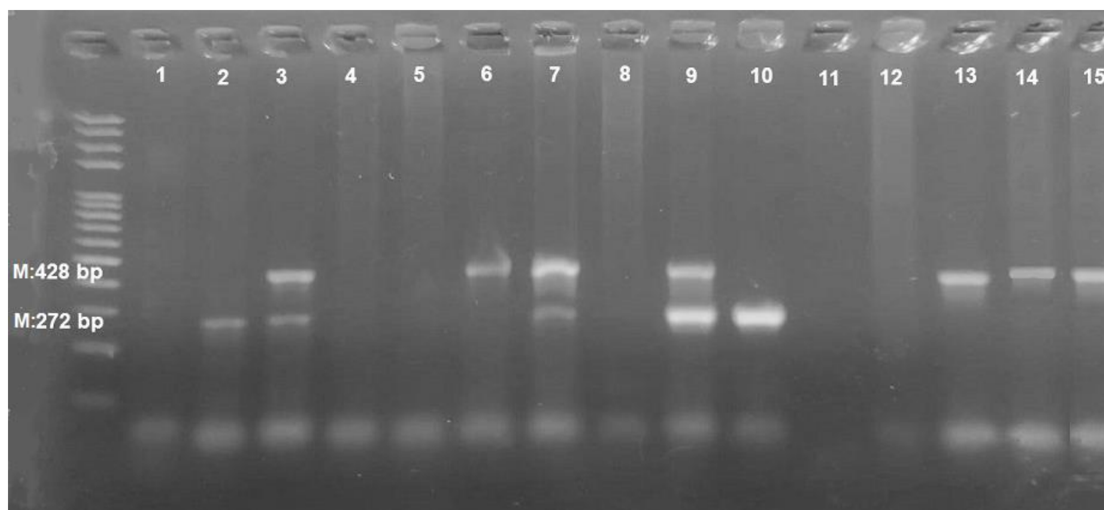


Figure 6. Detection of *pils2* gene (428 bp) and *CP44* gene (272 bp) in *P. aeruginosa* isolates. Lanes 3, 6, 7, 9, 13, 14, and 15 are positive samples for *pils2* gene. Lines 2, 3, 7, 9, and 10 are positive samples for *CP44* gene. M:100 bp ladder.

3.5.qRT-PCR expression analysis

The frequency of the *pilS2* gene in isolates of *P. aeruginosa* is shown in Table 4. The *pilS2* gene was identified in 46% (n = 7) of the *P. aeruginosa* isolates. This major subunit of Type IVb pili was detected among 50% (n = 2) of the *P. aeruginosa* strains isolated from sputum patients, 25% (n = 1) isolated from wound patients, 50% (n = 3) isolated from patients' urine, and 100% (n = 1) isolated from blood culture for patients.

On the other hand, 33% (n = 4) of the *P. aeruginosa* isolate was positive for pKLC102. The highest frequency of this element was detected among patients in urine culture (66%) and sputum culture (25%).

Table 4. Frequencies of *Pils2* and *pKlc102* genes among *P. aeruginosa* isolates.

Type of sample	No. Samples	*Pils2*	*pKlc102*
Sputum	4	2 (50%)	1 (25%)
Wound	4	1 (25%)	0 (0%)
Urine	6	3 (50%)	4 (66%)
Blood	1	1 (100%)	0 (0%)
Total	15	7 (46%)	5 (33%)

Additionally, the expression of four genes (*pils2, cp44, soj/4541 and int/soj*) relative to the *pbp-2* housekeeping gene in the two study groups is shown in Table 5 and Figure 7. The analysis revealed that *Pils2* gene expression was down-regulated by 0.045-fold in the disease groups comparison to the control group. This down-expression of the *Pils2* gene was statistically significant compared to the control group-P-value = 0.037. Similarly, *Cp44* gene expression was down-regulated by 0.032-fold in the disease groups compared to the control group.

In addition, this down-expression of the *Cp44* gene was statistically significant compared to the control group, with a P-value = 0.013. Likewise, *Soj/4541* gene expression was down-regulated by 0.059-fold in the disease groups compared to the control group. In addition, this down-expression of the *Soj/4541* gene was statistically significant compared to the control group, with a P-value = 0.041. In contrast, no statistically significant difference in *int/Soj* gene expression between the two study groups (P-value = 0.613).

Table 5. qRT-PCR data analysis for *the study genes* relative to *pbp-2* housekeeping gene in the two study groups.

