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Virulence Gene Profiles of Multidrug-Resistant *Pseudomonas aeruginosa* Isolated From Iranian Hospital Infections

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Background: The most common hospital-acquired pathogen is *Pseudomonas aeruginosa*. It is a multidrug resistant bacterium causing systemic infections.

Objectives: The present study was carried out in order to investigate the distribution of virulence factors and antibiotic resistance properties of *Pseudomonas aeruginosa* isolated from various types of hospital infections in Iran.

Patients and Methods: Two-hundred and seventeen human infection specimens were collected from Baqiyatallah and Payambaran hospitals in Tehran, Iran. The clinical samples were cultured immediately and samples positive for *P. aeruginosa* were analyzed for the presence of antibiotic resistance and bacterial virulence genes using PCR (polymerase chain reaction). Antimicrobial susceptibility testing was performed using disk diffusion methodology with Müeller–Hinton agar.

Results: Fifty-eight out of 127 (45.66%) male infection specimens and 44 out of 90 (48.88%) female infection specimens harbored *P. aeruginosa*. Also, 65% (in male specimens) and 21% (in female specimens) of respiratory system infections were positive for *P. aeruginosa,* which was a high rate. The genes encoding exoenzyme S (67.64%) and phospholipases C (45.09%) were the most common virulence genes found among the strains. The incidences of various β-lactams encoding genes, including bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M}, $bla_{DHA,}$ and bla_{VEB} were 94.11%, 16.66%, 15.68%, 18.62%, 21.56%, and 17.64%, respectively. The most commonly detected fluoroquinolones encoding gene was *gyrA* (15. 68%). High resistance levels to penicillin (100%), tetracycline (90.19%), streptomycin (64.70%), and erythromycin (43.13%) were observed too.

Conclusions: Our findings should raise awareness about antibiotic resistance in hospitalized patients in Iran. Clinicians should exercise caution in prescribing antibiotics, especially in cases of human infections.

Keywords:Pseudomonas aeruginosa; Virulence Factors; Hospital infections; Iran

1. Background

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen causing a wide range of human infections. It is a common hospital-acquired pathogen and responsible for urinary tract infections (UTIs), respiratory infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections, and a variety of systemic infections, particularly in patients with severe burns, bed ulcers, and in patients suffered from cancer or AIDS who are immunosuppressed (1-3). *P. aeruginosa* has been associated with a high rate of morbidity, and its mortality rate was reported from 18% to 61% in hospital-acquired infections (2, 4, 5).

P. aeruginosa virulence factors are related to its adhesions and other secreted toxins. The phenazine operons (*phzI* and *phzII*) and genes (*phzH*, *phzM,* and *phzS*) encode precursor proteins involved in the formation of three phenazine compounds passively secreted by *P. aeruginosa*: pyocyanin, 1-hydroxyphenazine, and phenazine-1-carboxamide (6, 7), which are responsible for increasing intracellular oxidative stress (6). *P. aeruginosa* also has a large number of other virulence factors such as exotoxin A (*exoA*), alkaline protease (*aprA*), exoenzyme S, U, and T (*exoS*, *exoU*, *exoT*), elastase and sialidase, which are *exoA* gene and virulence factor *exoS* secretions by a type III section system (8-10). A zinc metalloprotease called *lasB* has an elastolytic activity on human tissue and especially lung tissue (11). The gene called *nan*1 encodes sialidase, which is responsible for adherence to the cells (11). The phospholipids contained in pulmonary surfactants may be hydrolysed by two phospholipases C encoded by *plcH* and *plcN*. An extracellular neuraminidase is thought to play an important role in implantation of the bacterium (11).

Despite using potent antibiotics, invasive *P. aeruginosa* infection is associated with high mortality (12). In the past decade, acquired multidrug resistance, because of selective antibiotic pressure, has emerged in several countries; and in some cases, infections caused by multidrug resistant *P. aeruginosa* have been untreatable (13). A previous study addressed that the Iranian isolates of

P. aeruginosa were resistant to trimethoprim (100%), amoxicillin (100%), ampicillin (100%), tetracycline (100%), ticarcillin (100%), carbenicillin (90%), ceftazidime (80%), clavulanic acid (69.2 %), imipenem (60%), cefepime (52%), gentamicin (50%), and ciprofloxacin (40 %) (14). Similar incidence of antibiotic resistance among *P. aeruginosa* strains in other countries has been reported previously (12, 15-17). This high antibiotic resistance of *P. aeruginosa* was against commonly used antibiotics and especially β-lactams, including broad-spectrum cephalosporins, quinolones, chloramphenicol, and tetracyclines, mainly encoded by several antibiotic resistance genes, including bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M}, bla_{DHA} and bla_{VEB} (18).

