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Determine Biofilm Genes in *Pseudomonas aeruginosa* Isolated from Clinical and Environmental Samples

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Abstract

Objective: Isolates of *Pseudomonas aeruginosa* are an extremely adaptable bacterium that causes opportunistic diseases because of its varied metabolic pathways, genes, virulence factors, and considerable antibiotic resistance.

Methods: A total of 293 samples were collected from different places: 193 samples (% 65.87) of human samples and 100 samples (% 34.13) of wastewater samples in the period between 3rd September to 15th November 2023). Bacterial isolates were identified according to microscopic, cultural, and genetic characteristics. Antibiotic susceptibility of bacterial isolates was determined against twelve of the selected antibiotics. The biofilm production was done by using phenotypic ways (Congo red agar and Microtiter plate methods) as well as genotypic ways by detection of biofilm genes (algD, pelf, and pslD genes).

Results: Hundred-forty eight bacterial isolates were obtained, and sixty of these isolates were identified as Pseudomonas spp. (40.9%), twenty-six isolates of *E. coli* (17.9%), seventeen isolates of *K. pneumoniae* (11.3%), and forty-five isolates were beyond to other types of bacteria (30.1%), and out of sixty isolates of Pseudomonas spp., forty-two were identified as *P. aeruginosa* isolates. *P. aeruginosa* isolates revealed various resistance levels to antimicrobial agents gradually, ranging from 83.87% to Trimethoprim-sulfamethoxazole (SXT) to 3.22% to Aztreonam (AZT). Biofilm production by using the Congo red method showed that 27 isolates (64.28%) were positive results, while in the microtiter method, all forty-two isolates were positive (100%), the genetic detection showed that the *AlgD* gene was recognized in thirty-one isolates (73.8%), followed by *Pelf* and *PslD* genes in four isolates each (9.5%).

Conclusion: The isolation percentage showed a high occurrence of multi-drug resistance biofilm forming Pseudomonas spp. isolates which could be a critical indicator. Methods of biofilm detection showed that the microtiter plate method has accuracy more than the Congo red method; as well *AlgD* gene was prevalent compared with both other genes *Pelf*, and *PslD*.

Key words: *Pseudomonas aeruginosa*, Biofilm, *algD* gene, *pelf* gene, *pslD* gene.

Introduction

Isolates of *Pseudomonas aeruginosa* are an extremely adaptable bacterium that causes opportunistic human diseases because of its varied metabolic pathways and genes. Eliminating it can be difficult, particularly in those with cystic fibrosis due to nutritional diversity, virulence factors, and considerable antibiotic resistance.[1] Gram-negative *P. aeruginosa* is a common environmental bacterium causing severe illnesses, particularly in weakened immune systems. It is a primary cause of morbidity and mortality in cystic fibrosis patients and is resistant to various medications.[2] *P. aeruginosa*, an environmental bacterium, causes sporadic human illnesses due to its adaptability and resistance to various growing environments. It is challenging to eradicate due to dietary requirements and antibiotic resistance.[3] It is responsible for several medical conditions, including antibiotic resistance. Treatment is difficult because of biofilms, which increase the toxicity of the microorganism and lead to treatment failure, immune system evasion, and recurrent infections.[4] *P. aeruginosa* produces soluble pigments pyoverdine and pyocyanin for iron metabolism and suppurative infections. It forms non-lactose fermenting colonies on MacConkey agar and fluorescent pigment colonies on nutrient agar.[5] It is a multifactorial pathogen causing various infections, with three stages: adhesion, invasion, and widespread systemic illness.[4] *P. aeruginosa*, a bacterial species, colonizes host tissue using pili, flagella, exo-enzymes, and exopolysaccharides. Pathogenic factors include protease, exotoxin, phospholipase, exotoxin A, lipases, and phospholipases. *P. aeruginosa* colonies are distinguished by the blue-green pigment pyocyanin, which induces oxidative stress and triggers apoptosis in neutrophils,[6] It colonizes host tissue using glycoproteins with N-acetylglucosamine, GalNAc, D-mannose, L-fucose, and NeuAc sugar patterns. Pathogenic factors include protease, exotoxin-promoted infections, phospholipase, exotoxin A, lipases, and phospholipases. Its colonies are marked by pyocyanin, which induces oxidative stress and triggers apoptosis in neutrophils.[5] *P. aeruginosa* infections significantly increase morbidity and mortality due to their ability to express virulence factors, adapt to environmental changes, and develop antibiotic resistance. This leads to pan-drug-resistant (PDR) or multi-drug-resistant (MDR) isolates, resulting in longer hospital admissions, higher costs, and higher morbidity and mortality rates.[3] More than 120 species of rod-shaped, gram-negative, flagellated bacteria, such as those found in soil and water, belong to the genus Pseudomonas.[7] Generally, *P. aeruginosa* has a large bacterial genome, estimated at 7 million base pairs. Its accessory and core genomes make up 90% of the genome. Core gene discoveries vary due to strain sets, definitions, and annotations.[8] Another previous study found 5,109 protein-coding genes in all *P. aeruginosa* strains.[9]

