Original Article | Iran J Med Microbiol. 2022; 16(6): 557-565

10.30699/ijmm.16.6.557

Iranian Journal of Medical Microbiology | ISSN:2345-4342

A molecular New Update on the Biofilm Production and Carbapenem Resistance Mechanisms in Clinical *Pseudomonas aeruginosa* Isolates

Raziyeh Ramazani[1], Rabeeh Izadi Amoli[1*], Mojtaba Taghizadeh Armaki[2], Abazar Pournajaf[2], Hami Kaboosi[1]

1. *Department of Microbiology, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran*
2. *Infectious Disease and Tropical Medicine Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran*

ABSTRACT

Background and Aim: Carbapenem resistant- *Pseudomonas aeruginosa* (CRPA) is one of the most important causes of severe and persistent infections. The contributions of different resistance mechanisms to Carbapenems and biofilm formation among a collection of imipenem susceptible and non-susceptible *P. aeruginosa* isolates were investigated.

Materials and Methods: In this cross-sectional study, a total of 117 *P. aeruginosa* isolates were collected. The disc diffusion method assessed the susceptibility of isolates to various antimicrobials. The Carbazole method was used for the detection of alginate producers. Multiplex-PCRs were performed for the detection of biofilm and resistance genes. The expression mRNA levels of efflux pumps were assessed by phenotypic and genotypic (Quantitative Real-time PCR) approaches.

Results: The highest resistance rate was related to ceftazidime, chloramphenicol, ceftriaxone, tetracycline, and levofloxacin. MDR phenotype was observed in 8.4% of strains. The frequency of carbapenem resistance was also 24.7%. The Carbazole test was positive at 53.8%. In general, 62.4% of isolates were able to form a biofilm, 28.8% of which were resistant to carbapenem. The distribution of *algD* and *algU* genes were 41.8% and 26.5%, respectively. The frequency of MBL-encoded genes was as follows; *bla*IMP (62.1%), *bla*VIM (31.0%), and *bla*NDM (6.8%). The relative levels of MexX, MexC, MexB and MexA mRNA in CRPA strains with active efflux pump were 81.8%, 63.6%, 54.5%, and 36.4%, respectively.

Conclusion: The existence of different resistant mechanisms in *P. aeruginosa* can cause cross antibiotic resistance, lead to the appearance of resistant strains, and make the treatment difficult. Biofilm production is directly related to antibiotic resistance. Efflux pumps are actively expressed in carbapenem-resistant strains.

Keywords: Carbapenem, efflux pumps, biofilm, *Pseudomonas aeruginosa*

Received: 2022/03/10; **Accepted**: 2022/06/24; **Published Online:** 2022/09/09

Corresponding Information: **Rabeeh Izadi Amoli**, *Department of Microbiology, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran* Email: rabi-izadiamoli@yahoo.com

Use your device to scan and read the article online

Ramazani R, Izadi Amoli R, Taghizadeh Armaki M, Pournajaf A, Kaboosi H. A molecular new update on the biofilm production and carbapenem resistance mechanisms in clinical *Pseudomonas aeruginosa* isolates. Iran J Med Microbiol. 2022; 16 (6):557-65.

Download citation: BibTeX | RIS | EndNote | Medlars | ProCite | Reference Manager | RefWorks

Send citation to: Mendeley Zotero RefWorks

1. Introduction

Pseudomonas aeruginosa is a gram-negative, non-spore-forming opportunistic pathogen associated with healthcare infections (HAIs), particularly in immunocompromised patients **(1)**. As a global dilemma, the emergence of multidrug-resistance (MDR) strains has made medical therapy against *P. aeruginosa* difficult **(2)**. Furthermore, biofilm production in *P. aeruginosa* is the main factor in developing chronic infections, especially in cystic fibrosis and burn patients **(3)**. Furthermore, biofilm-forming *P. aeruginosa* strains are more resistant to antimicrobials and immune responses **(4)**. Biofilm is a complex community of microbial cells surrounded by an extracellular matrix composed of proteins, exopolysaccharides, and extracellular DNA (eDNA) **(5)**. This exopolysaccharide can impair the antimicrobials

entrance and act as a barrier against phagocytosis by host immune cells. Alginate, Psl (polysaccharide synthesis locus), and Pel (pellicle) are various exopolysaccharides produced in biofilm-forming *P. aeruginosa*. Psl is a mannose-rich polymer with an important role in the early stages of biofilm production, and Pel plays an essential role in the initial surface attachment and cell-to-cell connections **(6)**. Fifteen co-transcribed genes comprising pslA-O are essential for Psl synthesis. PslD protein, encoded by *pslD* gene, is anchored in the periplasm/outer membrane and essential for biofilm formation, possibly by transferring a biofilm-relevant exopoly-saccharide. A cellulose-sensitive exopolysaccharide is encoded by pel operon (pelA to pelG). PelF acts as a soluble glucosyltransferase and participates in the biosynthesis of Pel exopolysaccharide using UDP-glucose as a substrate **(7)**. Alginate is a linear unbranched polymer that so called 'mucoid' biofilms produce and act as an important virulence element **(8)**.

Treatment of *P. aeruginosa* infections is difficult due to its resistance to broad-spectrum antimicrobials, such as Carbapenems **(9)**. Metallo-β-lactamases (MBLs) are classified in class B and group III structural and functional classification systems, respectively and inhibited by metal chelators, such as ethylenediaminetetraacetic acid (EDTA) **(10)**. Numerous MBLs, including blaIMP, blaNDM, blaVIM, blaSPM, blaSIM, and blaGIM families, have been defined in carbapenem-resistant *P. aeruginosa* (CRPA) **(11)**. On the other hand, impermeability through alteration or loss of OprD, Overexpression of efflux pumps, and oxacillinases (Ambler class D β-lactamases) contribute to the emergence of CRPA **(12)**. Hyper-expression of MexAB-OprM efflux system is recognized to affect meropenem (MER) efficacy but not that of imipenem (IMP). In addition, the MexCD-OprJ and MexXY-OprM efflux systems reduce MER susceptibility **(13)**. Resistance of *P. aeruginosa* to numerous antimicrobials is mainly attributable to overexpression of the MexAB-OprM efflux pump **(4)**. This ribonucleoprotein efflux pump has three components, which include MexB (an inner membrane), MexA (a periplasmic membrane fusion protein) and OprM (a channel-forming outer membrane protein) **(14)**.

