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The Relation Between Biofilm Formation and Alginate Production in *Pseudomonas aeruginosa;* An Important and Neglected Topic

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ABSTRACT

Background and Aim: *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the most common opportunistic bacteria causing healthcare-associated infections (HAIs). Alginate as an adhesion substance plays role in its binding to inanimate surfaces and protecting against environmental conditions. Biofilm also increases the bacterial resistance to antimicrobials and immune system. The present study was aimed to survey the antimicrobial resistance pattern, biofilm formation, and alginate production in the clinical isolates of *P. aeruginosa*.

Materials and Methods: In this cross-sectional study in a period of one year 90 non-duplicative *P. aeruginosa* were isolated from the clinical samples. Antimicrobial susceptibility testing was performed by disk diffusion method. Biofilm formation and alginate production were measured by microtiter plate and carbazole assay, respectively. Polymerase chain reaction was conducted for detection of *pslA, pelA, ppyR, algD, algU* and *algL* genes.

Results: The highest resistance rate (74.4%) was related to ceftazidime. Alginate production was found in 87.8% of the isolates, which production levels were <250 μgml^{-1}, 250-400 μgml^{-1}, and >400 μgml^{-1} in 7.6%, 53.2%, and 39.2 of the isolates, respectively. The categories of biofilm formation in strains were as weak (11.1%), moderate (24.1%), and strong (64.8%). The prevalence of *ppyR, algD*, *algU, algL, pslA* and *pelA* genes were 100.0%, 92.2%, 86.6%, 67.7%, 85.2% and 42.5%, respectively.

Conclusion: The results highlighted an alarming trend in *P. aeruginosa* strains antibiotic resistance rates. A significant relationship was also observed between the alginate production and the biofilm formation. Thus, periodic monitoring, adherence to the antibiotic stewardship, avoiding arbitrary prescribing, and screening tests are unavoidable.

Keywords: Alginate, Antibiotic Resistance, Biofilm, PCR, *Pseudomonas aeruginosa*

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1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative flagellated bacterium that is considered as a main challenge in therapeutic failure, long-stay hospitalization, and imposing the cost burden on the health system **(1, 2)**. This bacterium is one of the causative agents for the serious infections, particularly in immunosuppressed cases such as cystic fibrosis, cancer, intensive care units (ICU), burns and wounds **(3)**. Todays, the appearance of multidrug resistant (MDR) isolates has become one of the most important complications in the treatment of these infections worldwide **(4)**. Drug resistance and biofilm formation are important factors of survival and colonization of this bacterium **(5)**.

Biofilm is a community of bacteria that is surrounded by an exopolysaccharide matrix (EPS), which acts as a supportive consortium. Extracellular

polymeric substance (EPS) extracellular DNA (eDNA), and proteins are the main components of biofilm **(6, 7)**. The *pslA* and *pelA* genes play an important role in biofilm formation as these elements showed overexpression in the biofilm-producing strains **(8)**. The *PelA* and *PslA* play essential role in the production of carbohydrate-rich structure of the biofilm matrix **(9)**.

Alginate is a linear un-branched polymer encoded by *algD*, *algU*, and *algL* genes, composed of 1–4 linked saccharides β-D mannuronic acid and a C-5 epimer of a-L-guluronic acid **(10)**. The *algU* plays an effective role in the expression of the *ppyR* gene (putative transmembrane protein) **(11)**. Alginate lyase (AlgL) is an enzymatic and structural protein that plays a role as a component of the alginate transporter system.

Studies have reported that resistance was significantly higher in biofilm-producing strains **(12)**. Hentzer et al **(10)** declared that the high production of alginate affects biofilm development on an abiotic surface. Biofilms produced by an alginate-overproducing isolate display a highly organized architecture and are significantly more resistant to the antimicrobials than a biofilm formed by an isogenic non-mucoid organism **(10)**. As a result, biofilm-related diseases are more difficult to eradicate and more prone to recurrence **(13)**. Abidi et al **(14)** concluded that biofilm formation is significantly higher in MDR strains.

In this study, we examined the relationship between antibiotic resistance profile, alginate production, and biofilm formation in the *P. aeruginosa* clinical strains isolated from two tertiary therapeutic hospitals in Babol, north of Iran.

2. Materials and Methods

2.1. Ethics Declaration

This cross-sectional study was approved by the Ethics Committee of Babol University of Medical Sciences (code number IR.IAU.AMOL.REC.1401.064).

