Original Article

Prevalence of Biofilm and Efflux Pump Genes Expression by PCR and Antibiotic Resistance Pattern in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is a significant pathogen responsible for nosocomial infections. *P. aeruginosa* is a multidrug-resistant (MDR) bacterium that is postulated to be the result of its plasmid-borne and intrinsic resistance to a number of pharmaceutical agents. This study examined the potential for biofilm formation, the distribution of the *psl*D, *pel*F, and *alg*D genes, and the expression of the *Mex*AB-*Opr*M efflux pump genes. Furthermore, the study examined the pattern of antibiotic resistance in multi-drug resistant *P. aeruginosa* isolates obtained from a range of clinical samples. A total of 76 strains of *P. aeruginosa* were obtained for this investigation from a range of clinical specimens. The susceptibility of the isolates to antibiotics was evaluated using the disk agar diffusion method. In conclusion, the term "multi-drug resistance" (MDR) is used to describe a specific pattern of resistance. The isolates were evaluated for the presence of three pivotal biofilm genes and their antimicrobial resistance patterns against ten standard antibiotic disks. The data were analyzed using version 25 of the SPSS statistical software. The examination of the isolates revealed that the most antibiotic sensitivity was associated with polymyxin, piperacillin, and ciprofloxacin. Additionally, the prevalence of biofilm-producing genes, specifically *psl*D, *pel*F, and *alg*D, was determined to be 68.4%, 80.3%, and 69.7%, respectively. The prevalence of *Mex*AB-*Opr*M efflux genes in the examined isolates was 89.5% for the *mex*A gene, 90.8% for the *mex*B gene, and 90.8% for the *opr*M gene. The majority of the isolates in this investigation exhibited the presence of efflux pump genes, as evidenced by the findings. Furthermore, a robust correlation was identified between a select number of efflux genes and biofilm formation or the antibiotics tetracycline, meropenem, amikacin, and polymyxin B.

Keywords: *Pseudomonas aeruginosa*, Efflux Pump, Biofilm, MDR.

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1. Introduction

Pseudomonas aeruginosa is a notable bacterial pathogen that has been linked to infections in hospitalized patients, individuals with compromised immune systems, and those with cystic fibrosis. The surveillance of nosocomial P. aeruginosa infections has revealed an alarming trend of rising antimicrobial resistance, including carbapenem resistance and multidrug resistance (1). *Pseudomonas aeruginosa* is a primary source of nosocomial infections, accounting for 10-15% of infections in patients hospitalized in intensive care units (ICUs) (2). *P. aeruginosa* exhibits the multidrug-resistant (MDR) phenotype, indicating its capacity to withstand the effects of multiple antimicrobial agents across three or more categories. (3) *Pseudomonas aeruginosa* exhibits natural resistance to a range of antibiotic classes, including aminoglycosides, carbapenems, beta-lactams, quinolones, and cephalosporins. Moreover, it can acquire new resistance genes through horizontal gene transfer, and the existence of diverse resistance mechanisms renders treatment more challenging (4). It is therefore evident that over 75% of burn-related mortalities are directly attributable to infection. Furthermore, the presence of multidrug-resistant (MDR) organisms has been demonstrated to increase mortality rates from 42% to 86%. The bacteria that reside in biofilms exhibit heightened resistance to antimicrobial agents and the human immune response, which can result in the establishment of persistent or chronic infections that are challenging to treat (6). Two extracellular polymeric matrices are produced by biofilm-forming bacteria, serving to bind the bacterial community to the biofilm. Polysaccharides are indispensable components of the biofilm matrix, as they provide structural support and confer resistance to antibacterial agents on the bacterial population (7). Alginate, Psl, and Pel are three exopolysaccharides that have been identified as key contributors to the formation of *P. aeruginosa* biofilms (8). There is now a substantial body of evidence supporting the view that biofilms play an important role in the ecological functioning of microbial communities and have a significant impact on human health through their involvement in the development of infections associated with healthcare settings. Estimates from the National Institutes of Health (NIH) suggest that bacterial biofilms are responsible for over 80% of chronic illnesses and 65% of microbiological disorders (9). Efflux pump systems are a significant factor in the antibiotic resistance of *Pseudomonas aeruginosa* isolates to various antibiotics (10). *P. aeruginosa* is capable of expressing twelve different Mex multidrug efflux pumps. The most significant efflux pumps in this family are the MexCD-MexXY-OprM, MexJK-OprM, MexAB-OprM, and OprJ pumps (11). The MexAB-OprM pump is the sole secretory pump present in all *Pseudomonas aeruginosa* isolates and is responsible for the bacteria's innate resistance to antibiotics (12).