Today, resistance to most antibiotics in *P. aeruginosa* has become a major challenge for physicians because it limits the choice of the appropriate antibiotic to treat these infections. On the other hand, knowing the mechanisms of resistance and limiting the transfer of resistance factors is inevitable due to the increasing number of carbapenem-resistant strains and the horizontal transfer of these resistance factors to susceptible strains, as well as cross-resistance to other antibiotic families. Therefore, this study was designed to determine the antimicrobial susceptibility profile, biofilm production and carbapenem resistance mechanisms in the clinical *P. aeruginosa* isolates.

2. Materials and Methods

Bacterial sampling

In a random simple sampling method, the sample size was calculated by the equation: n = z2P (1- P)/d2, where "n" was the sample size, "P" was the estimated prevalence proportion observed in a previous study (0.45), "z" was the probability (0.975), and "d" was the standard error (0.05). Thus, with these constraints and probabilities, a sample size of 117 non-duplicative clinical P. aeruginosa isolates. In general, from March 2020 to February 2021, 117 non-duplicative clinical samples were collected in this cross-sectional study from hospitalized patients admitted to the Rohani hospital, Babol, north of Iran. A positive P. aeruginosa culture results and age ⩾17 years were included. All isolates were identified as *P. aeruginosa* using biochemical and microbiological tests such as colony morphology and pigment production, Gram staining, oxidase, triple sugar iron (TSI), SIM, citrate, oxidative-fermentative (OF) media (Merck, Darmstadt, Germany), and growth at 42 °C. All strains were conserved in the brain-heart infusion broth (Becton Dickinson, Franklin Lakes, NJ) comprising 15% (v/v) glycerol (Merck Co., Germany) at -80 ˚C for further use. *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as positive and negative control, respectively.

Antimicrobial susceptibility testing (AST)

Susceptibility of isolates to different antibiotics, including piperacillin (PRL, 100 µg), piperacillin-tazobactam (PTZ, 100/10 µg), ticarcillin (TC, 75 µg), Ticarcillin-clavulanate (TCC, 75/10 µg) levofloxacin (LEV; 5 µg); ceftazidime (CAZ; 30 µg), ceftriaxone (CRO, 30 µg), imipenem (IPM; 10 µg), chloramphenicol (CHL, 30 µg), colistin (CS, 10 µg), gentamicin (GM; 10 µg), ciprofloxacin (CIP; 5 µg), and tetracycline (TET; 30 µg) (MAST Diagnostics, Merseyside, UK) was determined by Disk agar diffusion (DAD) method on the cation adjusted Mueller–Hinton plates (Merck, Darmstadt, Germany) according to the Clinical and Laboratory Standards Institute (CLSI) recommendations **(10)**.

Metallo β-lactamase (MBL)-producing strains

The combined double-disk synergy test (CDDST) was used to detect MBL- producing isolates. E-test strips of IPM ranging from; 4 to 256 µgmL^{-1} and IPM/EDTA (IMD) ranging from; 1 to 64 µgmL^{-1} (Liofilchem SRL, Rosetodegli Abruzzi, Italy) were used in agreement with the manufacturer's instruction (8).

Modified Hodge test (MHT)

Klebsiella pneumoniae carbapenemase (KPC)-producing *P. aeruginosa* isolates were identified using MHT. A 24h suspension of *E. coli* ATCC 25922 with equal turbidity to 0.5 McFarland standard solution was inoculated by a sterile cotton swab to the Mueller-Hinton Petri (Merck Co., Germany). Then, a 10 µg meropenem disk (MEM) was inserted at the center of the plate, and a test isolate was cultured from the margin of the disk to the edge of the plate in four directions. After incubation at 37˚C for 24h, the presence of a 'cloverleaf shaped' inhibition zone indicates a positive MHT test **(8)**.

Biofilm formation assay

Quantitative biofilm production was evaluated in vitro by a microtiter plate assay. Briefly, 200 µL of 1:100 dilutions of all overnight-grown microbial cultures in brain heart infusion (BHI) broth were inoculated into a 96-well flat-bottomed polystyrene Petri. After incubation at 37 °C overnight, the wells were washed twice with phosphate buffer saline (PBS, pH 7.2) to eliminate loosely attached and floating 'planktonic' isolates. Then the plates were forcefully shaken to remove all non-adherent strains. It was dried at room temperature to fix the attached biofilm. Biofilms were stained with 200 µL of crystal violet 0.1% (Sigma, St Louis, USA) for 6 min at 24°C and then washed with tap water and allowed to dry. The optical density (OD) was measured at 570 nm by an ELISA reader (BioTek, Bad Friedrich shall, Germany). Each test was done in triplicate, and a cut-off value (ODc) was determined. Biofilm formation was recorded as follows: non-biofilm forming (OD_{570}< 1), weak (1< OD_{570}<2), moderate (2< OD_{570}< 3) and strong (OD_{570}> 3) **(8)**.

Alginate production assay

The alginate production level was assessed using the Carbazole method. Briefly, after culturing each sample in Luria–Bertani broth, incubation was performed in a shaker incubator for 24 h at 37 ˚C and then centrifuged for 35 min at 12,000 ×g. To precipitate alginate, the supernatant was mixed with 95% ethanol (-70 ˚C) and centrifuged at 12,000 × g for 20 min. About 30 mL of alginate solution was mixed with 1.0 mL of borate–sulfuric acid (10 mM H_3BO_3 in concentrated H_2SO_4) and 300 mL of the 0.1% (w/v) Carbazole (Sigma-Aldrich, St. Louis, Missouri, United States). The mixture was heated at 50˚C for 30 min to develop the color reaction. Finally, the optical density was measured at 530 nm (OD_{530}) **(15)**.