2. Objectives

There was no comprehensive investigation about the molecular characterization and antimicrobial resistance properties of *P. aeruginosa* isolated from human clinical samples in Iran. Therefore, the present study was carried out in order to investigate the distribution of virulence and antibiotic resistance genes as well as the pattern of antibiotic resistance of *P. aeruginosa* isolated from Iranian hospitalized patients who suffered from UTIs, respiratory infections, burn infections, bed ulcers, and wound infections.

3. Patients and Methods

3.1. Samples and Identification of Pseudomonas aeruginosa

A total of 217 human clinical samples, including wound infections (n = 62), respiratory infections (n = 23), urinary tract infections (n = 41), bed ulcer (n = 39), and burn (n = 52) were collected from hospitalized male and females who were referred to Baqiyatallah and Payambaran hospitals, Tehran, Iran. All samples were transmitted to Microbiology and Infectious Diseases Research Center of the Islamic Azad University of Shahrekord in a cooler with ice-packs. Samples were placed on the selective medium of Pseudomonas Cetrimide Agar (PCA) (LABOBASI, Mendrisio, Switzerland) using a spreading technique. Plates were incubated for 18-24 hours and observed for suspected colonies of *P. aeruginosa*, which was identified by colony pigmentation, grape-like odor, motility and biochemical tests, including carbohydrate fermentation (-), citrate assimilation (+), lysine decarboxylase (-), indole (-), oxidase (+), beta-hemolysis on blood-agar (+), and DNase (-). Inocula from pure colonies on PCA were cultured on nutrient agar slants and kept at 4°C.

3.2. Antibiotic Susceptibility Test

P. aeruginosa isolates were incubated initially on the nutrient agar media (at 4°C) and their positive colonies were transferred to the Müeller-Hinton agar (HiMedia Laboratories, Mumbai, India). Antimicrobial susceptibility was performed on Mueller-Hinton agar by the standard disk diffusion method recommended by Clinical and Laboratory Standards Institute (19). This was done by dipping a sterile swab (stick into an overnight nutrient broth) and carefully swabbing the entire surface of Müeller–Hinton agar plates. The antibiotics used against the test bacteria were as follows: tetracycline (30 μg/disk); streptomycin (10 μg/disk); chloramphenicol (30 μg/disk); sulfamethoxazole (25 μg/disk); gentamicin (10 μg/disk); enrofloxacin (5 μg/disk); cephalothin (30 μg/disk); ciprofloxacin (5 μg/disk); trimethoprim (5 μg/disk); nitrofurantoin (300 μg/disk); ampicillin (10 u/disk); penicillin (10 u/disk), and erythromycin (15 μg/disk). Then, the antibiotic multidisk (Padtan Teb, Iran) was placed on the surface of the inoculated plates and gently pressed. The plates were incubated at 37°C for 18-24 h. The diameter of inhibition zone was measured in millimeters and isolates were scored as sensitive or resistant by comparing with values recommended on standard charts (19). *P. aeruginosa* ATCC 10145 was used as the quality control organism in antimicrobial susceptibility determination.

3.3. DNA Extraction and PCR Confirmation

Chromosomal DNA was extracted from each *P. aeruginosa* isolate by DNA extraction kit (DNPTM, CinnaGen, Iran) according to manufacturer's instruction. The bacteria were confirmed using the PCR method for *nan*1 gene of the *P. aeruginosa* (20). PCR was carried out with 2 μL template DNA, 0.25 μM of each primer (F: 5'-ATGAATACTTATTTTGATAT and R: CTAAATCCATGCTCTGACCC-3'), 0.2 mM deoxyribonucleoside triphosphates, 1X reaction buffer, 2 mM $MgCl_2$ and 1.5 U Taq DNA polymerase (Fermentas) in a total volume of 25 μL. The DNA was amplified using the following protocol: initial denaturation (94 °C for 5 min), followed by 25 cycles of denaturation (94°C for 35 s), annealing (53°C for 45 s) and extension (72°C for 1 min), with a single final extension of 7 min at 72°C.

3.4. PCR Amplification of Virulence Genes

Three different multiplex PCR assays were used in order to amplify various virulence genes. The programmable thermal cycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) PCR device was used in all PCR reactions. The first multiplex PCR reaction was used for the detection of *algD* and *algU* virulence genes of *P. aeruginosa* isolated from clinical samples. The PCR reaction was performed in a total volume of 50 μL, containing 2.5 μL of DNA template, 1.5 mM $MgCl_2$, 200 μM dNTP (Fermentas), 0.5 μM of each primer (*algD*F: 5'-AAGGCGGAAATGCCATCTCC-3' and *algD*R: 5'-AGGGAAGTTCCGGGCGTTTG-3' (21) as well as *algU*F: 5'-CGCGAACCGCACCATCGCTC-3' and *algU*R: 5'-GCCGCACGTCACGAGC-3') (22), 1.25 U Taq DNA polymerase (Fermentas), and 5 μL PCR buffer 10X . Reactions were initiated with 1 cycle, at 95°C for 2 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final elongation