2.2. Sample Collection and Bacterial Identification

The sample size was calculated by the formula **(15)**; $n= z^2P (1- P)/d^2$, “n” is the number of sample size, “P” is the estimated prevalence proportion ratios (PPR) (0.45), “z” is the probability (0.975), and “d” was the standard error of prediction (SEP) (0.05). Thus, in a period of one year (April 2022 to March 2023), in total 90 non-duplicative *P. aeruginosa* was collected from two large hospitals in Babol, north of Iran.

Pseudomonas aeruginosa strains were identified using colony morphology, Gram staining, oxidase and catalase tests, pyocyanin pigment, growth at 44°C, and oxidative-fermentative (OF) test, and then confirmed by the species-specific primers to amplify *oprL* gene with 504 bp **(16)**. All strains were stocked in 20% v/v glycerol-tryptic soy broth (Becton Dickinson, Franklin Lakes, NJ) at -70°C for further use. *P. aeruginosa* ATCC 27853 and 8821M were used as quality controls.

2.3. Antimicrobial Susceptibility Testing (AST)

Kirby-Bauer disk diffusion susceptibility test was performed in agreement with the clinical and laboratory standards institute guideline (CLSI; M100-S14), on the Mueller-Hinton agar (MHA) (Merck, Darmstadt, Germany) for ciprofloxacin (CIP, 5µg, S; ≥25 mm, I; 19-24 mm, R; ≤ 18 mm), ceftazidime (CAZ, 30µg, S; ≥18 mm, I; 15-17 mm, R; ≤ 14 mm), cefotaxime (CTX, 30µg, S; ≥18 mm, I; 15-17 mm, R; ≤ 14 mm), tetracycline (TET, 30µg, S; ≥15 mm, I; 12-14 mm, R; ≤ 11 mm), imipenem (IPM, 10µg, S; ≥16 mm, I; 16-18 mm, R; ≤ 15 mm), ampicillin (AMP, 10µg, S; ≥17 mm, I; 14-16 mm, R; ≤ 15 mm), cefepime (FEP, 30µg, S; ≥18 mm, I; 15-17 mm, R; ≤ 14 mm), aztreonam (ATM, 30µg, S; ≥22 mm, I; 16-21 mm, R; ≤ 15 mm), amikacin (AN, 30µg, S; ≥17 mm, I; 15-16 mm, R; ≤ 14 mm), gentamicin (GM, 10µg, S; ≥15 mm, I; 13-14 mm, R; ≤ 12 mm), and trimethoprim-sulfamethoxazole (SXT, 5µg, S; ≥16 mm, I; 11-15 mm, R; ≤ 10 mm) (Padtan Teb Co, Iran) **(17)**.

2.4. Alginate Production Assay

Alginate production level was determined using the carbazole method. In brief, each isolate was cultured in Luria–Bertani (LB) broth with aeration at 37˚C for 24 hr and then centrifuged for 30 min at 13,500 ×g. For alginate precipitation, the supernatant was mixed with cold 95% ethanol and then centrifuged at 13,500 × g for 13 min. Then, 70 ml of alginate solution was mixed with 600 ml of borate–sulfuric acid (24.74 gr of H_3BO_3 in 45 ml of 4 M KOH that was diluted in 100 ml distilled water) and 20 ml of 0.1% (w/v) carbazole (Sigma-Aldrich, St. Louis, Missouri, United States). For the chromogenic reaction, the mixture was heated at 50°C for 30 min. Finally, the optical density was recorded at 540 nm. The alginate standard curve was plotted and the amount of produced alginate was recorded per mg cell dry weight **(16)**.

2.5. Biofilm Formation Assay

A 96-well flat-bottom microtiter plate was used for the biofilm production evaluation. Briefly, 200 µl of fresh bacterial culture in the brain heart infusion (BHI) broth was poured to the wells. Following incubation for 24 hr at 37°C, each well was washed twice with phosphate buffer saline (PBS, pH 7.2) to remove detached, frail attached, and floating 'planktonic' strains. The plate was shaken to eliminate all non-

adherent isolates, and dried at the 25ºC for fixing the attached ones. The staining was done using crystal violet 0.1% (Sigma, St Louis, USA) for 6 min at room temperature, washed by tap water and allowed to dry. The optical density (OD_{570nm}) was recorded using a microplate ELISA reader (BioTek, Bad Friedrichshall, Germany). All tests were done in triplicate and cut-off value (OD_c) was determined. Biofilm formation values were measured as follows: non- biofilm forming ($OD_t < OD_c$), weakly ($OD_c < OD_t < 2_x\ OD_c$), moderately ($2_x\ OD_c < OD_t < 4_xOD_c$) and strongly ($OD_t \geq 4_xOD_c$) **(13)**.

