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Frequency of *cbrA*, *cbrB*, *ndvB*, and *phoBR* Genes in Relation to Biofilm Formation in *Pseudomonas aeruginosa* Clinical Isolates

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ABSTRACT

Introduction: After *Staphylococcus aureus* and *Escherichia coli*, *Pseudomonas aeruginosa* is the third cause of hospital-acquired infection (HAI). This bacteria's ability to colonize in different environments, especially in hospitals and biofilm formation, has added to its impact as an HAI. The molecular mechanism of biofilm formation is not well understood, but several genes contribute to this phenomenon. This study investigates the frequency of *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes in biofilm-forming *P. aeruginosa* isolates. **Methods:** Fifty *P. aeruginosa* clinical isolates were collected from various sources such as urine, ulcer, blood, secretions, and trachea in Milad Hospital, Tehran, from 2017 to 2018. Biofilm formation in the isolates was assessed by the microtiter plate assay, and the frequency of *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes was investigated by PCR. **Results:** Among the 50 isolates, 44% were strong biofilm former, 34% moderate biofilm former, 12% weak biofilm former, and 10% did not form biofilms. PCR revealed a frequency of 94% for the *cbrA* gene, 78% for *cbrB*, 96% for *ndvB*, and 48% for *phoBR*. The coexistence of all four genes was 68% in strong biofilm former isolates, 41% in moderate biofilm former isolates, 37% in weak biofilm former, and zero in the isolates that formed no biofilm. **Conclusion:** The high frequency of *ndvB* and *cbrA* genes and the coexistence of *ndvB* and *cbrB* suggest the contribution of these genes in the biofilm formation of *P. aeruginosa.*

INTRODUCTION

Biofilms are microbial populations surrounded by an extracellular polymeric matrix formed in different places or habitats [1, 2]. The formation of biofilms by pathogenic bacteria on tissues or medical equipment placed in the host body often leads to infections that exhibit high resistance to antimicrobials compounds and host immune responses. Investigations have shown that 80% of microbial infections are caused by bacterial biofilms [1]. Bacterial resistance in biofilms is up to 5000 times higher than their planktonic state or free-living bacteria [2, 3]. Several mechanisms contribute to this resistance. The first mechanism is that matrix creates a physical and chemical barrier against antibiotic penetration [3, 4]. The second hypothesis regarding bacterial resistance in biofilms is related to bacterial metabolic activity [5, 6]. Inactivity of bacteria in biofilms is critical in resisting different antibiotics that affect growing and dividing bacterial cells [7]. The third hypothesis is that mutation frequency in the bacterial population in biofilms is more remarkable than planktonic bacteria [8].

Pseudomonas aeruginosa is a Gram-negative, motile, aerobic, rod-shaped bacterium and grows in most environments. This bacterium is the most critical human opportunistic pathogen among the nosocomial infections agents and causes infections in urinary and respiratory tracts, soft tissues, bones, and joints [7][9]. One of the reasons for the chronic nature of infections caused by the opportunistic pathogen *P. aeruginosa* is its ability to form biofilms in which the bacteria are protected from host defense mechanisms and antibiotics [8]. Numerous genes are involved in *P. aeruginosa* biofilm formation; however, *cupA*, *cupB*, *cupC*, *cupD*, *cbrAB*, *phoBR*, and *ndvB* were shown to play a more critical role [10-12].

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This study investigates the frequency of *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes and their association with biofilm formation in *P. aeruginosa* clinical isolates.

MATERIAL AND METHODS

Isolation and identification of clinical isolates of *Pseudomonas aeruginosa*. This descriptive study was performed on 50 *P. aeruginosa* clinical isolates collected from hospitalized patients in Milad Hospital, Tehran, Iran from 2017 to 2018 from various sources, i.e., urine (n=15), wound (n=10), trachea (n=10), various secretions (n=5), and blood (n=10). Identity of *P. aeruginosa* isolates was obtained using Gram staining, growth on MacConkey agar (MAC), oxidase and catalase tests, triple sugar iron (TSI) agar medium, motility, production of indole and gas, growth at 42°C, and pyocyanin production on Müller Hinton agar [4].

