American Journal of Infectious Diseases 5 (4): 308-313, 2009
ISSN 1553-6203
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# Drug Susceptibility and Molecular Epidemiology of *Pseudomonas aeruginosa* Isolated in a Burn Unit

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**Abstract: Problem statement:** *Pseudomonas aeruginosa* is an opportunistic pathogen that still develops life-threatening infections in patients with immunological system defects, like burn patients, especially in developing countries. Since this bacterium is naturally resistant to many drugs and is able to get resistance to all effective antibiotics, the infection with this organism is particularly problematic condition for patients. This study was design to evaluated drug susceptibility for determination of multidrug-resistant isolates and molecular epidemiology of *P. aeruginosa* colonization for investigation of the isolates routes in the burn unit of Shahid Motahhari Hospital in Tehran. **Approach:** About 127 clinical and 2 environmental *P. aeruginosa* isolates were collected during 6 months. All *P. aeruginosa* isolates were analyzed for drug susceptibility by disk diffusion method and molecular epidemiology assessment were done by RAPD-PCR analysis. **Results:** Drug susceptibility tests were shown high resistance for ceftizoxime (86.8%), aztreonam (80.6%), kanamycin (79.8%) and tetracycline (78.3%); furthermore, low resistance for some antibiotics like imipenem (30.2%), piperacillin/tazobactam (34.1%) and amikacin (41.1%) was showed. In addition, 42 multidrug-resistant *P. aeruginosa* isolates were recovered from clinical specimens and one isolate from environmental samples. Molecular typing revealed eight different profiles that include two profiles, RAPD1 and RAPD4, with environmental resource. The major RAPD profile was RAPD1 profile (n = 64, 50.4%), which includes 31 (72.1%) multidrug-resistant isolates with an environmental reservoir. **Conclusion:** In summery, we were found three different profiles for multidrug-resistant strains. Different RAPD profiles suggested the different resources of infection, two environmental resources were found, that one of them was multidrug-resistant strain. These findings highlighted the need for further attention to disinfection inanimate hospital environment and controlled contact between staff with patients to limit transfer of *P. aeruginosa* in this BU; moreover, use of some antimicrobial agents must be restricted due to existence of high resistance and using of combined effective antibiotics is recommend.

**Key words:** *P. aeruginosa*, drug susceptibility, molecular epidemiology, MDR, RAPD-PCR, burn patients

## INTRODUCTION

One of the significant public health problems in many areas of the world is burn injury[5,9,13]. Burn patients are obviously at high risk for nosocomial infections[12]. The burn wound represents a site susceptible to colonization of opportunistic pathogens, e.g., *Pseudomonas aeruginosa*[8]. The occurrence of infections in patients with burn wounds have significantly decreased by present methods of burn wound care[17,21], however, severely burn patients may still develop life-threatening infections and continue to be a general complication in burn-related morbidity and mortality worldwide, particularly in developing countries. *P. aeruginosa* is a gram-negative human opportunistic pathogen that infects burn patients with

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immunological system defects and causes a wide range of infections. Since this bacterium is naturally resistant to many drugs and is able to get resistance to all effective antibiotics, the infection with this organism is particularly problematic condition for patients[6,8,14,15,19].

Multidrug-Resistant (MDR) bacteria have commonly been reported as the cause of nosocomial outbreaks of infection in burn units or as colonizers of the wounds of burn patients. Antimicrobial resistance has been reached to a troubling point in *P. aeruginosa* isolated from burn patients in Iran[1,8,15,20]. Previous studies demonstrated resistance for many antibiotics usually used to treatment of burn injuries that infection by *P. aeruginosa* in Iranian hospitals[11]. For example in one study, *P. aeruginosa* isolates were resistance to ceftizoxime (99%), ceftazidime (59.6%), ticarcilin (50%), ceftriaxone (44.3%) and cefoperazone (37.5%) and in another study, resistances of 75% for imipenem and 39% for ciprofloxacin in *P. aeruginosa* isolated from nosocomial source were showed[9]. It was showed *P. aeruginosa* as the main infectious agents in the Tohid Burn Center in Tehran with the frequency of 73.9[8] and it was reveal that these isolates were resistance over 95% for gentamicin, carbenicillin, co-trimoxazole, ceftizoxime and tetracycline, 90% for amikacin and 82% for ciprofloxacin. *P. aeruginosa* has been demonstrates as the leading cause of nosocomial infectious in Iranian BU[15]. Molecular epidemiologic studies have very important role in determination of transmission routes of pathogen for infection management. This kind of information can be used in clinical settings to separate continuing epidemics of an infectious agent from incidentally increased infection rates. DNA base typing methods known as the most appropriate approaches for epidemiological study. These methods may divided to two major categories, direct sequencing based techniques, Multilocus Sequence Typing (MLST) and DNA microarray and indirect methods of sequence analysis, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) and Pulsed Field Gel Electrophoresis (PFGE)[2,3,10,16].