Methods

Samples Collection: A total of 293 samples were collected from different places; 193 samples (% 65.87) of human samples, 58 samples (% 19.79) of wastewater, and 42 samples

(% 14.33) of chicken blood samples) in the period between 3rd September 2022 to 15th November 2023). Clinical samples were obtained from teaching laboratories in Medical City, AL-Shaheed Ghazi Al-Harari Hospital Laboratories, and AL-Yarmouk Hospital Laboratories in Baghdad while the environmental samples (wastewater) were collected from drain water of the same hospitals. Until they reached the laboratory, samples were transported on a transport medium containing sterile swabs. Thereafter, the samples were cultured on differential and selective media. Standard and confirmatory tests were subsequently performed on the developing isolates to ascertain further physical and biochemical characteristics.

Bacterial Identification: Bacterial samples were cultured in a pure and fresh medium prior to slide smear preparation, fixation, and gram stain testing, which involved observing the colour, size, shape, and arrangement of bacterial cells using a light microscope. The initial identification tests encompassed morphological analysis of bacterial growth on various media that had been inoculated beforehand, including MacConkey agar, Cetrimide agar, Nutrient agar, and Blood agar. These media were subjected to anaerobic conditions for approximately 18–24 hours at 37 degrees Celsius. Colony shape, texture, colour, hemolysis pattern, pigment generation, and edges were all investigated.[10] The biochemical tests were conducted according to previous studies.[11,12] These tests included catalase, oxidase, pyocyanin production, hemolysis, and proteolysis tests. Catalase was detected by dropping hydrogen peroxide on a colony. Oxidase was detected by forming a deep purple colour within 10–15 seconds. Pyocyanin production was detected by streaking pure bacterial isolates on various agar plates. Hemolysis was detected by streaking a pure colony with *P. aeruginosa* isolate on blood agar plates. Protease was detected by cultured *P. aeruginosa* isolates on skim milk agar, showing a clear zone surrounding the bacterial colony growth. These tests were conducted to determine the production and synthesis of bacterial enzymes.[11] The study used a polymerase chain reaction (PCR) to amplify the16SrRNA gene, which was used for identifying *P. aeruginosa* isolates, Table 1 illustrated the 16rRNA primers.[12]