2. Materials and Methods

A total of 76 isolates of *Pseudomonas aeruginosa* were collected over a seven-month period (February to October 2023) from clinical samples obtained from three laboratories in Tehran and Karaj. The samples included those from wounds, urine, and secretions. Following transfer to the TSB transport medium, the samples were delivered to the laboratory in a timeframe of slightly over two hours. The samples were cultivated for 24 hours at 37°C on plates containing Blood Agar (Ibresco, Iran) and MacConkey Agar (Ibresco, Iran). Subsequently, strains of *P. aeruginosa* were isolated through the application of conventional microbiological techniques. The susceptibility of the samples to the discs of tetracycline, amikacin, polymyxin B, meropenem, ticarcillin, ceftazidime, piperacillin, imipenem, ciprofloxacin, and gentamicin (Padtan Teb, Iran). The presence of the *psl*D, *pel*F, *alg*D, *mex*A, *mex*B, and *opr*M genes in *P. aeruginosa* isolates was determined using PCR and electrophoresis methods. The DNA of the isolates was extracted using the boiling method. The final optimized PCR reaction consisted of 1 µl of each primer (10 pmol), 0.5 µl dNTP (10 mM), 0.5 µl $MgCl_2$ (100 mM), 0.2 µl Taq DNA polymerase (1 unit), 2.5 µl PCR buffer (10×), and 0.5 µl of DNA template (100 µg/ml) in a total volume of 25 µl, with double distilled water. The temperature conditions for the *psl*D gene were as follows: denaturation for five minutes at 95°C, followed by 30 cycles of 95°C for one minute, 56°C for 40 seconds, 72°C for 45 seconds, and finally extension at 72°C for five minutes (13). The temperature conditions for the pelF gene are as follows: the process commences with heating at 94°C for 5 minutes, followed by 30 cycles of heating at 95°C for 60 seconds, 58°C for 40 seconds, and heating again at 72°C for 45 seconds. The final heating step is conducted at 72°C for 5 minutes (13). The temperature conditions for the algD gene are as follows: denaturation for five minutes at 94°C, followed by 30 cycles of 94°C for 45 seconds, 53°C for 45 seconds, 72°C for 45 seconds, and finally an extension at 72°C for seven minutes (14). The cycling program for efflux pump genes (*mex*AB-*opr*M) was modified as follows: an initial denaturation at 94 °C for 5 minutes was conducted, followed by 30 cycles of 94 °C for 45 seconds, 60 °C for 45 seconds, 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes (15-17). The molecular approach was optimized using *P. aeruginosa* ATCC 27853 as the control positive strain. The primer sequences and PCR conditions for the detection of genes are presented in Table 1.

2.1. Statistical Analysis

A model selection log-linear analysis was employed to ascertain the correlation between sociodemographic variables and the frequency of Pseudomonas aeruginosa isolation, utilising categorical data. All statistical analyses were conducted using the SPSS 25.0 software for Windows. A value of $P \leq 0.05$ was deemed statistically significant.

Table 1. Primers sequences as per standard reference.

Gene	Primer Sequences (5′ to 3′)	Product Size (bp)	annealing	Reference
***psl*D**	F: TGTACACCGTGCTCAACGAC R: CTTCCGGCCCGATCTTCATC	369	56°C	13
***pel*F**	F: GAGGTCAGCTACATCCGTCG R: TCATGCAATCTCCGTGGCTT	789	58°C	13
***alg*D**	F: ATG CGA ATC AGC ATC TTT GGT R: CTA CCA GCA GAT GCC CTC GGC	1310	53°C	14
***mex*A**	F: ACCTACGAGGCCGACTACCAGA R: GTTGGTCACCAGGGCGCCTTC	179	60 °C	15
***mex*B**	F: GTGTTCGGCTCGCAGTACTC R: AACCGTCGGGATTGACCTTG	244	60 °C	16
***opr*M**	F: CCATGAGCCGCCAACTGTC R: CCTGGAACGCCGTCTGGAT	205	60 °C	17

3. Results

3.1. Antibiotic Susceptibility

A total of 76 samples were collected, of which 53 were urine samples and 23 were from wounds. The respective proportions of these samples were 69.7% and 30.3%. The overall resistance of P. aeruginosa isolates to antimicrobial agents was 57.9% for meropenem, 44.7% for gentamicin, and 42.1% for tetracycline. The highest sensitivity was observed with regard to polymyxin, piperacillin, and ciprofloxacin, respectively. The overall antibiotic susceptibility pattern of the strains to antimicrobial agents is illustrated in Table 2. Figure 1 illustrates the prevalence of antibiotic resistance in clinical samples.

