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Molecular Detection of Genes Related to Biofilm Formation in Clinical *Pseudomonas Aeruginosa* Isolates

Detección Molecular De Genes Relacionados Con La Formación De Biopelículas En Aislados Clínicos De Pseudomonas Aeruginosa

[1]Doaa Mohammed Ridha, [2]Ali Jaffar Saleem, [3]Amal Aziz Kareem

[1]Biology department/ College of Education for Pure Science, University of Diyala

[2]Biology department/ College of Education for Pure Science, University of Diyala

[3]College of Health & Medical Technology - Baghdad, Middle Technical University, Baghdad, Iraq.

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Pseudomonas aeruginosa, biofilm, pslD, pelF, algD, fliC genes

ABSTRACT:

This study aimed to determine the biofilm formation ability and biofilm association genes (*pslD*, *pelF*, *fliC*, and *algD*) among *P. aeruginosa* clinical isolates. Fifty (50) *P. aeruginosa* isolates were identified out of 250 specimens from burn, wound swabs, urine, blood and sputum. The isolated bacteria were tested for their ability to form biofilm using a microtiter plate method. The expression of (*pslD*, *pelF*, *fliC*, and *algD*) genes in biofilm producers isolates was detected using PCR. The MTP detected 100% of isolates as biofilm producers, 32% were strong biofilm producers, 38% were moderate and 30% were weak. The results showed that all isolates have *16SrRNA* gene and the investigation of virulence factors associated genes revealed that isolates were harboring *algD*, *pslD*, *pelF*, and *fliC* gene of the biofilm producers were 96%, 100%, 88% and 58%, respectively.

Introduction

Pseudomonas aeruginosa is an environmentally widespread bacterium. It has emerged as one of the most important opportunistic pathogens of clinical relevance. It has been considered one of the largest general pathogens in hospitals, and has widely contributed to severe opportunistic infections, especially in immunocompromised patients (Abd Al-Mayali and Salman, 2020). These bacteria cause several types of infections, including wound, urinary, and respiratory infections, and may also colonize in healthy humans without causing disease. Infections with these bacteria are increasing worldwide due to survival, adaptation, and resistance mechanisms to different types of antimicrobials (Abdallah and Jabur, 2021).

The broad spectrum of infection caused by *Pseudomonas aeruginosa* depends on the presence of many virulence factors, such as the formation of biofilms. Biofilms are defined as organized assemblages of mono- or multi-species bacteria that adhere to living or non-living surfaces (Costa *et al*., 2021), and their ability to form biofilms on living and non-living surfaces is an important factor. It contributes to the pathogenesis of *P. aeruginosa* infection (Saffari *et al*., 2017).

Alginate biosynthesis is one of the essential components of biofilm formation in *P. aeruginosa*. The alg-D operon is responsible for the final production of alginates. Alginates, encoded by the *algD* gene, are a common type of polysaccharide and are found in the structure of biofilms (Farhan *et al*., 2023). The biosynthesis of exopolysaccharides, known as the polysaccharide coding locus (pel) and polysaccharide synthesis locus (psl), are among the most important essential exopolysaccharides that are exploited in the formation of biofilms in bacteria (Moradali *et al*,.

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2017).). The Psl operon contains 15 genes (pslA-O) involved in exopolysaccharide (EPS) synthesis, which is important for biofilm formation of these bacteria. Another important gene for biofilm formation, development, and maintenance is the Pel gene, which consists of seven genetic factors (pelA-G) and is involved in biofilm formation (Zimmer et al., 2013). The fliC gene is considered one of the key genes in flagella production due to its role in encoding the subunit protein, flagellin. The flagellar complex contributes to chemotaxis, random movement, and the acquisition of essential nutrients through attachment to surfaces, and contributes to causing infection (Suriyanarayanan et al., 2016).

Methods

Specimens collection and culture

A total of (250) clinical specimens from wounds, burns infection, urinary tract infection, sputum and ear swab specimens have been collected from three main hospitals / Diyala province, for the period from September 2022 to January 2023. Primary identification of the isolates was carried out using microscopical, cultural characterization on MacConkey agar, Cetrimide pseudomonas selective agar, Blood agar, and incubated aerobically at a temperature 37ºC for 24 hours. All culture media were ordered from Himedia/Iraq. Biochemical tests were performed and further identification was carried out by genotypic detection using 16SrRNA.

