# Screening of biofilm-producing genes from *Acinetobacter* isolates obtained from Covid-19 patients in ICU hospital section

## Abstract

Acinetobacter, recognized as a nosocomial pathogen, undergoes structural changes when exposed to various antibiotics, rendering it relatively resistant and posing challenges in disease treatment. This study aimed to identify two biofilm-related genes and assess the drug resistance profile of clinical strains. Clinical isolates were collected from the ICU of Afzalipour Hospital in Kerman, Iran, and phenotypically identified. Confirmation was achieved for 55 clinical Acinetobacter isolates. Antibiogram testing was conducted for meropenem, amikacin, ampicillin-sulbactam, cefotaxime, levofloxacin, rifampin, and tigecycline antibiotics. Biofilm formation ability was assessed using microtiter plates and crystal violet staining, followed by spectrophotometry at OD 490 nm. PCR was employed to determine the frequency of pslA and pelB genes. Analysis revealed that the highest age group affected was 1 to 15 years (19%), while the lowest was 26 to 35 years (5%). The frequencies of pslA and pelB genes were 34.5% and 65.5%, respectively, and drug resistance ranged from 72% to 100% for the mentioned antibiotics. Given the pelB gene's approximately twofold higher frequency compared to pslA, it suggests that in most studied isolates, Psl may often be disrupted or that intracellular c-di-GMP levels have significantly increased.



Keywords: Biofilms, *Acinetobacter*, Drug Resistance, Antibiotic, *pslA* and *pelB* genes

## 1. Introduction

The corona epidemic that started in the world in 2019 led to an increase in the number of patients in the ICU department of hospitals (1, 2). Corona virus caused many problems for patients due to the involvement of the respiratory system (1-4).

The corona pandemic caused a large number of people to be admitted to the intensive care units of hospitals. Due to the special closed system of these parts and the lack of proper air circulation, Spire equipment is a suitable place for the accumulation of commensal bacteria and the creation of biofilm (5, 6).

Biofilm is actually a collection of microbes that stabilize on a surface. Bacteria in biofilm structures have relationships with each other and their behavior is different from when they are alone. The resistance of bacteria to antimicrobial agents in the biofilm state is different from the planktonic state. Many genes are involved in the formation of biofilm, these genes act in a cascade manner and the activity of one gene depends on the activity of another gene (7).

In the biofilm structures, there are steps to form the biofilm structure, in which the initial attachment of the bacteria to the surface is done and the bacteria are colonized (8).

In the next stage, bacteria multiply on the surface and their number increases. And then they get trapped inside an extracellular matrix, which prevents the penetration of antimicrobial agents into the biofilm structure (9, 10).

*Acinetobacter* is a gram-negative bacterium without spores, which is one of the most important bacteria resistant to antimicrobial agents and antibiotics. These bacteria have a wide frequency in the intensive care units of the hospital and causes widespread problems (12-16).

*Acinetobacter* biofilms have been studied to a lesser extent. The purpose of this research is to investigate the effective genes in the production of *Acinetobacter* biofilm (18).

## 2. Materials and Methods

### 2. 1. Isolation and Identification

In this research, 47 *Acinetobacter* strains were isolated from clinical samples collected from Afzalipur Hospital, Kerman. Their initial identification was done by some biochemical tests such as Gram staining, oxidase, catalase and etc(19).

### 2. 2. Antibiogram Test

The antibiotic resistance pattern of the collected strains was determined by standard methods. The disk diffusion method was used to measure the antimicrobial effect. Discs containing antibiotics were placed on Mueller Hinton's medium, on which the bacteria had been previously cultured, and kept at 35 degrees for 24 hours, and then the halo of non-growth was measured (20).

### 2. 3. Biofilm Assay

Microtiter plate method was used to measure the ability of isolated *Acinetobacter* strains to form biofilm. In this method, the studied bacteria that had reached half McFarland turbidity were cultured in a microplate containing Mueller Hinton Broth medium and incubated for 24 hours at 37 degrees. After the incubation time, the contents of the plates were emptied and the biofilms formed by the bacteria were stained with 1% crystal violet. According to the following formula, the strength of the strains in biofilm formation was calculated. (21, 22, 23)

$$OD \leq OD_c = \text{not biofilm producer}$$

$$OD_c < OD \leq (2 \times OD_c) = \text{weak biofilm}$$

$(2 \times OD_c) < OD \leq (4 \times OD_c)$ = moderate biofilm

$(4 \times OD_c) \leq OD$ = strong biofilm

**Equation 1:** Classification of composed biofilms based on OD.

## 2.4. DNA Extraction and Polymerase Chain Reaction (PCR)

The DNA of studied strains were obtained using the genome extraction kit. Polymerase chain reaction was performed using special primers for two genes (PelB, PSlA), the conditions of the reaction are given in the Table 1, finally, the PCR product was loaded on a 1% gel and observed with a UV device. (UVitec, Cambridge, UK).

