Integron types, antimicrobial resistance genes, virulence gene profile, alginate production and biofilm formation in Iranian cystic fibrosis *Pseudomonas aeruginosa* isolates

Abazar Pournajaf[1], Shabnam Razavi[2,3], Gholamreza Irajian[2,3], Abdollah Ardebili[4,5], Yousef Erfani[6], Sana Solgi[2], Sajad Yaghoubi[7], Afsaneh Rasaeian[2], Yousef Yahyapour[8], Ramin Kafshgari[9], Saeed Shoja[10], Ramazan Rajabnia[8]

[1]Department of Microbiology, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran;
[2]Department of Microbiology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran;
[3]Microbial Biotechnology Research Center, Iran University of Medical Sciences, Tehran, Iran;
[4]Student Research Committee, Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran;
[5]Department of Microbiology, Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran;
[6]Department of Medical Laboratory Sciences, School of Allied Medical Sciences, Tehran University of Medical Sciences, Tehran, Iran;
[7]Asadabad Faculty of Medical Sciences, Asadabad, Iran;
[8]Infectious Diseases and Tropical Medicine Research Center, Babol University of Medical Sciences, Babol, Iran;
[9]Student Research Committee, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran;
[10]Infectious and Tropical Disease Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

SUMMARY

Cystic fibrosis (CF) patients commonly suffer from continuous and recurrent lung infections caused by *Pseudomonas aeruginosa*, the dominant pathogen in CF airways. This study aimed to determine the integron types, gene cassettes, virulence determinants, β-lactam resistance genes, biofilm formation and alginate production in *P. aeruginosa* isolated from Iranian CF patients. A total of 143 *P. aeruginosa* isolates were obtained from CF patients. Susceptibility of isolates to different antimicrobials was evaluated by disc diffusion method. ESBL, MBL and KPC production was assessed. Congo red agar and tissue culture plates were used for evaluation of biofilm formation. Alginate production was determined using the Carbazole assay. Integrase genes, resistance determinants (ESBLs, MBLs and KPC) and genes encoding virulence factors were evaluated by PCR. All isolates were susceptible to colistin, piperacillin-tazobactam and ticarcillin; 8.4% of isolates were considered as MDR phenotype. Out of 6.3% IPM-resistant isolates, prevalence of virulence genes was as follows: *lasB* (100%) and *plcB* (100%), *plcH* (96.5%). Biofilm formation and alginate production ability were found in 54.5% of isolates. The prevalence of the alginate-encoding genes was 92.3%, 86.7% and 67.1% for *algD*, *algU* and *algL* genes, respectively. *PpyR*, *pslA* and *pelA* genes were detected in 98.6%, 89.5% and 57.3% of the isolates, respectively. The high prevalence of colonization in CF lungs may increase the pathogenicity of *P. aeruginosa* due to their adhesion and protective properties caused by biofilm- and alginate-production. *LasB*, *plcB*, *plcH*, *exoS*, *toxA*, *algD*, *ppyR* and *pslA* genes were predominant in CF *P. aeruginosa* strains.

Keywords: antimicrobial resistance, virulence, biofilm, cystic fibrosis, *P. aeruginosa*.

Corresponding author
Ramazan Rajabnia
E-mail: ramazan69@yahoo.com

INTRODUCTION

Cystic fibrosis (CF) is a complex autosomal recessive genetic disorder with multiorgan effects and pulmonary manifestations. This disease is due to mutations in the cystic fibrosis transmembrane conductance regulator (*cftr*) gene encoding a chloride channel. Patients with CF are born with structurally normal lungs, but expand a progressive respiratory disease with recurrent chronic infections that lead to the formation of bronchiectasis and contribute to the respiratory failure, which is the major cause of death in these issues [1]. CF patients commonly suffer from continuous and recurrent lung infections caused by *Pseudomonas aeruginosa*, the dominant pathogen in CF airways. The acquisition of *P. aeruginosa* in children with CF leads to gradual deterioration of lung illness and is responsible for most of the mortality and morbidity related to this disease. The pathogenesis of *P. aeruginosa* is considered to be multifactorial including, flagella, pili, alginate, and extracellular proteases [2]. *P. aeruginosa* also has a large number of other virulence determinants such as, exotoxin A (*exoA*), alkaline protease (*AprA*), three exoenzyme S, U, and T (*exoS*, *exoU*, *exoT*), protease IV, elastase (*lasB*), pyocyanin (a redox-active toxin) and sialidase. These elements contribute to the invasion, and colonization of *P. aeruginosa* in the lungs of CF patients [3].