Biofilm formation assay

Biofilm formation was quantified using a microtiter plate test method described by (Dheep *et al*., 2011). Briefly, standard overnight cultures (1.5×108CFU/mL) were diluted 100-fold in brain–heart infusion broth. Bacterial suspension made of strong and moderate biofilm producer isolates. From each culture dilution, 200 µL [180 µL of Mueller-Hinton broth (MHB) and 20 µL of bacteria (5×105 CFU/mL)] were transferred into individual wells of a 96-well flat-bottomed polystyrene plate and incubated at 37◦C for 24 hrs. Negative control wells contained broth only. Thereafter, the content of each well was aspirated and the wells were washed three times with sterile physiological saline. Biofilm was fixed with 200 µl of methanol per well, and after 20 min the plates were emptied and left to air dry. The plates were stained with 200 µl per well of Crystal violet used for Gram staining) for 15 min, then washed three times with water. Add 200 microliters of 95% ethanol for 10 min, then read the optical density (OD) using an ELISA reader at a wavelength of (630) nm. Based on the optical densities of bacterial biofilms, all strains were classified into the following categories: no biofilm producers, weak, moderate, or strong biofilm producers, as previously described (Dheep *et al*., 2011).

Molecular detection for virulence genes

The positive colonies for the culturing and biochemical examination were extracted by DNA extraction kit (ABIOpure, USA) according to the manufacture instruction. polymerase chain reaction (PCR) is a simple and rapid method for detection of microorganisms. PCR was performed to detect the presence of *16SrRNA* gene and biofilm encoding genes (*algD*, *pslD*, *pelF* and *fliC*) using primers listed in Table 1.

Table 1. primers used in PCR amplification for genes detection.

Primer Name	Sequence 5`-3`	Annealing temp. ºC	Product size (pb)	Reference
16SrRNA-F	GGGGGATCTTCGGACCTCA	58	956	2021 Lahij *et al*.,
16SrRNA-R	TCCTTAGAGTGCCCACCCG			
algD-F	CTACATCGAGACCGTCTGCC	58	593	2022 Rajabi *et al*.,
algD-R	GCATCAACGAACCGAGCATC			
pelF-F	GAGGTCAGCTACATCCGTCG	58	789	
pelF-R	TCATGCAATCTCCGTGGCTT			
pslD-F	TGTACACCGTGCTCAACGAC	56	369	

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pslD-R	CTTCCGGCCCGATCTTCATC			
fliC-F	TGAACGTGGCTACCAAGAACG	56	180	Ghadaksaz *et al*., 2015
fliC-R	TCTGCAGTTGCTTCACTTCGC			

PCR reaction was carried out in 25µl of Premix (Promega, USA). 12.5 µl of Master premix were used with 3 ng template DNA,1 µM of five primer and the desired volume was fill by deionized distilled water. The thermal cycler programmer consisted of an initial denaturation at 95 ◦C for 5 min; 30 cycles of denaturation at 95 ◦C for 30 sec, annealing at 56-58 ◦C according to (table 1) for 30 sec and extension at 72◦C for 30 sec; followed by a final extension step at 72 ◦C for 7 min. PCR products were detected by electrophoresis on a 1.5 % agarose gel. Finally, the sizes of the PCR products were determined by comparing them with the migration of the DNA ladder (Xu *et al*., 2011).

Results and Discussion

Isolation of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa isolates appeared as pale, colorless, with irregular edges, on MacConkey agar medium and gray colonies appeared on the blood agar medium with complete hemolysis (β-hemolysis), also there is a clear growth on selective Cetrimide agar medium. The results of biochemical tests showed that all isolates were positive for the oxidase, catalase and motility test. The diagnosis of the isolates was confirmed molecularly using detection of the *16SrRNA* gene (figure 1). Bacterial isolates were selected from all sources and diagnosed.