2.6. Genomic DNA Extraction

Bacterial cells were lysed as follows: four to five microbial pure colonies were suspended in 25 µl of 0.25% sodium dodecyl sulfate (SDS)–0.05 N NaOH solutions and heated for 15 min. Then, 200 µl of ddH_2O was added to the microtube, and 5 µl of diluted mixture was used in PCR. The quality of the extracted DNA was checked by electrophoresis on 1.0% agarose gel, while the purity and concentration was assessed at $OD_{260/280nm}$ (Thermo Scientific Nanodrop 2000 Spectrophotometer). Template DNA was kept at -20°C for further analysis.

2.7. Polymerase Chain Reaction

PCR was carried out in total volume of 12 µl mixture, which included 1.3 µL of template DNA, 5 µL of pre-made master-mix (Ampliqon, Stenhuggervej, Denmark), 5.2 µL of DNase-free ddH2O and 0.3 µL of each primer (at concentration of 10 $pmol\mu L^{-1}$). The primer sequences and time-thermal condition are listed in Table 1. PCR amplicon products (4 µl) were loaded on 1.5% agarose gel prepared in 1X TBE (Tris/Borate/EDTA) buffer stained with SafeStain loading dye (CinnaGen Co., Iran) and imaged with Ultraviolet Illumination (Bio-rad, Hercules, USA).

Table 1. Oligonucleotide primer sequences and time-thermal condition used in this study **(16, 18)**.

Target site	Primer sequences	Product size (bp)	Time-thermal PCR condition
oprL	F=5'-ATGGAAATGCTGAAATTCGGC-3' R=5'-CTTCTTCAGCTCGACGCGACG-3'	504	Initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 60 s for 30 cycles and a final extension at 72°C for 10 min.
pslA	F=5′- TCCCTACCTCAGCAGCAAGC -3′ F=5′- TGTTGTAGCCGTAGCGTTTCTG -3′	656	Initial denaturation at 95°C for 6 min, denaturation at 95°C for 40 s, annealing at 57°C for 30 s, extension at 72°C for 60 s for 35 cycles and a final extension at 72°C for 10 min.
pelA	F=5′- CATACCTTCAGCCATCCGTTCTTC -3′ F=5′- CGCATTCGCCGCACTCAG -3′	786	Initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 60 s for 32 cycles and a final extension at 72°C for 7 min.
ppyR	F=5′- CGTGATCGCCGCCTATTTCC -3′ F=5′- ACAGCAGACCTCCCAACCG -3′	160	Initial denaturation at 95°C for 7 min, denaturation at 95°C for 35 s, annealing at 57°C for 40 s, extension at 72°C for 60 s for 33 cycles and a final extension at 72°C for 6 min.
algD	F:5′-AGAAGTCCGAACGCCACACC-3′ R:5′-CGCATCAACGAACCGAGCATC-3′	550	Initial denaturation at 95°C for 5 min, denaturation at 95°C for 55 s, annealing at 58°C for 45 s, extension at 72°C for 60 s for 32 cycles and a final extension at 72°C for 6 min.
algU	F=5′- CGATGTGACCGCAGAGGATG-3′ F=5′- TCAGGCTTCTCGCAACAAAGG-3′	292	Initial denaturation at 95°C for 7 min, denaturation at 95°C for 45 s, annealing at 57°C for 35 s, extension at 72°C for 60 s for 30 cycles and a final extension at 72°C for 6 min.
algL	F: 5′-CCGCTCGCAGATCAAGGACATC-3′ R: 5′-TCGCTCACCGCCCAGTCG-3′	432	Initial denaturation at 95°C for 6 min, denaturation at 95°C for 50 s, annealing at 58°C for 40 s, extension at 72°C for 55 s for 33 cycles and a final extension at 72°C for 8 min.

3. Results

Totally, 90 *P. aeruginosa* strains were obtained from 56.7% (n=51) male cases. The age range of the patients was from 18 to 89, with the mean age of 42.2 ± 1.3 years. The origin of samples was urine (n=21/90, 23.3%), wound (n=19/90, 21.1%), bronchoalveolar lavage (n=19/90, 21.1%), blood (n=11/90, 12.2%), sputum (n=9/90, 10.0%), stool (n=9/90, 10.0%), and cerebrospinal fluid (CSF) (n=2/90, 2.2%). The prevalence of strains according to the wards was as follows: intensive care unit [ICU] (n=23/90, 15.5%), infectious disease (n=20/90, 14.4%), neonatal intensive care unit [NICU] (n=16/90, 13.3%), emergency (n=16/90, 11/1%), internal medicine (n=10/90, 11.1%), surgery (n=3/90, 8.9%) and hematology-oncology (n=2/90, 3.4%).