The nucleic acid extraction of the *P. aeruginosa* isolates was performed using a DNA extraction kit (G-spin DNA extraction kit) according to the manufacturer's instructions. The PCR premix container has the following ingredients: 1 unit of freeze-dried Taq DNA polymerase, 250 micrograms of dNTPs, 10 milliliters of Tris-HCl (with a pH of 9.0), 30 milliliters of KCl, 1.5 milligrams of MgCl2, a stabilizing agent, and track dye. The reaction was conducted by establishing the following thermocycler conditions: an initial denaturation that occurred at 95°C for 5 minutes, followed by 25 cycles of denaturation at 95°C for 40 seconds, annealing at 58 °C for 45 seconds, and extension at 72°C for 60second. Finally, there was a final extension at 72°C for 10 minutes. The PCR results were analyzed using electrophoresis on a 1.5% agarose gel, subjected to ethidium bromide, and seen using a UV transilluminator. The lysate was incubated at 56°C for 10–30 minutes, followed by incubation at 70°C for 5 minutes. The sample tube was centrifuged at 13,000 rpm for 5 minutes to remove un-lysed tissue particles. The mixture was carefully applied to the spin column and centrifuged at 13,000 rpm for 1 minute. The DNA was stored in the deep freezer until PCR analysis. The concentration and purity of DNA were determined using a Nanodrop instrument, with an O.D of one corresponding to approximately 50 µg/ml for double-strand DNA. The final concentration of DNA was calculated using the formula DNA concentration (µg/ml) = O.D 260 x 50 x dilution factor.[13]

Antibiotic susceptibility test: The Kirby-Bauer method was employed to determine the susceptibility of different antibiotics to them. In contrast to the McFarland standards, *P. aeruginosa* colonies were seeded onto Mueller-Hinton agar. The plates were coated with antibiotic discs and incubated at 37°C for 18–24 hours.[14] Disc diffusion was employed to assess antibiotic sensitivity towards a variety of commercially available antibiotics, such as Gentamicin, Ciprofloxacin, Ceftazidime, Piperacillin, Cefotaxime/Clavulanic acid, Cotrimoxazole, Ticarcillin/Clavulanic acid, Levofloxacin, and Amikacin. The analyzers followed the CLSI 2022 recommendations.[15]

Phenotypic biofilm detection (Congo red agar method): Congo red agar method CRA was inoculated with the acquired bacterial strains and incubated at 37°C for 24–48 hours. CRA was prepared by supplementing BHI broth with 5 % sugar and Congo red stain (Congo red agar). The medium used in this experiment consisted of BHI (37 g/L), sucrose (50 g/L), agar (10 g/L), and Congo red stain (0.8 g/L). To manufacture the Congo red stain, a concentrated aqueous solution was utilized, which was autoclaved at 121 °C for 15 minutes before its addition to the cooled agar at 55 °C. Strong positivity was attributed to the presence of black colonies that possessed a dry, crystalline quality. When a dry, crystalline colonial morphology was absent, a darkening of the colonies indicated a biofilm producer with a moderate degree of positivity. Identifiable non-biofilm producers were colonies that maintained their pink colouration.[16]

Phenotypic biofilm detection (Microtiter plate method): The biofilm development assay was performed by

Table 1. **Oligonucleotide primers for amplification of *P. aeruginosa* genes**

Primers	Sequence	Amplicon size
16SrRNA	F 5- GGGGGATCTTCGGACCTCA -3	956bp
	R 5- TCCTTAGAGTGCCCACCCG -3	
AlgD	F 5-CTACATCGAGACCGTCTGCC-3	593 bP
	R 5-GCATCAACGAACCGAGCATC-3	
Pelf	F- GAGGTCAGCTACATCCGTCG-3	789 bp
	R 5-TCATGCAATCTCCGTGGCT-3	
PslD	F 5-TGTACACCGTGCTCAACGAC-3	369 bp
	R 5-CTTCCGGCCCGATCTTCATC-3	

the tissue culture plate method outlined by Babapour et al (2016). Bacterial isolates were cultivated on B.H.I. agar for 24 hours at a temperature of 37°C. A few colonies were diluted with normal saline and injected into a 96-well microtiter plate subsequent to the mixing process. In addition, control wells were vaccinated. After 24 hours of incubation at 37°C, the plates underwent three D.W. washes (7.2% pH) and dried. For fifteen minutes, adhering bacteria were fixed in 200 µl of 99% ethanol in each well. Following the application of a crystal violet solution coating, the plates underwent three washes with D.W. (pH 7.2) in order to eliminate the unbound dye. Using an ELISA reader, the absorbance of each well at 630 nm was determined, and the optical density (OD) value for the control well was subtracted. The adhesion capabilities of the bacterial test isolates were categorized into four distinct groups, with the cut-off value representing the optical density (ODc).[17]