Treatment of infections caused by *P. aeruginosa* is difficult due to its intrinsic and acquired resistance to a broad spectrum of antimicrobials, such as β-lactams [4]. The main mechanisms of resistance to these antimicrobials are overexpression of chromosomal cephalosporinases and production of plasmid-encoded Ambler class A (ESBLs), B (MBLs) and D (oxacillinases) β-lactamases [5]. Plasmids, conjugative transposons, integrons, integron-like elements, and insertion element common region (ISCR) determinants are vehicles and structures for the mobilization, acquisition, and spreading of resistance factors [6]. Integrons are not self-mobilizable factors, but contain an integrase (*IntI*)-encoding gene that allows the insertion of antimicrobials resistance gene cassettes between highly conserved nucleotide sequences. Biofilm formations within the CF airways is believed to mediate the infection, helping the bacteria to withstand diverse environmental conditions, including the aggressive antimicrobial disturbance and host immune response. The *pslA* and *pelA* genes play a significant role in formation of carbohydrate-rich structure of biofilm matrix. Psl produce a helical structure around *P. aeruginosa* cells which increases the cell-to-surface and cell-to-cell interactions essential for biofilm formation [7]. Also, alginate is a linear unbranched polymer encoded by *algD*, *algU* and *algL* genes, composed of 1-4 linked saccharides β-D mannuronic acid (M) and a C-5 epimer of α-L-guluronic acid (G) [7]. The alginate layer possibly impairs the ability of the immune system to battle *P. aeruginosa* infection of the CF-affected lung [8]. This study aimed to determine the integron types, gene cassettes, virulence determinants, β-lactam resistance genes, biofilm formation and alginate production in *P. aeruginosa* isolated from Iranian CF patients.

PATIENTS AND METHODS

Ethics statement

This project was approved by the Ethics Committee of The Iran University of Medical sciences (IUMS) in Ethical code number IR.IUMS.REC 1395.9221133207.

Study population

After collection of a comprehensive questionnaire, including clinical and family history, the CF disease was previously diagnosed in the patients studied by a pneumologist. The diagnosis of CF was normally carried out by clinical findings, according to a CF phenotype together with an elevated sweat chloride concentration. Diagnostic criteria for all patients were based on repeated positive sweat chloride tests and on typical findings of pulmonary/gastrointestinal illness. The sweat chloride test performed based on the pilocarpine method and considered positive if the results were above 60 mEq/L. The diagnosis of CF was established when relevant clinical manifestations were associated with a positive sweat chloride test. Clinical manifestations included respiratory signs, such as chronic cough or recurrent pneumonia and gastrointestinal symptoms in the form of chronic diarrhea or fatty diarrhea, failure to gain weight and failure to thrive (FTT) [9].

Sampling and bacterial isolation

P. aeruginosa isolates used in the present study were recovered from the sputum samples of the CF patients admitted to the several therapeutic centers of Iran's provinces (Tehran, Mazandaran, Fars, Razavi Khorasan, Yazd, Hormozgan, West Azerbaijan, and Lorestan). The geographic distribution of the studied provinces is shown in Figure 1. All isolates were identified as *P. aeruginosa* using the traditional biochemical and microbiological identification tests, including colony morphology, Gram staining, oxidase and catalase tests, pyocyanin pigment production, growth at 44°C, and oxidative-fermentative (OF) tests, and then confirmed by the API 20NE and API 32GN systems (bioMérieux, Marcy-l'Etoile, France). All strains were preserved in brain-heart infusion broth (Becton Dickinson, Franklin Lakes, NJ) containing 20% (v/v) glycerol (Merck Co., Germany) at -80 °C for further use. *P. aeruginosa* strain ATCC 27853 was used as a positive control in this study.

Antimicrobial susceptibility testing

Disk agar diffusion (DAD) test was performed for the following 14 antimicrobials: piperacillin-tazobactam (PTZ, 100/10 µg), piperacillin (PRL, 100 µg), ticarcillin (TC, 75 µg), ciprofloxacin (CIP, 5 µg), cefepime (CPM, 30 µg), ceftazidime (CAZ; 30 µg), imipenem (IPM; 10 µg), aztreonam (ATM, 30 µg), norfloxacin (NOR, 5 µg), ofloxacin (OFX, 5 µg), colistin (CO, 10 µg), gentamicin (GM, 10 µg), levofloxacin (LEV, 5 µg), and amikacin (AK, 30 µg) (MAST Diagnostics, Merseyside, UK) on the Mueller-Hinton agar (Merck Co., Germany) and interpreted in compliance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI document M100) [10]. MDR, XDR (extensively drug resistant microbes) and PDR (pan-drug resistance) isolates were recognized [11].