Multiplex Polymerase Chain Reactions

After overnight incubation, genomic DNA was extracted from the fresh colonies using the Bacteria Genome DNA Extraction Kit (TaKaRa, Dalian, China) and stored at –20 °C until used. The primer sequences are listed in Table 1. Multiplex PCRs were done in a final volume of 25 µL, include of 1.0 µL of template DNA (30ng/ µL), 12.5 µl Maxima Hot Start PCR Master Mix (2×) (Fermentas GmbH, St. Leon-Rot, Germany), 0.8 µL of each primer (10mM), and 9.9 µl of ddH_2O. The process of Multiplex PCR reactions was performed according to Table 2 in a Techne TC-512 thermocycler (Eppendorf, Hamburg, Germany). PCR amplicons were analyzed with UV light after running at 100 V for 60 min on a 1% agarose gel stained with DNA safe stain (SinaClon, Tehran, Iran).

Table 1. Oligonucleotide primer sequences used in this study (3, 9, 11, 13, 14, 19, 30)

Genes			Primer sequences (5′→3′)	Product size (bp)
Carbapenems-resistance genes	OXAs	*OXA-23-like*	F=5′- GATCGGATTGGAGAACCAGA -3′ R: 5- ATTTCTGACCGCATTTCCAT -3′	501
		OXA-24/40-like	F=5′- GGTTAGTTGGCCCCCTTAAA-3′ R: 5′ - AGTTGAGCGAAAAGGGGATT-3′	246
		OXA-58-like	F= 5′- AAGTATTGGGGCTTGTGCTG -3′ R=5′- CCCCTCTGCGCTCTACATAC -3′	599
		OXA-48-like	F=5′- TTGGTGGCATCGATTATCGG-3′ R=5′- GAGCACTTCTTTTGTGATGGC-3′	744
	MBLs	*IMP*	F=5′- TGAGCAAGTTATCTGTATTC-3′ F=5′- TTAGTTGCTTGGTTTTGATG-3′	740
		SPM	F=5′- CCTACAATCTAACGGCGACC-3′ F=5′- TCGCCGTGTCCAGGTATAAC-3′	674
		VIM	F=5′- GATGGTGTTTGGTCGCATA-3′ F=5′- CGAATGCGCAGCACCAG-3′	390
		NDM	F=5′- GGGCAGTCGCTTCCAACGGT-3′ F=5′- GTAGTGCTCAGTGTCGGC AT-3′	475
	KPC	KPC	F=5′- ATGTCACTGTATCGCCGTCT-3′ F=5′- TTACTGCCCGTTGACGCCC-3′	882
	Efflux-encoded gene	*MexA*	F=5′- ACCTACGAGGCCGACTACCAGA -3′ F=5′- GTTGGTCACCAGGGCGCCTTC -3′	179
		MexB	F=5′- GTGTTCGGCTCGCAGTACTC -3′ F=5′- AACCGTCGGGATTGACCTTG -3′	244

Genes			Primer sequences (5′→3′)	Product size (bp)
Biofilm and alginate formation genes		*MexC*	F=5′- GTACCGGCGTCATGCAGGGTTC -3′ F=5′- TTACTGTTGCGGCGCAGGTGACT -3′	164
		MexX	F=5′- TGAAGGCGGCCCTGGACATCAGC-3′ F=5′- GATCTGCTCGACGCGGGTCAGCG-3′	326
		OprM	F=5′- CCATGAGCCGCCAACTGTC -3′ F=5′- CCTGGAACGCCGTCTGGAT -3′	205
	Housekeeping gene	*rpsL*	F=5′- AAGCGCATGGTCGACAAGA-3' F=5′- CTGTGCTCTTGCAGGTTGTGA-3'	201
		pslA	F=5′- TCCCTACCTCAGCAGCAAGC -3′ F=5′- TGTTGTAGCCGTAGCGTTTCTG -3′	656
		pelA	F=5′- CATACCTTCAGCCATCCGTTCTTC -3′ F=5′- CGCATTCGCCGCACTCAG -3′	786
		ppyR	F=5′- CGTGATCGCCGCCTATTTCC -3′ F=5′- ACAGCAGACCTCCCAACCG -3′	160
		algU	F=5′- CGATGTGACCGCAGAGGATG-3′ F=5′- TCAGGCTTCTCGCAACAAAGG-3′	292
		algD	F= 5′-AGAAGTCCGAACGCCACACC-3′ R= 5′-CGCATCAACGAACCGAGCATC-3′	550

Table 2. Multiplex PCRs programs and cycles in the present study

Reaction set	Amplified genes	Reaction compounds	M-PCR program	Cycles of amplification
S1	*OXA-23-like* *OXA-24/40-like* *OXA-58-like* *OXA-48-like*	1.5 µL of template, 12.5 µL of PCR Master Mix, 1.0 µL of each primer, and 9.0 µL of ddH2O.	Initial denaturation at 94°C for 6 min, denaturation at 95°C for 35 s, annealing at 58°C for 60 s, extension at 72°C for 60 s and a final extension at 72°C for 6 min.	30
S2	IMP SPM VIM NDM KPC	1.0 µL of template DNA, 13.5 µL of CinnaGen PCR Master Mix, 1.1 µL of each primer, and 8.3 µL of ddH2O	initial denaturation at 95°C for 7 min, denaturation at 94°C for 50 s, annealing at 57°C for 50 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min.	
S3	*mexA* *mexB* *mexC* *mexX* *oprM*	1.2 µL of template DNA, 12.6 µL of CinnaGen PCR Master Mix, 0.9 µL of each primer, and 9.7 µL of ddH2O	initial denaturation at 94°C for 5 min, denaturation at 95°C for 55 s, annealing at 56°C for 50 s, extension at 72°C for 60 s and a final extension at 72°C for 5 min.	32
S4	*pslA* *pelA* *ppyR* *algU* *algD*	1.8 µL of template DNA, 13.6 µL of CinnaGen PCR Master Mix, 1.5 µL of each primer, and 6.6 µL of ddH2O.	initial denaturation at 95°C for 5 min, denaturation at 95°C for 60 s, annealing at 55°C for 45 s, extension at 72°C for 60 s and a final extension at 72°C for 5 min.	33