As shown in Table 2, the highest resistance rate was related to CAZ (n=67/90, 74.4%), followed by ATM (n=57/90; 63.3%), FEP (n=49/90, 54.4%), TET (n=45/90, 50.0%) and CIP (n=33/90, 36.7%). Resistance to IPM showed that the frequency of carbapenem-resistant strains (CRPA) was 63.3% (n=57/90).

Alginate production ability was found in 87.8% (n=79/90), in which 7.6% (n=6/79) were <250 μgml^{-1}, 53.2% (n=42/79) between 250-400 μgml^{-1}, and 39.2% (n=31/79) >400 μgml^{-1}. Alginate production level in *P. aeruginosa* 8821M strain was 470 μgml^{-1}. Among 35.5% (n=32/90) of MDR isolates, 15.6% (n=5/32) produced <250 μgml^{-1}, 59.4% isolates (n=19/32) produced 250-400 μgml^{-1}, and 25.0% (n=8/32) produced >400 μgml^{-1} alginate. The level of alginate production was not significantly related to the antibiotic resistance (P>0.05), but it was significantly related to the biofilm production (P<0.05). The alginate-encoding genes, *algD*, *algU*, and *algL*, were detected in 92.2% (n=83/90), 86.6% (n=78/90), and 67.7% (n=61/90) of the isolates, respectively. Compared to the alginate-free strains, the frequency of these genes was significantly higher in alginate-producing strains.

In general, 60.0% (n=54/90) of the isolates were able to form biofilm. Among 60.0% biofilm-producing isolates, 51.8% (n=28/54) were MDR. There was a significant relationship between biofilm production and antibiotic resistance (P<0.05). According to the OD cut-offs, category of biofilm formation was as follows; weak (n=6/54, 11.1%), moderate (n=13/54, 24.1%) and strong (n=35/54, 64.8%). A significant relationship was observed between the level of alginate production (>400 μgml^{-1}) and intensity of biofilm formation, so that in strong biofilm-forming strains ($OD_t \geq 4_xOD_c$), more alginate levels were produced (P<0.05). All biofilm-producing isolates were positive for *ppyR* gene. The *pslA* and *pelA* genes were present in 85.2% (n=46/54) and 42.5% (n=23/54) of the biofilm producing isolates, respectively. The prevalence of *pslA* and *pelA* genes in alginate-producer isolates were 35.4% (n=28/79) and 17.7% (n=14/79), respectively.

Table 2. Resistance profiling, biofilm formation and alginate production

Strain number	Antibiotic resistance	Biofilm phenotype	Biofilm-encoded genes	Alginate-production
1	ATM/ FEP/ TET/ CIP	Weak	*ppyR / pelA/ algD/ algU/ algL*	<250 μgml^{-1}
2	CAZ/ TET / CIP/ SXT	Moderate	*ppyR / pelA/ algD/ algU/ algL*	250-400 μgml^{-1}
3	CAZ/ATM/ IPM / CTX/ AN	Strong	*ppyR / pslA/ algD/ algU/ algL*	>400 μgml^{-1}
4	ATM/ FEP/ CIP/ IPM	Strong	*ppyR / pslA/ pelA/ algD/ algL*	>400 μgml^{-1}
5	CAZ/ TET / IPM/ SXT/ AN	non- Biofilm	*algD/ algU/ algL*	250-400 μgml^{-1}
6	CAZ/ATM/ FEP / TET/ CIP/ IPM/ CTX/ AMP	Strong	*ppyR/ pslA/ pelA/ algD/ algU/ algL*	>400 μgml^{-1}
7	ATM/ TET/ IPM	non- Biofilm	*algD*	250-400 μgml^{-1}
8	ATM/ FEP/ CIP/ AMP	Weak	*ppyR / pelA/ algD/ algU/ algL*	<250 μgml^{-1}
9	CAZ/ TET / CIP/ SXT/ AN/ GM	Strong	*ppyR / pslA/ algD/ algU/ algL*	250-400 μgml^{-1}
10	CAZ/ FEP/ TET / IPM/ AMP/ AN/ GM	Strong	*ppyR/ pslA / algD/ algU/ algL*	>400 μgml^{-1}
11	CAZ/ATM/ TET / IPM	Moderate	*ppyR / pelA/ algD/ algU/ algL*	<250 μgml^{-1}
12	CAZ/ATM/ FEP/ TET/ CIP / IPM/ AMP	Strong	*ppyR / pelA/ algD/ algU/ algL*	>400 μgml^{-1}
13	CAZ/ FEP/ CIP/ IPM / GM	Strong	*ppyR / pslA/ algD/ algU/ algL*	>400 μgml^{-1}
14	ATM/ CIP/ IPM/ AN	non- Biofilm	*algD/ algL*	Non-alginate
15	CAZ / CIP/ IPM/ SXT	Weak	*ppyR / pelA/ algD/ algU/ algL*	250-400 μgml^{-1}