Phenotypic detection of ESBL

Double disc diffusion synergy test (DDST) was used for detection of ESBLs-producing *P. aeruginosa*. Briefly, the method was performed by both

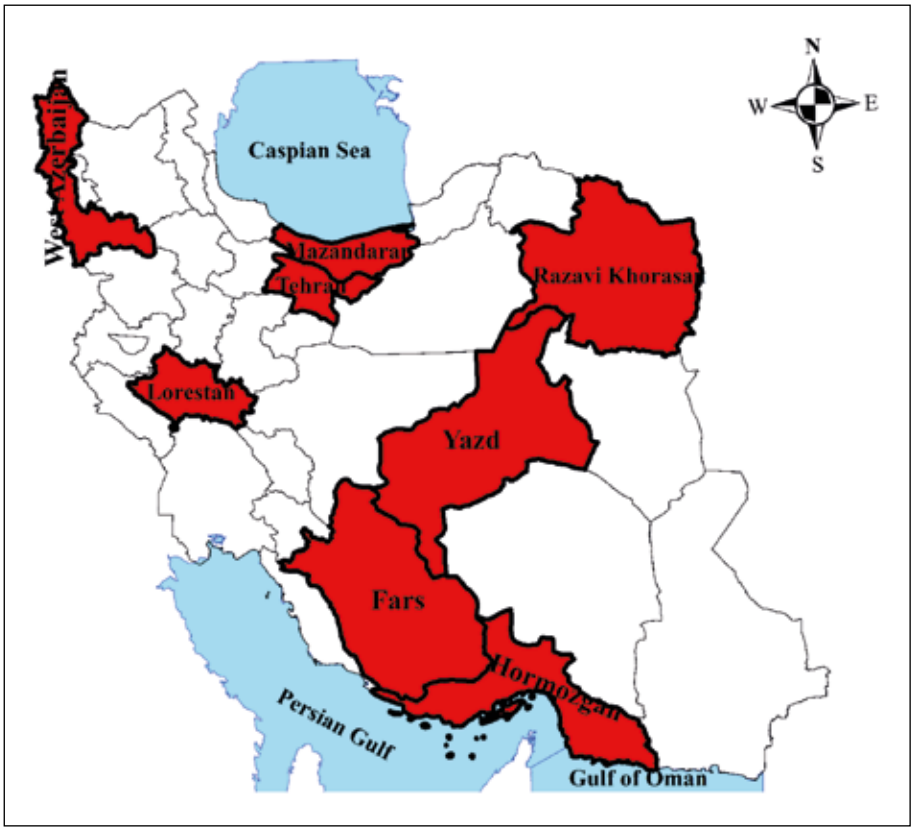

Figure 1 - Geographic locations of provinces surveyed in this study.

CTX and CAZ alone and in combination with clavulanic acid (CLA). A ≥5 mm increase in the zone diameter of inhibition (ZDI) for either CAZ and/or CTX in combination with CLA against to its ZDI when tested alone is a criterion for an ESBL-producing strain [11].

Phenotypic detection of the Metallo β-lactamase (MBL)
In agreement with the Anwar et al. combined disk test (CDT) was used for the phenotypic detection of MBLs in carbapenem resistant *P. aeruginosa*. So, MBL E-test (IPM [ranging from 4 to 256 µg/mL] and IPM/EDTA (IMD) [ranging from 1 to 64 µg/mL]) (Liofilchem SRL, Roseto degli Abruzzi, Italy) was performed in concordance with the manufacturer's recommendations [12].

Modified Hodge test (MHT) for carbapenemase detection
The IPM-resistant *P. aeruginosa* strains were subjected to the MHT for identification of carbapenemases. An overnight suspension of *Escherichia coli* ATCC 25922 with an adjusted turbidity to 0.5 McFarland standard was inoculated using a sterile cotton swab on the Mueller-Hinton agar (MHA) (Merck Co., Germany). Then, a 10 µg meropenem disk (HI-MEDIA, Mumbai, India) was placed at the center of the Petri dish and test isolate was cultured from the edge of the disk to the border of the plate in four different directions. All plates were incubated overnight at 37°C. The presence of a "cloverleaf shaped" ZDI due to the carbapenemase producing organisms (CPOs) by the test strain was considered as positive.