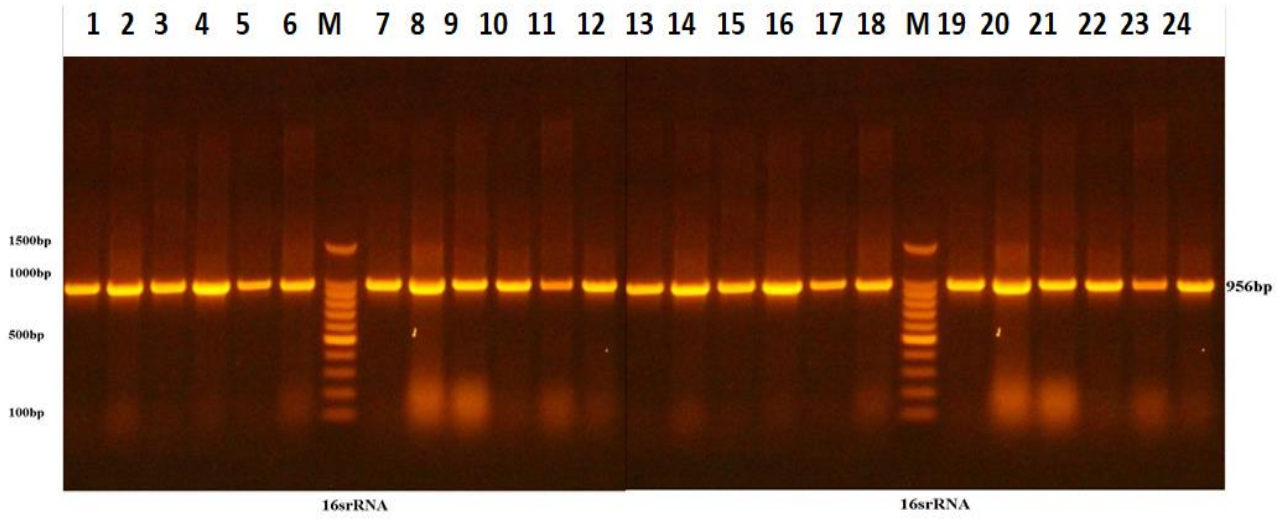

Figure 1: Electrophoresis of *16SrRNA* gene product (956 bp) on an agarose gel using PCR technology for *P. aeruginosa* at a voltage of 100 V, a current of 100 mA, and a time of 60 min.

Fifty (20%) *P. aeruginosa* isolates were obtained after confirming their diagnosis phenotypically, microscopically, biochemical tests, and genetically. The isolation rate result is close to Farhan *et al* (2023) result which was 28%.

Biofilm formation

Pseudomonas aeruginosa is one of the most important pathogens causing serious infections in patients. Biofilm is an important virulence factor in *P. aeruginosa* that plays a role in infection, evading immune defense mechanisms and protecting the bacteria from antibiotics effect (AL-Wrafy *et al*., 2017).

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The biofilm formation assay among all isolates demonstrated that 50 isolates (100%) were biofilm producers. In biofilm-producing isolates, 32% were strong biofilm producers, 38% were moderate biofilm producers and 30% were weak biofilm producers. The result is compatible with Al-draghi and Saeed (2020) result which was 100% biofilm producers, table 1.

Table 1: Biofilm production ability of isolates

Biofilm	No. of isolates	Percentages %
Strong	16	32
Moderate	19	38
Weak	15	30
Total	50	100%

The high percentage of *P. aeruginosa* isolates in the current study on the production of the biofilm explains the high resistance of these isolates against the antibiotic.

In determining the antibiotic needed for treatment, it is necessary to know the concentration necessary to prevent the growth of the bacteria that make up the biofilm, because the usual concentrations of antibiotics kill the cells surrounding the biofilm, while the cells within the polysaccharide remain intact and act as a center for re-growth and re-infection periodically (Saxena *et al*., 2014).