**Table 1:** PCR methods

| **Gene** | **Primer** | **Product size (bp)** | **PCR program *** | **Ref** |
|---|---|---|---|---|
| *PelB* | F: 5′- CGCCTGCTCTGGTTCTACAT -3′<br>R: 5′- AGTCGTTGGGATTGGACTTG -3′ | 400 | Initialization: 5 sec- 95 °C<br>Denaturation: 30 sec- 95 °C<br>Annealing: 45 sec- 51 °C<br>Elongation: 45 sec- 72 °C<br>Final elongation: 5 sec- 95 °C | (25) |
| *PslA* | F: 5′- CACTGGACGTCTACTCCGACGATAT -3′<br>R: 5′- GTTTCTTGATCTTGTGCAGGGTGTC -3′ | 163 | | (25) |
| 16S rRNA (Specific to *P. aeruginosa*) | F: 5′- CTACGGGAGGCAGCAGTGG -3′<br>R: 5′- TCGGTAACGTCAAAACAGCAAAGT-3′ | 600 | | (25) |

*: The PCR program was performed for 35 cycles.

# 3. Results

## 3. 1. Antibiogram Test

The results of the antibiogram test are shown in Table (2). As can be seen in this table, there is resistance to most of the antibiotics mentioned in the table, and the highest resistance to Rifampin antibiotics and the lowest resistance to Ampicillin-Sulbactam

**Table 2:** Antibiogram test results (%).

| Antibiotics | Sensitive | Intermediate | Resistance |
|---|---|---|---|
| Meropenem | 0 | 0 | 100 |
| Amikacin | 0.93 | 0.93 | 98 |
| Ampicillin-Sulbactam | 0.93 | 28 | 71 |
| Cefotaxime | 0.93 | 0 | 99 |
| Levofloxacin | 0 | 0.93 | 99 |
| Rifampin | 0 | 0 | 100 |
| Tigecycline | 5 | 23 | 72 |

## 3.2. Biofilm Assay

Figure 1 shows image of the biofilm created by *Acinetobacter* strains. This image is after coloring by Crystal Violet. As can be seen in this picture, 36 strains of *Acinetobacter* were strong biofilm forming, 13 strains were medium biofilm and 6 strains had low potential for biofilm formation.

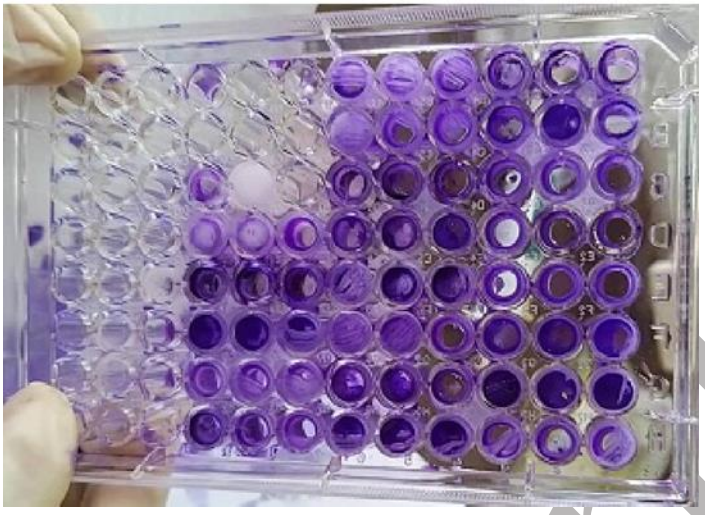

**Fig 1:** Biofilm formation by *Acinetobacter* strains.

### 3.3. PCR and Gel Electrophoresis

The results of the screening of biofilm producing genes are shown in Figure 2, which is actually the product of PCR using gene-specific primers. As can be seen in this figure, 19 strains have PslA gene and 36 strains have PelB gene, and in general, the frequency of these two genes is 34 and 65%, respectively.