Quantitative biofilm production assay
Tissue culture plate (TCP) method defined by Christensen et al. as gold standard assay, was used for identification of biofilm formation (22). Strains from agar plates were inoculated in 10 mL in TSB supplemented with 1% Glc (TSBglu) media and incubated for 24h at 37°C in stationary phase and diluted 1:100 with fresh medium. Single wells of sterile 96 well-flat bottom polystyrene tissue culture plates (Sigma-Aldrich, Costar, and USA) were filled with 200 µl aliquots of the diluted cultures. The control microorganisms were also incubated, diluted and included to TCP. Negative quality control (NQC) wells contained inoculated sterile TSBglu broth. All plates were incubated at 37°C for 24 h and then, substances of each well were gradually removed by tapping the plates. The wells were rinsed four times with 0.2 mL of phosphate buffer saline (PBS, pH 7.2) to remove loosely attached and floating "planktonic" organisms. Biofilms formed by adherent "sessile" organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Additional stain was removed by washing with sterile distilled water and plates were retained for drying. Adherent *P. aeruginosa* cells commonly formed biofilm on all side wells and were regularly stained with crystal violet (1%). The quantitative analysis of biofilm formation was done by adding the 200 µl of 95% ethanol to decolorize the wells. Optical density (OD) of stained adherent strains were measured with a micro ELISA auto reader (model 680, Bio-Rad, UK) at a wavelength of 570 nm (OD_{570} nm). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Biofilm formation was scored as follows: non-biofilm forming (A_{570} <1); +, weak (1 <A_{570} <2); ++, moderate (2 <A_{570} < 3); +++, strong (A_{570} >3) [13].

Qualitative biofilm production assay
Congo red agar (CRA) method described by Freeman et al. was used to screen biofilm formation in all bacterial isolates [14]. This method uses the brain heart infusion agar (BHI) medium supplemented with 5% sucrose (BHI) and Congo red. The medium consists of 37 gm/L BHI (Merck Co., Germany), 50 g/L sucrose, 10 g/L of agar No.1 and 0.8 g/L of Congo red stain (Oxoid, UK). At first, it is necessary to prepare Congo red indicator as concentrated aqueous solution, then to autoclave at 121°C for 15 min, distinctly from other medium factors and then added when the agar had cooled to 50°C. All plates were inoculated and incubated aerobically for 24 h to 48h at 37°C. Positive results were shown by black-grown colonies with a dry crystalline consistency. In addition, weak biofilm producers showed pink colonies; however, darkening at the centers of colonies were occasionally seen. The test was performed in triplicate for this aim [14].

Alginate production assay
According to the Carbazole method, alginate production level was determined [3]. In brief, all specimens were cultured in Luria-Bertani (LB) broth with fast aeration at 37°C overnight and then centrifuged for 30 min at 13,000 × g. To pre-

cipitate alginate, the supernatant was mixed with 95% ethanol (-70°C) and then centrifuged for 15 min at 13,000 × g 70 mL of alginate solution was combined with 600 mL of borate–sulfuric acid (24.74 g of H3BO3 in 45 mL of 4 M KOH that was diluted in 100 mL distilled water) and 20 mL of the 0.1% (w/v) Carbazole (Sigma-Aldrich, St. Louis, Missouri, United States). To develop the color reaction, the mixture was heated at 52°C for 30 min. Eventually, the optical density was assessed at 530 nm (OD_{530}). The standard curve of alginate concentration was provided and the amount of alginate produced was measured per mg cell dry weight. The test was repeated three times and the mean value for OD was recorded.

Polymerase chain reaction

Template DNA was extracted from the colonies grown on the brain heart infusion (Merck Co., Germany) agar plates by the Bacteria Genome DNA Extraction Kit (TaKaRa, Dalian, China), and then kept on -20°C until used. The primer sequences used in this study are listed in Table 1 [3, 15-21]. The PCR reaction was carried out in a final volume of 25 µl, including the following contents: 1.0 µL of template DNA, 13 µL Maxima Hot Start PCR Master Mix (2×) (Thermo, Waltham, Massachusetts, United States), 0.9 µL of each primer, and 10.1 µL of nuclease-free water. The samples were amplified in a Techne TC-512 thermo cycler (Eppendorf, Hamburg, Germany) as follows: one cycle represents initial denaturation at 95°C for 5 min followed by 33 cycles of denaturation (95°C for 30 s), annealing (65°C for 60s) extension (72°C for 90s) and finally the process ends with a final extension at 72°C for 5 min. PCR amplicon products were loaded on 1.0% agarose gel, stained with Gel Red™

