Original Article

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Evaluation of Biofilm Formation, Alginate Production, Pattern of Drug Resistance, and the Presence of Efflux Pump *MexAB-OprM, MexXY* (-OprA), and *AmpC* Gene in Clinical Isolates of *Pseudomonas aeruginosa*

Saeid Besharati [1], Mohammad Rahbar [2], Neda Soleimani [1]

[1] Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran, [2] Departments of Microbiology, Iranian Reference Health Laboratory Research Center, Ministry of Health and Medical Education, Tehran, Iran

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Correspondence to: Soleimani N
Address: Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
Email address: N_Soleimani@sbu.ac.ir

Background: One of the most significant factors contributing to multidrug resistance in *Pseudomonas aeruginosa* infections is the formation of biofilms and the production of alginate. This study aimed to evaluate the overexpression of efflux pumps *MexAB-OprM*, *MexXY* (-OprA), and the *AmpC* gene and investigate biofilm and alginate in *P. aeruginosa* clinical isolates.

Materials and Methods: One-hundred isolates of *P. aeruginosa* were collected two government-specialized hospitals from February 2024 to June 2024 in Tehran, Iran. The disk diffusion method was used for antimicrobial susceptibility and detecting the pattern of antibiotics. We used a microtiter plate and carbazole assay to investigate biofilm formation and alginate production, respectively. We investigated the efflux pump *MexAB-OprM*, *MexXY* (-OprA), and the *AmpC* gene expression with real-time PCR and its correlation with biofilm, alginate, and antibiotic resistance.

Results: 30 multidrug-resistant (MDR) isolates were detected, and 27 antibiotic patterns were obtained. A significant relationship between biofilm formation and resistance to PRL was observed ($P<0.01$). All of the samples with more than 250µg/ml level of alginate production were resistant to Piperacillin-Tazobactam (PTZ), which was significant ($P<0.05$). Also, the relationship between alginate production and strong biofilm formation was significant. The expression of resistance-nodulation-division (RND) efflux pumps *MexAB-OprM*, *MexXY* (-OprA), and *AmpC* gene in MDR isolates of *P. aeruginosa* was significantly increased.

Conclusion: High prevalence of MDR, along with high expression of efflux pump genes, was concerning. High production of biofilm formation and its relationship with alginate were observed in *P. aeruginosa* clinical isolates. To prevent the spread of antibiotic resistance, implementing monitoring methods and not overusing and abusing antibiotics is necessary.

Keywords: *Pseudomonas aeruginosa*; Biofilm; Alginate; Efflux pump; Antibiotic resistance

INTRODUCTION

Pseudomonas aeruginosa is a gram-negative bacillus found in various habitats. This feature, along with a high and diverse level of resistance to antibiotics, makes it a deadly organism (1, 2). This bacterium causes a variety of bacteremia infections, hospital-acquired infections, pneumonia, bloodstream infections, and urinary tract infections. Biofilm formation is a major cause of chronic