Genes	Group	*Pbp-2* CT	*gene* CT	ΔCT	ΔΔCT	RQ = $2^{-\Delta\Delta Ct}$	P-value
Pils2	Control	20.0 (19.4-20.4)	28.0 (26.4-29.4)	8.0 (6.3-9.6)	0.009 (-1.6-1.6)	1.07 (0.51-8.2)	0.037*
	disease	30.6 (30.1-31.0)	18.1 (17.5-18.9)	12.4 (11.6-13.2)	4.48 (3.73-5.24)	0.045 (0.026-0.075)	
Cp44	Control	18.0 (17.4-18.6)	25.0 (23.6-26.6)	7.2 (6.4-8.0)	0.000 (-0.08-0.77)	1.02 (0.66-2.15)	0.013*
	disease	18.8 (17.9-19.7)	31.6 (29.6-33.0)	12.2 (10.05-14.1)	5.02 (2.85-6.91)	0.032 (0.009-0.142)	
int/Soj	Control	15.2 (14.9-15.7)	35.4 (34.8-36.7)	20.2 (19.1-21.7)	1.09 (0.0-2.6)	0.470 (0.26-1.30)	0.613
	disease	18.94 (17.9-20.1)	39.5 (36.6-42.3)	19.5 (18.3-22.8)	0.38 (-0.82-3.7)	0.143 (0.032-0.947)	
Soj/4541	Control	17.2 (16.4-18.1)	30.7 (29.8-31.7)	13.5 (13.1-13.9)	0. (-0.39-0.39)	1.02 (0.76-1.3)	0.041*
	disease	18.8 (17.9-19.3)	36.5 (33.4-37.3)	17.7 (13.8-19.2)	4.1 (0.25-5.6)	0.059 (0.02-0.84)	

Values represent the median (Interquartile Range). The P-value is calculated by a Mann-Whitney test. * Significant at < 0.05.

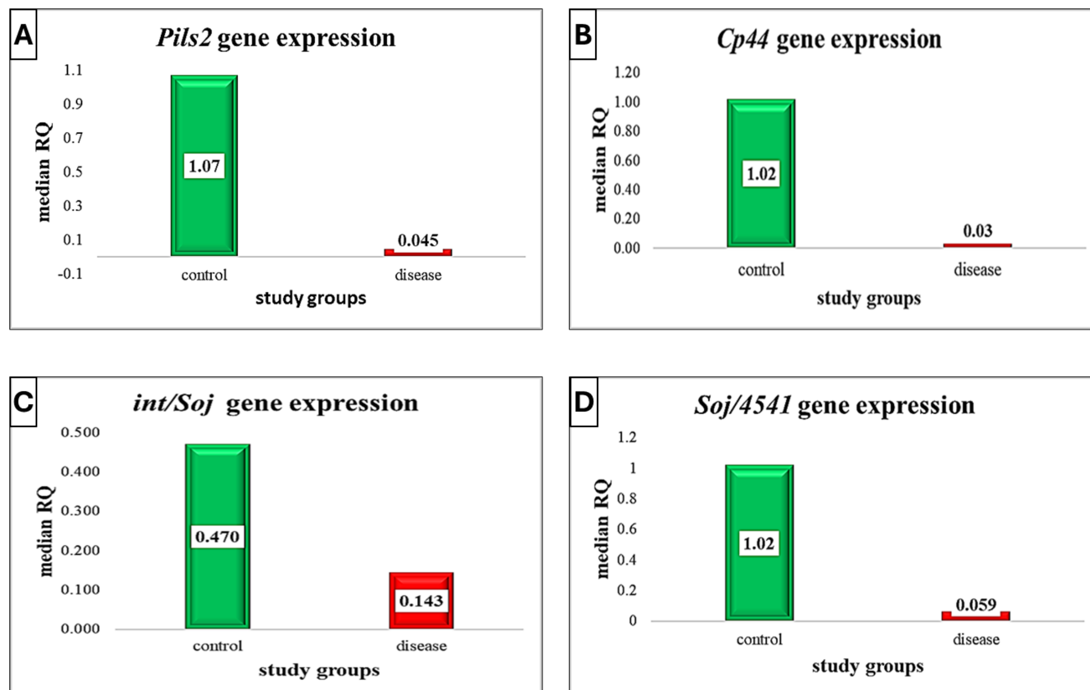


Figure 7. The fold change in the gene's expression relative to the *pbp-2* housekeeping gene in the two study groups.