Table 1 - Oligonucleotide primer sequences used in this study

Genes			*Primer sequences (5′→3′)*	*Product size (bp)*
Classes of integrons	*intI*		F:5′-GGTGTGGCGGGCTTCGTG-3′ F:5′-GCATCCTCGGTTTTCTGG-3′	457
	intII		F:5′-CACGGATATGCGACAAAAAGGT-3′ F:5′-GTAGCAAACGAGTGACGAAATG-3′	789
	intIII		F:5′-GCCTCCGGCAGCGACTTTCAG-3′ F:5′-ACGGATCTGCCAAACCTGACT-3′	980
β- lactamase genes	ESBLs	*PER*	F:5′-ATGAATGTCATTATAAAAGC-3′ R:5′-AATTTGGGCTTAGGGCAGA-3′	926
		CTX-M	F:5′-GGTTAAAAAATCACTGCGTC-3′ R:5′-TTGGTGACGATTTTAGCCGC-3′	863
		SHV	F:5′-TGGTTATGCGTTATATTCGCC-3′ R:5′-GCTTAGCGTTGCCAGTGCT-3′	867
		TEM	F:5′-ATAAAATTCTTGAAGAC-3′ R:5′-TTACCAATGCTTAATCA-3′	1075
		GES	F:5′-ATGCGCTTCATTCACGCAC-3′ R:5′-CTATTTGTCCGTGCTCAGG-3′	863
		VEB	F:5′-ATTTCCCGATGCAAAGCGT-3′ R:5′-TTATTCCGGAAGTCCCTGT-3′	542
	MBLs	*IMP*	F:5′-TGAGCAAGTTATCTGTATTC-3′ F:5′-TTAGTTGCTTGGTTTTGATG-3′	740
		SPM	F:5′-CCTACAATCTAACGGCGACC-3′ F:5′-TCGCCGTGTCCAGGTATAAC-3′	674
		VIM	F:5′-AAAGTTATGCCGCACTCACC-3′ F:5′-TGCAACTTCATGTTATGCCG-3′	815
	KPC	*blaKPC*	F:5′-CTTGCTGCCGCTGTGCTG-3′ F:5′-GCAGGTTCCGGTTTTGTCTC-3′	489

Genes		Primer sequences (5′→3′)	Product size (bp)
Virulence gene profiles	*exoU*	F:5′-GATTCCATCACAGGCTCG-3′ F:5′-CTAGCAATGGCACTAATCG-3′	3308
	exoT	F:5′-CAATCATCTCAGCAGAACCC-3′ F:5′-TGTCGTAGAGGATCTCCTG-3′	1159
	exoY	F:5′-TATCGACGGTCATCGTCAGGT-3′ F:5′-TTGATGCACTCGACCAGCAAG-3′	1035
	plcN	F:5′-TCCGTTATCGCAACCAGCCCTACG-3′ F:5′-TCGCTGTCGAGCAGGTCGAAC-3′	481
	aprA	F:5′-TGTCCAGCAATTCTCTTGC-3′ F:5′-CGTTTTCCACGGTGACC-3′	1017
	PhzI	F:5′-CATCAGCTTAGCAATCCC-3′ F:5′-CGGAGAAACTTTTCCCTC-3′	392
	phzII	F:5′-GCCAAGGTTTGTTGTCGG-3′ F:5′-CGCATTGACGATATGGAAC-3′	1036
	phzS	F:5′-TCGCCATGACCGATACGCTC-3′ F:5′-ACAACCTGAGCCAGCCTTCC-3′	1752
	phzM	F:5′-ATGGAGAGCGGGATCGACAG-3′ F:5′-ATGCGGGTTTCCATCGGCAG-3′	875
	phzH	F:5′-GGGTTGGGTGGATTACAC-3′ F:5′-CTCACCTGGGTGTTGAAG-3′	1752
	pvdA	F:5′-GACTCAGGCAACTGCAAC-3′ F:5′-TTCAGGTGCTGGTACAGG-3′	1281
	lasA	F:5′-GCAGCACAAAAGATCCC-3′ F:5′-GAAATGCAGGTGCGGTC-3′	1075
	pilA	F:5′-ACAGCATCCAACTGAGCG-3′ F:5′-TTGACTTCCTCCAGGCTG-3′	1675
	exoS	F:5′-CTTGAAGGGACTCGACAAGG-3′ F:5′-TTCAGGTCCGCGTAGTGAAT-3′	504
	plcH	F:5′-GAAGCCATGGGCTACTTCAA-3′ F:5′-AGAGTGACGAGGAGCGGTAG-3′	307
	lasB	F:5′-GGAATGAACGAGGCGTTCTC-3′ F:5′-GGTCCAGTAGTAGCGGTTGG -3′	300
	pilB	F:5′-ATGAACGACAGCATCCAACT-3′ F:5′-GGGTGTTGACGCGAAAGTCGAT-3′	826
	Nan1	F:5′-ATGAATACTTATTTTGATAT-3′ F:5′-CTAAATCCATGCTCTGACCC-3′	1317
	plcB	F:5′-CAGCTCCGCATGATATTGAC-3′ F:5′-CTGCCAAAGTTTGCTGTGAA-3′	723
	algU	F:5′-CGATGTGACCGCAGAGGATG-3′ F:5′-TCAGGCTTCTCGCAACAAAGG-3′	292
	algD	F:5′-AGAAGTCCGAACGCCACACC-3′ R:5′-CGCATCAACGAACCGAGCATC-3′	550
	algL	F:5′-CCGCTCGCAGATCAAGGACATC-3′ R:5′-TCGCTCACCGCCCAGTCG-3′	432
	exoA	F:5′-TGCTGCACTACTCCATGGTC-3′ F:5′-ATCGGTACCAGCCAGTTCAG-3′	190
Biofilm formation genes	*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

