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Detection of Mutations of Antimutator Gene *pfpI* in *Pseudomonas aeruginosa* Species Isolated from Burn Patients in Tehran, Iran

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ABSTRACT

Introduction: *Pseudomonas aeruginosa* is an opportunistic pathogen of clinical importance, particularly in immunocompromised and burn patients. This bacterium is becoming resistant to many antibiotics via intrinsic or acquired mechanisms. Mutations in anti-mutator genes, such as *pfpI*, can be a potential intrinsic mechanism of antibiotic resistance. This study aimed to evaluate the possible effects of mutations of this gene on coding proteins of multi-drug resistant *P. aeruginosa* isolates. **Methods:** The antibiotic resistance pattern of 50 *P. aeruginosa* isolates against 9 anti-*pseudomonas* antibiotics was determined by the disk diffusion method. PCR, followed by sequencing, detected the mutations in the *pfpI* gene. The retrieved sequences were translated to the corresponding amino acid sequences using an online protein database. The amino acid sequences in mutated isolates were compared with the reference sequence using a multiple alignment method. **Results:** Out of 50 isolates, 43 (86%) were resistant to all antibiotics. Sequencing and multiple alignment analyses showed that amino acids in positions 21, 24, and 57 of *pfpI* gene were changed in resistant isolates, and all these mutations were observed in each isolate. Homology modeling showed that these amino acids were part of a cleft on the protease. The other point mutations resulted in amino acid changes were in positions 67, 83, and 165. **Conclusion:** The data obtained in this study showed that the *pfpI* gene of *P. aeruginosa* might have a significant effect on response to antibiotics. Further epidemiologic and comprehensive studies are required to confirm these findings.

INTRODUCTION

Pseudomonas aeruginosa is a common cause of acute hospital-acquired infections in burn patients. The acquisition of resistance mechanisms is on the rise, and resistant strains of *P. aeruginosa* are spreading worldwide [1]. Tissue damages in burn patients can become aggravated by the infections caused by resistant *P. aeruginosa* strains resulting in a delay in the wound healing process [3]. Burn injuries are one of the common types of traumas [1] and can provide a suitable environment for bacterial growth, leading to prolonged hospitalization [2].

Most *P. aeruginosa* strains are resistant to the routine antibiotics, and the number of hypermutant species of *Pseudomonas* is on the increase. The bacterium can quickly become resistant to antibacterial agents due to mutations in specific genes. *P. aeruginosa* is a hyper mutant bacterium mainly due to an insufficiency in antimutant genes. These genes can repair the damaged DNA or activate a system to prevent DNA damage. Hyper mutant species do exist in different bacterial populations, and there is a clear link between hypermutation and resistance to the host immune system, and drug resistance [4]. Recently, the role of the antimutant gene, *pfpl*, in the spread of hyper mutant strains of *P. aeruginosa* in cystic fibrosis patients was documented. The product of this gene is a protease that plays a role in resistance to environmental stress, such as oxidation, heat, salt, and ultraviolet radiation. Mutations in *pfpl* gene make the bacterium highly susceptible to spontaneous mutations in other genes, *e.g.*, *mutT*, *mutY*, and *mutM* which can be

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responsible for drug resistance. The primary role of *pfpl* gene is to prevent mutations caused by hydrogen peroxide [4].

The present study investigated the relationship between mutations in *pfpl* gene and the emergence of resistant *P. aeruginosa* strains in burn patients.

MATERIAL AND METHODS

Bacterial sample collection. From October 2011 to November 2012, 50 clinical isolates of *P. aeruginosa* were collected from the wound samples of burn patients hospitalized in Tehran Shahid Motahari Burn Hospital, Iran, Each isolate was obtained from the wound sample of an individual patient. The identity of the isolates was confirmed by standard biochemical tests including oxidation of glucose, xylose, mannitol, lactose, sucrose, and maltose; nitrate; Christensen urea; gelatin liquefaction; catalase; Simmons citrate; motility; and indole and H_2S production and cultivation on Mueller-Hinton agar and observation of blue-green colonies as described elsewhere [5].

Antibiotics susceptibility assay. Antibiotic susceptibility test was performed by the disk diffusion method based on the CLSI 2012 guidelines [6] with *P. aeruginosa* ATCC 27853 type strain as the control. The isolates were grown on Mueller Hinton agar (MERCK-Germany) and a panel of antibiotics listed in Table 1 (Mast company, UK) including imipenem (IPM-10 µg), ceftizoxime (CT-30 µg), cefotaxime (CTX-30 µg), amikacin (AN-30 µg), gentamycin (GM-10 µg), tobramycin (TOB-10 µg), carbenicillin (CAR-100 µg), piperacillin (PIP-100 µg), and ciprofloxacin (CP-5 µg) was used to show the antibiotic susceptibility of the isolates.