Biofilm production assessment by microtiter plate method. The *P. aeruginosa* isolates were cultured overnight in trypticase soy agar (TSA) with 2% glucose [13, 14]. Bacterial culture turbidity was adjusted to 0.5 MacFarland standard in trypticase soy broth, and 200 µL from each isolate were transferred into 3 wells of two 96-well polystyrene plates (SPI Co. Korea). *Pseudomonas aeruginosa* ATCC strain 27853 as the positive control and TSA with 2% glucose without the bacteria as negative control were included in the assay. Plates were incubated at 37°C for 24 h before being washed twice with phosphate buffer saline (pH 7, PBS). After drying at room temperature, the wells were fixed with 200 µL of 95% methanol for 15 min and stained with crystal violet (1%) for 5 min. The plates were washed with distilled water, the wells were filled with 100 µL glacial acetic acid (33%), and optical densities in wells were measured using an ELISA reader (Biotech, USA) at 570 nm. The optical density cut-off (ODc) for biofilm production was calculated using the optical density (OD) obtained from the mean OD of negative control plus three folds of the negative control deviation [15]. Based on the intensity of biofilm production, the strains were divided into four groups: strong (OD > 0.872), intermediate (OD, 0.36 - 0.576), weak (OD, 0.218 - 0. 288), and non-biofilm producers (OD <0.218) (Table 2).

PCR Amplification of *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes. DNA was extracted from *P. aeruginosa* isolates grown on nitrite agar by the phenol-chloroform method [16]. The presence of *cbrA* and *cbrB*, *phoBR*, and *ndvB* genes was examined by PCR using specific primers (Table 1). PCR was performed in a 10 µL reaction mixture containing 1 mM $MgCl_2$, 0.25 mM dNTP, forward and reverse primers (200 pM/µL), 100 ng of DNA, and 1 unit *Taq* DNA polymerase. PCR amplifications were programmed for an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were run on 1.5% agarose gels (100 volts for 45 min), and the results were visualized after staining with ethidium bromide by a gel documentation device (Iran Kia Gene, Iran).

Table 1. The primers used for amplification of *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes.

Target genes	Sequences (5'→3')		Product length	Reference
ndvB	Forward primer	ACAGCAGGAGCATCCACGAAG	250 bp	This study
	Reverse primer	GCCCTGTTCGTCTTCAAGGT		
phoBR	Forward primer	GACGAACTGACCGTCGACAT	464 bp	This study
	Reverse primer	GAGAAACGATAGCCGGTCCC		
cbrA	Forward primer	CACGCTGTTCATGCTGATCG	838 bp	This study
	Reverse primer	GTTGGAGAGGTCCTGTTCGG		
cbrB	Forward primer	CAGCAAGATTCGCAAGGTCG	710 bp	This study
	Reverse primer	CCAGCTCGATGTCGATACCC		

RESULTS

Biofilm formation.

Table 2. Biofilm formation of *P. aeruginosa* isolates by the microtiter plate method.

ODc	Mean optical density (OD_{570})	Degree of biofilm formation
Strong	> 0.872	OD > 4×ODc
Moderate	0.436 < OD ≤ 0.872	ODc×2 < OD ≤ 4×ODc
Weak	0.218< OD ≤ 0.436	ODc < OD ≤ 2×ODc
Negative	≤ 0.218	OD ≤ ODc

Standard deviation (SD) = 0.031; Mean=0.125; Mean OD of negative control wells + (3 × standard deviations of negative control wells) = optical density cut-off (ODc); ODc=0.218.

Of the 50 isolates, 22 (44%) were strong biofilm formers, 17 (34%) moderate biofilm formers, 6 (12%) weak biofilm formers, and 5 (10%) formed no biofilm (Table 2).