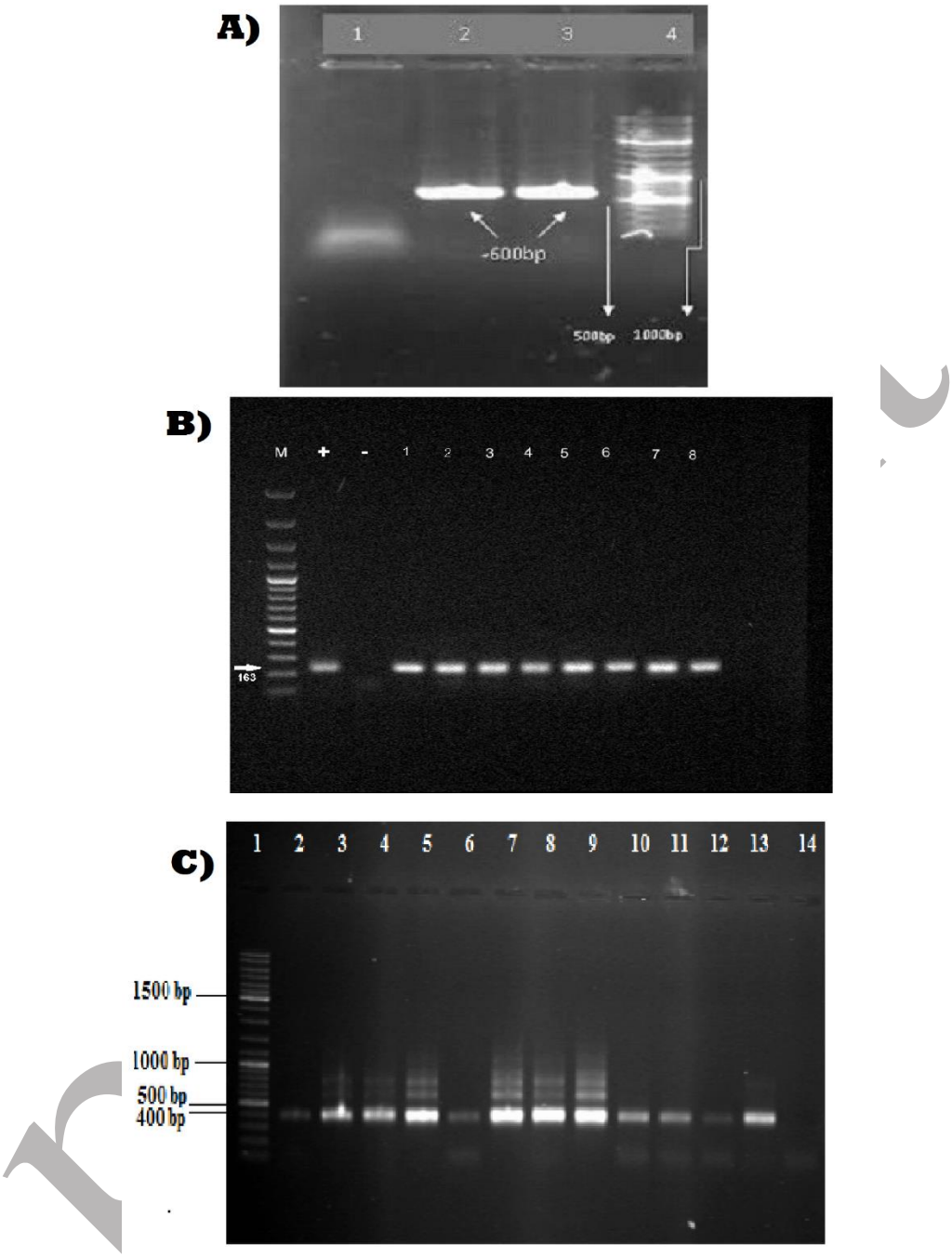


**Fig 2:** PCR product by specific primers: A) Determination of PCR reaction specificity, column 1 of sterile distilled water as the negative control, column 2 of positive control, column 3 of *Acinetobacter* gene, column 4 of the ladder. B) *PslA* and C) *PelB*.

