Original Article

Screening of Biofilm-Producing Genes from *Acinetobacter* Isolates Obtained from Covid-19 Patients in ICU Hospital Section

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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

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1. Introduction

The global corona epidemic that commenced in 2019 resulted in an escalation in the number of patients in the intensive care unit (ICU) departments of hospitals (1, 2). The corona virus has been implicated in numerous respiratory system complications (1-4).The corona pandemic has led to a substantial increase in the number of individuals requiring admission to the intensive care units (ICUs) of hospitals. The unique environment of these units, characterised by its closed system and inadequate air circulation, has been identified as a conducive habitat for the proliferation of commensal bacteria and the formation of biofilm (5, 6).Biofilm, in essence, constitutes a collection of microbes that adhere to a surface, exhibiting a distinct behaviour compared to their planktonic state. These bacteria within biofilm structures exhibit a relationship with each other, leading to an altered phenotype in terms of their resistance to antimicrobial agents when compared to their planktonic state. The formation of biofilm is a complex process involving numerous genes that act in a cascading manner, where the activity of one gene is dependent on the activity of another (7).The process of biofilm formation involves several stages, including initial attachment of bacteria to a surface, followed by bacterial colonization (8). In the subsequent stage, bacteria multiply on the surface, leading to an increase in their population. The subsequent stage is the entrapment of bacteria within an extracellular matrix, which hinders the penetration of antimicrobial agents into the biofilm structure (9, 10).Acinetobacter is a gram-negative bacterium devoid of spores, which is among the most significant bacteria in terms of its resistance to antimicrobial agents and antibiotics. These bacteria are prevalent in the intensive care units of hospitals and are a major cause of concern (12-16).Acinetobacter biofilms have been the subject of only a limited number of studies. The objective 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 study, a total of 47 Acinetobacter strains were isolated from a variety of clinical samples obtained from Afzalipur Hospital in Kerman. The initial identification of these strains was conducted through the implementation of several biochemical tests, including Gram staining and the assessment of oxidase and catalase activities, as outlined in the literature (19).

2. 2. Antibiogram Test

The antibiotic resistance pattern of the collected strains was determined by standard methods. The antimicrobial effect was measured using the disk diffusion method. 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. The halo of non-growth was then measured (20).

2. 3. Biofilm Assay

The microtiter plate method was utilised to assess the capacity of isolated Acinetobacter strains to form biofilm. In this method, the studied bacteria, which had attained half McFarland turbidity, were cultivated in a microplate containing Mueller Hinton Broth medium and incubated for 24 hours at 37 degrees.Subsequent to the incubation period, the contents of the plates were decanted and the biofilms formed by the bacteria were stained with 1% crystal violet. The strength of the strains in terms of biofilm formation was then calculated using the following formula: (21, 22, 23).

$OD \leq OD_c$ = not biofilm producer
$OD_c < OD \leq (2 \times OD_c)$ = weak biofilm
$(2 \times OD_c) < OD < (4 \times OD_c)$ = moderate biofilm
$(4 \times OD_c) < OD$ = strong biofilm

Equation 1: Classification of composed biofilms based on OD.

2.4. DNA Extraction and Polymerase Chain Reaction (PCR)

The deoxyribonucleic acid (DNA) of the studied strains was extracted using a genome extraction kit. A polymerase chain reaction (PCR) was then performed using special primers for two genes (PelB, PSlA). The conditions of the reaction are given in Table 1. Finally, the PCR product was loaded on a 1% agarose gel and observed with a UV device (UVitec, Cambridge, UK).

3. Results

3. 1. Antibiogram Test

The results of the antibiogram test are presented in Table 2. As illustrated in the table, the majority of the antibiotics mentioned are resistant to at least one antibiotic, with the highest levels of resistance observed against Rifampin and the lowest levels against Ampicillin-Sulbactam.

3.2. Biofilm Assay

As illustrated in Figure 1, the image of the biofilm created by Acinetobacter strains has been coloured using Crystal Violet. The figure demonstrates that 36 strains of Acinetobacter were strong biofilm formers, 13 strains were medium biofilm formers, and 6 strains exhibited low potential for biofilm formation.