Strain number	Antibiotic resistance	Biofilm phenotype	Biofilm-encoded genes	Alginate-production
16	CAZ/ FEP/ TET / AN	Moderate	*ppyR / pslA/ algD/ algU/ algL*	250-400 µgml^{-1}
17	CAZ/ATM/ CIP / SXT	Moderate	*ppyR / pslA/ pelA/ algD/ algU/ algL*	250-400 µgml^{-1}
18	ATM/ TET/ CIP/ IPM	non- Biofilm	*algD/ algU/ algL*	Non-alginate
19	CAZ/ FEP / TET/ IPM	non- Biofilm	*algD/ algU*	Non-alginate
20	CAZ/ATM / CIP/ IPM	Moderate	*ppyR / pslA/ pelA/ algD/ algU/ algL*	250-400 µgml^{-1}
21	CAZ/ FEP / CIP/ IPM/ AMP/ GM	Strong	*ppyR / pslA/ pelA/ algD/ algU/ algL*	>400 µgml^{-1}
22	CAZ/ FEP/ TET / IPM	non- Biofilm	*algD/ algU/ algL*	250-400 µgml^{-1}
23	ATM/ FEP/ CIP/ AMP	Moderate	*ppyR/ pslA/ pelA/ algD/ algU/ algL*	250-400 µgml^{-1}
24	FEP/ TET/ CIP/ AMP	non- Biofilm	*algD/ algL*	Non-alginate
25	ATM/ TET/ IPM/ AN/ GM	Strong	*ppyR/ pslA/ pelA/ algD/ algU/ algL*	>400 µgml^{-1}
26	CAZ/ FEP / CIP/ IPM	non- Biofilm	*algD*	250-400 µgml^{-1}
27	CAZ/ATM / IPM/ CTX/ GM	non- Biofilm	*algD/ algL*	<250 µgml^{-1}
28	CAZ/ FEP/ TET / IPM/ GM	Strong	*ppyR/ pslA/ pelA/ algD/ algU*	>400 µgml^{-1}
29	CAZ/ FEP / TET/ IPM	non- Biofilm	*algD/ algL*	250-400 µgml^{-1}
30	CAZ/ATM/ FEP/ CIP / CTX/ AMP	Strong	*ppyR/ pelA/ algD/ algU/ algL*	>400 µgml^{-1}
31	CAZ/ FEP / TET/ CTX	non- Biofilm	*algD/ algL*	250-400 µgml^{-1}
32	CAZ/ FEP / IPM/ AMP/ AN	Strong	*ppyR/ pslA/ pelA/ algD/ algU/ algL*	>400 µgml^{-1}
33	CAZ/ATM / CIP/ CTX/ AN	non- Biofilm	*algD/ algU*	250-400 µgml^{-1}
34	CAZ/ FEP / TET/ IPM	non- Biofilm	*algD/ algL*	Non-alginate
35	CAZ / CIP/ SXT/ AMP	Weak	*ppyR / pslA/ algD/ algU/ algL*	<250 µgml^{-1}
36	CAZ, ATM/ FEP/ SXT	Moderate	*ppyR / pslA/ algD/ algU/ algL*	250-400 µgml^{-1}
37	FEP/ TET/ CIP/ SXT/ GM	Strong	*ppyR / pslA/ algD/ algU/ algL*	>400 µgml^{-1}
38	ATM/ FEP/ IPM/ SXT/ CTX	non- Biofilm	*algD*	Non-alginate
39	CAZ/ FEP / IPM/ CTX	Moderate	*ppyR / pslA/ pelA/ algD/ algU/ algL*	250-400 µgml^{-1}
40	CAZ / TET/ CIP/ IPM/ CTX/ AMP	Strong	*ppyR / pslA/ algD/ algU/ algL*	>400 µgml^{-1}
41	CAZ/ATM/ FEP / IPM	Strong	*ppyR / pslA/ algD/ algU/ algL*	250-400 µgml^{-1}
42	ATM/ FEP/ TET/ IPM/ AMP/ AN	Strong	*ppyR / pslA/ pelA/ algD/ algU/ algL*	>400 µgml^{-1}
43	CAZ/ATM / CIP/ CTX	Strong	*ppyR / pslA/ algD/ algU/ algL*	>400 µgml^{-1}
44	CAZ/ FEP/ SXT/ AN/ GM	Strong	*ppyR / pslA/ algD/ algU/ algL*	250-400 µgml^{-1}
45	CAZ/ATM/ FEP/ TET/ GM /IPM / SXT	Strong	*ppyR / pslA/ pelA/ algD/ algU/ algL*	>400 µgml^{-1}
46	CAZ/ATM/ FEP/ CIP/ IPM/ SXT	Strong	*ppyR/ pslA/ algD/ algU/ algL*	>400 µgml^{-1}
47	CAZ/ATM/ FEP	non- Biofilm	*algD/ algU/ algL*	250-400 µgml^{-1}
48	CAZ/ATM/ CIP/ IPM	Moderate	*ppyR / pslA/ algD/ algU/ algL*	250-400 µgml^{-1}