## 4. Discussion

*Acinetobacter*, known for its opportunistic nature and numerous virulence factors, has a significant capacity to form biofilms. These biofilms can diminish the effectiveness of antimicrobial drugs, leading to chronic infections. According to a study by Saxena et al. (2014), 80 Acinetobacter isolates were collected from patients with lower respiratory tract infections in India (6, 7). The findings, obtained through a method comparable to the current study, revealed high resistance rates to amoxiclav (97%) and levofloxacin (74%), while resistance to amikacin was notably lower at 33% (26).

A study conducted in Iran on 55 clinical Acinetobacter isolates reported a resistance rate of 98%. The notable disparity in amikacin resistance between the clinical isolates from Iran and India might be explained by differences in local lifestyles and the varied hospital wards from which the samples were obtained. Additionally, the biofilm analysis of the 80 isolates from Lucknow, India, indicated that 20% formed strong biofilms, 21.25% moderate biofilms, and 58.75% weak biofilms (27).

In similar studies conducted in Iran, the current study observed the highest frequency of strong biofilm formation, with only 10.90% of the isolates demonstrating weak biofilm formation. According to Heydari and Eftekhar (2015), in their investigation of Acinetobacter isolates from a burn unit in Iran, 66.7% of the isolates formed strong biofilms, while 33.3% formed weak biofilms (28). Their findings also revealed that all biofilm samples tested positive, and 14% of the samples negative for biofilm formation possessed the PslA gene. In a recent study by Kamali et al. (2020), examining 80 Acinetobacter isolates, the biofilm formation ratios were reported as 16.25% strong, 33.75% moderate, 33.75% weak, and 16.25% incapable of forming biofilms. Additionally, 12.5% of the isolates were resistant to amikacin. (29).

In Acinetobacter, genes associated with biofilm formation include ppyR, pslA, and pelA, along with genes related to alginate production such as algD, algU, and algL. Mucoid strains that overproduce alginate can facilitate lung colonization in cystic fibrosis (CF) patients, often leading to fatal outcomes (30, 31). Before mucoid strains appear, non-mucoid strains that produce Psl and Pel biofilms tend to colonize the patients' lungs. Strains that produce matrix IV result in stable rugose small-colony variants (RSCV), which are prolific producers of Pel and Psl. Generally, mutations that increase intracellular levels of c-di-GMP can boost the expression of Pel and Psl genes, leading to the RSCV phenotype (32, 33). This RSCV phenotype is also more resistant to antibiotics and the immune system. Notably, the RSCV phenotype was observed in 33 out of 86 CF patients with P. aeruginosa over a two-year period. (34).

Cho et al. (2018) investigated 82 carbapenem-resistant Acinetobacter isolates from various hospital wards in South Korea and found that approximately 93% of the biofilm-forming isolates possessed the PslA gene. In contrast, the current study found a PslA gene presence of about 34.5%, although the initial screening of isolates was not based on carbapenem resistance.

Typically, when both Psl and Pel are present, Psl tends to dominate, with Pel having only a limited impact on biofilm phenotypes. However, in cases where the Psl operon is absent or disrupted, such as in PA14, or when c-di-GMP levels are significantly increased, Pel plays a more prominent role in biofilm formation. This study's findings indicated that the frequency of the pelB gene was about twice that of the pslA gene. Colvin et al. (2012) also studied the PAO1 strain, which mainly relies on Psl for biofilm formation, and reported that mutations in psl result in the formation of weak biofilms (35), which eventually strengthen after a prolonged period due to Pel rearrangement. Similarly, Emami et al. (2015) found that 70% of Pseudomonas isolates could form biofilms, with the pslA gene present in approximately 43% of them.(36, 37).

## 5. Conclusion

This study identifies the pel and psl genes as key contributors to the formation of strong, moderate, and weak biofilms in pathogenic *Acinetobacter* bacteria. The presence of these genes enhances the bacterium's pathogenicity and its resistance to antibiotics. Therefore, research aimed at inhibiting these two genes could not only reduce the biofilm-forming ability and proliferation of *Acinetobacter* but also improve the effectiveness of various antibiotics in treating diseases caused by this bacterium.

**Ethics Declarations**

**Competing Interests:** There are not any conflicts of interest among the authors.

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**Author contributions:**

**Data Availability:** Data will be available after publication

**Ethics Approval:** All authors approve the ethics in this study

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