Genotypic biofilm detection (biofilm production genes): The study used three pairs of primers, *algD, pelf*, and *pslD* as shown in Table 1, and a reaction mixture of 12.5 µl of iNtRON 2X PCR Master mix (i-Taq), 1 µl of each primer, 5 µl of DNA template, and 3.5 µl of nuclease-free water. The reaction was carried out in 30 cycles, with initial denaturation taking 95 seconds, denaturation taking 60 seconds, and 30 cycles of extension. The DNA extraction samples and PCR products were detected using gel electrophoresis, which was visualized using a safe gel stain and UV transilluminator (320) nm. The process involved melting agarose, adding ethidium bromide, and preparing the sample. The wells were loaded with 5 µl of PCR product, and DNA ladder was loaded in the middle single well. The gel was then exposed to a UV transilluminator and photographed using a digital camera.[13]

Results

Bacterial Isolation and Identification Results

From 3rd September to 15th November 2023, a total of 293 samples were collected from various sources. Clinical sources included swabs from burns and wounds, sputum, urine, pus, blood, ear swabs, nasal swabs, prostatic secretions, and soft tissue swabs while environmental sources included wastewater from different hospitals. Out of 293 samples which were collected in the current study; hundred-forty- eight bacterial isolates were obtained, and sixty of these isolates identified as *Pseudomonas spp*. (40.9%), twenty-six isolates of *E. coli* (17.9%), seventeen isolates of *K. pneumoniae* (11.3%), and forty-five isolates were beyond other types of bacteria (30.1%), As shown in Figure 1.

MacConkey agar showed colonies of *Pseudomonas spp.* with a pale, smooth, spherical, non-fluorescent bluish pigment and pyocyanin-producing colonies. Cetrimide agar showed them as smooth, greenish-yellow colonies with pyocyanin pigment, a blue hue produced by *P. aeruginosa*. HiFluoro Pseudomonas agar base was used as a chromogenic selective medium for *P. aeruginosa* isolation. Biochemical tests were employed to identify *P. aeruginosa* isolates, which were positive in catalase, oxidase, protease enzymes, the citrate test, and growth at 42°C.[8,18] A microscopic analysis identified *Pseudomonas aeruginosa* as a Gram-negative, red-bacilli bacterium (0.5–1.0) (1.5–5.0) m in length, either alone, in pairs, or short chains. The bacterium was either straight or slightly curved. It is propelled by a polar flagellum, and certain strains