DNA extraction and amplification of *pfpl* gene. DNA extraction from *P. aeruginosa* isolates was performed as follows: A loopful of each *P. aeruginosa* culture was transferred into a tube containing 200 µl sterile distilled water and mixed by vortexing to obtain a homogeneous bacterial suspension. The tubes were submerged in a water bath for 10 min at 95°C followed by centrifugation at 8,960 x*g* for 5 min. The supernatants containing the DNA were recovered and kept at 4°C until used. The 25 µl PCR reaction mixture contained 17.25 µl sterile double distilled water, 2.5 µl 10x PCR buffer, 3 mM $MgCl_2$, 200 µM dNTPs, 10 ρmol of each forward and reverse primers (Table 2), 0.125 µl Taq DNA polymerase 5 u/µl, 2.5 µl template DNA 20 ng/µl (Tuba Negin, Iran). Amplification of *pfpl* gene was performed in an Eppendorf MasterCycler (Hamburg, Germany) programmed for 5 min initial denaturation at 95°C, 38 cycles of 1 min denaturation at 95°C, 1 min annealing at 62°C, 1 min extension at 72°C, and a final 10 min extension at 72°C.

The PCR product was resolved by gel electrophoresis on a 1.5% agarose gel, and visualized by staining with ethidium bromide under UV in a gel documentation instrument (Maham Azma Pharmed, Iran). The PCR products were sent to Alpha Sequencing Company (USA), and sequencing was performed in both directions using the same primers used for amplification following a purification procedure to remove salts, impurities, and primers.

Determination of point mutations and enzyme active site. Point mutations were detected in resistant isolates by comparing the sequence data with the standard *pfpl* sequence of *P. aeruginosa* PAO1, obtained from the NCBI website (https://www.ncbi.nlm.nih.gov/). In the resistant strains, we searched for the mutations that were absent in the susceptible ones. If a mutation occurred only in the resistant isolates, it was assumed responsible for resistance. The mutations were identified by performing *pfpl* multiple alignments against the reference strain and susceptible strains using ClustalW2 software (www.ebi.ac.uk-/tools/msa). For predicting the enzyme active site, the *P. aeruginosa* (RefSeq: NP_249046.1) PfpI enzyme from the NCBI database was used as the reference sequence. The homology model and active site prediction of the enzyme was performed using the I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The iterative threading assembly refinement (I-TASSER) server is an integrated platform for automated protein structure and function prediction based on the sequence-to-structure-to-function paradigm. Starting from an amino acid sequence, I-TASSER first generates three-dimensional (3D) atomic models from multiple threading alignments and iterative structural assembly simulations [18]. Pymol (v2.3.2) was used for visualizing PDB files.

RESULTS

Patients and cause of burn injuries. Seventy-two percent of burn patients were men and twenty eight percent women. The average age was 32.9 years. Most of the cases (75.3%) had a combination of 2nd and 3rd-degree burns. Flame burns caused by petrol and gas were the most common cause of the injuries (21%) followed by boiling water, fire, hot oil, and electricity. The average length of hospitalization was 25 days, with the overall mortality rate of 30%.

Antibiotic susceptibility profile. Results from the antibiotic susceptibility test by disk diffusion method showed that ciprofloxacin was the most effective antibiotic against *P. aeruginosa* among the commonly used antibiotics, and ceftizoxime was the least effective antibiotic against the bacteria. Among the *P. aeruginosa* isolates from burn wounds 76% were resistant to ciprofloxacin, 80% to piperacillin, 84% to imipenem, 86% to amikacin, tobramycin, and gentamycin, 88% to carbenicillin, and 96% to ceftizoxime. The detailed results are shown in Table 3.

Detection of *pfpl*. Knowing that all *P. aeruginosa* isolates have *pfpl* gene, our PCR results in the presence of the gene in all the isolates. To interpret the results of *pfpl* gene sequencing and correlate it to the antibacterial test, we divided the antibiogram results into two groups: susceptible and resistant.