Efflux pump inhibition (EPI)

In order to evaluate the phenotypic activity of the efflux pump, the minimum inhibitory concentration (MIC) of IPM ranging between 2-256 µgmL^{-1} was determined in the presence and absence of Cyanide 3-Chlorophenyl-hydrazone (CCCP; C2759 Sigma-Aldrich, France) as an EPI at a final concentration of 25 µgmL^{-1} based on Azimi *et al*. **(14)** The strains were confirmed as efflux pumps hyper-expressed when the MICs value in the presence of EPI were at least quadruple less than the MICs in the absence of CCCP. The *P. aeruginosa* PAO1 was used as a reference strain.

Total RNA extraction and cDNA synthesis

A Thermo RNA extraction kit (cat. No. K0732) was used for total RNA extraction. Templet RNA was treated with RNase-free DNaseI (SinaClon) and quantified by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA purity was assessed in an absorbance ratio of 260 nm vs. 280 nm. Complementary DNA (cDNA) was synthesized using the M-MuL V reverse transcriptase (SinaClon) by adding 5 µg DNA-free RNA and random hexamer primer based on the manufacturer's guidelines.

Quantitative real-time PCR (qRT-PCR)

SYBR Premix EX TaqII (Tli RNaseH Plus) (Takara Bio Inc.) was used for amplification of *mexA*, *mexB*, *mexC*, *mexX* and *oprM* genes in duplicate repeats at the Rotor-Gene Real-Time PCR machine (Corbett

Research, Sydney, Australia; Model RG 3000). The *rpsL* gene was used as a house-keeping gene. The primer sequences are shown in Table 1. Relative overexpression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Normalized expression of all genes was calibrated compared with corresponding mRNA expression by PAO1; results are present as an mRNA relative expression compared with the wild type PAO1. Downexpression of *oprM* was considered relevant when it was 30% compared to the *P. aeruginosa* PAO1 quality strain. Each qRT-PCR reaction was run in triplicate with DEPC-treated water as a negative control.

Statistical analysis

Chi-Square test was done on the relationship between categorical variables, counting biofilm characteristics and antimicrobial resistance by SPSS, 22.0 (SPSS Inc., Chicago, IL, USA). A $P_{\text{-value}} < 0.05$ was considered statistically significant.

3. Results

In total 117 non-repetitive *P. aeruginosa* isolates were collected from 58.9% (n=69) male and 41.1% (n=48) female. The mean age of the cases was 43.4±1.1 years, ranging from 17 to 81 years. The origin of strains was urine (n = 37, 31.6%), respiratory tract (bronchoalveolar lavage [BAL], sputum and endotracheal tube [ETT]) (n = 32, 27.4%), wound (n = 18, 15.4%), blood (n = 11, 9.4%), skin lesions (11; 9.4%), eye swabs (*n* = 3, 2.6%), cerebrospinal fluid (CSF) (n = 2, 1.7%), ear swab (n = 1, 0.8%), heart biopsy (n = 1, 0.8%) and orthopedic device (n = 1, 0.8%). The distribution of strains according to hospital wards were as follows: intensive care unit [ICU] (n; 22, 18.8%), neonatal intensive care unit [NICU] (n; 19, 16.2%), infectious disease (n; 16, 13.7%), urology (n; 14, 11.9%), gastroenterology (n; 11, 9.4%), surgery (n; 11, 9.4%), hemodialysis (n; 10, 8.5%), Ear, Nose and Throat [ENT] (n; 5, 4.3%) hematology-oncology (n; 4, 3.4%), gynecology (n; 2, 1.7%), neurosurgery (n; 1, 0.8%) ophthalmology (n; 1, 0.8%) and orthopedics (n; 1, 0.8%).

As shown in Table 3, all isolates were susceptible to CS. The highest resistance rate was related to CAZ (n; 67, 57.3%), CHL (n; 57; 48.7%), CRO (n; 49, 41.9%), TET (n; 45, 38.5%) and LEV (n; 33, 28.2%). Twenty-nine (24.7%) isolates were resistant to IMP, considered CRPA. The prevalence of patients infected with CRPA in different wards were as follows; ICU (n; 11, 37.9%), infectious disease (n; 8, 27.6%), surgery (n; 4, 13.7%), NICU (n; 2, 6.8%), urology (n; 2, 6.8%), gastroenterology (n; 1, 3.4%), and hematology-oncology (n; 1, 3.4%). The frequency of MDR and XDR strains were 43.6% (n; 51) and 9.4% (n; 11), respectively.