Among the 15 urine isolates, six (40%) were strong biofilm formers, four moderate (26.6%), four weak (26.6%), and one (6.6%) showed no biofilm formation. Of 10 isolates from wounds, four (40%) were strong biofilm formers, three (30%) moderate, two (20%) weak,

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and one (10%) formed no biofilm. Among the 10 tracheal isolates, six (60%) formed strong biofilms, three (30%) moderate, and one (10%) had a weak biofilm. All five (100%) secretion samples formed strong biofilms. Of the 10 blood specimens, seven (70%) produced strong biofilms, two (20%) moderate, and one (10%) showed weak biofilm formation (Fig. 1). As observed, most of the isolates obtained from body fluids were strong biofilm formers, indicating their ability for systemic survival.

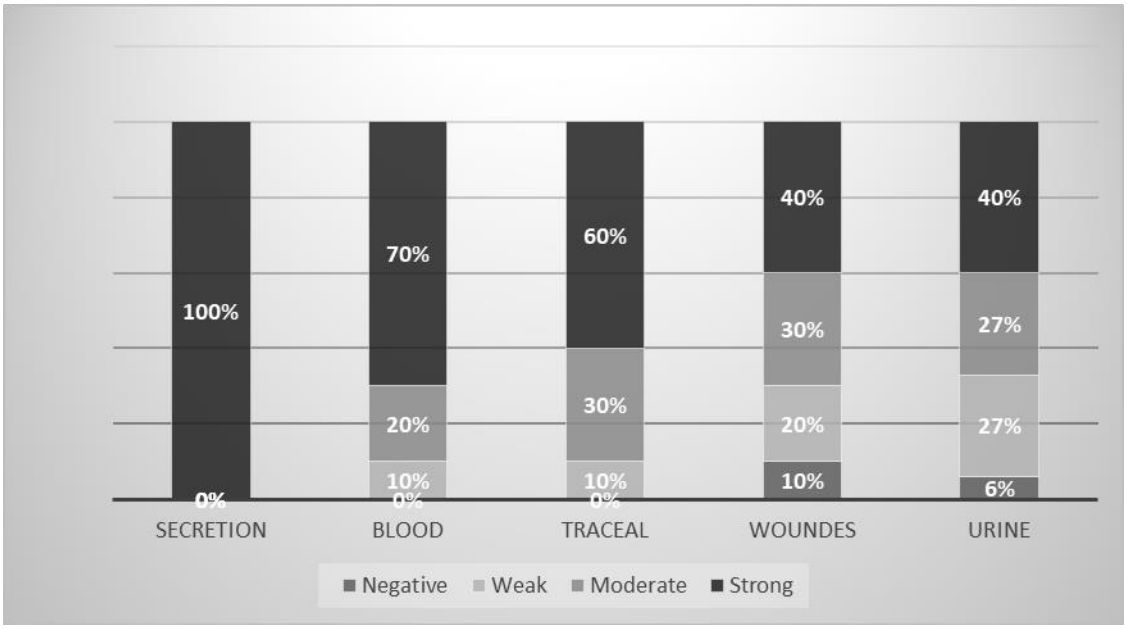

Fig. 1. Frequency of *P .aeruginosa* biofilm formation in clinical specimens measured by the microtiter plate method

PCR amplification of *cbrA, cbrB, phoBR,* and *ndvB* genes. Amplification of *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes in P. *aeruginosa* clinical isolates yielded amplicons of various sizes (Fig. 2).