infections, and alginate plays a role in cystic fibrosis, inflammatory response, and protection against phagocytosis (3-6). *P. aeruginosa* has a wide range of intrinsic or acquired resistance mechanisms against antipseudomonal antibiotics, among them biofilm formation, alginate production, membrane permeability change, and efflux pump can be mentioned. This mechanism, with extrusion of antibiotics from the bacterial cell, can cause a multidrug-resistant (MDR) phenotype (7). One of the common mechanisms of antibiotic resistance in bacteria (especially Gram-negative) is the efflux pumps, which are often coded by chromosomes (8, 9). Among the efflux pumps, reports have shown that some of them, including *MexAB-OprM*, *MexXY* (-OprA), along with the overexpression of chromosomal cephalosporinase *AmpC,* play an important role in increasing antibiotic resistance (10). *AmpC* enzymes are a group of beta-lactamases whose overexpression can cause resistance to third-generation cephalosporins (3GCs) and piperacillin/tazobactam (11, 12). Efflux pumps are tripartite protein complexes that are composed of three different proteins. *MexA, MexX*; periplasmic membrane fusion protein, *MexB, MexY*; a resistance-nodulation-cell division transporter (RNDt), *OprM, OprA*; a channel-forming outer membrane factor (OMF) (13). The operon *MexAB-OprM* was the first and the main contributor to antibiotic resistance, which is controlled by the repressor genes *mexR* [134], *nalC* [135], and *nalD* (7). Expression of *MexAB-OprM* increased in antibiotic-resistant *P. aeruginosa* strains and exported antibiotics such as macrolides, quinolones, chloramphenicol, lincomycin, tetracycline, novobiocin, and most β-lactams out of the pump. As the drug concentration increases, the conformational change caused by *MexB* pushes the active molecules towards the tunnel formed by *MexA* and *OprM* (14). Expression of *MexXY* (-OprA) increased in antibiotic-resistant *P. aeruginosa* strains and export antibiotics, such as aminoglycoside resistance. The cooperation of *OprA*, *MexXY,* and the OMF *OprA* in some *P. aeruginosa* strains can cause resistance to carbenicillin and sulbenicillin (15). The operon *MexXY* (-OprA) is a contributor to antibiotic resistance, which is controlled mainly by the repressors *MexZ*, *ParRS*, and *ArmZ* (7). The efflux pumps and biofilm have a positive relationship with each other, and the expression of the efflux pump can increase the formation of biofilm. The study conducted on the efflux pump showed that its inhibitors can significantly reduce the biofilm (16-18).

This study aimed to evaluate biofilm formation and the expression of resistance-nodulation-division (RND) efflux pumps *MexAB-OprM*, *MexXY* (-OprA), *AmpC* genes in clinical isolates of *P. aeruginosa*.

MATERIALS AND METHODS

Sample collection and identification

One hundred clinical isolates of *P. aeruginosa* were collected from two government-specialized hospitals (Milad and Mehr hospitals) from February 2024 to June 2024. The sources of collection isolates were sputum, blood, wounds, urine, and tracheal. All of the isolates were identified by their production of blue-green pigment on Mueller-Hinton agar (Merck Co., Germany), with a distinctive odor, Gram staining, and the ability to produce oxidase and catalase. They also exhibited a reaction (K/K) on TSI agar slants and were able to grow at 42°C on Nutrient agar (Merck Co., Germany). All isolates were stored at -70°C in nutrient broth medium (Merck, Germany) containing 15% glycerol until analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility to Piperacillin 100 µg (PRL), Piperacillin-Tazobactam 100/10 µg (PTZ), Ceftazidime 30 µg (CAZ), Aztreonam 30 µg (ATM), Doripenem 10 µg (DOR), Imipenem 10 µg (IMI), Tobramycin 10 µg (TN), Amikacin 30 µg (AK), Gentamicin 10 µg (GM), Ciprofloxacin 5 µg (CIP), Norfloxacin 5 µg (NOR) and Ofloxacin 5 µg (OFX) (Mast Diagnostics Group Ltd., UK) was performed by disc agar diffusion method. *P. aeruginosa* ATCC 27853 was used as the reference strain for antibiotic susceptibility testing. *P. aeruginosa* isolates adjusted to 0.5 McFarland, inoculated on Muller-Hinton agar (Merck, Germany) (24 hours at 37◦C). Based on

respective zone diameter breakpoints (mm) according to CLSI 2023, phenotypes "susceptible", "intermediate", and "resistant" were defined. Isolate with resistant to at least three different classes of antimicrobial agents were defined as MDR (19).