Moreover, Figure 8 illustrates the melting curve of the four study genes expression. It was done to validate the expected PCR product's specific generation and avoid primer dimmers.

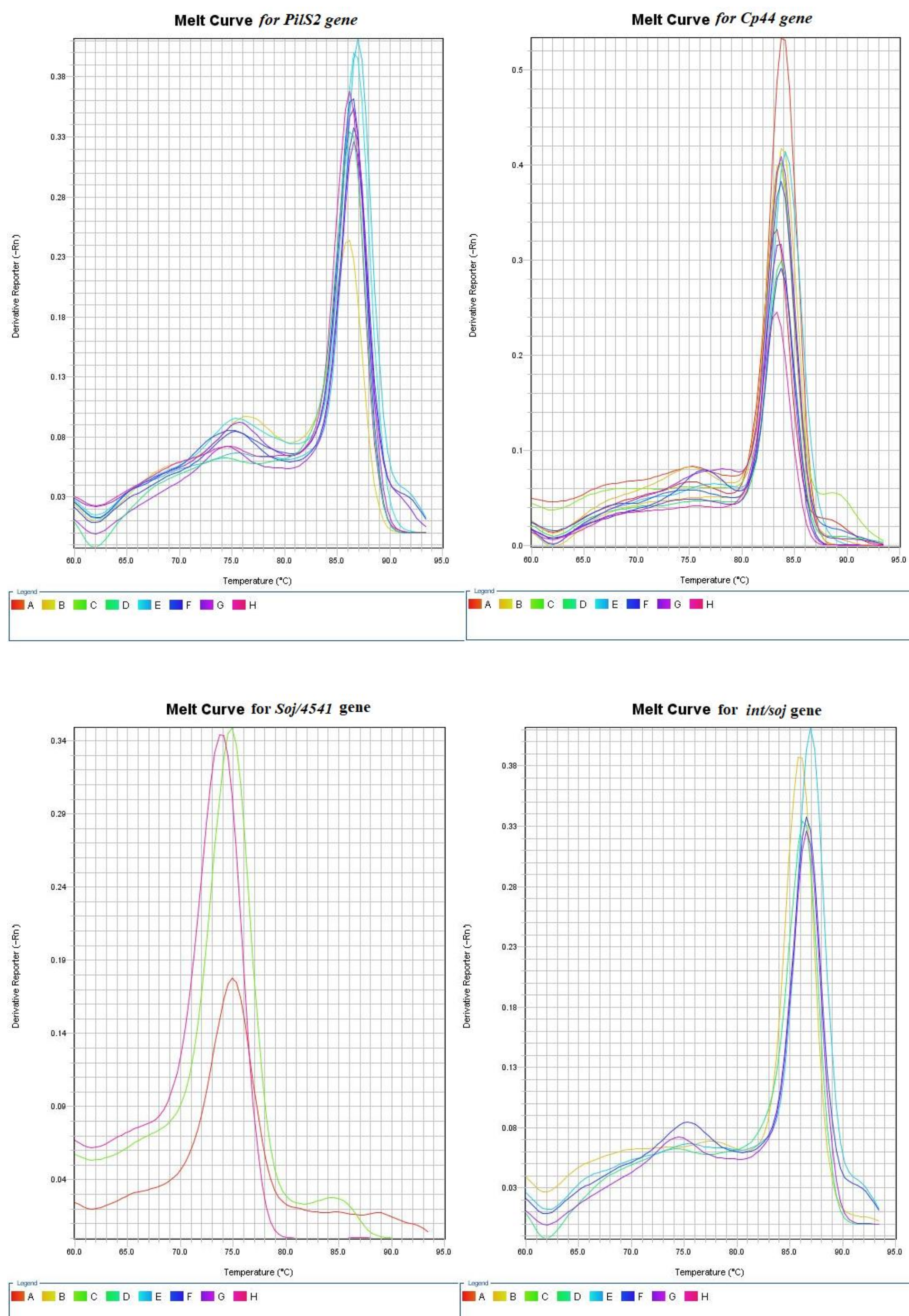


Figure 8. Melting curves of all genes in the study

3.6. Comparison of genes expressions among the disease groups

As shown in Figure 9, the comparison of the three genes *int/Soj*, *Pils2*, and *Soj/4541* expression among the disease group did not show a significant difference ($p > 0.05$). Similarly, no significant difference was found among the four groups, ($p > 0.05$). The gene expression did not seem to influence the results of the bacterial resistance.