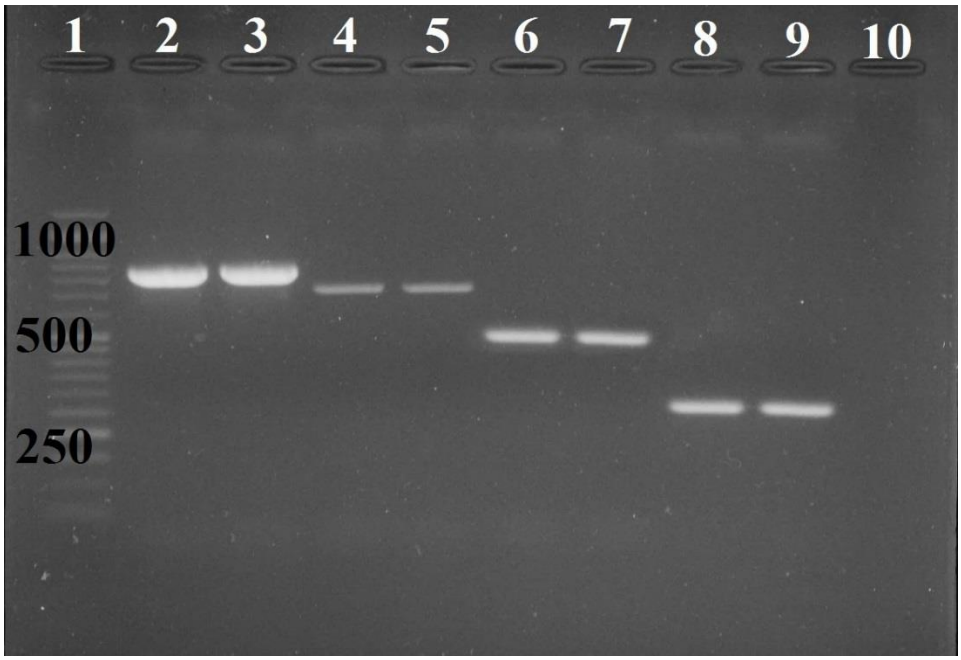

Fig. 2. Agarose gel (1.5%) electrophoresis of amplified *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes. Lane1, size marker (50bp); lanes 2 and 3, *cbrA* gene; lanes 4 and 5, *cbrB* gene; lanes 6 and 7, *phoBR* gene; lanes 8 and 9, *ndvB* gene; lanes 10, NTC (non-template control).

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The frequency of the *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes were 94%, 78%, 48%, and 96%, respectively. No significant relationship was found between biofilm formation and the presence of *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes in isolates from different clinical sources ($P = 0.1$) (Table 3).

The frequency of individual genes and their coexistence in different clinical isolates is reflected in Table 4. Statistical analysis showed a significant relationship between biofilm intensity and the presence of each of these genes. ($P = 0.05$).

Table 3. Frequency of *cbrA, cbrB, phoBR, and ndvB* genes in isolates from different clinical sources.

clinical sources	*cbrA*	*ndvB*	*cbrB*	*phoBR*
	n (%)	n (%)	n (%)	n (%)
Urine n=15	15 (100)	14 (93)	11 (73)	7 (46.6)
Wounds n=10	9 (90)	9 (90)	6 (60)	4 (40)
Tracheal n=10	10 (100)	10 (100)	7 (70)	5 (50)
Secretions n=5	5 (100)	5 (100)	4 (80)	3 (60)
Blood n=10	10 (100)	10 (100)	7 (70)	5 (50)

Table 4. Frequency of *ndvB*, *phoBR*, *cbrA*, and *cbrB* genes presence and their coexistence in the biofilm-forming P. *aeruginosa* clinical isolates.

Biofilm formation	*cbrA*	*cbrB*	*phoBR*	*ndvB*	*cbrA& cbrB*	*cbrA& phoBR*	*cbrA& ndvB*	*cbrB& phoBR*	*phoBR& ndvB*	Coexsistence of all four genes
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Strong (n=28)	28 (100)	28 (100)	19 (68)	28 (100)	28 (100)	19 (68)	28 (100)	19 (68)	19 (68)	19 (68)
Moderate (n=12)	12 (100)	9 (76)	5 (41)	12 (100)	9 (76)	6 (50)	12 (100)	5 (41)	5 (41)	5 (41)
Weak (n=8)	8 (100)	4 (50)	3 (37)	7 (87)	4 (50)	3 (37)	5 (62)	3 (37)	3 (37)	3 (37)
Negative (n=2)	1 (50)	0	0	2 (100)	_	_	1 (50)	_	_	_

Based on our findings, the presence of these genes is not specific to biofilm formation but may participate in other vital activities such as swarming and cytotoxicity.