Strain number	Antibiotic resistance	Biofilm phenotype	Biofilm-encoded genes	Alginate-production
49	ATM/ FEP/ TET/ IPM	Strong	*ppyR / pslA/ pelA/ algD/ algU/ algL*	>400 µgml^{-1}
50	ATM/ TET/ CIP/ AMP	non- Biofilm	*algD/ algU/ algL*	250-400 µgml^{-1}
51	CAZ / TET/ SXT/ CTX/ GM	non- Biofilm	*algD/ algU/ algL*	250-400 µgml^{-1}
52	CAZ/ATM / CIP/ IPM	non- Biofilm	*algD/ algU*	250-400 µgml^{-1}
53	CAZ/ATM/ FEP/ TET/ SXT / CTX/ AMP/ AN	Strong	*ppyR / pslA/ algD/ algU/ algL*	>400 µgml^{-1}
54	ATM/ TET/ IPM/ SXT/ CTX	Strong	*ppyR / algD/ algU/ algL*	>400 µgml^{-1}
55	CAZ / TET/ CIP/ IPM	Moderate	*ppyR / pslA/ algD/ algU*	>400 µgml^{-1}
56	CAZ/ATM / IPM/ CTX	non- Biofilm	*pslA/ algD/ algU/ algL*	250-400 µgml^{-1}
57	ATM/ FEP/ TET/ IPM	non- Biofilm	*algD*	250-400 µgml^{-1}
58	ATM/ TET/ CIP/ IPM/ GM	Strong	*ppyR/ pslA/ algD/ algU/ algL*	>400 µgml^{-1}
59	CAZ/ATM/ FEP / SXT	non- Biofilm	*algD/ algU/ algL*	>400 µgml^{-1}
60	ATM/ FEP/ IPM/ CTX/ AMP/ GM	Strong	*ppyR / pelA/ algD/ algU*	250-400 µgml^{-1}
61	CAZ/ATM / IPM/ SXT/ AMP/ AN	Strong	*ppyR / pelA/ algD/ algU/ algL*	>400 µgml^{-1}
62	CAZ/ATM/ FEP/ TET	Moderate	*ppyR/ pslA / algD/ algU*	250-400 µgml^{-1}
63	CAZ/ATM / IPM/ CTX/ AN	non- Biofilm	*pslA/ algD/ algU*	250-400 µgml^{-1}
64	CAZ/ATM/ FEP/ CIP	non- Biofilm	*algD*	250-400 µgml^{-1}
65	CAZ/ATM/ TET/ IPM/ CTX/ GM	Strong	*ppyR/ pslA/ algD/ algU*	250-400 µgml^{-1}
66	CAZ / TET/ IPM/ CTX	non- Biofilm	*algD/ algU/ algL*	>400 µgml^{-1}
67	CAZ/ FEP / IPM/ AMP/ AN/ GM	Strong	*ppyR / pslA/ algD/ algU/ algL*	>400 µgml^{-1}
68	CAZ/ATM/ FEP / CIP/ IPM/ CTX/ AMP/ GM	Strong	*ppyR / pslA/ algD/ algU*	250-400 µgml^{-1}
69	CAZ / TET/ IPM/ AN	non- Biofilm	*algD/ algU*	Non-alginate
70	ATM/ TET/ IPM/ SXT	Weak	*ppyR/ pslA/ algU/ algL*	<250 µgml^{-1}
71	CAZ/ FEP / CIP/ IPM/ SXT	non- Biofilm	*algD/ algU*	250-400 µgml^{-1}
72	CAZ / TET/ IPM/ GM	non- Biofilm	*algU*	250-400 µgml^{-1}
73	CAZ / TET/ SXT/ GM	Moderate	*ppyR / pslA/ algD/ algU/ algL*	>400 µgml^{-1}
74	CAZ/ATM/ IPM / CTX/ AN/ GM	Strong	*ppyR / pslA/ algD/ algU*	Non-alginate
75	CAZ/ATM/ FEP/ SXT / CTX/ AMP/ AN	Strong	*ppyR / pslA/ algD/ algU*	Non-alginate
76	CAZ/ATM/ TET/ IPM	non- Biofilm	*algU*	250-400 µgml^{-1}
77	CAZ/ATM/ FEP/ CTX/ GM	non- Biofilm	*algD/ algU*	250-400 µgml^{-1}
78	CAZ/ATM/ TET/ IPM	non- Biofilm	*algU*	250-400 µgml^{-1}
79	ATM/ FEP/ TET/ SXT/ AN	non- Biofilm	*algU*	Non-alginate
80	CAZ / TET/ CIP/ IPM/ SXT/ CTX	Strong	*ppyR/ pslA/ algD/ algU/ algL*	>400 µgml^{-1}
81	CAZ / TET/ SXT/ AN	non- Biofilm	*algU*	250-400 µgml^{-1}
82	ATM/ FEP/ TET/ CTX	Weak	*ppyR/ pslA/ algD/ algU*	Non-alginate
83	CAZ/ATM / IPM/ SXT/ GM	non- Biofilm	*algU*	250-400 µgml^{-1}
84	ATM/ FEP/ SXT/ CTX/ AMP/ AN/ GM	Strong	*ppyR/ pslA/ algD/ algU/ algL*	>400 µgml^{-1}
85	CAZ/ FEP / IPM/ SXT	non- Biofilm	*algD/ algU*	250-400 µgml^{-1}
86	CAZ/ATM / IPM/ CTX/ AN	non- Biofilm	*algD/ algU*	250-400 µgml^{-1}