(Biotium, Landing Pkwy, Fremont, CA, USA) and photographed with ultraviolet illumination (Bio-Rad, Hercules, USA).

RESULTS

From March 2016 to February 2017, a total of 143 non-duplicated *P. aeruginosa* isolates were obtained from the unrelated families who had an affected child with CF. 54.5% (n = 78) and 45.5% (n = 65) of isolates were recovered from male and female, respectively. The mean age of the patients was 9.5 years, with a range of 6 to 13 years. Distribution of patients by provinces was as follows: Tehran (n = 32, 27.4%), Mazandaran (n = 21, 14.6%), Fars (n = 19, 13.3%), Razavi Khorasan (n = 18, 12.5%), Yazd (n = 16, 11.2%), Hormozgan (n = 14, 9.7%), West Azerbaijan (n = 12, 8.4%), and Lorestan (n = 11, 7.7%). 8.4% (n = 12/143) of *P. aeruginosa* isolates were resistant to three or more antimicrobials (MDR). No isolates showed XDR or PDR phenotype. Results of the antimicrobial susceptibility testing are shown in Table 2. All isolates were susceptible to CO, PTZ and TC. Out of 143 isolates, 6.3% (n = 9) showed resistance to IPM, of which 22.2% (n = 2) were positive for MBL production by either DDST or E-test method. All the IPM-susceptible isolates were MBL negative by the E-test method. One of 2 MBL-producing isolates was found to carry *blaIMP* gene. Other MBL-encoding genes were not identified. No isolate was positive for production of ESBLs and KPC. Genetic distribution of MGEs showed that Class 1 and class 2 integrons were present in 18.9% (n = 27) and 2.8% (n = 4) isolates, respectively. No class 3 integron was detected among the isolates.

Table 2 - Antimicrobial susceptibility profile.

Antimicrobial agents	*Antimicrobial susceptibility profile No. (%)*		
	S	*I*	*R*
PTZ	143 (100)	0 (0.0)	0 (0.0)
PRL	134 (93.7)	1 (0.7)	8 (5.6)
TC	143 (100)	0 (0.0)	0 (0.0)
CIP	101 (70.6)	1 (0.7)	41 (28.7)
CPM	126 (88.1)	0 (0.0)	17 (11.9)
CAZ	110 (76.9)	0 (0.0)	33 (23.1)
IPM	134 (93.7)	0 (0.0)	9 (6.3)
ATM	136 (95.1)	0 (0.0)	7 (4.9)
NOR	122 (85.3)	2 (1.4)	19 (13.3)
OFX	132 (92.3)	1 (0.7)	10 (7)
CO	143 (100)	0 (0.0)	0 (0.0)
GM	107 (74.8)	3 (2.1)	33 (23.1)
LEV	128 (89.5)	0 (0.0)	15 (10.5)
AK	117 (81.8)	2 (1.4)	24 (16.8)

PTZ, piperacillin-tazobactam; PRL, piperacillin; TC, ticarcillin; CIP, ciprofloxacin; CPM, cefepime; CAZ, ceftazidime; IPM, imipenem; ATM, aztreonam; NOR, norfloxacin; OFX, ofloxacin; CO, colistin; GM, gentamicin; LEV, levofloxacin; AK, amikacin. S, susceptible; I, intermediate; R, resistant.