Biofilm formation assay

The microtiter plate method was used for investigating biofilm formation in *P. aeruginosa* isolates. Briefly, isolates were cultured in MHB for 24 h at 37°C, diluted (1:100), and 200 µL was inoculated into 96-well flat-bottomed (SPL, South Korea) with triplicate. Washed with PBS three times and dried, fixed with methanol, and stained with crystal violet. Finally, acetic acid was measured at 570 nm by an ELISA reader (elx808, BioTek, USA). The biofilm formation levels were classified: $OD_{test\ (t)} \leq OD_{control\ (c)}$ non-biofilm, $OD_c < OD_t < 2xOD_c$ weak biofilm, $2x\ OD_c < OD_t < 4xOD_c$ moderate biofilm, $OD_t \geq 4xOD_c$ strong biofilm (Figure 1).

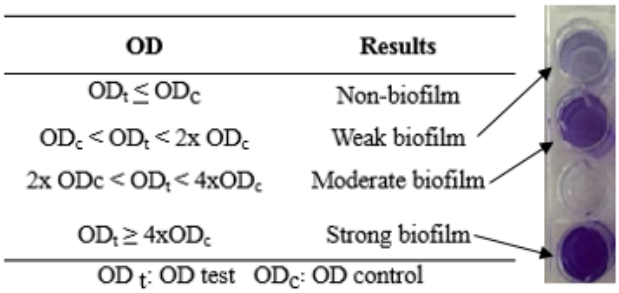

Figure 1. Interpretation of result of OD to detect the level of biofilm formation (42).

Alginate assay

The Knutson method with modification of the carbazole method (20) used for the investigation of alginate in *P. aeruginosa* isolates. Briefly, incubated 24 hours at 37°C, adjusted to 0.5 McFarland, incubated 48 hours at 37°C, culture mixed with borate-sulfuric acid and carbazole (with vortex). Incubated suspension for 30 min at 55°C. Finally, OD was measured at 540 nm by an ELISA reader (elx808, BioTek, USA).

RNA extraction, cDNA synthesis, and primer design

Primers were designed with Vector NTI version 11.5 for used real-time PCR (Table 1). The following cycling conditions were used for the PCR program: initial activation step 1 cycle of 10 min at 95°C, denaturation 40 cycles of 15 sec at 95°C, annealing step 40 cycles of 30 sec at 60 °C, extension 40 cycles of 30 sec at 72 °C, melting curve 1 cycles of 30,20,20 sec at 95,60,95°C respectively. Total RNA from *P. aeruginosa* isolates was extracted from exponentially grown bacteria (0.5 McFarland at OD 600 nm) using RNA extraction kit (GeneAll Biotechnology Co. Ltd, Korea) based on the manufacturer's instructions. The RNAs were then treated with RNase-free DNase I. The purity of the products was determined by a NanoDrop spectrophotometer (ND-1000, Wilmington, USA). Synthesis of cDNA was performed using (ATR-MED, Iran). Briefly, the following compounds (Template RNA, Random Hexamer, diethylpyrocarbonate (DEPC)-treated) were added to a sterile 0.2 µl microtube free of any nuclease, on ice. Then mixed gently and incubated at 70°C for 5 minutes. cDNA Synthesis Mix (include: strand buffer, dNTPs, RNasein, M-MLV). The following compounds were added to the previous reaction product to bring the total volume of the reaction product to 20 µl. The resulting mixture was incubated for 60 minutes at 37°C, then 5 minutes at 70°C, and then transferred to -20°C.

Table 1. Primers used for real-time PCR

Gene	Sequence of primers	Product size	Ref
mexAB	F:CTGCTCAAGGTCAATCCCG R:GCGTTGATGCTGTAGTCCTG	89bp	Designed in this study
mexXY	F:CGATCAGGAAGGTGGTCAG R:CAGGTACATCACGGCGAAC	147bp	Designed in this study
AmpC	F:CAGAAGGACCAGGCACAGAT R:CGATGCTCGGGTTGGAATAG	93bp	Designed in this study
16s rRNA	F: GCAATTGCGTGTTTCTCCG R: CCTGGACTTTGTGGATAACC	174bp	Designed in this study