3.3. PCR and Gel Electrophoresis

The results of the screening of biofilm-producing genes are presented in Figure 2, which is the result of a polymerase chain reaction (PCR) using gene-specific primers. As demonstrated in this figure, 19 strains were found to have the PslA gene, and 36 strains were found to have the PelB gene. The frequency of these two genes is generally 34% and 65%, respectively.

4. Discussion

Acinetobacter, a microbe known for its opportunistic nature and numerous virulence factors, has a significant capacity to form biofilms, which have been shown to diminish the effectiveness of antimicrobial drugs, leading to chronic

Table 1. PCR methods.

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

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

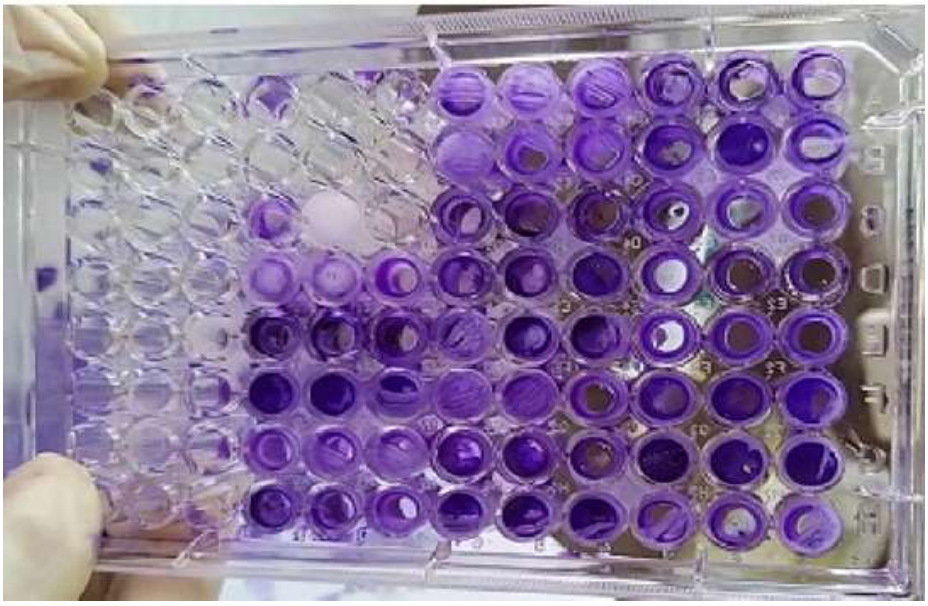

Figure 1. Biofilm formation by *Acinetobacter* strains.