Simultaneous frequency of *cbrA*, *cbrB*, *phoBR*, *and ndvB* genes was 68% in strong biofilm-former isolates, 41% in moderate biofilm-formers, 37% in weak biofilm formers, and 0% in negative biofilm isolates. These results show that the interaction of all four genes may be an essential factor in biofilm formation. On the other hand, the presence of *cbrA*, *cbrB*, and *ndvB* genes, even in negative biofilm isolates, may indicate their role in other functions than biofilm formation, such as swarming and cytotoxicity.

The co-presence of *cbrA* and *ndvB* genes in negative biofilm-forming strains (50%) may also indicate their role in functions other than biofilm formation.

DISCUSSION

We investigated biofilm formation in *P. aeruginosa* isolated from various clinical specimens and its association with *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes presence. Overall, the rate of strong biofilm formation was higher in urine, blood, and secretions specimens compared to the isolates from other sources. In a study in Iran, *P. aeruginosa* isolates from urine showed strong biofilm formation, whereas blood isolates formed weak

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and moderate biofilms [17]. Our results showed that the frequencies of *ndvB*, *cbrA*, and *cbrB* genes were 100% in strong biofilm producers. Takayuki et al. (2001) examined the *cbrA* and *cbrB* mutants in *P. aeruginosa* PAO strain and showed that the *cbrA-cbrB* system controlled the expression of several catabolic pathways, possibly regulating the intracellular nitrogen balance along with the *nrB-ntrC* system [18].

Yeung et al. (2011) showed that, under the swarming conditions, *cbrA* regulates the expression of many genes, including *phoPQ*, *pmrAB*, *arnBCADTEF*, *dnaK*, and *pvdQ* [19]. The *cbrA* is a kinase sensor and produces pyoverdine. The CbrB protein is a transcriptional activator for the σ_{54} RNA polymerase. Genetic evidence suggests that *cbrB* increases the expression of the *crcZ* gene in *P. aeruginosa* [20].

The two-component system, *phoR-B,* is activated by phosphate limitation. When phosphate levels in a bacterium are reduced, PhoR phosphorylates PhoB, which acts as a transcription factor by binding to Pho-Box promoters. In the absence of phosphate, PhoB up-regulates the *rhlR* expression, which results in rhamnolipid production that promotes hyper swarming.[21].

Safari et al. (2017) evaluated the frequency of *ndvB* and *Tssc1* genes among 92 *P. aeruginosa* isolates from patients with eye infections in Farabi Hospital, Tehran, Iran. All isolates could form biofilms and 96.7% and 90.2% harbored *ndvB* and *Tssc1* genes, respectively, showing the significance of these genes in biofilm production [22]. The *ndvB* gene codes a glycosyltransferase essential for the formation of periplasmic glucose. Glucans keep antibiotic molecules away from their target cells and result in antibiotic resistance in the bacterial populations of biofilms [17]. A study on the expression of the *ndvB* gene in biofilm-producing *P. aeruginosa* isolates showed that biofilm formation was significantly higher among urine, endotracheal tube aspirate, and burn isolates than those from wound specimens [22].

Tahseen et al. (2020) showed the *ndvB* gene in 100% biofilm-producing *P. aeruginosa* clinical specimens. This study also concluded that *ndvB* gene expression was responsible for antibiotic resistance in *P. aeruginosa* biofilms [23]. The coexistence of *cbrA*, *cbrB*, *phoBR*, and *ndvB* genes was 68% in strong biofilm formers, 41% in moderate biofilm formes, and 37% in weak biofilm strains. However, the concurrent frequency of *cbrA* and *ndvB* genes in 50% of negative biofilm strains indicates their roles in mechanisms other than biofilm formation. To date, the role of the *ndvB* gene has not been shown n biofilm formation in *P. aeruginosa*,. However, it may play other vital roles such as response to environmental conditions, osmolarity, and bacterial motility [16-20]

Since few clinical studies have been performed on *ndvB*, *phoBR*, *cbrA*, and *cbrB* genes, more extensive studies are needed to investigate their role in biofilm formation in clinical isolates and their possible effect on antibiotic resistance in *P. aeruginosa*.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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