step at 72°C for 7 min. The second multiplex PCR reaction was used for detection of *lasB*, *toxA*, *plcH*, *plcN,* and *exoS* virulence genes of *P. aeruginosa* isolated from clinical samples. List of the primers is shown in Table 1 (23) 18. The PCR reaction was performed in a total volume of 50 μL, containing 25 ng of DNA template, 10 mM Tris-HCl, 50 nmol KCl, 1.5 mM $MgCl_2$, 200 μM dNTP (Fermentas), 12.5 pmol of each primer, 1 U Taq DNA polymerase (Fermentas) and 5 μL PCR buffer 10X . Reactions were initiated at 1 cycle at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 55 for 1 min, 72°C for 1.5 min and a final elongation step at 72°C for 5 min. The third multiplex PCR reaction based on the method of Finnan et al. (7) and was used for the detection of *exoT*, *exoY*, *exoU*, *apr*, *phzH*, phzM, *phzI*, *phzII*, *phzS*, *lasA*, *pvdA*, *pilA* and *pilB* virulence genes. Table 1 shows the list of primers.

Table 1. Oligonucleotide Primers Used for Virulence Genes Amplification

Gene	Sequence (5'–3')	Size of Product (bp)
lasB	*lasB* F: GGAATGAACGAAGCGTTCTCCGAC	284
	lasB R: TGGCGTCGACG*AACA*CCTCG	
toxA	*toxA* F: CTGCGCGGGTCTATGTGCC	270
	toxA R: GATGCTGGACGGGTCGAG	
plcH	*plcH* F: GCACGTGGTCATCCTGATGC	608
	plcH R: TCCGTAGGCGTCGACGTAC	
plcN	*plcN* F: TCCGTTATCGCAACCAGCCCTACG	481
	plcN R: TCGCTGTCGAGCAGGTCGAAC	
exoS	*exoS* F: CGTCGTGTTCAAGCAGATGGTGCTG	444
	exoS R: CCGAACCGCTTCACCAGGC	
exoT	*exoT* F: CAATCATCTCAGCAGAACCC	1159
	exoT R: TGTCGTAGAGGATCTCCTG	
exoY	*exoY* F: TATCGACGGTCATCGTCAGGT	1035
	exoY R: TTGATGCACTCGACCAGCAAG	
exoU	*exoU* F: GATTCCATCACAGGCTCG	3308
	exoU R: CTAGCAATGGCACTAATCG	
apr	*apr* F: TGTCCAGCAATTCTCTTGC	1017
	apr R: CGTTTTCCACGGTGACC	
phzII	*phzII* F: GCCAAGGTTTGTTGTCGG	1036
	phzII R: CGCATTGACGATATGGAAC	
phzM	phzM F: ATGGAGAGCGGGATCGACAG	875
	phzM R: ATGCGGGTTTCCATCGGCAG	
phzS	*phzS* F: TCGCCATGACCGATACGCTC	1752
	phzS R: ACAACCTGAGCCAGCCTTCC	
phzI	*phzI* F: CATCAGCTTAGCAATCCC	392
	phzI R: CGGAGAAACTTTTCCCTC	
phzH	*phzII* F: GGGTTGGGTGGATTACAC	1752
	phzII R: CTCACCTGGGTGTTGAAG	
lasA	*lasA* F: GCAGCACAAAAGATCCC	1075
	lasA R: GAAATGCAGGTGCGGTC	
pvdA	*pvdA* F: GACTCAGGCAACTGCAAC	1281
	pvdA R: TTCAGGTGCTGGTACAGG	
pilA	*pilA* F: ACAGCATCCAACTGAGCG	1675
	pilA R: TTGACTTCCTCCAGGCTG	
pilB	*pilB* F: TCGAACTGATGATCGTGG	408
	pilB R: CTTTCGGAGTG*AACA*TCG	

