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Molecular Epidemiology and Drug Susceptibility of *Pseudomonas aeruginosa* Strains Isolated From Burn Patients

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Abstract

Background: *Pseudomonas aeruginosa* is 1 of the major agents of nosocomial infections in burn centers. Our objectives were to determine the genetic diversity of *P. aeruginosa* isolated from burn patients.

Methods: One hundred thirty-one *P. aeruginosa* isolates were collected from the burn center of Shahid Motahhari Hospital in Tehran. Phenotypic screening for drug susceptibility was performed by disk diffusion method according to Clinical Laboratory Standards Institute guidelines, and genetic diversity of all isolates was determined by the pulsed-field gel electrophoresis (PFGE) technique.

Results: Antimicrobial susceptibility testing showed that the majority of *P. aeruginosa* strains were resistant to ceftizoxime (87%) and aztreonam (80.2%). Pulsed-field gel electrophoresis revealed 11 profiles in which environmental strains were included in PFGE1 and PFGE7 patterns. The major PFGE profile was PFGE1 containing 18 (42.9%) MDR isolates and including an environmental MDR bacterium.

Conclusions: Our findings highlighted that further attention needs to be focused on the disinfection of this burn unit.

Keywords: *P. aeruginosa*, pulsed-field gel electrophoresis, DNA fingerprinting, drug susceptibility, multidrug resistance

Pseudomonas aeruginosa is 1 of the major agents of nosocomial infections in burn centers, especially in Iran.[1,2] This opportunistic and highly resistant bacterium causes severe problems for hospitalized burn patients.[3] Infections of *P. aeruginosa*, particularly multidrug-resistant (MDR) strains, in burn patients is commonly discussed as a general complication. These patients are obviously at high risk for difficult-to-treat or untreatable infections.[4] Severely burned patients with immunological system defects develop life-threatening infections frequently, and as a result, this gram-negative bacterium continues to be a general complication in burn-related morbidity and mortality worldwide.[5-7]

Multidrug-resistant bacteria have commonly been reported as the cause of nosocomial outbreaks of infection in burn units (BU) or as colonizers of the wounds of burn patients. Resistance to many drugs has been reached to a worrying point in *P. aeruginosa* isolated from burn patients in Iran.[8-10] Previous studies confirmed resistance to many antibiotics used routinely for treatment of burn wounds infected by *P. aeruginosa* in Iranian hospitals.[2,11,12] For example, Hadadi and colleagues have shown *P. aeruginosa* isolates were resistant to ceftizoxime (99%), ceftazidime (59.6%), ticarcilin (50%), ceftriaxone (44.3%), and cefoperazone (37.5%).[12] In another study, resistances of 75% for imipenem and 39% for ciprofloxacin in *P. aeruginosa* isolated from nosocomial source was reported.[11] It was shown that *P. aeruginosa* is the main infectious agent at the Tohid Burn Center in Tehran with a frequency of 73.9%, and it was revealed that 95% of isolates were resistant to gentamicin, carbenicillin, co-trimoxazole, ceftizoxime, and tetracycline, 90% for amikacin and 82% for ciprofloxacin.[2] *P. aeruginosa* has been demonstrated as the leading cause of nosocomial infections in Iranian BU.[1] Molecular epidemiologic studies have a very important role in the determination of transmission routes of the pathogen for managing infection. This type of information can be used in clinical settings to separate continuing epidemics of an infectious agent from incidentally increased infection rates. DNA typing methods are known as the most suitable approaches for epidemiological studies.[13,14] Pulsed-field gel electrophoresis (PFGE) is 1 of the most powerful techniques and is used as the gold standard for the typing of many microorganisms like *P. aeruginosa*.[15-17] In this study, PFGE was applied for molecular typing, and the results were used for detailed analysis of the routes of *P. aeruginosa* colonization in the BU. Since effective management of nosocomial infections, especially in BU, needs to inform about infection transmission routes and drug susceptibility of pathogens, this study was conducted to investigate antibiotic susceptibility and molecular epidemiology of *P. aeruginosa* isolated in the BU of Shahid Motahhari Hospital, 1 of the referral BUs in Tehran, Iran, between February 2008 and June 2008.