Analysis of efflux pump gene expression and the *AmpC* gene

Real-Time PCR was performed by a Real-Time PCR machine (Stratagene MX3000), and the expression of *mexA, mexX,* and *AmpC* with the housekeeping *16s rRNA* gene was run in triplicate. DEPC was considered a negative control. *P.aeruginosa* isolates were considered to have efflux pump genes overexpression when the transcriptional

levels of *MexAB-OprM*, *MexXY* (–OprA), and *AmpC* were at least 20, 40, and >40-fold higher than strain PAO1 (the wild-type reference), using the $2^{-\Delta\Delta CT}$ method (21).

Statistical analysis

The results were statistically analyzed using GraphPad Prism (version 8.0.2) and SPSS (version 25).The results of expressing gene isolate *P.aeruginosa* with the control bacteria by the t-test. The correlation between antibiotic resistance patterns, biofilms, and Alginate using Pearson's Chi-square test. P value of <0.05 was considered to be statistically significant.

RESULTS

Clinical samples

A total of 100 isolate *pseudomonas aeruginosa* were collected during the study period from different clinical samples (45 were from urine (45%), 31 from sputum (31%), 13 from tracheal (13%), 8 from wound (8%), and 3 from blood (3%)). The patients were admitted from two hospitals from February 2024 to June 2024. Among this sample, 54 isolates (54%) were male, and 46 isolates (46%) were female. Fifty-three isolates were obtained from inpatients, and 47 isolates were from outpatients. *Pseudomonas aeruginosa* ATCC 27853 was used as the antibiogram control strain, *P. aeruginosa* 8821M as the alginate control strain, and *P. aeruginosa* PAO1 as the biofilm formation and real-time PCR control strain.

Pattern of antibiotic resistance

A total of 12 antibiotics were evaluated by the disk diffusion method. 27 patterns of antibiotics showed (Table 2). Resistance to Ofloxacin (30%) was the highest, and resistance to Piperacillin/Tazobactam (18%) was the lowest. 59 isolates were susceptible to all of the antibiotics (pattern 1), and 11 isolates were resistant to all of the antibiotics (pattern 27). Non-susceptibility to at least one antibiotic in three or more different antibiotic families was considered as multiple drug resistance (MDR). 21.2% of isolates from outpatients (10 /47) were MDR, and 37.7% isolated from inpatients (20/53) were MDR. In total, from 30 MDR isolate 66.7% (20/30) were inpatients and 33.3% (10/30) was outpatient. Although MDR isolates from inpatient was more than from outpatients, this difference was not significant ($P = 0.07$).

Biofilm formation product

The results of biofilm formation in several patterns of antibiotics and isolates are shown in Table 2. In total, 100 isolates of *P. aeruginosa*, all of isolates were considered biofilm producers (33% strong, 53% moderate, and 14% weak). Most of the strong biofilm production was related to the urine sample, with 47% (47/100), and then the sputum sample with 29% (29/100). In a total of 47 urine samples, only 6.3% (47/3) of them had weak biofilm, and 93.7% (47/44) had moderate and strong biofilm, which was significant (P <0.05).

Biofilm formation and MDR

All of the samples with moderate and strong biofilm were resistant to PRL. A significant relationship between biofilm formation and resistance to PRL was observed (P=0.01). No significant relationship between biofilm formation and other antibiotics was observed. Among 30 MDR isolates, 14 isolates (46.6%) produced strong biofilm, 15 isolates (50%) produced moderate biofilm, and 1 isolate (3.33%) produced weak biofilm formation. Among MDR isolates, the percentage of strong biofilm formation was higher than that of weak biofilm. The amount of strong and moderate biofilm formation, 96.6% (29/30) in MDR isolates, was significantly higher than weak biofilm (3.33%, 1/30). Significant correlations between biofilm formation and MDR have been observed (P <0.05).