Molecular study

Four genes for biofilm formation (*algD, pelf, pslD, fliC*) were detected in the isolates under study by polymerase chain reaction (PCR) for 24 *P. aeruginosa* isolates from wounds, burns, blood, urine, sputum and ear swab. All genes under study are involved in biofilm formation, contributing to surface adhesion, microcolony formation and dissemination.

The three exopolysaccharides (Psl, Pel, and alginate) are highly involved in surface binding, formation, and stability of biofilm structure, also they are the most important exopolysaccharides exploited in biofilm formation (da Silva *et al.* , 2019). Although *Pel* or *Psl* is often strain-specific, many isolates can switch between Pel and Psl synthesis in response to stress to maintain infection in the host and to respond to ambient conditions (Thi *et al*., 2020).

The results of the detection of genes in the current study showed in (table 2) revealed that the presence of the *algD* gene amplicons was found in 96% of *P. aeruginosa* isolates, and the size of amplified DNA is 593 bp. This result was consistent with the results of Farhan et al (2023) with rate 95.8 %, (figure 2). These results supported previous results obtained from phenotypic detection, which showed mucous, smooth, and slimy phenotypes for local isolates as a result of the spread of the *algD* gene among these isolates, which suggests that the process of biofilm formation for these isolates depends on the production of alginate or Slime, which is a major virulence factor that plays an important role in causing infection.

Table 2: virulence gene PCRs performed upon biofilm-forming isolates

Sources of samples	algD	pslD	pelF	fliC
Burns	7 (29.1%)	7 (29.1%)	7 (29.1%)	4 (17%)
Wounds	6 (25%)	6 (25%)	4 (17%)	1 (4%)
Urine	4 (17%)	5 (21%)	4 (17%)	4 (17%)
Blood	2 (8.3%)	2 (8.3%)	2 (8.3%)	2 (8%)
Sputum	2 (3.8%)	2 (3.8%)	2 (8.3%)	2 (8%)
Ear	2 (.38%)	2 (.38%)	2 (8.3%)	1 (4%)
Total	23 (96%)	24 (100%)	21 (88%)	14 (58%)

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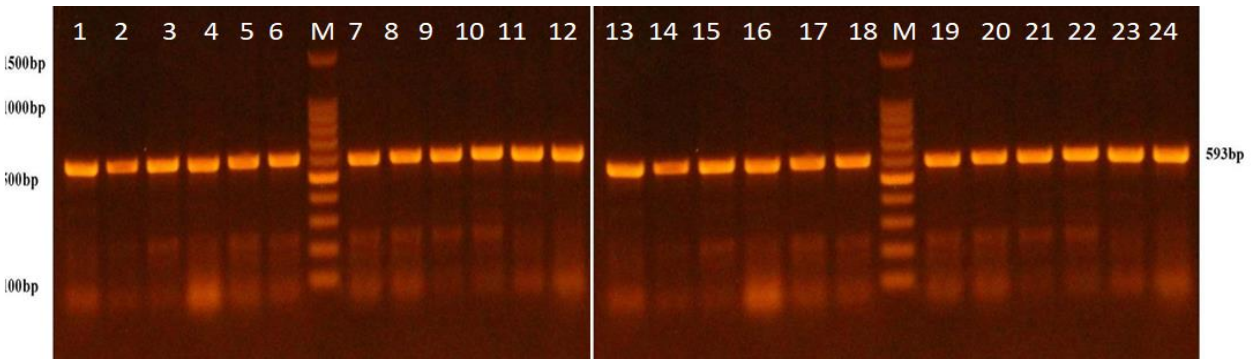

Figure 2: Electrophoresis of *algD* gene product (593 bp) on an agarose gel using PCR technology for *P. aeruginosa* at a voltage of 100 V, a current of 100 mA, and a time of 60 min.

The results of gene detection also showed that 24 (100%) isolates from various sources were carriers of the *pslD* gene amplicons and the size of amplified DNA is 369 bp. The result agree with Ugwuanyi et al. (2021) which found that all of biofilm producing isolates harbor *pslD* gene, (figure 3). It has been observed that the *Pel* gene, which is produced by *P. aeruginosa*, performs the function of maintaining the bacteria's ability to continue producing biofilms even if other exopolysaccharide genes lose their ability to express (Colvin *et al*., 2012).