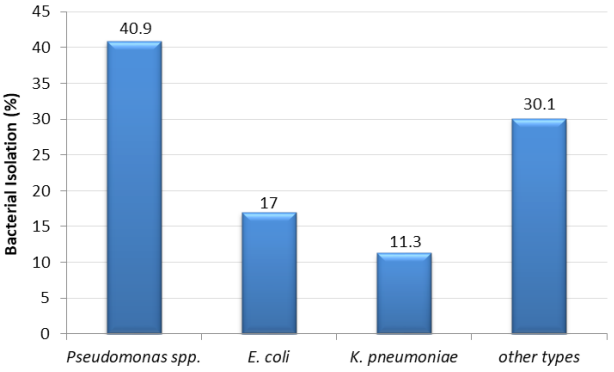

Fig.1 **Isolation percentage of the bacterial isolates.**

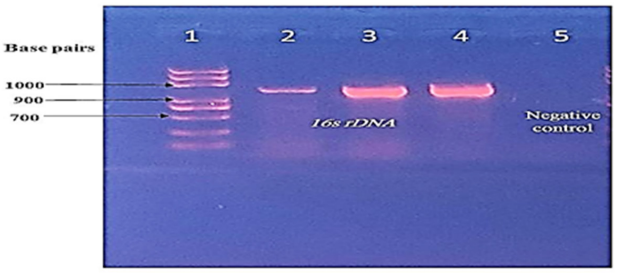

Fig. 2 **Genetic identification of *P. aeruginosa* isolates.**

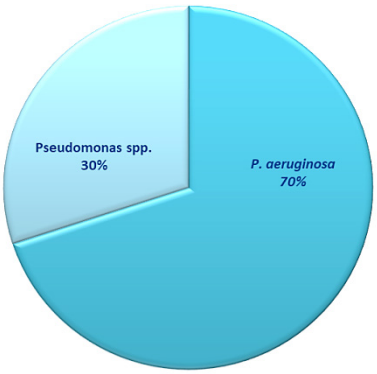

Fig. 3 **Isolation percentage of *P. aeruginosa* by 16S rRNA.**

may possess as many as two or three of these flagellums. *Pseudomonas aeruginosa* are virulent aerobes that have a broad temperature tolerance (5–32°C), with an optimal growth range of 37°C. Nevertheless, these organisms have the capacity to endure temperatures between 10 and 42°C, albeit with modifications to their outer membrane proteins, lipopolysaccharides (LPS), and lipids.[19]

The current research recorded that *16S* rRNA PCR amplification was efficacious in the detection of *P. aeruginosa* isolates; expected amplicons of 956 bp confirmed their diagnosis. Precise PCR primers and a PCR machine were utilized in the investigation. The species of the isolates was validated via DNA sequencing, hence verifying its phenotypic analysis. Out of sixty isolates of *Pseudomonas spp*., forty-two were identified as *P. aeruginosa* isolates, Figures 2 and 3 illustrated the genetic identification of *P. aeruginosa* isolates and their percentage respectively.

Results of antibiotic susceptibility test: *P. aeruginosa* isolates were tested for the susceptibility tests as routinely performed by disc diffusion (Kirby- Bauer) method using Muller Hinton agar. The size of the clear inhibition of the growth zone determined whether the bacterium was resistant, intermediate, or susceptible to the recommended antibiotics and the results were interpreted according to the recommendation of CLSI (2023). *P. aeruginosa* isolates revealed various resistance levels to 12 antimicrobial agents belonging to different antimicrobial classes. The results were explained according to the recommendation of CLSI (2023). *P. aeruginosa* isolates revealed various resistance levels to antimicrobial agents gradually, as follows: Trimethoprim-sulfamethoxazole SXT (83.87%), Piperacillin PIP (67.74%), Ceftazidime CAZ (64.51%), Gentamycin GEN (61.29%), Ciprofloxacin CIP (51.61%), Levofloxacin LVX (48.38%), Amikacin AMK and Ticarcillin-clavulanate TIM (45.16%), Imipenem IMP (35.48%), Colistin CST (32.25%), Piperacillin-tazobactam TZP (25.8%), Aztreonam AZT (3.22%), as shown in Figure 4.

Results of Biofilm Formation

Congo red method: The qualitative screening method for biofilm identification, the Congo red agar test, provides a more streamlined and expedient approach. Through the cultivation of 42 *Pseudomonas aeruginosa* isolates on Congo red agar, biofilm formation is detected. Positive outcomes signify robust biofilm development, as 27 (64.28%) of the samples demonstrated biofilm production capability, while 15 (35.82%) yielded negative results, Figure 5.

Microtiter plate method: Using the microtiter plate method, the capacity of pathogenic bacterial isolates to form biofilms was evaluated. The biofilm formation potential of 42 isolates was evaluated by comparing the optical density (OD) values of attached dyed bacterial cells. The findings indicated that the rate of moderate biofilm production peaked at 19 (45.23%), with weak production following at 16 (38.09%). Strong biofilm production ranked third at 7 (16.67%), as well as no weak biofilm producer isolates, Figure 6.