Table 3. No [(%)] antimicrobial resistance pattern in CRPA and CSPA isolates

Clinical isolates		PRL	PTZ	TC	TCC	LEV	CAZ	CRO	IPM	CHL	GM	CIP	TET	CS
CRPA (n= 29)	S	25 (86.2)	28 (96.5)	27 (93.1)	27 (93.1)	11 (37.9)	8 (27.6)	9 (31.0)	0 (0.0)	10 (34.5)	18 (62.1)	18 (62.1)	8 (27.6)	29 (100.0)
	I	0 (0.0)	1 (3.4)	0 (0.0)	1 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (10.3)	1 (3.4)	0 (0.0)	0 (0.0)
	R	4 (13.7)	0 (0.0)	2 (6.8)	1 (3.4)	18 (20.5)	21 (72.4)	20 (68.9)	29 (100.0)	19 (65.5)	8 (27.6)	10 (34.5)	21 (72.4)	0 (0.0)
CSPA (n=88)	S	84 (95.5)	85 (96.6)	88 (100.0)	86 (97.7)	70 (79.5)	36 (40.9)	50 (56.8)	0 (0.0)	46 (52.3)	81 (92.0)	78 (88.6)	61 (69.3)	88 (100.0)
	I	1 (1.1)	3 (3.4)	0 (0.0)	2 (2.3)	3 (3.4)	6 (6.8)	9 (10.2)	0 (0.0)	4 (4.5)	5 (5.6)	6 (6.8)	3 (3.4)	0 (0.0)
	R	3 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	15 (17.1)	46 (52.3)	29 (32.9)	88 (100.0)	38 (43.2)	2 (2.3)	4 (4.5)	24 (27.3)	0 (0.0)

In total, 24.1% (n; 7 of 29) and 17.2% (n; 5 of 29) of CRPA isolates were MBL- positive with the CDDST and E-test methods, respectively. Carbapenem-susceptible *P. aeruginosa* (CSPA) isolates have no MBL phenotype in both CDDST and E-test methods. In all CRPA isolates, the presence of KPC was phenotypically and genotypically positive in 17.2% (n; 5 of 29) and 10.3% (n; 3 of 29), respectively. Carbazole test results showed that 53.8% (n; 63 of 117) of strains were alginate-producing isolates, of which 37.9% (n; 11 of 29) were CRPA. The *algD* and *algU* genes were 41.8% (n; 49 of 117) and 26.5% (n; 31 of 117), respectively. Most CRPA strains carried *alg* genes. *algD* and *algU* genes were present simultaneously in 49.1% (n; 26 of 53) strains.

In general, 62.4% (n; 73 of 117) of isolates were able to form a biofilm, 28.8% (n; 21 of 73) of which were CRPA. Compared with the cut-off value, the phenotype of biofilm formation was as follows; weak (n; 11/73, 15.1%), moderate (n; 19/73, 26.0%) and strong (n; 43/73, 58.1%). Out of all biofilm-producer CRPA strains, 85.7% (n; 18 of 21) and 14.3% (n; 3 of 21) were strong and moderate biofilm-producer. There is

a significant relationship between biofilm production and carbapenem resistance (P-$_{value}$ <0.05). Fifty-eight isolates were able to produce biofilm and alginate simultaneously. All biofilm-producing strains carried *ppyR* gene. So, 90.4% (n; 66 of 73) and 43.8% (n; 32 of 73) of biofilm-producer isolates were positive for *pslA* and *pelA* genes, respectively. Eighteen isolates carried *pslA* and *pelA* genes simultaneously. The prevalence of *pslA* and *pelA* genes in the biofilm-producer CRPA isolates was 90.5% (n; 19 of 21) and 52.4% (n; 11 of 21), respectively. The frequency of MBL genes were *bla*IMP (62.1%, n = 18/29), *bla*VIM (31.0%, n = 9/29), and bla_{NDM} (6.8%, n = 2/29). Out of all CRPA isolates, 20.6% (n: 6 of 29), 6.8% (n: 2 of 29), 3.4% (n: 1 of 29) and 3.4% (n: 1 of 29) simultaneously carried bla_{IMP}/bla_{VIM}, bla_{IMP}/bla_{NDM}, bla_{VIM}/bla_{NDM} and bla_{IMP}/bla_{VIM}/bla_{NDM} genes, respectively. On the other hand, 75.8% (n: 22 of 29), 10.3% (n: 3 of 29), and 3.4% (n: 1 of 29) of CRPA isolates harbored OXA-48-like, OXA-23-like, and OXA-24/40-like, respectively. SPM and OXA-58-like were not found in any isolates.

According to the phenotypic assessments, it was found that 37.9% (n; 11/29) of CRPA isolates have active efflux pumps by adding CCCP. Compared to the PAO1 strain, the relative levels of MexX, MexC, MexB and MexA mRNA in CRPA strains with active efflux pump was 81.8% (n; 9/11), 63.6% (n; 9/11), 54.5% (n; 6/11) and 36.4% (n; 4/11), respectively. Furthermore, 27.3% (n; 3/11) isolates showed a hyperexpression of both mexXY-oprM and mexCD-oprJ efflux genes. Among the MDR- and XDR- CRPA isolates, the mean gene expression of *mexX, mexC, mexB,* and *mexA,* were 61.54 (CI 95%: 14.24 to 107.15), 10.32 (CI 95%: 2.01 to 21.56), 7.14 (CI 95%: 1.80 to 14.50) and 2.23 (CI 95%: 1.00 to 4.11), respectively. 5 and 9 strains overexpressed only one or two active efflux pumps. Fifty-eight percent of the isolates overexpressed at least one efflux pump. Surprisingly, all CRPA isolates with active efflux pumps had reduced *oprM* expression. The relative reduction in expression of oprM in MDR isolates was significantly higher than in non-MDR CRPA strains (p-value <0.002). According to the statistical analysis, the hyperexpression of efflux genes had a significant correlation with resistance to all antibiotics (P-value < 0.05) except CS, PTZ, TC and TCC.

4. Discussion

Introduction emerging strains of *P. aeruginosa* resistant to different antibiotics, especially carbapenem-producing isolates, are major therapeutic problems worldwide. CS is the last line of treatment for infections caused by CRPA (9). In agree with Pournajaf et al., **(8)** our AST result showed that CS is also the best choice in treating CRPA infections **(8)**. In agreement with Javiya *et al.,* **(16),** *Pseudomonas* species demonstrated marked resistance against monotherapy of cephalosporins, penicillins, fluoroquinolones, macrolides, and tetracyclines (16). In a 4-year follow-up in the US (2012 to 2015), Sader *et al.,* **(17)** showed that the only antimicrobials with higher than 90% susceptibility rates were CS, ceftazidime-avibactam, and amikacin **(17)**.