Alginate production

In 100 isolates of *P. aeruginosa*, the level of alginate production in 10 isolates was <250 µg/ml, in 53 isolates was between 250-400 µg/ml, and in 37 isolates was >400 µg/ml. The highest level of alginate production was related to urine, with 47% (47/100), and the sputum, with 29% (29/100). In 47 urine samples, 93.6% (44/47) had alginate production >250 µg/ml, and only 6.4% (3/47) had <250 µg/ml.

Table 2. Pattern of antibiotics of *P. aeruginosa* isolates

Number of isolate (N=100)	Groups	MDR	Pattern of antibiotic (S, I, R)	Biofilm	Alginate
59	**1**	N	Sensitive to all antibiotic	11A, 33B, 15C	6A, 28B, 25C
3	**2**	N	IMI(I)	2C, 1B	2B,1C
1	**3**	N	IMI(I) ,OFX(I)	B	B
1	**4**	N	ATM(I), IMI(I)	B	B
1	**5**	N	CAZ(I), ATM(I), OFX(I)	A	B
1	**6**	N	IMI(I), DOR(I), OFX(I)	B	C
1	**7**	N	PRL(I), CAZ(I), ATM(I), DOR(I), OFX(I)	A	A
1	**8**	N	IMI(R), GM(I), TN(I)	B	B
1	**9**	N	PRL(I), CAZ(I), ATM(R), IMI (R), TN(I), OFX(I)	C	A
1	**10**	N	PRL(I), CAZ(I), ATM(R), IMI(R),TN(I), OFX(I)	C	C
1	**11**	P	IMI(R), AK (I), GM(R), TN(R), CIP(I), OFX(R)	C	A
1	**12**	P	PRL(I), PTZ(I), CAZ(R), IMI(I), DOR(R), GM(R), TN(R), CIP(R), NOR(R), OFX(R)	C	A
1	**13**	P	PRL(I), ATM(R), GM(R), TN(R), AK(I), CIP(I)	C	C
1	**14**	P	CAZ(R), IMI (I), DOR(R), GM(R), TN(R), CIP(R), NOR(R), OFX(R)	B	A
1	**15**	P	PRL(I), PTZ(I), ATM(R), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	B	B
1	**16**	P	PRL(I), PTZ(I), CAZ(R), IMI(R), DOR(R), GM(R), TN(R), CIP(R), NOR (R), OFX(R),	C	B
1	**17**	P	CAZ(R), ATM(R), IMI(R), DOR(I), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	C	B
1	**18**	P	PRL(I), CAZ(R), IMI(R), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	C	C
1	**19**	P	PRL(I), CAZ(R), ATM(R), IMI(R), DOR(R), GM(R), TN(R), AK(I), CIP(R), NOR(R), OFX (R)	A	B
1	**20**	P	PRL(R), PTZ(I), ATM(I), IMI(R), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	B	B
1	**21**	P	PRL(R), PTZ(R), CAZ(I), ATM(I), IMI(R), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(I), OFX(R)	B	B
1	**22**	P	PRL(I), PTZ(R), ATM(I), IMI(R), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	B	B
1	**23**	P	PRL(R), PTZ(I), CAZ(I), ATM(I), IMI(R), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	B	B
2	**24**	P	PRL(I), PTZ(R), CAZ(R), ATM(I), IMI(R), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	2C	B
1	**25**	P	PRL(R), PTZ(I), CAZ(R), ATM(I), IMI(I), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	B	A
3	**26**	P	PRL(R), PTZ(R), CAZ(R), ATM(R), IMI(I), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	2B, 1C	3B
11	**27**	P	PRL(R), PTZ(R), CAZ(R), ATM(R), IMI(R), DOR(R), GM(R), TN(R), AK(R), CIP(R), NOR(R), OFX(R)	4C, 7B	4B, 7C