Determination of biofilm production genes: The study tested the main biofilm formation genes (*AlgD, Pelf*, and *PslD*) in 42 isolates of *P. aeruginosa*. Results showed a high prevalence of *AlgD* in 31 isolates (73.8%), followed by *Pelf* and *PslD* in 4 isolates (9.5%), as illustrated in Figure 7.

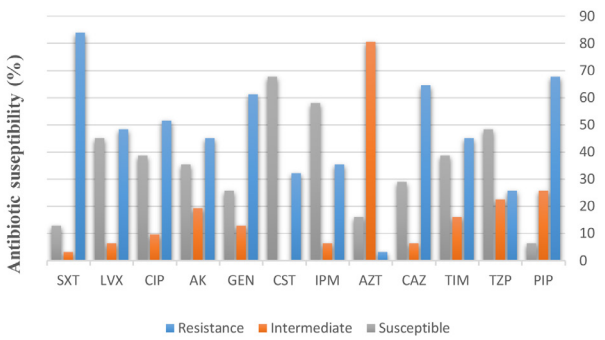

Fig. 4 **The percentage of antibiotic susceptibility tests. Where SXT, Trimethoprim-sulfamethoxazole; LVX, Levofloxacin; CIP, Ciprofloxacin; AK, Amikacin; GEN, Gentamycin; CST, Colistin; IMP, Imipenem; AZT, Aztreonam; CAZ, Ceftazidime; TIM, Ticarcillin-clavulanate; TZP, Piperacillin-tazobactam; PIP, Piperacillin.**