Alginate production test showed that 88.8% (n = 127/143) of the isolates had the ability to produce alginate. The alginate-encoding genes, *algD*, *algU* and *algL*, were detected in 92.3% (n = 132), 86.7% (n = 124) and 67.1% (n = 96) of isolates, respectively. The simultaneous presence of alginate-encoding genes in Iranian CF *P. aeruginosa* isolates were as follows; *algD/algU* (n = 117/127, 92.1%), *algD/algL* (n = 74/127, 58.3%), *algU/algL* (n = 48/127, 37.7%) and *algD/algU/algL* (n = 23/127, 18.1%). The TCP method showed that 78.3% (n = 112) of all isolates were able to biofilm formation, including weak (n = 17/112, 15.2%), moderate (n = 31/112, 27.6%) and strong (n = 64/112, 57.1%) types. By CRA method, 7.7% (n = 11/143) of isolates produced black colonies and were able to form biofilm, of which 63.6% (n = 7/11), 18.2% (n = 2/11) and 18.2% (n = 2/11) were strong-, moderate-, and weak- biofilm producers, respectively. 54.5% (n = 78) of all isolates produce both biofilm and alginate, simultaneously. In addition, *ppyR*, *pslA* and *pelA* genes responsible for biofilm formation, were detected in 98.6% (n = 141/143), 89.5% (n = 128/143), and 57.3% (n = 82/143) of the isolates, respectively. The distribution of biofilm formation genes in the biofilm-producing isolates were as follows; *ppyR* (87.9%, n = 124/141), *pslA* (94.5%, n = 121/128) and *pelA* (89%, n = 73/82). All biofilm-producing isolates were carrying *ppyR* gene. The predominant pattern of biofilm formation genes in our study was, *ppyR/pslA* (n = 113), *ppyR/pela* (n = 61) and *ppyR/pslA/pelA* (n = 48). Molecular analysis of the virulence-related factors showed that all isolates harbored *lasB* and *plcB* genes. *PlcH* and *plcN* genes were found in 96.5% (n = 138/143) and 32.8% (n = 47/143) of isolates, respectively. The prevalence of the type III effector proteins-encoding genes was

as follows: *exoS* (n = 129/143, 90.2%), *exoU* (n = 57/143, 39.8%), *exoT* (n = 41/143, 28.6%) and *exoY* (n = 36/143, 25.2%). 92.3% (n = 132/143) isolates were positive for *toxA* gene. Results of PCR for the phenazin-encoding gene region revealed that 70.6% (n = 101/143) and 56.6% (n = 81/143) of isolates were positive for *phzII* and *phzI*, respectively. The frequency of *phzH* and *phzS* genes was the same in all isolates tested (n = 92/143, 64.3%). The prevalence of the *nan1*, *lasA*, *pilA*, *apr*, *pvdA* and *pilB* genes was 37.7% (n = 54/143), 26.6% (n = 38/143), 16.1% (n; 23/143), 12.6% (n = 18/143), 9.8% (n = 14/143) and 9.1% (n = 13/143), respectively.

DISCUSSION

In the present study, isolates exhibited a variety of virulence genes and some resistance genes were investigated. The CR-PA frequency in the present study was 6.3% in the period 2016 to 2017, being lower than frequencies reported by Najafi et al. and Varaiya et al. [22, 23]. In addition, 8.4% of isolates were identified as MDR, a figure that is lower than that reported in a previous study [24]. However, all isolates were susceptible to CO, PTZ and TC, indicating the importance of antimicrobial susceptibility testing in choosing antibiotics for treatment of infections.

In our study, 2 CR-PA (22.2%) were MBL-producer, of which one isolate was associated with the presence of the bla_{IMP} gene. In addition, ESBL- and KPC-producing isolates were not detected. Although in the present study two *P. aeruginosa* isolates were found to be MBL-phenotype, their detection in patients with CF underlying disease is of concern as MBL-producer CR-PA can spread rapidly. In a study directed by Mustafa et al. out of 153 *P. aeruginosa* isolated from CF patients from the United Kingdom, Belgium, and Germany, ESBLs-, MBLs- and carbapenemases-producing isolates were not detected [25]. However, the rate of MBL, ESBL, and other resistance determinants vary greatly among different studies. Our results showed also that class I integron was the most prevalent in our strains which was in agreement with Nikokar et al. and in contrast with report from Rojo-Bezares et al. [26, 27]. Such discrepancies in the prevalence of resistance determinants reflect differences in infection control policies and other factors, including geographical distance, and kind of infections and specimen source.

Molecular analysis of the virulence-related determinants showed that the highest and lowest virulence factors were related to *plcH* and *pilB*, respectively, comparable to studies performed by Lanotte et al. Mitov et al. and Fazeli et al. [28-30]. In addition, all *P. aeruginosa* isolates were positive for *plcB* and *lasB* genes. With respect to the potential role of PlcB in pathogenesis, it should be noted that the main lipid component of pulmonary surfactant is phosphatidylcholine (PC). Moreover, the levels of both PC and phosphatidylethanolamine (PE) are raised in the bronchoalveolar lavage of young adults with CF in comparison with age matched controls without CF. Consequently, it is possible that these increased levels of phospholipids could serve as a factor enhancing chemokinesis and/or a chemoattractant for *P. aeruginosa,* which has primarily colonized the upper respiratory tract in CF patients [31]. In the present study, a significant isolates were found to carry corresponding phenazine genes. Wilson et al. showed that phenazines with high concentrations are available in the respiratory tract of patients with CF [32], suggesting the role of phenazine in the pathogenesis of CF.