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Abbreviations

PFGE, pulsed-field gel electrophoresis; MDR, multidrug-resistant; BU, burn units; MP, multi-purpose; UPGMA, unweighted pair group method using arithmetic averages

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Materials and Methods

Sampling and Patient Demographics

The intensive care BU of the Shahid Motahhari Hospital is a referral center for patients with severe burn injuries. Between February 2008 and June 2008, 129 *P. aeruginosa* isolates from burn patients and 2 isolates from the hospital environment were collected. Patients hospitalized in the BU had different types of burn injuries. They included 14 patients (10%) under 15 years old and 126 patients (90%) over 15 years old, 108 (77.1%) were male, and 32 (22.9%) were female. The clinical samples included burn wound swabs, blood, and biopsy specimens, and the environmental samples included water from faucets, antiseptics, hand-washing solutions and swabs from sinks, hydrotherapy equipment, floors, and other damp surfaces with a potential for cross-contamination throughout the BU.

Bacterial Analysis

All samples were cultured in Mueller-Hinton agar, and *P. aeruginosa* were isolated from the samples by standard microbiology procedures. Each isolate originated from a single colony of each patient's culture and was identified as *P. aeruginosa* by API 20NE (bioMérieux, Lyon, France). *Pseudomonas aeruginosa* isolates were stored in Luria-Bertani broth medium (Merck KGaA, Darmstadt, Germany) containing 30% glycerol at -80°C.

Drug Susceptibility Testing

Drug susceptibility testing and interpretation were performed according to Clinical Laboratory Standards Institute guidelines.[18] Tests were completed for all isolates by disk diffusion method for 13 antimicrobial agents, including amikacin, aztreonam, cefotaxime, ceftazidime, ceftizoxime, ciprofloxacin, gentamicin, imipenem, kanamycin, meropenem, piperacillin, piperacillin-tazobactam, and tetracycline (Mast Diagnostics, Mast Group, Merseyside, UK). Multidrug resistant *P. aeruginosa* isolates were resistant to ceftazidime and at least 3 of the following antibiotics: amikacin, aztreonam, ciprofloxacin, gentamicin, imipenem, piperacillin, and aminoglycosides. *Pseudomonas aeruginosa* ATCC 27853 was used as a control.

PFGE Method

Pulsed-field gel electrophoresis was performed according to a previously described protocol by Gautom with some modifications.[16] Briefly, *P. aeruginosa* bacteria were grown overnight on Mueller-Hinton agar plates and then suspended directly with sterile cotton swabs in about 2 to 3 mL of TE buffer (100 mM Tris and 100 mM EDTA). The cell suspensions were adjusted with TE buffer to 20% transmittance by using a Bio-Rad spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Aliquots of 100 µL of the cell suspensions were transferred to 1.5 mL microcentrifuge tubes. Lysozyme and proteinase K were added to a final concentration of 1 mg/mL each and mixed by pipetting. The bacterial suspensions were incubated at 37°C for 10 to 15 minutes. Multi-purpose (MP) agarose (Roche Diagnostics GmbH, Mannheim, Germany) was prepared in water to a final concentration of 1.2% and maintained at 55°C in a water bath. Following the lysozyme-proteinase K incubation, 7 µL of 20% sodium dodecyl sulfate and 140 µL of 1.2% MP agarose were mixed with each bacterial suspension using a pipette. This bacterium-agarose mixture was immediately added to plug molds (Pharmacia Biotech, Stockholm, Sweden). The plugs were allowed to solidify for 5–10 minutes at 4°C and then transferred to 2 mL round-bottom tubes containing 1.5 mL of ESP buffer (0.5 M EDTA, pH 9.0; 1% sodium lauryl sarcosine; 1 mg of proteinase K per mL). These were incubated in a water bath at 55°C for 2 hours. After the completion of proteolysis, the plugs were transferred to 50 mL tubes containing 8 mL to 10 mL of sterile, preheated (50°C) distilled water and incubated for 10 minutes at 50°C with a gentle mixing in a shaker water bath. Subsequently, 4 50°C washes were done in a shaker water bath for 15 minutes each with 8 mL to 10 mL of preheated (50°C) TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH=8). The plugs were then cooled to room temperature in a TE buffer. At this point, they could be used immediately or stored for 3 to 4 weeks at 4°C in 1 mL of TE. For restriction endonuclease digestion, 2 1 mm thick slices of each plug were incubated at 37°C for 3 hours with 50 U of XbaI enzyme, in 100 mL of the appropriate (1×) restriction enzyme buffer.