PRL: piperacillin; PTZ: piperacillin-tazobactam; CAZ: ceftazidime; ATM: asteronam; IMI: imipenem; DOR:doripenem; GM: gentamicin TN: tobramycin; AK: amikacin; CIP: ciprofloxacin; NOR:norfloxacin OFX:ofloxacin. **Biofilm**: A: Weak, B: Moderate, C: Strong. **Alginate**: A: <250 µg/ml, B: 250-400 µg/ml, C: >400 µg/ml. N: negative, P: positive, **Pattern of antibiotic** S: sensitive , I: intermediate , R: resistance

Alginate and MDR

All of the samples with more than 250 µg/ml level of alginate production were resistant to PTZ; a significant relationship between level of alginate production and resistance to PTZ was observed (*P* =0.03). We had no significant relationship between the level of alginate production and other antibiotics. Among 30 MDR isolates, 4 isolates had alginate production levels of less than 250 µg/ml, 13 isolates had levels between 250 and 400 µg/ml, and 13 isolates had levels greater than 400 µg/ml.

Alginate and biofilm formation

In a study of 33 isolates that demonstrated strong biofilm production, 93.9% (31 out of 33) had an alginate production level exceeding 400 µg/ml. In another group of 41 isolates with an alginate production level of 400 µg/ml, 90.2% (37 out of 41) exhibited moderate to strong biofilm formation. A significant relationship was found between alginate production and strong biofilm formation. Specifically, the level of alginate production greater than 400 µg/ml was significantly higher in isolates that formed strong biofilms compared to those with weak or moderate biofilms (P < 0.03).

Efflux pump and *AmpC* gene with MDR

The investigation showed that the expression of efflux pump genes *mexAB*, *mexXY*, and *ampC* in MDR isolates *P. aeruginosa* was significantly increased that show in figure (*P* <0.00) (Figure 2).

Efflux pump and *AmpC* gene with biofilm formation

The results revealed that out of 14 strong biofilm isolates of *P. aeruginosa*, the expression of the mexAB gene increased more than 40 times in 8 samples. Additionally, 4 samples showed expression between 21 and 40 times, while 2 samples had expression levels below 20 times. In total, among 29 isolates with moderate to strong biofilms, 18 cases demonstrated mexAB gene expression exceeding 40 times.

Moreover, from the 14 strong biofilm isolates, 4 cases exhibited increased expression of the mexXY gene by more than 40 times, 6 cases were in the range of 21 to 40 times, and 4 cases were below 20 times. In total, out of the 29 isolates, 7 cases of moderate to strong biofilm showed mexXY gene expression greater than 40 times.

Although the frequency of strong biofilms was higher, there was no significant correlation with mexAB (P = 0.9) and mexXY (P = 0.5). Among the 14 cases of strong biofilm, only 1 case had gene expression exceeding 40 times, and in 4 cases, the AmpC gene expression was between 21 and 41 times. These findings were not significant (P > 0.05).

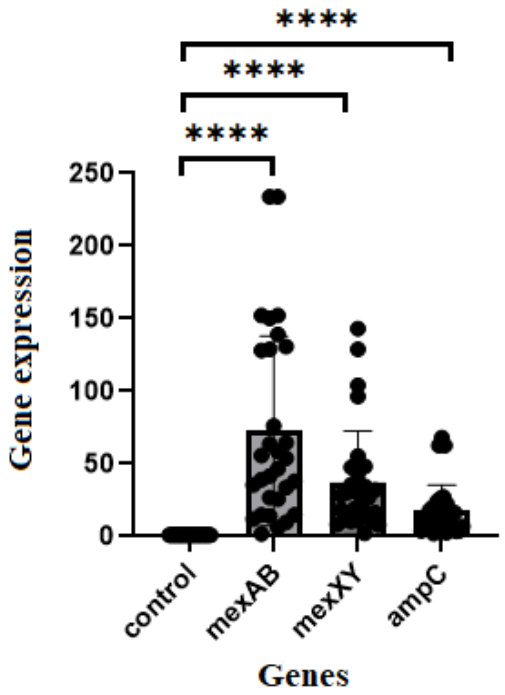

Figure 2. The expression of efflux pump genes and *ampC* in MDR isolates of *P. aeruginosa*. **** = significant *p value*, *P* >0.00