Consistent with Lanotte et al. the frequency of *exoS* among the *P. aeruginosa* isolates was high (93.8%) [28]. Feltman et al. reported an 85% prevalence of *exoS* among strains from sputa of CF patients [33]. In addition, 28.6% and 46.2% of CF *P. aeruginosa* isolates were positive for *exoU* gene in the studies by Mitov et al. in Bulgaria and by Feltman et al. in USA, respectively [29, 33]. In contrast to our study, Firouzi-Dalvand et al. showed that *exoU* is commonest virulence gene in the *P. aeruginosa* isolated from urinary tract infections (UTIs) (73% vs. 39.8%) [34]. This contrast may be due to the differences in the studied patients (UTI vs. CF), and personal characteristics (adults vs. children).

Yousefi-Avarvand et al. and Sabharwal et al. reported that 90.4% and 100% of *P. aeruginosa* strains studied had *toxA* gene, respectively [35, 36]. In agreement with these researchers, we found *toxA* gene in 92.3% of Iranian CF patients. Sialidase encoded by *nan*1 gene is responsible for adherence to the respiratory tract. In agreement with Mitov et al. 37.7% of CF- isolates were carrying *nan1* gene [29]. The study by Mitov et al. showed that the prevalence of *nan1* was signifi-

cantly higher in CF isolates (38.1%) than that in non-CF isolates (16.9%) [29]. Similar to Bulgaria CF isolates surveyed by Mitov et al. the prevalence of *nan1* among Iranian CF isolates was lower than in French CF *P. aeruginosa* isolates examined by Lanotte et al. [29, 28] (67.1%) which was related to their predominant stable clinical status. Moreover, 26.6% of CF isolates were carrying *lasA* gene, which was approximately similar to the Najafi et al. study (30%) [22]. In consent with Finnan et al. and Mitov et al. the prevalence of *pilB* and *pilA* among the studied CF *P. aeruginosa* was low, suggesting the role of non-pilus adhesins, such as alginate in this group of strains [29, 37].

Infection with alginate-producing *P. aeruginosa* in CF lungs has been related to an overactive immune response and a poor clinical condition, suggesting that alginate production is a vital virulence element in these patients. In the present study, alginate production was detected in 88.8% of the Iranian CF *P. aeruginosa* strains. Interestingly, our result is similar to Ghadaksaz et al. (89.4%) [3]. However, 39% of strains isolated from various clinical samples other than CF patients were positive for alginate production in the study conducted by Zaranza et al. [38]. In agreement with the Ghadaksaz et al. the prevalence of the alginate-encoding genes in our study was 92.3%, 86.7%and 67.1% for *algD*, *algU* and *algL* genes, respectively [3]. Furthermore, 78.3% and 7.7% of isolates were able to produce biofilm in TCP and CRA methods, respectively, which is comparable with Hassan et al. [39]. In agreement with Hassan et al. our study showed that TCP is a quantitative and reliable gold-standard method to detect biofilm-forming bacteria [39]. In the study conducted by Ghadaksaz et al. the prevalence of the *ppyR*, *pslA*, *pelA* genes was 99%, 83.7% and 45.2%, respectively [3]. However, Hou et al. [40] reported that no *P. aeruginosa* isolates were phenotypically positive for biofilm formation in CRA and TCP methods, while 31% of isolates contained the *pslA* gene. This inconsistency may be due to alteration and/or mutation in biofilm regulatory genes.

CONCLUSION

To the best of our knowledge, this is the first report of molecular characterization of Iranian CF *P. aeruginosa* isolates. Early infection caused by *P. aeruginosa* in CF patients usually occurs in childhood and infection is lifelong and persistent. Once chronic infection of *P. aeruginosa* has become established in the respiratory tracts of CF patients, alginate-producing and biofilm-producing strains can harshly be eliminated by antibiotic therapy. In addition, antimicrobial-resistant *P. aeruginosa* infection deteriorates the prognosis of CF patients, also increasing the need for care and the use of medications, hospital and therapeutic resources.

ACKNOWLEDGMENTS

This research work was collaboratively supported by the Iran university of Medical sciences (contract number; 27733) and Babol university of medical sciences (contract number; 9542125).

Conflict of interest

The authors have no conflict of interests.

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