Multi-purpose agarose at a concentration of 1.2% provided the desired resolution of DNA fingerprints. The plug slices of the samples were loaded and electrophoresed in 1.2% MP agarose with 2.5 liters of standard 0.5× TBE running buffer. The electrophoresis was performed with the Gene Navigator System (Pharmacia Biotech). Electrophoresis run conditions were designed for a run time of 24 hours. In these runs, the initial and final switch times were 5 seconds and 90 seconds, respectively; all other parameters remained identical with those of the standard procedure.

Following electrophoresis, the gels were stained for 20 minutes in 500 mL of sterile distilled water containing 50 µL of ethidium bromide (10 mg/mL) and destained in 3 washes of 30 minutes each in 1 liter of distilled water. The gels were analyzed under UV transilluminator (UVItec, Cambridge, UK), and TIFF files were saved for analyzing with GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis of the Dice similarity indices based on the unweighted pair group method using arithmetic averages (UPGMA) was done to generate a dendrogram describing the relationship among pulsotypes. A difference of at least 1 restriction fragment in the profiles was considered the criterion for discriminating between clones. Visual analysis was completed based on Tenover criteria.[19]

Results

Drug Susceptibility Testing

Drug susceptibility tests by the disk diffusion method showed many isolates were resistant to ceftizoxime (87%), aztreonam (80.2%), kanamycin (79.4%), tetracycline (78.6%), and ceftazidime (74.8%), but few isolates were resistant to imipenem (16%), piperacillin/tazobactam (19.1%), and amikacin (35.1%). Forty-two MDR *P. aeruginosa* isolates were recovered from clinical samples, and 1 isolate was recovered from the environment. The results of the drug susceptibility tests are shown in **Table 1**.

PFGE Fingerprinting

Genotyping by PFGE reveals different profiles (**Figure 1**) that by GelCompar software classified 11 profiles, PFGE1 to PFGE11 (**Table 2**). Five PFGE profiles included MDR strains

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Table 1_*In Vitro* Susceptibilities of 131 *P. aeruginosa* Isolates to 13 Antimicrobial Agents

No. (%)			
Resistant	Intermediate	Sensitive	Antibiotic
46 (35.1)	8 (6.1)	77 (58.8)	Amikacin
105 (80.2)	7 (5.3)	19 (14.5)	Aztreonam
66 (50.4)	10 (7.6)	55 (42.0)	Cefotaxime
98 (74.8)	1 (0.8)	32 (24.4)	Ceftazidime
114 (87.0)	12 (9.2)	5 (3.8)	Ceftizoxime
66 (50.4)	15 (11.4)	50 (38.2)	Ciprofloxacin
99 (75.6)	2 (1.5)	30 (22.9)	Gentamicin
21 (16.0)	19 (14.5)	91 (69.5)	Imipenem
104 (79.4)	2 (1.5)	25 (19.1)	Kanamycin
48 (36.7)	2 (1.6)	81 (61.8)	Meropenem
90 (68.7)	13 (9.9)	28 (21.4)	Piperacillin
25 (19.1)	19 (14.5)	87 (66.4)	Piperacillin-Tazobactam
103 (78.6)	9 (6.9)	19 (14.5)	Tetracycline