Interestingly, Goli *et al.,* **(18)** and Farajzadeh Sheikh *et al.,* **(19)** showed that resistance to CS in *P. aeruginosa* strains was increasing (2%, n =2 of 100 isolates in Goli *et al,* and 1.3%, n = 5 of 269 in Farajzadeh Sheikh *et al.*, studies) (18, 19). The researchers concluded that the emergence of CS-resistant *P. aeruginosa* is increasing, which indicates a great challenge in treating infections caused by MDR P. aeruginosa, antibiotic stewardship, and their rational administration are suggested.

On the other hand, CRPA is considered the most severe hospital pathogens menacing public health, and the World Health Organization has subjected them to a global priority pathogens list of antibiotic-resistant microorganisms (20). In line with Azimi *et al.,* **(14)** and Dogonchi *et al.,* **(11)**, 24.7% (n=29) of our collected isolates were resistant to IPM (11, 14). In contrast to our study, Goncalves *et al.,* **(21)** showed that out of 157 collected *P. aeruginosa* strains, 43.9% were CRPA **(21)**. The sources of collected bacteria and geographical distance may justify this difference in frequency. As you can see in Table 4, the frequency of resistance and virulence genes was higher in CRPA strains (P-$_{value}$ <0.05). Regardless of the similar distribution of bla_{NDM} genes in Dogonchi *et al.,* (2018) study, the frequencies of bla_{IMP} and bla_{VIM} genes were 90% and 40%, respectively, which differed from our study **(11)**. Genetic differences between strains and the localization of the geographical distribution of resistance genes may be justified.

Table 4. Molecular distribution of virulence factors and resistance genes in CRPA and CSPA isolates

Strains	*OXA-23-*	OXA-24/40	*OXA-58*	*OXA-48*	*IMP*	*VIM*	*NDM*	*KPC*	*pslA*	*pelA*	*ppyR*	*algU*	*algD*
CRPA (n= 29)	3 (10.3)	1 (3.4)	0 (0.0)	22 (75.8)	15 (51.7)	8 (27.6)	2 (6.9)	3 (10.3)	19 (65.5)	11 (37.9)	29 (100.0)	27 (93.1)	23 (73.9)
CSPA (n=88)	0 (0.0)	0 (0.0)	0 (0.0	0 (0.0	3 (3.4)	1 (1.1)	0 (0.0)	0 (0.0)	47 (53.4)	21 (23.8)	44 (50.0)	4 (4.5)	26 (29.5)

No [(%)] Distribution of virulence and resistance genes.

Virulence genes distribution showed that the prevalence of *pslA*, *pelA*, *ppyR*, *algU* and *algD* genes were 65.5%, 37.9%, 100%, 93.1%, and 73.9% in CRPA isolates. In a study of Iranian cystic fibrosis patients by Pournajaf *et al.,* (8), the frequency of *algD*, *algU*, *ppyR*, *pslA,* and *pelA* genes were 92.3%, 86.7%, 98.6%, 89.5%, and 57.3%, respectively **(8)**. The slight difference observed may be due to the important role of the biofilm and alginate formation in the CF airways. Based on PCR data from Ghadaksaz *et al.,* **(15)**, the distribution of the genes in the clinical *P. aeruginosa* isolates was as follows: *ppyR* (99%), *pslA* (83.7%), *pelA* (45.2%), *algU* (90.4%), and, *algD* (87.5%) **(15)**. In contrast with Płókarz *et al.,* **(22)** 28.8% of our strains were biofilm producers **(22)**. The study design and source of samples (human vs. animals) could be the reason for this discrepancy. In our study, antibiotic resistance was higher in strong biofilm strains. Gajdács *et al.,* **(23)** showed that the distribution of isolates with different biofilm-forming capacities did not show noticeable differences among the MDR and non-MDR groups **(23)**. As inferred from our results, 24.1% and 17.2% of CRPA isolates were MBL- positive with the CDDST and E-test methods, respectively. In agreement with Sachdeva *et al.,* **(24),** the MHT has the least sensitivity and specificity **(24)**. In concord with Galani *et al.,* **(25),** the combination disc test (CDT) with imipenem/imipenem plus 0.5 M EDTA or ceftazidime/ceftazidime plus 0.2 M EDTA and the double-disc synergy test (DDST) with imipenem 10 mm apart from EDTA are the most effective methods for detection of MBLs **(25)**. So, Ranjan *et al.,* **(26)**, Šuto *et al.,* **(27),** and Cherak *et al.,* **(28)** demonstrated that DDST was more specific in detecting MBLs in comparison to CDT **(26-28)**. This inconsistency in results may be due to changes in the population structure of MBL genes between various geographical regions. Moreover, false-positive MBL producers might produce weaker and unknown β-lactamases, which should be differentiated.

In this study, 37.9% of CRPA strains had an active efflux pump. Increased expression of MexX, MexC, MexB and MexA in CRPA strains was 81.8%, 63.6%, 54.5% and 36.4%, respectively. In a study directed by Rodríguez-Martínez *et al,* (2009) the overexpression of MexAB-OprM, MexCD-OprJ and MexXY-OprM were 28%, 31% and 37.5% in the CRPA isolates, respectively **(12)**. The results of Gutierrez *et al.,* **(29)** showed a partial association between the relative level of MexAB-OprM mRNA and resistance to meropenem **(29)**. In contrast with our data, Pourakbari *et al,* **(30)**, declared that of 45 *P. aeruginosa* isolates the frequency of genes overexpression was as follows: *mexA* (n=25, 55.5%), *mexB* (n=24, 53.3%) and *oprM* (n=16, 35.5%) (30). In contrast with our findings, Azimi *et al.,* **(14)** found that 20% (n= 28/140) of CRPA had active efflux pumps by adding CCCP **(14)**. In the real-time PCR assay process, 54% (n= 15/ 28) of *P. aeruginosa* with positive IEPs showed overexpression of mexX, mexC, and mexA genes. Goli *et al,* **(13)** showed that 68.4% (n= 39) and 75.4% (n=43) isolates exhibited an overexpression of mexAB-oprM and mexXY-oprM, respectively **(13)**. One of the most important reasons for the difference is the origin of the samples (burn and ICU isolates vs. other samples). As an achievement of the present study and agrees with Goli *et al*., **(13),** the hyperexpression of efflux pumps had a significant correlation with resistance to all antibiotics, excluding CS, PTZ, TC, and TCC **(13)**. So, it can be inferred that efflux pumps were probably not the main cause of high-level resistance to these antimicrobials.