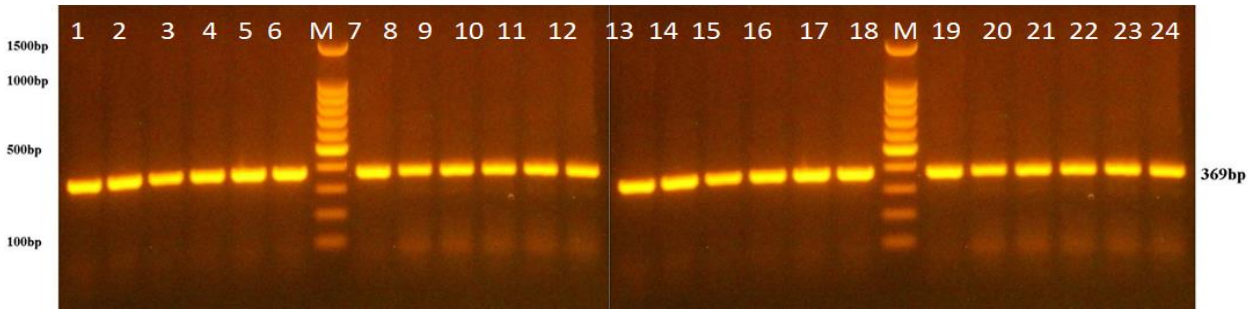

Figure 3: Electrophoresis of *pslD* gene product (369 bp) on an agarose gel using PCR technology for *P. aeruginosa* at a voltage of 100 V, a current of 100 mA, and a time of 60 min.

The results showed that the percentage of *pelF* gene presence among the P. *aeruginosa* isolates under study was 88%. The result close to Rajabi et al. (2022) result which demonstrated that (70.5%) were harbor *pelF* gene, (figure 4).

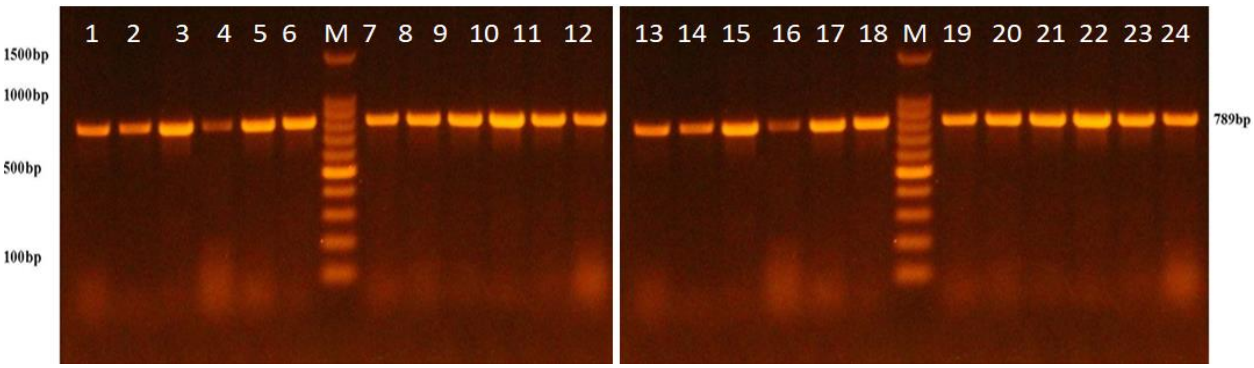

Figure 4: Electrophoresis of *pelF* gene product (789 bp) on an agarose gel using PCR technology for *P. aeruginosa* at a voltage of 100 V, a current of 100 mA, and a time of 60 min.

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Cohen *et al.*, (2015) noted that deletions of the *pelF* and *pslD* genes abolished biofilm formation, and also pointed out the regulatory role of the intracellular signaling molecule bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) for gene expression for biofilm matrix synthesis, including extracellular polysaccharides (pel and psl) and adhesion.

The results also showed that the percentage of *fliC* gene expression in *P. aeruginosa* isolates under study was 58%.