resistant to multiple classes of antibiotics; these MDRs were resistant to similar classes of drugs. The PFGE1 with 42 isolates was the major PFGE group that included 18 MDR clinical sample isolates and 1 environmental MDR strain. The PFGE2 profile had 23 isolates and 13 MDR isolates, and the PFGE3 profile had 17 isolates with 6 MDR strains. Other profiles comprising PFGE4, 5, 6, 7, 8, 9, 10, and 11 have 13, 8, 4, 9, 5, 5, 3, and 2 isolates, respectively. The PFGE1 to PFGE5 profiles included MDR isolates (**Table 2**). Two environmental isolates (E1 and E2) were classified in PFGE1 and PFGE7 profiles, respectively. It was shown that E1 was an MDR isolate.

Discussion

Pseudomonas aeruginosa infection is a major cause of mortality and morbidity in hospitalized patients of developing countries.[1] One of the most important aspects for choosing an efficient method to prevent this infection is determining the relationship between genotype and drug susceptibility. In this study, the relationship between isolates, genotypes, and drug susceptibility patterns of *P. aeruginosa* isolates were investigated. The results may be useful for achieving an appropriate approach to eliminate infections. Pulsed-field gel electrophoresis analysis is 1 of the best genotyping methods for *P. aeruginosa* fingerprinting and is sometimes mentioned as the gold standard method for this bacterium.[13] We used PFGE for typing all *P. aeruginosa* isolates obtained from hospitalized patients in Shahid Motahari BU and environmental isolates. All of the *P. aeruginosa* isolates were typeable, and 11 PFGE profiles were identified. These PFGE profiles were analyzed for any possible relationship to environmental and/or MDR isolates.

Multidrug-resistant bacteria have commonly been reported as the cause of nosocomial outbreaks of infection in BUs or as colonizers of the wounds of burn patients.[1,11] *Pseudomonas aeruginosa* has been demonstrated to be a leading cause of nosocomial infections in Iranian burn patients, and antimicrobial resistance has reached a critical point.[2,8-10] In previous studies, resistance to many antibiotics used to treat burn injuries infected by *P. aeruginosa* in Iranian hospitals were shown.[1,2,8-12] For example in 1 study, resistance of *P. aeruginosa* isolates to ceftizoxime, ceftazidime, ticarcilin, ceftriaxone, and cefoperazone were 99%, 59.6%, 50%, 44.3%, and 37.5%, respectively.[2] In another study, 75% resistance for imipenem and 39% for ciprofloxacin in *P. aeruginosa* isolates from a nosocomial source were shown.[12] In the Tohid Burn Center in Tehran, *P. aeruginosa*