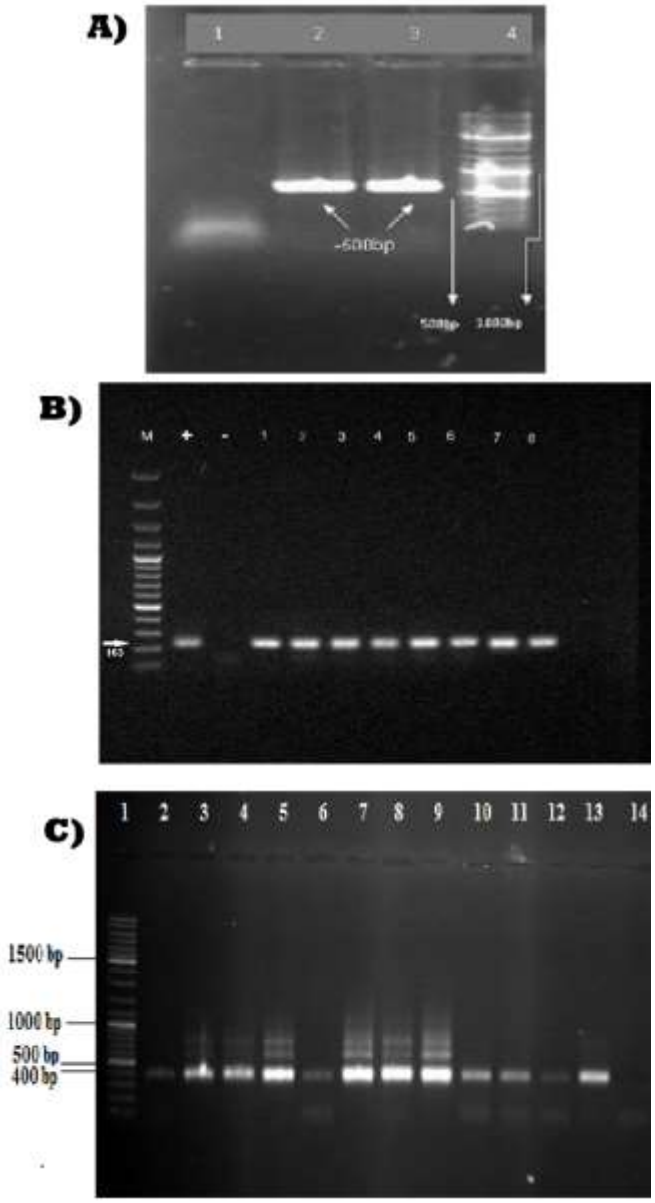

Figure 2. PCR product by specific primers: A) Determination of PCR reaction specificity, column 1 of sterile distilled water as the negative control, column 2 of positive control, column 3 of *Acinetobacter* gene, column 4 of the ladder. B) *PslA* and C) *PelB*.

infections (Saxena et al., 2014). In a study of 80 Acinetobacter isolates from patients with lower respiratory tract infections in India, 6 and 7 were found to be particularly prevalent (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. Furthermore, the biofilm analysis of 80 isolates from Lucknow, India, revealed that 20% formed strong biofilms, 21.25% formed moderate biofilms, and 58.75% formed weak biofilms (27).In analogous 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. In a separate investigation of Acinetobacter isolates from a burn unit in Iran, Heydari and Eftekhar (2015) found that 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 subsequent study by Kamali et al. (2020), which examined 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 (29). Additionally, 12.5% of the isolates were resistant to amikacin. In the genus Acinetobacter, genes associated with the formation of biofilms include ppyR, pslA, and pelA, along with genes related to alginate production such as algD, algU, and algL. Mucoid strains that overproduce alginate have been observed to facilitate lung colonization in cystic fibrosis (CF) patients, often leading to fatal outcomes (30, 31). Prior to the manifestation of mucoid strains, non-mucoid strains that produce Psl and Pel biofilms characteristically colonize the patients' lungs.Strains that produce matrix IV give rise to stable rugose small-colony variants (RSCV), which are prolific producers of Pel and Psl. The expression of Pel and Psl genes is known to be enhanced by mutations that increase intracellular levels of c-di-GMP (32, 33). This RSCV phenotype has been observed to be more resistant to antibiotics and the immune system (34). Notably, the RSCV phenotype was observed in 33 out of 86 CF patients with P. aeruginosa over a two-year period. 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 (Cho et al., 2018).In contrast, the present study found a PslA gene presence of about 34.5%, although the initial screening of isolates was not based on carbapenem resistance (Smith et al., 2022). 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 (Colvin et al., 2012).This study found 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. Emami et al. (2015) also found that 70% of Pseudomonas isolates could

form biofilms, with the pslA gene present in approximately 43% of them (36, 37). 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 has been demonstrated to enhance 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.

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Authors' Contribution
Study concept and design: A. MF.
Acquisition of data: H. A.
Analysis and interpretation of data: S. K.
Drafting of the manuscript: H. M.
Critical revision of the manuscript for important intellectual content: B. Z.
Statistical analysis: SB. G.
Administrative, technical, and material support: H. M.

Ethics
All authors approve the ethics in this study.

Conflict of Interest
There are not any conflicts of interest among the authors.

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Data Availability
Data will be available after publication.

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