3.5. PCR Amplification of Antibiotic Resistance Genes

Various β-lactamase genes and the genes that encode DNA *gyrA*se (*gyrA*) and topoisomerase IV (*parC*) were detected using two separate multiplex PCR assays. Primers *gyrA*-F (5'-GTGTGCTTTATGCCATGAG-3') and *gyrA*-R (5'-GGTTTCCTTTTCCAGGTC-3') (24) were used to amplify 287 bp of the fluoroquinolone resistance-determining region of the *gyrA* gene. Primers *parC*-F (5'- CATCGTCTACGCCATGAG-3') and *parC*-R (5'-AGCAGCACCTCGGAATAG-3') (24) were used to amplify 267 bp of the fluoroquinolone resistance-determining region of *parC*. PCR amplification was performed in a 50 μL mixture, containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM mix of deoxynucleotide triphosphates (Fermentas), 10 pmol of each primer, 1 U of Taq DNA Polymerase (Fermentase), and 150 ng of the DNA template. Amplification of the target regions was performed in 35 cycles consisting of initial heat activation at 95 °C for 6 min, denaturation at 95 °C for 45 s, annealing at 51°C for 45 s and elongation at 72 °C for 1 min, with a final elongation at 72°C for 7 min. List of primers used for detection of various β-lactamase genes is shown in Table 2 18. The PCR reaction was performed in a total volume of 25 μL, containing 2 μL of DNA template, 1.4 mM $MgCl_2$, 150 μM of each dNTP (Fermentas), 0.3 μM of each primers and 1 U Taq DNA polymerase (Fermentas). The cycling parameters used were as previously described (18).

3.6. Gel Electrophoresis

Fifteen microliter of PCR products were resolved on a 1.5% agarose gel containing 0.5 mg/mL of ethidium bromide in Tris–borate–EDTA buffer at 90 V for 1 hour, also using suitable molecular weight markers. The products were examined under ultraviolet illumination.

3.7. Statistical Analysis

The results were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA) for analysis. Statistical analysis was performed using SPSS/16.0 software (SPSS Inc., Chicago, IL) for finding significant relationships among incidences of bacteria in males and females, virulence gene and pattern of resistance of *P. aeruginosa* isolated from clinical samples. $\chi 2$ test and Fisher exact 2-tailed test analysis were performed in this study. Statistical significance was regarded at a P value < 0.05.

3.8. Ethical Considerations

The present study was accepted by the ethics committees of the Baqiyatallah and Payambaran hospitals, Tehran, Iran and Microbiology and Infectious Diseases Center of the Islamic Azad University of Shahrekord Branch, Iran. Written informed consents were obtained from all the study patients or their parents.

4. Results

All the human clinical samples were examined using culture and PCR techniques. Out of 217 clinical samples, 102 (47%) were positive for *P. aeruginosa* (Table 3). In addition, 58 out of 127 male clinical samples (45.66%) and 44 out of 90 female clinical samples (48.88%) were positive for *P. aeruginosa*. We found that the patients with respiratory infections had the highest incidence of *P. aeruginosa* (65.21%), while the patients with UTIs had the lowest incidence (21.95%). Table 4 presents the antimicrobial resistance in the *P. aeruginosa* isolates from the human clinical samples. Bacterial strains exhibited the highest level of resistance to penicillin (100%), followed by tetracycline (90.19%), streptomycin (64.70%), and erythromycin (43.13%).

Table 2. Oligonucleotide Primers Used for β-Lactamase Genes Amplification

Antimicrobial Agent	Resistance Gene	Sequence (5'–3')	Size of Product (bp)
β-lactamase	bla_{TEM}	F: ATGAGTATTC*AACA*TTTCCG	867
		R: GGACTCTGC*AACA*AATACGC	
		R: CTGACAGTTACCAATGCTTA	
β-lactamase	bla_{SHV}	F: GGTTATGCGTTATATTCGCC	867
		R: TTAGCGTTGCCAGTGCTC	
β-lactamase	bla_{OXA}	F: ACACAATACATATCAACTTCGC	814
		R: AGTGTGTTTAGAATGGTGATC	
β-lactamase	bla_{CTX-M}	F: ATGTGCAGYACCAGTAARGT	593
		R: TGGGTRAARTARGTSACCAGA	
β-lactamase	bla_{DHA}	F: CACACGGAAGGTTAATTCTGA	970
		R: CGGTTARACGGCTGAACCTG	
β-lactamase	bla_{VEB}	F: CGACTTCCATTTCCCGATGC	642

Table 3. Distribution of *Pseudomonas aeruginosa* in Various Source of Infection According to the Gender [a]

Source of Isolation	Number of Patients	Number of Positive Samples, No.(%)
Wound infection		
M	41	20 (48.78)
F	21	16 (76.19)
Respiratory infection		
M	14	8 (57.14)
F	9	7 (77.77)
Urinary tract infection		
M	25	6 (24)
F	16	3 (18.75)
Bed ulcer		
M	22	10 (45.45)
F	17	6 (35.29)
Burn		
M	25	14 (56)
F	27	12 (44.44)
Total		
M	127	58 (45.66)
F	90	44 (48.88)

[a] Abbreviations: M, male; F, female.