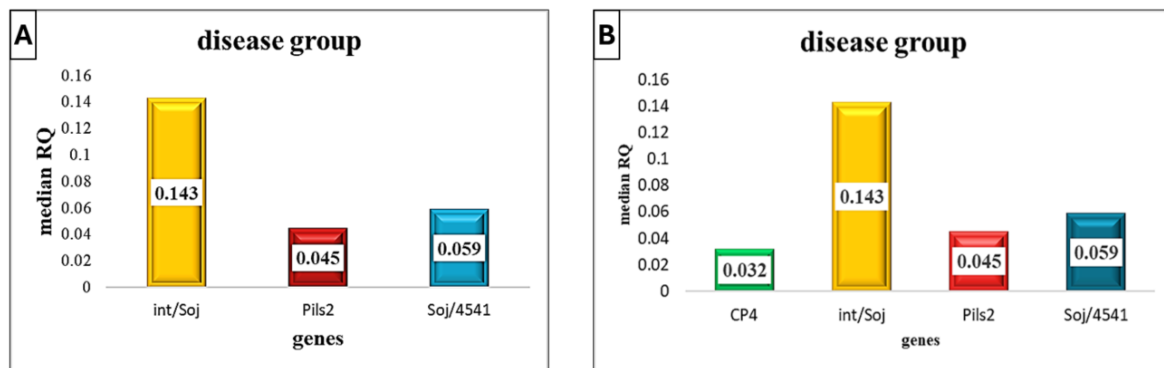


Figure 9. (A) Gene expression of *int/Soj, Pils2*, and *Soj/4541* in the disease groups. (B) Gene expression of *Cp44, int/Soj, Pils2*, and *Soj/4541* in the disease groups.

3.7. The association between the *pilS2* gene and *pKlc102*

The association between the *pilS2* gene and *pKlc102* is shown in Table 12. The *pilS2* gene was associated with *pKLC102* in 33% of the isolated sample from 9 positive isolates for the *Pils2* gene or *pKLC102* gene. However, no statistically significant association was found (P-value > 0.05).

Table 6. The association between the *pilS2* gene and *pKlc102*

		pKlc102		**Total**	**P-value**
		negative	positive		
pils2	Negative	6 (75.0%)	2 (25.0%)	8 (100.0%)	0.608
	Positive	4 (57.1%)	3 (42.9%)	7 (100.0%)	
Total		10 (66.7%)	5 (33%)	15 (100.0%)	

Values represent numbers (percentages), and a Fisher Exact test calculates the P-value.

4. Discussion

Pseudomonas aeruginosa is a major pathogen known for causing diverse infections in various environments and patient groups. Its capacity to form biofilms enhances its virulence and antibiotic resistance, posing significant challenges for eradication, particularly in conditions such as Cystic Fibrosis, where it causes considerable morbidity and mortality. The bacterium exhibits a range of resistance patterns, including multidrug resistance, which is a serious concern in healthcare settings and complicates treatment approaches (Yin et al., 2022). The results of this study revealed concerning levels of antibiotic resistance among *Pseudomonas aeruginosa* isolates, with some antibiotics exhibiting particularly high resistance rates. Notably, Ampicillin/Sulbactam and Chloramphenicol show 100% resistance, indicating a complete lack of efficacy against these isolates. (Azimi et al., 2019) While certain antibiotics like Aztreonam and Meropenem demonstrate relatively lower resistance rates of around 25-

30%, others like Ceftazidime and Amoxicillin/Clavulanic Acid show higher resistance rates exceeding 65%. This variation suggests differences in the effectiveness of antibiotics against *P. aeruginosa* isolates.

Biofilm formation is important for the progression of *P. aeruginosa* infections, especially in cases involving infections associated with medical devices and chronic lung infections in patients with cystic fibrosis. (Tuon et al., 2022).