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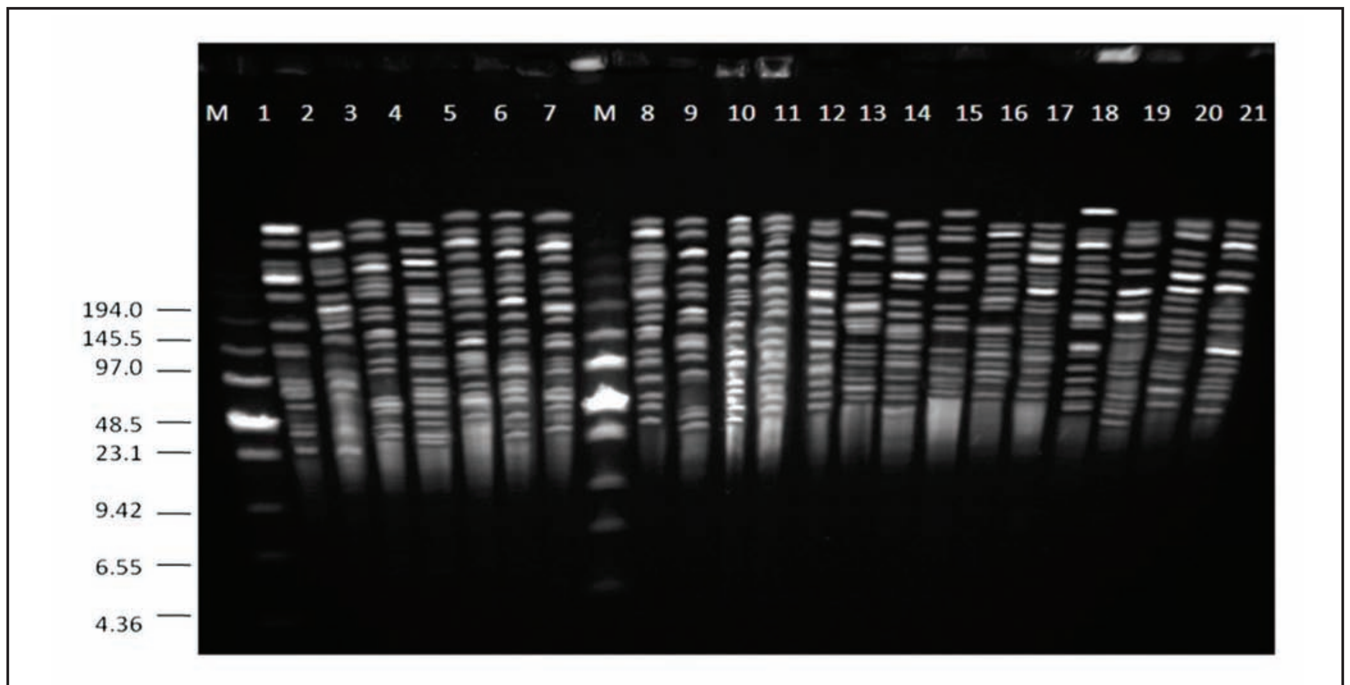

Figure 1_*XbaI*-PFGE profiles of *P. aeruginosa* strains isolated from burn patients. M; Pulse Marker 0.1–200 kb (Sigma). Lines 5, 6, 7, and 18 refer to the PFGE1 profile; lines 3, 8, 10, and 11 refer to the PFGE2 profile; lines 4 and 20 refer to the PFGE3 profile; lines 2 and 12 refer to the PFGE4 profile; lines 16 and 17 refer to the PFGE5 profile; line 15 refers to the PFGE6 profile; lines 14 and 21 refer to the PFGE7 profile; line 13 refers to the PFGE8 profile; line 19 refers to the PFGE9 profile; line 9 refers to the PFGE10 profile; and line 1 refers to the PFGE11 profile.

Table 2_Classification of *P. aeruginosa* PFGE Profiles and the Relationship Between Profiles and MDR Isolates

PFGE Pattern	Frequency (Isolates Number)	Frequency of MDR (Isolates Number)
PFGE1	42 (1, 3, 8-12, 14, 18, 28, 35, 40, 47-50,54, 57, 60, 62, 67-69, 78, 79, 86, 88-90, 92, 96, 100, 101, 105, 107, 108, 114, 117, 119, 122, 127, E1*)	18 (1, 3, 9, 12, 14, 28, 35, 40, 49, 50, 67, 86, 88, 100, 107, 114,122, E1)
PFGE2	23 (2, 13, 15, 17, 19, 33, 34, 46, 59, 61, 63, 70, 76, 81, 85, 87, 95, 99, 102, 106, 116, 120, 129)	13 (13, 15, 17, 19, 33, 34, 59, 61, 76, 81, 87, 95, 102)
PFGE3	17 (6, 7, 25, 26, 36, 38, 44, 58, 64, 65, 72, 74, 93, 98, 110, 128)	6 (7, 26, 36, 58, 110, 128)
PFGE4	13 (24, 27, 30, 41, 51, 71, 84, 91, 94, 103, 109, 11, 123)	3 (24, 41, 91)
PFGE5	8 (4, 31, 53, 56, 66, 75, 104, 113)	3 (31, 53, 75)
PFGE6	4 (29, 43, 55, 124)	0
PFGE7	9 (22, 32, 45, 52, 77, 82, 118, 125, E2*)	0
PFGE8	5 (23, 37, 80, 115, 121)	0
PFGE9	5 (21, 39, 42, 97, 126)	0
PFGE10	3(16, 20, 83)	0
PFGE11	2 (5, 112)	