Table 4. Antimicrobial Resistance Properties in *Pseudomonas aeruginosa* Isolated From Clinical Infections in Human [a,b]

Source of Isolation	*P. aeruginosa* Isolates	TE30	S10	C30	SXT	GM10	NFx5	CF30	CIP5	TMP5	F/M300	AM10	P10	E15
Wound infection														
M	20	19	17	3	10	5	5	5	4	8	2	4	20	6
F	16	15	12	2	6	7	4	4	3	6	1	8	16	3
Respiratory infection														
M	8	8	5	2	4	2	3	3	3	1	1	2	8	4
F	7	7	3	1	2	3	2	2	1	2	-	-	7	2
Urinary tract infection														
M	6	5	3	1	5	-	2	2	2	3	-	-	6	1
F	3	2	2	1	1	1	2	2	1	-	-	1	3	1
Bed ulcer														
M	10	8	5	2	3	4	3	3	2	7	1	4	10	5
F	6	5	3	1	-	2	2	2	1	2	-	2	6	3
Burn														
M	14	12	10	2	4	10	3	3	3	3	1	6	14	12
F	12	11	6	2	8	6	2	2	2	6	1	3	12	7
Total														
M	58	52	40	10	26	21	16	16	14	22	5	16	58	28
F	44	40	26	7	17	19	12	12	8	16	2	13	44	16

[a] Abbreviations: M, male; F, female.
[b] TE30 = tetracycline (30 μg/disk); S10 = streptomycin (10 μg/disk); C30 = chloramphenicol (30 μg/disk); SXT = sulfamethoxazole (25 μg/disk); GM10 = gentamicin (10 μg/disk); NFX5 = enrofloxacin (5 μg/disk); CF30 = cephalothin (30 μg/disk); CIP5 = ciprofloxacin (5 μg/disk); TMP5 = trimethoprim (5 μg/disk); F/M300 = nitrofurantoin (300 μg/disk); AM10 = ampicillin (10 u/disk); P10 = penicillin (10 u/disk); E15 = erythromycin (15 μg/disk).

Distribution of antimicrobial resistance genes within the *P. aeruginosa* isolates from the human clinical samples is shown in Table 5. Genes that encode resistance to β-lactams antibiotics such as bla_{TEM} (94.11%), bla_{DHA} (21.56%), and $bla_{C\text{-}TX\text{-}M}$ (18.62%) and the genes that encode resistance to fluoroquinolones, like *gyrA* (15. 68%) were the most common antibiotic resistance genes in the human clinical samples. Interestingly, we found that males had the highest frequency of antibiotic resistance genes. Table 6 shows the distribution of virulence genes in the *P. aeruginosa* isolates from the human clinical samples. The most commonly detected virulence genes of *P. aeruginosa* isolates from the human clinical samples were *exoS* (67.64%), *plcH* (45.09%), *phzM* (36.27%), *exoT* (36.27%), *toxA* (35.29%), and *pilA* (34.31%). Moreover, we found that isolates of burn infection had the highest frequency of virulence genes. The PCR results for detection of some virulence and antibiotic resistance genes are shown in Figures 1, 2, and 3.

Table 5. Distribution of Antimicrobial Resistance Genes in *Pseudomonas aeruginosa* Isolated from Clinical Infections in Human [a]

Source of Isolation	*P. aeruginosa* Isolates	bla_{TEM}	bla_{SHV}	bla_{OXA}	$bla_{CTX\text{-}M}$	bla_{DHA}	bla_{VEB}	*gyrA*	*parC*
Wound infection									
M	20	20	2	-	1	2	-	1	-
F	16	16	4	2	2	1	-	2	1
Respiratory infection									
M	8	7	1	2	1	2	1	-	-
F	7	6	2	-	-	1	-	-	-
Urinary tract infection									
M	6	5	-	4	1	2	1	2	-
F	3	3	1	-	1	2	-	-	1
Bed ulcer									
M	10	10	2	3	4	4	1	2	-
F	6	5	-	1	-	-	2	2	1
Burn									
M	14	14	3	4	7	6	7	5	-
F	12	10	2	-	2	2	6	2	-
Total									
M	58	56	8	13	14	16	10	10	-
F	44	40	9	3	5	6	8	6	3

[a] Abbreviations: M, male; F, female.

Table 6. Distribution of Virulence Genes in *Pseudomonas aeruginosa* Isolated From Clinical Infections in Human [a]