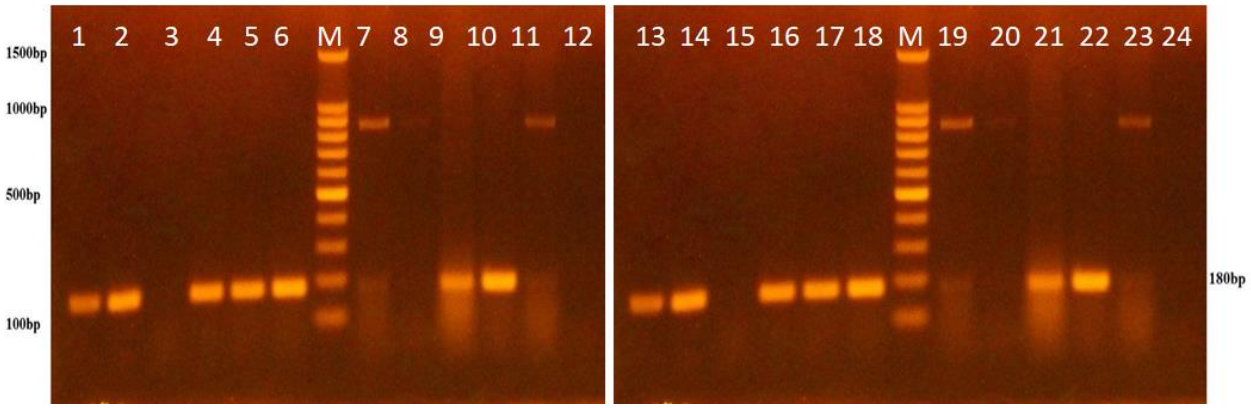

Figure 5: Electrophoresis of *fliC* gene product (180 bp) on an agarose gel using PCR technology for *P. aeruginosa* at a voltage of 100 V, a current of 100 mA, and a time of 60 min.

The *fliC* gene is a critical component in the formation of bacterial flagella, due to the subunit encoding the B-type flagellin protein. Flagella are highly immunogenic, essential for the development of infection and the *fliC* gene plays a vital role in the initial attachment of *P. aeruginosa* when creating a biofilm. Moreover, the *fliC* gene regulates the timing and rate of attachment and separation but does not affect the structure of biofilms, in addition to its role in the process of biofilm attachment (Płókarz *et al*., 2022).

The current study showed the existence of several genetic patterns for the genes under study that are responsible for the formation of biofilms, table 3. Where 58% (n = 14) of the isolates showed the pattern algD-pslD-pelF-fliC from different isolate sources. The genetic patterns algD-pslD-pelF appeared from different sources in 7 isolates (29%), and the genetic patterns algD-pslD appeared in two isolates (8.3%) and from the source of wounds only. Finally, one isolate from the source of urine showed the pattern *pslD* gene only.

Table 3: genotypic patterns of biofilm related genes.

Genetic patterns	No. of isolates %	Isolation source
algD-pslD-pelF-fliC	14 (58%)	Different isolation sources
algD-pslD-pelF	7 (29%)	Different isolation sources
algD-pslD	2 (8.3%)	Wounds
pslD	1 (4.2%)	Urine

In agreement with other studies conducted by Banar (2016), Ghadaksaz (2015), and Kamali (2020), which showed a significant correlation between the ability to form biofilms and the presence of genes related to biofilm formation. The ability to produce biofilms, despite the absence of biofilm genes studied, suggests

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the presence of other biofilm-specific genetic factors that contribute to matrix development in *Pseudomonas aeruginosa* (Moradali *et al*., 2017). In contrast, the absence of biofilm production in the presence of genes may be due to chromosomal mutations in various regulatory systems that affect the production of functional proteins associated with biofilms (Kamali *et al*., 2020).

Conclusion

In this study, a high prevalence of biofilm producer isolates, implicated in hospitalized patients, is a serious problem that makes the treatment of *P. aeruginosa* infections difficult and complicated. The *algD*, *pslD*, *pelF*, and *fliC* genes have a significant role in biofilm formation.

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