5. Conclusion

The current study identified IMP-resistant P. aeruginosa strains from different clinical samples. Different resistant mechanisms in *P. aeruginosa* can cause cross antibiotic resistance, lead to the appearance of resistant strains, and make the treatment difficult. Biofilm production is directly related to antibiotic resistance. Efflux pumps are actively expressed in carbapenem-resistant strains. Therefore, it is essential to find a way to inhibit efflux pumps from controlling the cross-resistance and the emergence of these hard-to-treat strains.

Acknowledgment

We appreciate the sincere collaboration of the Microbiology Department, Ayatollah Amoli branch of Islamic Azad University, Department of Medical Microbiology and Biotechnology, School of Medicine, Babol University of medical sciences and Department of medical mycology and parasitology, School of Medicine, Babol University of Medical Sciences for assistance in the conduct of the study.

Ethics approval

This project was approved by the Ethics Committee of the Babol Branch, Islamic Azad University, with the Ethical code number IR.IAU.BABOL. REC 1399.090.

Funding

This study was financially supported by the Ayatollah Amoli Branch, Islamic Azad University, Amol, north of Iran. The funding body had no role in the project's design, sampling, analysis, interpretation of data, or writing and editing of the manuscript.

Conflict of Interest

The authors declared no conflict of interests.

Reference

1. Chaudhury N, Paul R, Misra RN, Chaudhuri SS, Mirza S, Sen S. Evaluating the trends of bloodstream infections by nonfermenting Gram negative Bacilli among the patients in a tertiary Care Hospital of western part of India and its antibiogram. Int J Curr Microbiol Appl Sci. 2019; 8(1):1149-62. [DOI:10.20546/ijcmas.2019.801.121]
2. Kunz Coyne AA-O, El Ghali AA-O, Holger DA-O, Rebold NA-O, Rybak MA-OX. Therapeutic Strategies for Emerging Multidrug-Resistant Pseudomonas aeruginosa. Infect Dis Ther. 2022; 11(2):661-82. [DOI:10.1007/s40121-022-00591-2] [PMID] [PMCID]
3. Rouhi S, Ramazanzadeh R. Prevalence of blaOxacillinase-23and blaOxacillinase-24/40-type Carbapenemases in Pseudomonas aeruginosa Species Isolated from Patients with Nosocomial and Non-nosocomial Infections in the West of Iran. Iran J Pathol. 2018;13(3).
4. Tuon FF, Dantas LR, Suss PH, Tasca Ribeiro VS. Pathogenesis of the Pseudomonas aeruginosa Biofilm: A Review. Pathogens. 2022;11(3):300. [DOI:10.3390/pathogens11030300] [PMID] [PMCID]
5. Noori HG, Tadjrobehkar O, Moazamian E. Biofilm formation capacity of Pseudomonas aeruginosa is significantly enhanced by sub-inhibitory concentrations of Tomatidine. Gene Rep. 2022; 27:101570. [DOI:10.1016/j.genrep.2022.101570]
6. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. Curr Opin Microbiol. 2007;10(6):644-8. [PMID] [PMCID] [DOI:10.1016/j.mib.2007.09.010]
7. Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C, et al. The Pel and Psl polysaccharides provide Pseudomonas aeruginosa structural redundancy within the biofilm matrix. Environ Microbiol. 2012;14(8):1913-28. [PMID] [PMCID] [DOI:10.1111/j.1462-2920.2011.02657.x]
8. Pournajaf A, Razavi S, Irajian G, Ardebili A, Erfani Y, Solgi S, et al. Integron types, antimicrobial resistance genes, virulence gene profile, alginate production and biofilm formation in Iranian cystic fibrosis Pseudomonas aeruginosa isolates. Infez Med. 2018;26(3):226-36.
9. Azizian K, Alikhani MY, Mashouf RY, Gholizadeh P, Noshak MA. Antibiotic resistance patterns and frequency of metallo-beta-lactamases in Klebsiella pneumoniae in Hamadan, Iran. J Contemp Clin Pract. 2019;5(1):10-7.
10. Weinstein MP. Performance standards for antimicrobial susceptibility testing: Clinical and Laboratory Standards Institute; 2021.
11. Dogonchi AA, Ghaemi EA, Ardebili A, Yazdansetad S, Pournajaf A. Metallo-β-lactamase-mediated resistance among clinical carbapenem-resistant Pseudomonas aeruginosa isolates in northern Iran: A potential threat to clinical therapeutics. Tzu Chi Med J. 2018;30 (2):90-6. [DOI:10.4103/tcmj.tcmj_101_17] [PMID] [PMCID]
12. Rodríguez-Martínez J-M, Poirel L, Nordmann P. Molecular Epidemiology and Mechanisms of Carbapenem Resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2009;53(11):4783-8. [DOI:10.1128/AAC.00574-09] [PMID] [PMCID]
13. Goli HR, Nahaei MR, Rezaee MA, Hasani A, Kafil HS, Aghazadeh M, et al. Role of MexAB-OprM and MexXY-OprM efflux pumps and class 1 integrons in resistance to antibiotics in burn and Intensive Care Unit isolates of Pseudomonas aeruginosa. J Infect Public Health. 2018;11(3): 364-72. [DOI:10.1016/j.jiph.2017.09.016] [PMID]
14. Azimi L, Fallah F, Karimi A, Shirdoust M, Azimi T, Sedighi I, et al. Survey of various carbapenem-resistant mechanisms of Acinetobacter baumannii and Pseudomonas aeruginosa isolated from clinical samples in Iran. Iran J Basic Med Sci. 2020;23(11):1396.
15. Ghadaksaz A, Fooladi AAI, Hosseini HM, Amin M. The prevalence of some Pseudomonas virulence genes related to biofilm formation and alginate production among clinical isolates. J Appl Biomed. 2015;13(1):61-8. [DOI:10.1016/j.jab.2014.05.002]
16. Javiya VA, Ghatak SB, Patel KR, Patel JA. Antibiotic susceptibility patterns of Pseudomonas aeruginosa at a tertiary care hospital in Gujarat, India. Indian J Pharmacol. 2008;40(5):230. [DOI:10.4103/0253-7613.44156] [PMID] [PMCID]
17. Sader HS, Huband MD, Castanheira M, Flamm RK. Pseudomonas aeruginosa antimicrobial susceptibility results from four years (2012 to 2015) of the international network for optimal resistance monitoring program in the United States. Antimicrob Agents Chemother. 2017;61 (3):e02252-16. [DOI:10.1128/AAC.02252-16] [PMID] [PMCID]
18. Goli HR, Nahaei MR, Ahangarzadeh Rezaee M, Hasani A, Samadi Kafil H, Aghazadeh M.