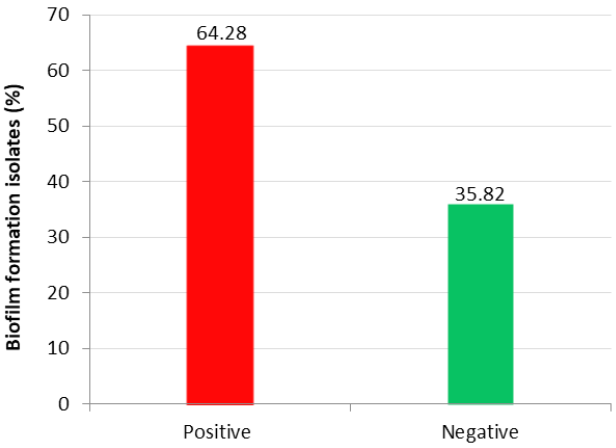

Fig. 5 **Biofilm formation percentage by Congo red method.**

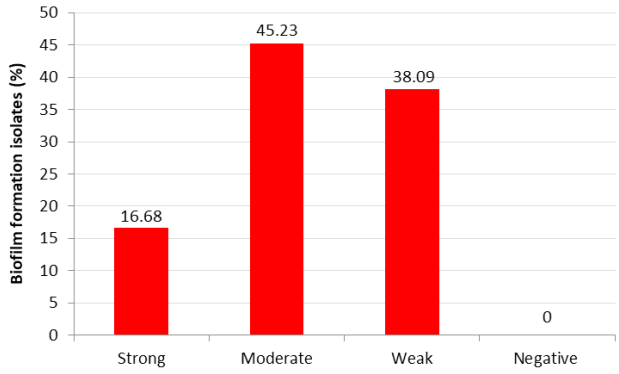

Fig. 6 **Biofilm formation percentage by Microtiter plate method.**

Discussion

According to the results, the antibiotic resistance in isolates showed multidrug resistance (MDR) phenotypes that isolates resist at least one antibiotic from more than three different classes.[20] MDR *P. aeruginosa* poses a grave threat to public health due to its frequent involvement in potentially fatal healthcare-associated infections like ventilator-associated pneumonia, bacteremia, and UTI.[21] It further contributes to substantial morbidity and mortality through wound and soft tissue infections. *P. aeruginosa* can become resistant to several kinds of antibiotic drugs even while receiving therapy, which makes treatment difficult. *P. aeruginosa* possesses a natural defense against antibiotics due to its intrinsic resistance, a multi-layered obstacle course for these drugs. This includes inducible AmpC cephalosporinase and MexAB-OprM/MexXY efflux pumps that neutralize or expel antibiotics, combined with a tightly packed outer membrane and OXA-type oxacillinase enzymes. Furthermore, its inherent propensity to acquire new resistance genes adds another layer of complexity to overcome.[22] Bacterial biofilms also pose a significant threat due to their inherent resistance to antibiotics. This resistance stems from three key factors: the physical barrier of the biofilm itself, the altered metabolic state of biofilm bacteria, and the presence of dormant "persister" cells that evade antibiotic action.[23] Intrinsic resistance is caused by low outer membrane permeability, efflux pumps, and antibiotic inactivation enzymes. Adaptive resistance involves