Strain number	Antibiotic resistance	Biofilm phenotype	Biofilm-encoded genes	Alginate-production
87	CAZ/ATM/ SXT	Moderate	*ppyR / pslA/ pelA/ algD/ algU/ algL*	250-400 µgml^{-1}
88	ATM/ FEP/ TET/ IPM/ SXT/ CTX/ AN/ GM	Strong	*ppyR / pslA/ algD/ algU/ algL*	>400 µgml^{-1}
89	CAZ/ FEP / SXT/ CTX	non- Biofilm	*algD/ algU/ algL*	250-400 µgml^{-1}
90	CAZ/ATM/ FEP/ SXT / AN/ GM	Strong	*ppyR/ pslA/ algD/ algU/ algL*	>400 µgml^{-1}

Ciprofloxacin (CIP), ceftazidime (CAZ), cefotaxime (CTX), tetracycline (TET), imipenem (IPM), ampicillin (AMP), cefepime (FEP), aztreonam (ATM), amikacin (AN), gentamicin (GM), and trimethoprim-sulfamethoxazole (SXT).

4. Discussion

Since the emergence of resistant strains of *P. aeruginosa*, the treatment of these infections has become a major challenge worldwide **(3)**. Antimicrobial resistance and biofilm formation are considered the most important challenges in the treatment of these infections **(4)**.

Antimicrobial susceptibility testing results showed the highest resistance rate related to CAZ (74.4%), ATM (63.3%), FEP (54.4%), TET (50.0%), and CIP (36.7%). Thus, 63.3% of the isolates were carbapenem-resistant strains (CRPA). These data are in agreement with Ramazani et al **(15)** and Davarzani et al **(16)** results. The emergence of CRPA strains around the world is a major challenge, because carbapenems are considered as reliable and widely used treatment options in the treatment of these infections. In contrast with our data, the prevalence of CRPA was 24.7% in Ramazani et al **(15)** and 6.3% in Pournajaf et al **(18)** studies. One of the reasons for the increase in carbapenem resistance can be mentioned the excessive use and transfer of resistance genes by transposable elements (TEs) such as plasmid, integron, and transposon.

In a 10-years longitudinal study in Taiwan (SMART 202-2021), Karlowsky et al **(19)** reported 17.3% (n=520/3013) CRPA isolates. The frequency of CRPA increased from 11.5%–12.3% (2012–2015) to 19.4%–22.8% (2018–2021) (P≤0.0001). Vaez et al **(20)** reported a different distribution of resistance to IPM in different provinces of Iran (from 76.1% in Isfahan to 7.5% in Hamedan).