Source of Isolation	*P. aeruginosa* isolates	*exoT*	*exoY*	*exoS*	*exoU*	*phzH*	*apr*	*phzII*	*phzM*	*phzI*	*phzS*	*lasA*	*lasB*	*pvdA*	*pilA*	*pilB*	*toxA*	*algD*	*algU*	*plcH*	*plcN*	*nan1*
Wound infection																						
M	20	5	7	18	-	1	2	-	1	-	1	-	1	2	10	2	4	2	5	11	6	1
F	16	2	6	11	2	-	1	8	3	2	-	2	3	8	1	6	3	1	2	7	11	2
Respiratory infection																						
M	8	1	3	6	2	2	-	2	4	1	1	1	2	1	2	1	-	-	1	4	3	-
F	7	4	1	3	-	-	2	1	2	-	1	2	3	1	3	2	5	-	-	5	2	2
Urinary tract infection																						
M	6	3	-	2	1	-	1	3	3	1	1	1	-	2	1	-	4	-	3	-	2	-
F	3	1	1	1	-	2	-	-	1	-	1	1	1	-	-	1	2	-	-	-	-	-
Bed ulcer																						
M	10	7	4	7	3	4	-	4	6	2	1	5	-	2	4	-	8	-	7	4	-	6
F	6	2	-	2	-	1	2	1	1	2	-	2	1	1	2	1	-	-	2	2	1	2
Burn																						
M	14	10	5	11	4	7	4	6	9	3	8	7	2	6	7	3	9	2	10	8	6	8
F	12	2	6	8	2	4	3	4	7	2	6	6	5	2	5	2	1	3	2	5	3	3
Total																						
M	58	26	19	44	10	14	7	15	23	7	12	14	5	13	24	6	25	4	26	27	17	15
F	44	11	4	25	4	7	6	14	14	6	8	13	13	12	11	12	11	4	6	19	17	9

[a] Abbreviations: M, male; F, female.

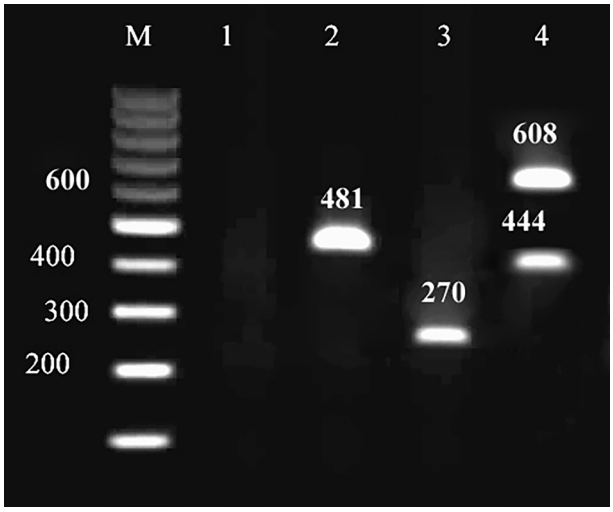

Figure 1. Agarose Gel Electrophoresis of PCR Products Amplified With a Multiplex PCR Method for the *plcN* (481 bp), *toxA* (270 bp), *exoS* (444 bp), and *plcH* (608 bp) Genes From *Pseudomonas aeruginosa*. M = 100-bp DNA Ladder.

Figure 2. Agarose Gel Electrophoresis of PCR Products Amplified With a Multiplex PCR Method for the *exoT* (1159 bp), *exoU* (3308 bp), apr (1017 bp), *phzM* (875 bp), *phzS* (1752 bp), and *pilB* (408 bp) genes From *Pseudomonas aeruginosa*. M = 1-kb DNA Ladder.

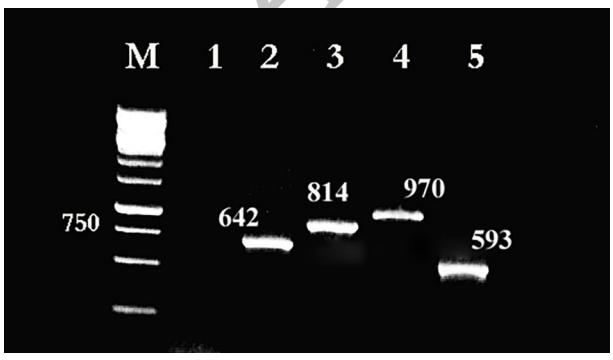

Figure 3. Agarose Gel Electrophoresis of PCR Products Amplified With a Multiplex PCR Method for the *blaVEB* (642 bp), *blaOXA* (814 bp), *blaDHA* (970 bp), and *blaCTX-M* (593 bp) Genes From *Pseudomonas aeruginosa*. M = 1-kb DNA Ladder.

5. Discussion

The results of the present study showed that multidrug resistant *P. aeruginosa* is one of the main active pathogens in human clinical samples, especially wound infections, respiratory infections, UTIs, bed ulcer, and burn infections. Also, our results showed that antibiotics were used in an irregular manner in Iranian hospitals. These two findings may lead to the emergence of resistant *Pseudomonas* strains, which can infect patients and even healthy people in hospitals.