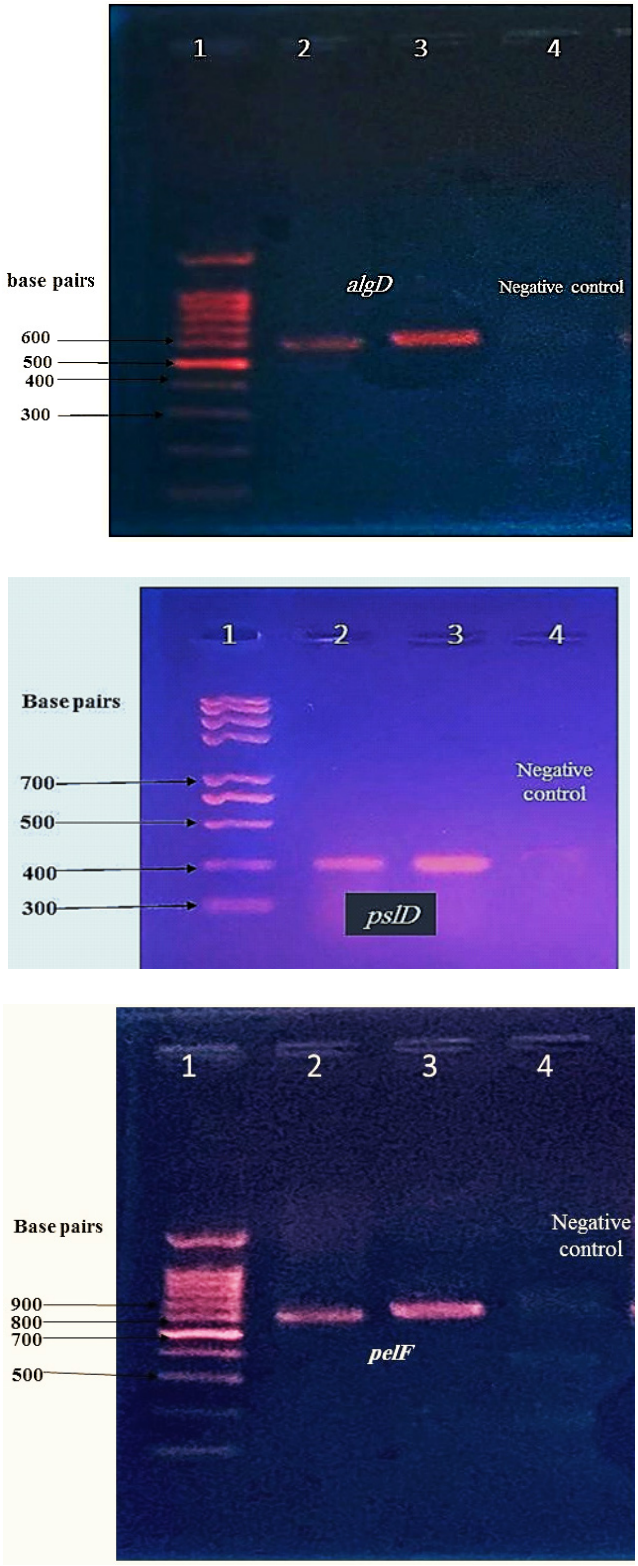

Fig. 7 **Gel electrophoresis of *AlgD, pslD,PslD* genes in *P. aeruginosa*.**

the production of biofilm in the lungs of infected patients, limiting antibiotic availability. Multidrug-tolerant persisters can withstand antibiotics, resulting in persistent and recurring infections in CF patients. Fluoroquinolones are used to treat *P. aeruginosa* infections but have increased resistance due to chromosomal point mutations in the quinolone resistance determining region (QRDR) genes, efflux pump overexpression, and QRDR mutations.[7] The increasing resistance to key antipseudomonal antibiotics, including carbapenems, aminoglycosides, and fluoroquinolones, is implicated in the alarming emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *P. aeruginosa* strains, creating a grave therapeutic challenge. In conclusion, the resistance of *P. aeruginosa* isolates to various antibiotics is a complex issue that requires further research and understanding. Further research is needed to develop effective strategies to combat this resistance and improve the quality of antibiotics used in the treatment of *P. aeruginosa* isolates.[23] The current result agreed with a local study conducted by Hamid Salman Marah Al-delfey who reported that 66/100 (66%) of his *P. aeruginosa* isolates produced a strong slim layer indicated by the formation of black colonies.[24] Another research was done at Al-Mosul University, it reported that 31/49 (63.27%) isolates were biofilm producers.[25] Another study conducted by Abdulhaq N. et al. at Faisalabad University was disagreed with our study results, they mentioned that 23/52 (44.23%) samples showed biofilm formation, and 29/52 (55.77%) showed negative results.[20] In another study by Sultan A. *et al.* done in Egypt, their result was 63/145 (43.4%) were biofilm produceres, and 82/145 (56.6%) were not biofilm produceres.[26] Results were reported in another local investigation conducted by Hussein Abed W and Mohammed Kareem, which yielded intermediate production results of 46 (92%) and strong biofilm producer findings of 4 (8 %).[27] Generally, all the collected isolates of *P. aeruginosa* (100%) possess the ability to produce biofilm layers of different thicknesses. Most isolates possess the *AlgD* gene, which produces alginate, a linear polymer crucial for biofilm stability and protection. The *Pelf* gene encodes proteins related to polysaccharide biosynthesis components, essential for the synthesis of a glucose-rich matrix material. Pellicle production is controlled by the *pel* operon, which consists of seven genes (*pelA* through *pelG*). *Psl*, a neutral polysaccharide, is produced by *P. aeruginosa*, isolates from the environment and aids in biofilm creation and protection. The *psl* operon consists of 15 co-transcribed genes (*pslA* to *pslO*). PslD protein is essential for biofilm formation, likely by exporting a biofilm-relevant exopolysaccharide.[25] Biofilms are formed from planktonic cells in a complex, regulated process. Factors such as carbon source, iron limitation, and glycoprotein mucin influence biofilm formation in *P. aeruginosa*. The biofilm life cycle is dependent on filamentous prophages and the extracellular matrix. Alginate overproduction, expression of polysaccharide loci, and extracellular DNA contribute to structured biofilm formation. The process entails a synchronized series of activities, which include adhering to a surface, forming micro colonies, maturing, expanding, and spreading. High-throughput screening tests have made it easier to study the genetic components that play a role in the formation of biofilms.[28]

Conclusion

The isolation percentage of the collected samples showed that high occurrence of Pseudomonas spp. isolates compared with other bacterial isolates which could be a critical indicator due to their ability to resist many kinds of antibiotic as well as possessing many virulence factors. Most

of the collected isolates were MDR *P. aeruginosa*, and that makes it grave threat to public health due to its frequent involvement in potentially fatal healthcare-associated infections for both humans and animals. Methods of biofilm detection showed that the microtiter plate method has accuracy more than the Congo red method, as well as *AlgD* gene was prevalent compared with both other genes *Pelf*, and *PslD*.

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Conflicts of Interest

The authors have no conflicts of interest to declare for this study.

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