The qualitative biofilm assay revealed that *P. aeruginosa* is capable of forming biofilms. Biofilms are complex populations of bacteria coated in a matrix of extracellular polymeric substances (EPS) (Saxena et al., 2014). The red staining observed indicates the presence of biofilm formation, which is a critical virulence factor associated with chronic infections and antibiotic resistance. (Abdulhaq et al., 2020)

In our study, the quantitative evaluation illustrated the temporal evolution of biofilm development. Significant biofilm growth was evident after 36 hours, highlighting the vigorous attachment and proliferation of *P. aeruginosa* on the surface. Nevertheless, a decline in adherence to biofilm formation was noted after 48 hours, implying a possible alteration in bacterial activity or external factors impacting biofilm formation. The evaluation of biofilm development in isolates of *Pseudomonas aeruginosa* offers valuable insights into the virulence, pathogenicity, and possible therapeutic targets for addressing infections associated with biofilm (Islam et al., 2023). The study performed by (Al-Kafaween et al., 2019) Biofilm formation of *P. aeruginosa* on a 96-well plate was effectively demonstrated using MHB following a three-day cultivation period.

(Žiemytė et al., 2021) investigate a higher absorption shows that the biofilm has grown larger. Panel c shows biofilm formation in ibiTreat 96-well plates after 36 hours, with a darker color indicating greater biofilm mass.

In this study, utilizing gel electrophoresis, it was observed that the *pilS2* and *CP44* genes were present in the *P. aeruginosa* isolates, as evidenced by bands corresponding to their respective sizes in base pairs. The identification of the *pilS2* gene indicates a potential involvement in bacterial attachment and pathogenicity, while *CP44* might play a role in virulence (Bahramian et al. 2019).

The frequency study revealed that 46 % of P. aeruginosa isolates carried the pilS2 gene. The presence of this gene varied across differ ent sample types, indicating possible changes in pathoge nicity and adaptation to host conditions. (Bahramian et al., 2019).

The *pilS2 gene* was identified at variable frequencies across different sample types, with the highest frequency detected in blood cultures (100%) and urine samples (50%), followed by sputum samples (50%), and the lowest frequency in wound samples (25%).

Analysis of the *pKlc102 plasmid (Cp44 gene)* found its presence in 33% of *P. aeruginosa* isolates, with urine cultures having the highest frequency (66%). This plasmid may carry genes associated with antibiotic resistance and virulence, which

influence the pathogenic potential of the bacteria. (Khosravi et al., 2016)

The expression of the *pilS2 gene* relative to the *pbp-2* housekeeping gene was examined between the control and illness groups using qRT-PCR. The findings revealed a significant decrease in *pilS2 gene* expression in the illness group compared to the control group, with a fold change of 0.045. This study reveals a possible relationship between decreased *pilS2* expression and disease pathology, emphasizing the role of this gene in P. aeruginosa pathogenicity. The analysis provides valuable insights into the prevalence and expression of the *pilS2 gene* in *P. aeruginosa* isolates (Evseev et al., 2023) and provides insight into its potential function in bacterial pathogenicity and illness progression. (Edward et al., 2023)

In our study, the analysis of *Cp44 gene* expression relative to the *pbp-2* housekeeping gene revealed that the illness group had significantly lower *Cp44 gene* expression than the control group. Specifically, the illness group had a significantly lower median expression level of the Cp44 gene (Mdn = 0.032) than the control group (Mdn = 1.02), with a statistically significant P-value of 0.013. That suggests a potential link between lower *Cp44 gene* expression and illness status, indicating that Cp44 may play a role in *P. aeruginosa* pathogenicity. (Kolobkov et al., 2022).

The results based on the frequency of the *pKlc102 plasmid* and the expression of the *Cp44 gene* provide insight into the pathogenic mechanisms of *P. aeruginosa* infections. The presence of the *pKlc102 plasmid* and the downregulation of *Cp44 gene* expression in disease conditions may lead to improved virulence or antibiotic resistance, potentially affecting disease severity and treatment results. (Qin et al., 2022)

The *int/Soj gene* is known to be involved in a variety of biological activities, such as chromosome segregation and cell division. The study compared *int/Soj gene* expression to the *pbp-2* housekeeping gene in two groups: control and disease. Table 9 and Figure 4 illustrate the median expression levels of the *int/Soj gene* in both groups.

The analysis revealed no statistically significant change in *int/Soj gene* expression between the control and illness groups, with a P-value of 0.613. Although the disease group had lower median expression of the int/Soj gene (Mdn = 0.143) than the control group (Mdn = 0.470), this difference was not statistically significant. The lack of significant variations in *int/Soj gene* expression shows that the regulatory mechanisms involving this gene are not strongly related to the illness phenotype investigated. While there may be some differences in gene expression levels between the two groups, they do not appear to be significant enough to influence disease status.