Determination of point mutations and enzyme active site. Amino acids at positions 21, 24, and 57 in PfpI protein of the reference strain as well as the susceptible strains were changed from valine, threonine, and glutamic acid to isoleucine, isoleucine and aspartic acid in all resistant strains, respectively. Mutations at positions 21, 24, and 57 had coincided. Also, in many resistant isolates, aspartic acid at position 67 in the PfpI sequence of reference and susceptible strains substituted with asparagine. In the resistant isolates

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exhibiting asparagine at position 67, there were no mutations at positions 21, 24, and 57 (Table 4). Likewise, the strains with mutations at positions 21, 24, and 57 had no changes at position 67.

Amino acids at positions 83 and 165 in the PfpI sequence of reference, as well as susceptible isolates, were substituted with valine and asparagine in many resistant strains, respectively. One or both mutations together at positions 83 and 165 seem to be responsible for the resistance. The positions of the mutations are summarized in Table 4.

The I-TASSER server predicted the tertiary structure of the enzyme. The model with the highest C-score was selected for further analysis. Residues 52, 53, 81, 82, 112, 113, 132 had the highest C-score (0.22). C-score is the confidence score of the prediction. C-score ranges between 0 to 1, where a higher score indicates a more reliable prediction, so they were selected as the catalytic site and shown in Figure 1.

DISCUSSION

This is the first report on the mutations at several sites of the *pfpl* gene of *P. aeroguinosa*. The *pfpI* gene is a newly described gene in *P. aeroginosa* that has a regulatory role in spontaneous and H_2O_2-induced mutations. Besides, the gene product (PfpI) can protect bacteria against other types of extracellular stress. Also, inactivation of this gene has shown to affect biofilm formation [4], which is essential to the pathogenesis of *P. aeruginosa*. The original work of Rodríguez *et al.* (2009) reported the presence of this gene in *P. aeruginosa*. However, the role of the gene in antibiotic resistance has remained unknown [4].

Table 1. The list of antibiotics used in this study

Number	Antimicrobial agent	Code	Conc.	Susceptible zone diameter		
				Resistance	Intermediate	Sensitive
1	Imipenem	IPM	10 µg	13 ≤	14-15	16 ≥
2	Ceftizoxime	CT	30 µg	14 ≤	15-19	20 ≥
3	Cefotaxime	CTX	30 µg	14 ≤	15-22	23 ≥
4	Amikacin	AN	30 µg	14 ≤	15-16	17 ≥
5	Gentamycin	GM	10 µg	12 ≤	13-14	15 ≥
6	Tobramycin	TOB	10 µg	12 ≤	13-14	15 ≥
7	Carbenicillin	CAR	100 µg	13 ≤	14-16	17 ≥
8	Piperacilin	PIP	100 µg	17 ≤	_	18 ≥
9	Ciprofloxacin	CP	5 µg	15 ≤	16-20	21 ≥

Table 2. The sequence of the PCR primers used in this study

Primer name	Nucleotide sequence
PfpI (F)	5'-GGCTTCTGTTGGAAAGCGG-3'
PfpI (R)	5'-GCATTATAGCGCAGCGACG-3'

Table 3. The antibiotic resistance pattern of *Pseudomonas aeruginosa* species isolated from burn wounds

Antibiotic	Susceptible		Intermediate		Resistant	
	Number	Percentage	Number	Percentage	Number	Percentage
Amikacin	7	14%	0	0%	43	86%
Piperacillin	10	24%	0	0%	40	80%
Gentamycin	7	14%	0	0%	43	86%
Tobramycin	7	14%	0	0%	43	86%
Imipenem	8	20%	0	0%	42	84%
Carbenicillin	0	0%	6	12%	44	88%
Ciprofloxacin	8	16%	4	8%	38	76%
Cefotaxime	0	0%	3	6%	47	94%
Ceftizoxime	0	0%	2	4%	48	96%

Table 4. The positions of the mutated amino acids

Position	Amino acid in the reference strain	Amino acid in the antibiotic sensitive strains	Amino acid in the antibiotic-resistant strains
21	Valine	Valine	Isoleucine
24	threonine	threonine	Isoleucine
57	Glutamic acid	Glutamic acid	Aspartic acid
67	Aspartic acid	Aspartic acid	Aspargine
83	Isoleucine	Isoleucine	Valine
165	Aspartic acid	Aspartic acid	Aspargine