E1 and E2 are environmental isolates.

was the main infectious agent, with a frequency of 73.9, and it was revealed that these isolates were resistant more than 95% for gentamicin, carbenicillin, co-trimoxazole, ceftizoxime, and tetracycline, 90% for amikacin, and 82% for ciprofloxacin.[2]

Drug susceptibility tests of *P. aeruginosa* isolates were completed, and some isolates resistant to many antibiotics were determined. Forty-three MDR isolates with 5 PFGE profiles (PFGE1-PFGE5) were found in this study. These results reveal different potential sources for MDR isolates. Our results have shown 2 environment sources for FGE1 and PFGE7 profiles, which were found in the tap water and sink drains, respectively. However, we may have missed some important outsource agents for other PFGE patterns in this study.

In this study some isolates, including the MDR PFGE1 strains, showed resistance to amikacin, aztreonam, ceftazidime, imipenem, meropenem, and piperacillin, which are the first-line antibiotics used in BU. This may illustrate the importance of the selective pressure of antibiotics in the emergence and selection of MDR epidemic strains. Nowadays, outbreaks with MDR *P. aeruginosa* strains have become rather frequent, and the persistence of MDR *P. aeruginosa* clones in BUs have been reported.[10,11]

Pseudomonas aeruginosa colonization may originate from endogenous sources such as the intestinal tract or from exogenous sources such as contaminated equipment or other patients with *P. aeruginosa*. Understanding the routes of colonization is critical to the development of efficient preventive measures against infection. Even if the overall rate of *P. aeruginosa* colonization is not significantly reduced, it is important to recognize cross-infecting strains, especially if they exhibit resistance to a variety of antibiotics and give rise to severe infections. Colonized patients represent a continuous reservoir of (epidemic) strains from which other patients can be colonized via cross-acquisition. In contrast with some studies,[3] we isolated 2 *P. aeruginosa* strains from the inanimate hospital environment that were an important source of the patients' infections. The large number of unique genotypes observed in the patients, however, suggests that most of the patients were colonized from an exogenous source. On the other hand, 42 patients were colonized with the PFGE1 strain, 23 patients were colonized with the PFGE2 strain, and 17, 13, 8, 4, 9, 5, 5, 3, and 2 patients were colonized with PFGE3 to PFGE11 isolates, respectively. There was minimal overlap between the hospitalization periods of patients. In addition, a thorough survey of the inanimate hospital environment successfully identified 2 ongoing reservoirs of PFGE1 and PFGE7 strains found in the tap water and sink drains, respectively.

Several studies have demonstrated that cross acquisition can play an important role in the epidemiology of nosocomial colonization and infection with *P. aeruginosa*.[14,20-22] Nikbin and colleagues have shown that a few isolates were distributed widely at 2 hospitals and the environment in Tehran.[21]

In this study, the transmission of some patients with PFGE1 and PFGE7 profiles may have originated from environmental sources, and other isolates may have originated from the staff's hands, equipment, or other unknown sources in the BU. These results emphasize the importance of completing routine drug susceptibility tests and molecular fingerprinting to monitor routes of infections and changes in the drug resistance of infectious agents for successful management of infections.

Conclusion

In conclusion, our findings show that environmental sources may have a significant role in the transmission of *P. aeruginosa* in this BU. This study highlighted the need for additional attention to the disinfection of inanimate objects in the hospital environment to limit the transfer of *P. aeruginosa* in this BU. Moreover, the use of some antimicrobial agents must be restricted, due to the high resistance to them. LM

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