3.2.Gene Pattern Characterization

In this study, the presence of antibiotic resistance and biofilm-related genes was evaluated in P. aeruginosa isolates by means of a polymerase chain reaction (PCR) method. The presence of biofilm genes, specifically *psl*D, *pel*F, and *alg*D, was investigated in all isolates. The prevalence of the genes *psl*D, *pel*F, and *alg*D was found to be 68.4%, 80.3%, and 69.7%, respectively. Additionally, *P. aeruginosa* isolates were examined for efflux pump genes, and it was determined that the prevalence of *mex*A, *mex*B, and *opr*M genes was 89.5%, 90.8%, and 90.8%, respectively. The results of the polymerase chain reaction (PCR) for the genes from the *P. aeruginosa* isolates are presented in Figure 2. The frequency of biofilm-producing genes in clinical samples was as follows: the *alg*D gene was observed in 65.2% of wound samples and 71.7% of urine samples. The *pls*D gene was identified in 78.3% of wound samples and 64.2% of urine samples. Additionally, the *pel*F gene was observed in 82.6% of wound samples and 79.2% of urine samples. No statistically significant difference was observed between the frequency of biofilm genes in clinical samples ($P > 0.05$). The frequency of *Mex*AB-*Opr*M efflux pump genes in clinical samples was as follows: The frequency of the *mex*A gene in wound and urine samples was 95.7% and 86.8%, respectively. Additionally, the frequency of the *mex*B gene in wound and urine samples was 95.7% and 88.7%, respectively. The frequency of the *opr*M gene was 95.7% in wound samples and 88.7% in urine samples. No significant difference was observed between the frequency of efflux pump genes in clinical samples ($P > 0.05$). However, a significant relationship was identified between the *opr*M gene and tetracycline resistance ($P < 0.05$), as well as between the *alg*D gene and amikacin resistance ($P < 0.05$), and between the *psl*D gene and meropenem resistance ($P < 0.05$). Additionally, a significant relationship was observed between the *opr*M and *mex*B genes and polymyxin antibiotics ($P < 0.05$).

Table 2. Antimicrobial susceptibility pattern of *P. aeruginosa* isolates.

Antibiotics	Sensitive (%)	Intermediate (%)	Resistance (%)
Tetracycline	39 (51.3)	5 (6.6)	32 (42.1)
Amikacin	43 (56.6)	8 (10.5)	25 (32.9)
Plymyxin B	74 (97.4)	---	2 (2.6)
Meropenem	28 (36.8)	4 (5.3)	44 (57.9)
Ticarcillin	60 (78.9)	3 (3.9)	13 (17.1)
Ceftazidime	49 (64.5)	3 (3.9)	24 (31.6)
Piperacillin	64 (84.2)	---	12 (15.8)
Imipenem	33 (43.4)	4 (5.3)	39 (51.3)
Ciprofloxacin	53 (69.7)	7 (9.2)	16 (21.1)
Gentamicin	39 (51.3)	3 (3.9)	34 (44.7)

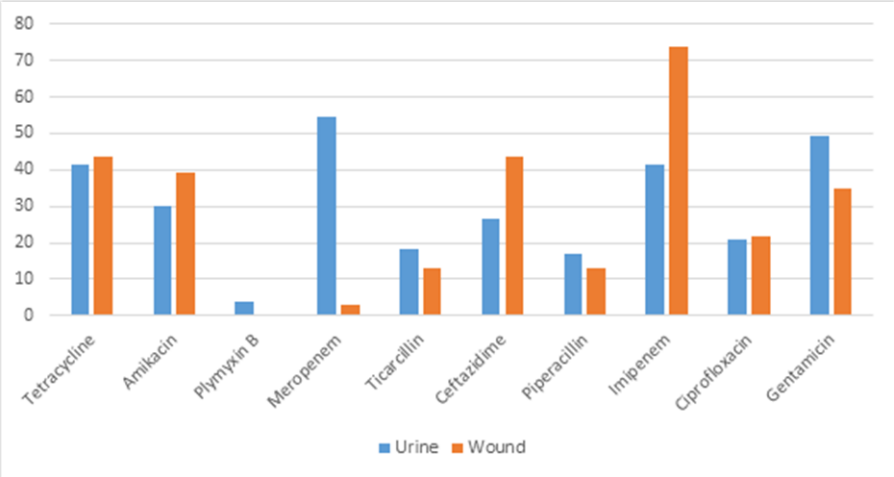

Figure 1. Percentage of antibiotic resistance in clinical samples.