Efflux pump and *AmpC* gene with alginate

The results indicated that out of 13 isolates of *P. aeruginosa* with alginate concentrations greater than 400 µg/ml, the expression of the mexAB gene increased more than 40 times in 8 isolates. In 4 cases, the mexAB gene expression ranged between 21 and 41 times, while the remaining isolate had expression levels below 20 times. Additionally, of the 13 isolates with alginate levels exceeding 400 µg/ml, 2 exhibited mexXY gene expression greater than 40 times, and 6 cases showed expression between 21 and 41 times, with the other isolates under 20 times. However, there was no significant correlation found

for mexAB (P > 0.05) or mexXY (P > 0.05). Among the 13 isolates with alginate concentrations over 400 µg/ml, none had gene expression exceeding 40 times, and 4 cases of AmpC gene expression were observed to be between 21 and 41 times. Again, there was no significant correlation (P>0.05).

DISCUSSION

P. aeruginosa is a pathogenic bacterium, and the widespread occurrence of antibiotic resistance has raised this bacterium as one of the concerns in the field of health and treatment. Biofilm and alginate are only part of the effective mechanisms in pathogenesis and play an effective role in the occurrence of MDR. All of the clinical isolates of *P. aeruginosa* were able to produce biofilm, alginate, and multidrug resistance (MDR). Biofilm formation in *P.aeruginosa* isolates has been investigated in several studies that showed a high rate of biofilm production (22, 23). This result was consistent with our studies. The present study, out of a total of 47 urine samples, only 6.3% (47/3) had weak biofilm, and 93.7% (47/44) had moderate and strong biofilm ($P< 0.05$), which was consistent with the study of de Sousa and Nadar, who showed a high rate of strong biofilm formation in urine samples (24, 25). The amount of strong and moderate biofilm formation in MDR isolates was significantly higher than weak biofilm (P=0.05), which was consistent with other studies (26, 27).

In this study, strong biofilm in PRL-resistant isolates was 31.5% ($P= 0.01$), which was consistent with the study of Heydari et al. (28). The highest percentage of alginate production above 400 µg/ml was observed for isolates isolated from urine (48.9%) and sputum (37.9%). Alginate production in isolates with PTZ (norfloxacin) resistance was 61.1% (11/18). The results of other studies were consistent with our study and showed the highest antibiotic resistance against the PTZ (29). There was a direct relationship between alginate production and biofilm formation, so that increasing alginate production increased strong biofilm formation, which was statistically significant ($p < 0.05$). This relationship was shown in several studies (30, 31). This study showed the important role of strong biofilm formation in the increase of antibiotic resistance. Also, it has been shown that the formation of biofilm with alginate can significantly increase the resistance to antibiotics and challenge the treatment process.

This study showed a significant correlation between biofilm formation and antibiotic resistance patterns. Out of a total of 47 urine samples, only 6.3% (3/47) had weak biofilm, and 93.7% (44/47) had moderate and strong biofilm, which was significant (P=0.03). In this study, by examining 12 antibiotics, 27 patterns of drug resistance were obtained. Without a doubt, the study of all antibiotics referred to CLSI of different antibiotics can provide a wider range of MDR, Extensively Drug-Resistant (XDR), and totally drug-resistant (TDR), which is a valuable step to identify resistant antibiotics isolate *P. aeruginosa*. This finding also showed that the MDR rates are high among *P. aeruginosa* clinical strains in Iran, which is similar to the antibiotic resistance values reported by Mirzaei et al. (32) from Northeast of Iran, and Goli et al. from Iran (33). A study by Rashid Mahmood et al**.** showed high resistance to gentamicin 83.87% (n=26/31), amikacin 54.83% (n=17/31), and imipenem 22.58% (n=7/31), which was more than this report (34). Also, according to Saeli et al., the antibiotic resistance of *P. aeruginosa* isolates: tobramycin 45.5% (91/200) and amikacin 43% (86/200) was higher resistance to tobramycin and amikacin than the present study (35). Despite the concerns about the increase in the indiscriminate use of antibiotics, special attention to antibiogram before starting treatment and supportive policies to reduce the over-prescription of antibiotics are important factors to reduce antibiotic resistance in recent years in Iran.