Similar results have been reported by Zavascki et al. (25, 26), Lanini et al. (27), and Pitten et al. (28). Statistical analyses showed a significant ($P < 0.01$) association between the incidence of *P. aeruginosa* in respiratory infections (65.21%) and UTIs (21.95%). The high incidences of *P. aeruginosa* in respiratory infections have been previously reported (29-31). In the Viedma et al. (32) study, the main clinical wards in which drug-resistant *P. aeruginosa* strains were isolated included internal medicine (31.1%), surgery (17.5%), intensive care (13.1%), pulmonology (11.5%), and hematology (8.2%). Also, Viedma et al. (32) showed that out of 183 patients, 143 (78.1%) were considered infected, including 36 (19.7%) patients with lower respiratory tract infection, 30 (16.4%) patients with urinary tract infection, 28 (15.3%) patients with bacteremia, and 22 (12%) patients with intra-abdominal infection.

There were significant differences ($P < 0.01$) in the incidence of *P. aeruginosa* strains between male and female patients. Al-Hasan et al. (33) reported that 62.80% of *P. aeruginosa* strains were isolated from hospitalized males. Also, gender-wise prevalence showed 61.78% male and 38.22% females of the study of Khan et al. (34) were infected by *P. aeruginosa,* which was similar to our results. One possible explanation for the high prevalence of *P. aeruginosa* in male patients lies in their jobs, which usually included hard works outside the house, while Iranian women are typically housewives. Therefore, they are away from contaminated work environments.

The overall incidence of *P. aeruginosa* in human clinical samples of our study was 47%. Similar incidence rates have been reported from India (29.6%) (35), Georgia (31.5%) (36), Norway and Sweden (25.8 to 45.9%) (37), Turkey (16.4%) (38), and Brazil (37.3%) (25). The overall incidence for *P. aeruginosa* was 6.4 cases/100,000 population in England, Wales and Northern Ireland (39). Infections were reported more among those aged 75 years and older, especially among male patients with a rate of 52.0 cases/100,000 population compared with 19.7 cases/100,000 for female patients (the same age group) (40). Tacconelli et al. (40) reported that out of 358 patients with *P. aeruginosa* bacteremia, 133 (37%) were hospitalized in medical wards, 103 (29%) in ICUs, 97 (27%) in surgical wards, and 25 (7%) in neonatology wards, 45 (12%) patients had HIV infection and 28 (8%) had hematologic malignancies.

Similar investigations have been done on Iranian hospitals infections (41-50). Bacterial strains of our investiga-

tion were multidrug resistant. All isolates were resistant to penicillin which shows the irregular and extreme prescription of this antibiotic. There were significant differences ($P < 0.01$) in the distribution of antimicrobial resistance among penicillin, nitrofurantoin, and chloramphenicol ($P < 0.05$) and among resistance to penicillin and ampicillin, ciprofloxacin, enrofloxacin, and gentamicin. Majority of the *P. aeruginosa* strains of the Japoni et al. (51) investigation were resistant to more than 5 antibiotics which was similar to our results. Shiny et al. (52) reported that out of the 500 pus and 500 urine samples screened, the percentage positivity of *P. aeruginosa* were 12.8% (53) and 4% (20), respectively and all (100%) samples were sensitive to imipenem, while resistance was maximum to cefotaxime (93.75%). In a study of Viedma et al. (32), which was conducted from 2007 to 2010, the prevalence of *P. aeruginosa* had increased from 2.8% to 15.3% over this period and all the isolates were only susceptible to colistin (100%) and amikacin (75%). High antibiotic resistances against penicillin, tetracycline, streptomycin, and erythromycin have been previously reported too (15, 17). Our results showed that 39.21% and 21.56% of bacterial strains were resistant to gentamicin and ciprofloxacin, respectively. Fazeli et al. (54) showed that 29% and 32.2% of the *P. aeruginosa* strains were resistant to ciprofloxacin and gentamicin, which was similar to our results. Ciprofloxacin has been stated to be the most potent available drug for the treatment of *P. aeruginosa* infections (55). Resistance of *P. aeruginosa* to ciprofloxacin was 21.56%, compared with 26.8% in Latin America (56) and 10%-32% in Europe (57, 58).

The antibiotic resistant pattern found by Akingbade et al. (15) investigation showed that *P. aeruginosa*, on one hand, had high resistant to amoxicillin (92.7%), ampicillin (90%), cloxacillin (88.2%), cotrimoxazole (77.3%), erythromycin (72.7%), tetracycline (70.9%), streptomycin (65.5%), and ofloxacin (690%), and on the other hand, had low resistant to ceftazidime (20%), gentamicin (26.4%), levoxin (30.9%), ceftriaxone (34.5%), and ciprofloxacin (35.5%), which was similar to our results. Our results also showed that 16.66% and 6.86% of our bacterial strains were resistant to chloramphenicol and nitrofurantoin, respectively. Similar results have been reported by Lim et al. (18) from Malaysia and Smith et al. (59) from Nigeria. Chloramphenicol and nitrofurantoin are banned antibiotics and the slight antibiotic resistance to these drugs detected in our study indicates that irregular and unauthorized use of them may have occurred in Iran.