Emergence of colistin resistant Pseudomonas aeruginosa at Tabriz hospitals, Iran. Iran J Microbiol. 2016;8(1):62-9.

19. Sheikh AF, Shahin M, Shokoohizadeh L, Halaji M, Shahcheraghi F, Ghanbari F. Molecular epidemiology of colistin-resistant Pseudomonas aeruginosa producing NDM-1 from hospitalized patients in Iran. Iran J Basic Med Sci. 2019;22 (1): 38.

20. Armin S, Fallah F, Azimi L, Kafil HS, Ghazvini K, Hasanzadeh S, et al. Warning: spread of NDM-1 in two border towns of Iran. Cell Mol Biol. 2018; 64(10):125-9. [DOI:10.14715/cmb/2018.64.10.20] [PMID]

21. Gonçalves IR, Dantas RCC, Ferreira ML, Batistão DWdF, Gontijo-Filho PP, Ribas RM. Carbapenem-resistant Pseudomonas aeruginosa: association with virulence genes and biofilm formation. Braz J Microbiol. 2017;48(2):211-7. [PMID] [PMCID] [DOI:10.1016/j.bjm.2016.11.004]

22. Płókarz D, Czopowicz M, Bierowiec K, Rypuła K. Virulence Genes as Markers for Pseudomonas aeruginosa Biofilm Formation in Dogs and Cats. Animals. 2022;12(4):422. [DOI:10.3390/ani12040422] [PMID] [PMCID]

23. Gajdács M, Baráth Z, Kárpáti K, Szabó D, Usai D, Zanetti S, et al. No correlation between biofilm formation, virulence factors, and antibiotic resistance in Pseudomonas aeruginosa: results from a laboratory-based in vitro study. Antibiot. 2021;10(9):1134. [PMID] [PMCID] [DOI:10.3390/antibiotics10091134]

24. Sachdeva R, Sharma B, Sharma R. Evaluation of different phenotypic tests for detection of metallo-β-lactamases in imipenem-resistant Pseudomonas aeruginosa. J lab physicians. 2017; 9(04):249-53. [DOI:10.4103/JLP.JLP_118_16] [PMID] [PMCID]

25. Galani I, Rekatsina PD, Hatzaki D, Plachouras D, Souli M, Giamarellou H. Evaluation of different laboratory tests for the detection of metallo-β-lactamase production in Enterobacteriaceae. J Antimicrob Chemother. 2008;61(3):548-53. [DOI:10.1093/jac/dkm535] [PMID]

26. Ranjan S, Banashankari GS, Babu PRS. Evaluation of phenotypic tests and screening markers for detection of metallo-β-lactamases in clinical isolates of Pseudomonas aeruginosa: A prospective study. Med J DY Patil Vidyapeeth. 2015;8(5):599. [DOI:10.4103/0975-2870.164977]

27. Šuto S, Bedenić B, Likić S, Kibel S, Anušić M, Tičić V, et al. Diffusion of OXA-48 carbapenemase among urinary isolates of Klebsiella pneumoniae in non-hospitalized elderly patients. BMC Microbiol. 2022;22(1):1-15. [PMID] [PMCID] [DOI:10.1186/s12866-022-02443-y]

28. Cherak Z, Loucif L, Moussi A, Bendjama E, Benbouza A, Rolain J-M. Emergence of Metallo-β-Lactamases and OXA-48 carbapenemase producing Gram-negative bacteria in hospital wastewater in Algeria: A potential dissemination pathway into the environment. Microbial Drug Resistance. 2022;28(1):23-30. [DOI:10.1089/mdr.2020.0617] [PMID]

29. Gutiérrez O, Juan C, Cercenado E, Navarro F, Bouza E, Coll P, et al. Molecular epidemiology and mechanisms of carbapenem resistance in Pseudomonas aeruginosa isolates from Spanish hospitals. Antimicrob Agents Chemother. 2007;51(12):4329-35. [DOI:10.1128/AAC.00810-07] [PMID] [PMCID]

30. Pourakbari B, Yaslianifard S, Yaslianifard S, Mahmoudi S, Keshavarz-Valian S, Mamishi S. Evaluation of efflux pumps gene expression in resistant Pseudomonas aeruginosa isolates in an Iranian referral hospital. Iran J Microbiol. 2016;8 (4):249.