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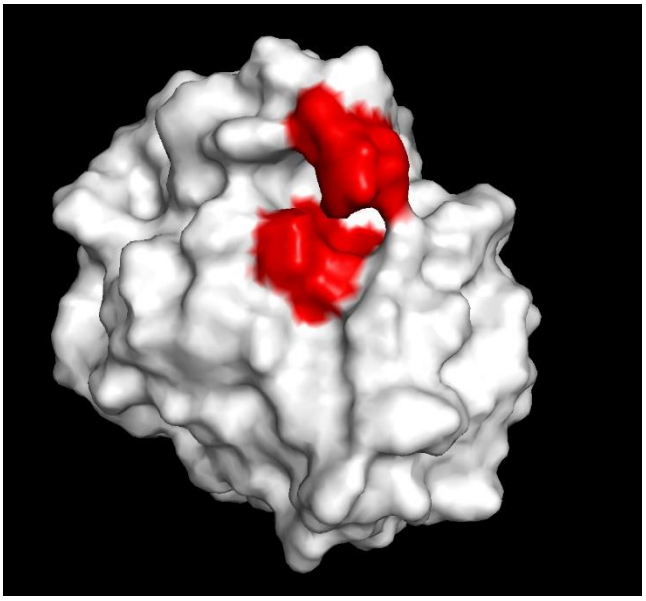

Fig. 1. Three-dimensional structure of the PfpI enzyme. The 3-D structure was determined using the I-TASSER server. Positions 52, 53, 81, 82, 112, 113, and 132 had the highest C-score which is the confidence score of the prediction are shown in red.

Upon exposure to stressors, including antibiotics, oxidative stress, heat, and UV, *P. aeruginosa* paradoxically uses opposing mechanisms to react to external stress. On the one hand, some mutator genes (*mutT, mutY, and mutM*), become activated to change some specific nucleotides on the target sequence of DNA (17); thus evading the deleterious effects of the stressors. On the other hand, some antimutator genes, including *pfpI* are activated/upregulated to balance the situation out and to keep the target DNA and the gene product intact and normal. Harboring intact *pfpI* gene makes *P. aeruginosa* strains susceptible to the antibiotics, while mutations on the gene render most of the strains antibiotic-resistant.

Hypermutation, by creating antibiotic resistance or resistance to reactive oxygen species (ROS), maybe a useful survival mechanism for the bacteria in the short-term, but in the long-run, hypermutator bacteria are prone to extinction due to the accumulation of deleterious stressors such as ROS in the bacterial cells leading to other types of harmful mutations. Therefore, different hypermutator bacteria use a different mechanism to compensate for the hypermutation consequences. For example, in a study on hypermutator *Escherichia coli*, it was found that the expression of NorM (a member of efflux pumps) can export specific oxidants out of the bacterial cell and reduce the level of intracellular oxidative stress [15]. This mechanism that *E. coli* uses is different from the antimutator mechanism explained for *P. aeruginosa* in this study.

Specific genes occur naturally in hypermutator bacteria [7], and the presence of such bacteria can cause antibiotic resistance and disruption of host immunological defenses [8, 9]. However, the presence of such genes may not be as specific in other bacteria as it is in *P. aeruginosa*. For example, an examination of 222 Enterobacteriaceae isolates originated from the urinary tract and bloodstream infections showed no relationship between the presence of hypermutable phenotype and the ability to initiate an antibiotic-resistant biofilm, which is contrary to what is known for hypermutator *P. aeruginosa* strains isolated from cystic fibrosis patients [14].

The PFPI-like genes occur in eukaryotic microorganisms as well, *e.g.*, a gene coding for a PfpI-like protein was identified in *Leishmania major* and *Leishmania mexicana*, *Leishmania braziliensis*, and *Leishmania infantum* the causative agents of different types of leishmaniasis. The gene in the *Leishmania* species has been degenerated into a pseudogene due to reductive genome evolution and has lost its biological function. However, an open reading frame, which is responsible for the expression of a biologically active PFP1 in *L. major* may contribute to the tissue tropism and, consequently, disease progression [16].

There are very few studies on the nature of hyper-mutated genes, and their clinical importance in antibiotic resistance is not much evident [8]. Some studies have revealed that the frequency of hypermutator variants of *P. aeruginosa* in chronic inflammations was much higher than the frequency of non-hypermutator isolates (8, 10). Typically 65% of *P. aeruginosa* isolates showed to be defective in mismatch repair systems in the genes like *mutS*, *mutL*, and *uvrD*, while defects in the *mutS* gene were more common [10, 11].

There are reports on multi-drug resistant *P. aeruginosa* strains isolated from burn patients in Iran [12, 13]. The results of the present study show that the antimutator *pfpI* gene in *P. aeruginosa* isolates from burn patients had a fundamental function in antibiotic resistance. Further epidemiological studies with a larger sample size are required to confirm the correlation between the frequency of antimutator *pfpI* gene and the extent of chronic *P. aeruginosa* infections.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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