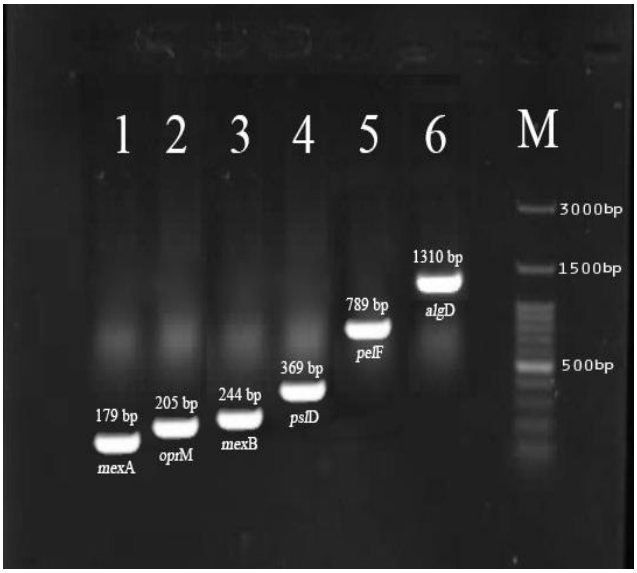

Figure 2. Amplification of *psl*D, *pel*F, *alg*D, *mex*A, *mex*B, and *opr*M genes from *P. aeruginosa* isolates. Lane M, DNA marker (100 bp); Lane1, *mex*A (179 bp); Lane 2, *opr*M (205 bp); Lane3, *mex*B (244 bp); Lane 4, *psl*D (369 bp); Lane 5, *pel*F (789 bp) and Lane 6 *alg*D (1310 bp).

4. Discussion

In this study, 76 isolates of *Pseudomonas aeruginosa* were examined to determine the prevalence of biofilm-producing genes in clinical samples. The results indicated that *psl*D 52 was present in 68.4% of the samples, *pel*F in 61 (80.3%), and *alg*D in 53 (69.7%). Additionally, the prevalence of *Mex*AB-*Opr*M efflux pump genes in clinical samples was observed, with *mex*A, *mex*B, and *opr*M genes present in 68 (89.5%), 69 (90.8%), and 69 (90.8%) samples, respectively. The antibiotic resistance pattern results demonstrated that meropenem exhibited the highest resistance (57.9%), while polymyxin B demonstrated the lowest resistance (2.6%). The prevalence of multidrug-resistant (MDR) Pseudomonas aeruginosa isolates has increased worldwide in recent years (19). In the study conducted by Begi et al. the resistance rate to imipenem and meropenem antibiotics was found to be 45.45% and 39.39%, respectively. These findings differ from those of the present study. Additionally, the resistance results for imipenem and meropenem antibiotics differ from those reported by Siasi et al. (21), who conducted their study in Tehran. In a study conducted by Aminizadeh et al. on *Pseudomonas aeruginosa* isolated from the ICU, the highest and lowest levels of resistance were observed, respectively, to ceftazidime (87%) and imipenem (5.6%) (22). In a study conducted by Fazeli et al. in Isfahan on 66 isolates of *Pseudomonas aeruginosa* isolated from the ICU

department, 75.8% of the isolates demonstrated resistance to the antibiotic ceftazidime, and 72.7% of the isolates exhibited resistance to piperacillin (23). In the study conducted by Rajabi et al., the prevalence of the *alg*D, *pel*F, and *psl*D genes was 78.6%, 70.5%, and 36.6%, respectively. The prevalence of the *alg*D and *pel*F genes was similar to that observed in our study (24). In the study conducted by Nawaz et al., the resistance rates to imipenem, meropenem, and gentamicin antibiotics were found to be 90.3%, 92.3%, and 61.5%, respectively. These findings differ from those observed in the present study. A review of existing studies indicates that the prevalence of resistant *P. aeruginosa* is highly dependent on geographical area, biological patterns, and the use of common antibiotics in the region. This is the reason for the discrepancy in the results of the studies. Consequently, an investigation into the alteration of antibiotic resistance patterns over specific time periods may prove an efficacious approach to the treatment of *P. aeruginosa* infections. The use of an antibiogram test is currently being recommended as a means of preventing the development of antibiotic resistance. It appears that the identification of safe strains through PCR testing is a crucial step. Based on the available evidence, the potent component of the bacterial attack's biofilm organization capacity and cytotoxicity impact may offer promising avenues for treatment beyond the conventional antimicrobial approach. Moreover, the pharmaceutical industry may be encouraged to provide unused antimicrobials to elucidate the resistance issue if the isolated *P. aeruginosa* exhibits a high prevalence of antimicrobial resistance.

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Note Applicable.

Authors' Contribution
Literature review and research, conceptualization, methodology, supervision, project administration, writing-reviewing and editing, methodology, investigation, studies analysis: A.S.B., L.S.M.M., A.F.M., Writing original draft preparation, writing-reviewing and editing, and methodology: F.T.P., R.K.R., S.F.A., investigation. Validation and Reviewing: R.K.R., A.S.B.

Ethics
Not Applicable.

Conflict of Interest
The authors certify no conflict of interest.

Data Availability
All data generated or analyzed during the course of this study has been incorporated into this published article.

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