Soj/4541, also known as the chromosomal partitioning protein soj, is essential for chromosome segregation during bacterial cell division. Down-regulation of this gene in a disease group may influence bacterial fitness and virulence by affecting chromosomal stability and cell division processes. (Reinhart & Oglesby-Sherrouse, 2016)

Our study showed a significant down-regulation of *Soj/4541 gene* expression in the disease group compared to the control group, with a fold change of 0.059. This shows that the expression of the *Soj/4541 gene* may be

related to the disease state, indicating a possible role in *P. aeruginosa* pathogenicity. (Qin et al., 2022)

The observed difference in *Soj/4541 gene* expression between the research groups was statistically significant (P = 0.041). This suggests that the down-regulation of the *Soj/4541 gene* in the disease group is unlikely to have occurred by chance and could have biological significance. (Finnan et al., 2004)

In our study, the detection of gene expression by RT-PCR was performed after *Pseudomonas aeruginosa* isolates were exposed to antibiotics (beta-lactams, fluoroquinolone, and carbapenem), which showed the highest resistance. The downregulation of gene expression may be a result of carbapenem or beta-lactam treatment. (Lister, 2002)

Similarly, Aurilio et al. (2022) and Tängdén et al. (2013) revealed that the production of extended-spectrum beta-lactamases can lead to reduced expression of cell membrane porins.

Recently, a study by Cao et al. (2023) demonstrated that the expression level of the studied genes (*sicX* gene) had a low level during standard laboratory growth and was the most down-regulated gene (*sicX* gene) when a chronic infection is spread to induce acute septicemia.

The current study investigated the prevalence of the *pilS2 gene*, which encodes the crucial subunit of the Type IVb pili, and its association with bacterial pathogenicity in *P. aeruginosa* isolates from patients. The results revealed that the pilS2 gene was detected in 7 (46%) isolates. *PAPI-1-attB integration* was observed in 8 (53.3%) isolates, while the circular form of PAPI-1 was discovered in 6 (40%) isolates. Additionally, 5 (33%) strains were tested positive for *pKLC102*.

In a study of the frequency and consequences of the *Pseudomonas aeruginosa* plasmid *pKLC102*, it was associated with antibiotic resistance and virulence factors. Bahramian et al. (2019) provided fundamental insights into the prevalence of *pilS2 gene* associations with *pKLC102* among various *P. aeruginosa* strains collected from patients suffering from various infections, indicating a significant presence (70.5%) of *pKLC102* in these isolates, and 85% of the *P. aeruginosa* strains encoded the *pilS2 gene*.

The study that was performed by Dabiri et al (Hossein Dabiri et al., 2014) The investigation focused on Type IV pilin subgroups within *P. aeruginosa* strains, revealing the presence of the *PilS2 gene* in varying percentages across different types of isolates: 57.5% in environmental strains, 67.5% in cystic fibrosis strains, and 73.3% in burned isolates.

Research conducted by Sadeghifard et al. (2012) examined the occurrence of *PAPI-1* in clinical isolates of *P. aeruginosa*. The findings revealed that 35% of the isolates harbored this genomic island. Interestingly, the study noted that most of the *PAPI-1*-positive isolates demonstrated notable levels of resistance to antibiotics.

Klockgether et al. (2007) investigated the variety within the *pKLC102/PAGI-2* family across different *P. aeruginosa* strains, demonstrating a large distribution and high mobilization rate, emphasizing its possible significance in horizontal gene transfer

events leading to genetic variability within bacterial populations.

The study by Hernández-Ramírez et al. (2018) on plasmid-encoded mobile genetic elements highlighted how such elements could confer both heavy metal resistance and increased virulence to *P. aeruginosa* strains, emphasizing the multifaceted impact of plasmids like pUM505, which shares similarities with *pKLC102,* on bacterial adaptability and pathogenicity.

5. Conclusions

In this study, we found that the high level of virulence in *tested Pseudomonas aeruginosa* isolates from various disease types may be associated with the presence of *pils2 genes*, *plasmid PKLC102, and Soj/4541 genes* from *PAPI-1*. These isolates exhibited significant down-regulation of gene expression in the disease groups when compared to the control group, as assessed using RT-PCR. This gene expression pattern was especially evident in isolates with high levels of antibiotic resistance.

Therefore, further research is necessary to examine how often *PilS2, PAPI-1, and pKLC102* occur and how they relate to antibiotic resistance and biofilm formation in *Pseudomonas aeruginosa* isolates.

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