Several genes which encode resistance to β-lactams antibiotics such as bla_{TEM} (94.11%), bla_{DHA} (21.56%), bla_{OXA} (15.68%), bla_{VEB} (17.64%), bla_{SHV} (16.66%), and $bla_{\text{CTX-M}}$ (18.62%) and the genes that encode resistance to fluoroquinolones like *gyrA* (15. 68%) and *parC* (2.94%) were detected in the bacterial strains of our study. There were statistically significant differences ($P < 0.05$) amongst the incidences of genes encoding resistance to β-lactams and those encoding resistance to fluoroquinolones. Also, statistical analyses showed a significant ($P < 0.05$) association between the incidence of bla_{TEM} and other genes encoding resistance to β-lactams. There were no significant differences between the incidence of *gyrA* and *parC* genes. Of 82 *P. aeruginosa* stains isolated from human clinical specimens, 15%, 74%, 74%, 76%, 82%, and 76% had *tetA*, *tetB*, bla_{VIM2a}, bla_{VIM2b}, *aacA,* and bla_{OXA}, respectively. A recent study performed within our region in Egypt reported that the majority (97%) of *P. aeruginosa* isolates were beta-lactamase producers (3). *P. aeruginosa* isolates of the Du et al. (60) study showed the high incidence of antibiotic resistance genes, including $bla_{\text{TEM-1}}$ (100%), $bla_{\text{PSE-1}}$ (100%), $bla_{OXA\text{-}2}$ (96.2%), $bla_{SHV\text{-}18}$ (91.3%), $bla_{OXA\text{-}17}$ (78.3%), $bla_{\text{VIM-3}}$ (26.1%), $bla_{OXA\text{-}10}$ (21.7%), and $bla_{SHV\text{-}1}$ (8.7%). In the human isolates, the most prevalent extended-spectrum β-Lactamases (ESBLs) in *P. aeruginosa* are $bla_{SHV\text{-}5}$ and $bla_{SHV\text{-}12}$ in Taiwan (61). However, in the present study, we found that bla_{TEM} (94.11%) and bla_{DHA} (21.56%) were the most commonly detected antibiotic resistance genes.

The results of disk diffusion method were confirmed the results of PCR amplification of resistance genes. Our results indicated the high presence of virulence factors in *P. aeruginosa* isolates. Totally, *exoS* (67.64%) and *plcH* (45.09%) were the most commonly detected virulence genes. There were statistically significant differences ($P < 0.05$) amongst the incidences of *exoS* and *exoT* and *exoU*, also $P < 0.05$ amongst the incidences of *phzM* and *phzI*, *phzS* and *phzH* genes. There were no significant differences amongst the incidences of *lasA*, *lasB, plcH,* and *plcN*. Also, statistical analyses showed a significant ($P < 0.05$) association between the incidences of *pilA*, *pilB, algD,* and *algU*. The *exoS* gene is directly translocated into eukaryotic cells by the contact-dependent type III secretory process and, as such, it provides the bacterium with a mechanism for manipulating the eukaryotic cells it encounters. In support of *exoS* contributing to *P. aeruginosa* pathogenicity, bacterial translocation of *exoS* into epithelial cells results in a general inactivation of cellular function, as recognized by the inhibition of DNA synthesis, loss of focal adhesion, cell rounding, and microvillus effacement (62, 63). High importance of the *exoS*, *exoU,* and *exoT* genes of *P. aeruginosa* in the pathogenicity of lung diseases has been reported before (10). Also, the *exoS* gene contributed to dissemination in burn, lung diseases and keratitis (10, 53, 64). Our results showed that the *exoS* gene had the highest incidence in human clinical samples, including wound, respiratory and urinary tract infections, bed ulcer, and burn. The *plcH* gene is responsible for proinflammatory activities (9), virulence in animal models (66), pulmonary inflammation (9), and inhibition of oxidative burst of neutrophils (65). Similar results have been reported previously from Malaysia (66), Australia (67), Jamaica (68), and Lebanon (17).

As far as we know, this investigation is the most comprehensive report of virulence factors and antibiotic resistance properties of *P. aeruginosa* isolated from Iranian human clinical samples. Our results revealed that all the

exo, *apr*, *phz*, *las*, *pvd*, *pil*, *tox*, *alg*, *plc,* and *nan* virulence genes are predominant in human infections. Antibiotic resistance against penicillin, tetracycline, and streptomycin were high. Prescription of ciprofloxacin, nitrofurantoin, and chloramphenicol can be effective for the treatment of human infections due to *P. aeruginosa* in our area. Hence, judicious use of antibiotics is required by clinicians.

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Authors' Contributions

DNA extraction, PCR, manuscript preparation, statistical analysis, and project support were all performed by Hassan Momtaz. Sample collections and coordination was performed by Nastaran Fazeli. All authors have read and approved the final manuscript.

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