Ardabil and Tehran are both provinces in Iran. Studies conducted in Ardabil have shown high resistance rates among various antibiotics, with penicillins showing resistance as high as 94%, carbapenems at up to 66.7%, monobactams at 42.9%, cephems up to 50%, and fluoroquinolones reaching 76.2% (35, 36). In contrast, the

current study conducted in Tehran revealed lower resistance rates: piperacillin at 19%, carbapenems (specifically IMI at 25% and DOR at 27%), monobactams at 19%, and fluoroquinolones with OFX at 30%, NOR at 27%, and CIP at 29%. One of the effective factors in the high levels of resistance in Ardabil hospitals is their extensive use in the treatment of a wide range of infections, and another reason for the difference in antibiotic resistance compared to the recent study is the diverse geographical areas for sampling patients.

A study conducted in the northwest region of Iran in 2024 revealed that 30% of clinical *Pseudomonas aeruginosa* isolates were multidrug-resistant (MDR) (37). This percentage is notably higher than the findings of Lee et al., which reported that MDR *P. aeruginosa* accounted for 22.1% of isolates in Asia and 7.3% in Oceania in 2019 (38). The rising incidence of multidrug resistance (MDR) in Asian countries is concerning.

Our study revealed a correlation between the overexpression of efflux pumps and MDR. Specifically, we found that 96% (29 out of 30) of our MDR isolates of *P. aeruginosa* exhibited overexpression of AmpC and mexB, while 100% (30 out of 30) showed overexpression of mexY. This result is much higher than reported by Xavier et al. in Brazil (39), Matsumoto et al. in Japan (40), and Rahbar et al. in Iran (41).

This study demonstrates a significant correlation between the overexpression of efflux pump genes mexAB, mexXY, and AmpC in the MDR phenotype of *P. aeruginosa* clinical isolates ($p < 0.05$). This result is in line with previous studies by Elbrolosy et al. from Egypt (42), and Goli et al. from Iran (33). In the current study, we measured the expression of efflux pump genes (*MexAB-OprM*, *MexXY*(-*OprA*)) and *AmpC* along with biofilm formation and alginate production. Most of the studies conducted in Iran on the expression of efflux pump genes and biofilm production were concentrated, and limited studies in Iran investigated efflux pump, biofilm, and alginate together.

CONCLUSION

The present study showed the important role of strong biofilm formation and alginate in the increase of MDR. Also, our results showed that *P. aeruginosa* isolates with antibiotic resistance overexpressed efflux pump genes *mexAB*, *mexXY*, and *ampC* genes. As a result of all the efforts made to control antibiotic resistance, the spread of MDR strains isolated from clinical samples is worrying. Genotyping of *P. aeruginosa* isolates in epidemiological studies, and a wide range of antibiotics to identify MDR and XDR, is useful to deal with it.

Limitations

Our limitation in this research project was the lack of funding. If there were more funds, it could be developed in more areas with more diverse samples.

Declaration of Competing Interest

There is no conflict of interest.

Funding

No specific funding has been provided for research.

Ethics approval and consent to participate

This article is the output of a PhD thesis. This study was approved by the Ethics Committee (IR.SBU.REC.1403.115) of Shahid Beheshti University, Tehran, Iran. The study protocol was approved by the Faculty of Microbiology and Biotechnology at Shahid Beheshti University (Tehran, Iran).

Consent for publication

Not applicable.

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