This wide distribution of CRPA strains has become a major challenge in treatment, which indicates a lack in adherence of antibiotic stewardship and proper monitoring of rational antibiotic prescribing. Therefore, based on the previous studies, poly-therapy and alternative treatment is suggested instead of mono-therapy **(21, 22)**.

Consistent with Pournajaf et al **(18)** and Ghadaksaz et al **(23)**, alginate production was found in 87.8% of our isolates, which consist of 7.6% <250 µgml^{-1}, 53.2% between 250-400 µgml^{-1}, and 39.2% >400 µgml^{-1}. In agreement with Davarzani et al **(16)**, the level of alginate production was not significantly related to the antibiotic resistance, but it was related to the biofilm production. Also, alginate production was <400 µgml^{-1}, 250-400 µgml^{-1} and >250 µgml^{-1} in 39.0% (n=39/100), 51.0% (n=51/100) and 10.0% (n=10/100) of the isolates, respectively.

In contrast with our data, Valadbeigi et al **(24)** and Davarzani et al **(16)** showed a high distribution of alginate production in burn and urine samples, respectively. While in line with Pournajaf et al **(18)**, the highest level of alginate was found in respiratory samples. This could explain the role of non-pilous adhesions such as alginate in the colonization of microbes in the airways.

Similar with Pournajaf et al **(18)** and Ghadaksaz et al **(23)**, the prevalence of *algD*, *algU* and *algL* genes in our isolates was 92.2%, 86.6% and 67.7%, respectively. The prevalence of *algD* gene in the studies directed by Elogne et al **(25)** and Rajabi et al **(26)** was 90.7% (n=129/151) and 78.6% (n=66/85), respectively. The variation in the distribution of alginate-coding genes is mostly related to the type and volume of the samples.

Alginate protects bacteria from the adversities surrounding environment and also increases adhesion to the solid surfaces. Therefore, it plays an important role in the early stages of infection and colonization. The presence of this layer prevents the clearance of the organism by the immune system **(27)**.

The microtiter biofilm formation assay is a simplified quantitative and reliable method that is comparable among different laboratories to detect biofilm-forming bacteria **(18, 28, 29)**. Overall, 60.0% of our isolates were able to form biofilm, including weak (11.1%), moderate (24.1%) and strong (64.8%) producers. Kamali et al **(30)** showed 83.7% (n=67/80) of the isolates produced biofilm; 16.2% (n=13/80) strong, 33.7% (n=27/80) moderate, and 33.7% (n=27/80) weak biofilm producers.

In line with Davarzani et al **(16)**, there was a significant relationship between the level of alginate production (>400 $\mu g ml^{-1}$) and strong biofilm-forming strains. Molecular distribution of biofilm-encoded genes showed that all biofilm-producing isolates were positive for *ppyR* gene. The *pslA* and *pelA* genes were present in 85.2% and 42.5% of biofilm producing isolates, respectively. Ghadaksaz et al **(23)** showed that 99.0%, 83.7% and 45.2% of the isolates carried *ppyR*, *pslA*, *pelA* genes, respectively. Soleymani-Fard et al **(31)** showed that the prevalence of *pslA* and *pelB* genes was 34.5% and 65.5%, respectively. The difference in distribution of the biofilm-coding genes can be caused by the type and size of the sample, geographical distance, and genetics of the strains.

5. Conclusion

The results highlight an alarming trend in *P. aeruginosa* strains antibiotic resistance rate. Thus, periodic monitoring, adherence to the antibiotic stewardship, avoiding arbitrary drugs prescribing, and screening tests are necessary and unavoidable. The formation of biofilm and alginate plays an important role in pathogenesis. Furthermore, poly-therapy based on the appropriate anti-pseudomonal antimicrobials with anti-biofilm agents can be used to enhance the treatment of biofilm-associated illnesses.

6. Declarations

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Ethical Considerations

This cross-sectional study was approved by the Ethics Committee of Babol University of Medical Sciences (code number IR.IAU.AMOL.REC.1401.064).

Authors' Contributions

Maryam Shafigh and Abazar Pournajaf conceived and designed the experiments. Abazar Pournajaf wrote the main manuscript text. Hami Kaboosi, Rabie Izadi Amoli, and Yousef Yahyapour performed the experiments. Maryam Shafigh analyzed the data. Maryam Shafigh and Abazar Pournajaf reviewed and finalized the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of Interest

The authors declare no conflict of interest.

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