As we mentioned some drug susceptibility studies for *P. aeruginosa* isolates in Iran, the resistance degree for some antibiotics were reached to threatening level. Despite the very significance of molecular epidemiological studies, unfortunately, there are only few studies about molecular epidemiology of *P. aeruginosa* in Iran[11].

We applied RAPD-PCR analysis; a discriminatory and reproducible genotyping technique, to do a detailed analysis of the routes of *P. aeruginosa* colonization in the BU. Since determining the transmission routes of infection and drug susceptibility of the pathogens have the major impact on effective management of nosocomial infections in burn injuries, this study was planning to investigate drug susceptibility and routes of transmission by molecular epidemiology in *P. aeruginosa* isolated in BU of Shahid Motahhari Hospital, one of the referral BU in Tehran (Iran), between February 2008 and June 2008.

## MATERIALS AND METHODS

**Sampling and patients demographics:** The intensive care burn unit of the Shahid Motahhari Hospital is a referral center for patients with severe burn injury in Tehran, Iran. Between February 2008 and June 2008, 127 *P. aeruginosa* isolates from burn patients and 2 isolates from Hospital environment were collected. Patients were hospitalized in BU because of different types of burn injuries. They include; 14 (11%) under 15 year-old and 113 (90%) over 15 year-old patients and in all patients, 98 (77.2%) were men, the remained (n = 29, 22.8%) were women. The main causes of burn injuries in this study were as follows: Gasoline (n = 41, 32.3%), oil (n = 24, 18.9%), liquid gas (n = 22 17.3%), boiling water (n = 17, 13.4%), electrical (n = 7, 5.5%), flame (n = 5, 4%), tar (n = 4, 3.1%), alcohol (n = 4, 3.1%) and acid (n = 3, 2.4%). The clinical samples included burn wound swabs or biopsy specimens and environmental samples included water from faucets, antiseptics, hand-washing solutions and swabs from sinks, hydrotherapy equipment, floors and other damp surfaces with potential for cross-contamination throughout the burn unit.

**Bacterial analysis:** All samples were cultured on the Mueller-Hinton agar and the *P. aeruginosa* were isolated from 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). *P. aeruginosa* isolates were stored in Luria-Bertani broth medium (Merck KGaA, Darmstadt, Germany) containing 30% glycerol at -80°C.

**Drug susceptibility testing:** The drug susceptibility tests were done for all isolates by Bauer-Kirby agar disk diffusion method for thirteen antimicrobial agents including, amikacin, aztreonam, cefotaxime, ceftazidime, ceftizoxime, ciprofloxacin, gentamicin, imipenem, kanamycin, meropenem, piperacillin, piperacillin-tazobactam and tetracycline (Mast Diagnostics, Mast Group Ltd, Merseyside, UK). MDR

*P. aeruginosa* isolates were resistant to ceftazidime and at least three of following antibiotics; amikacin, aztreonam, ciprofloxacin, gentamicin, imipenem, piperacillin and aminoglycosides. CLSI breakpoints were used to determine susceptibility.

**Isolation of genomic DNA:** Genomic DNA of the isolates was extracted from 2 mL of cultures acquired from a single colony as follows: The cells were harvested by centrifugation (3000×g, 8 min), the bacterial pellet was suspended in 567 µL of TE buffer (50 mM Tris, 50 mM EDTA, pH 8.0) plus 30 µL 10% SDS and 3 µL of proteinase K (20 mg $mL^{-1}$) and incubated 1 hr at 37°C. Then 80 µL of 10% CTAB in 0.7% NaCl was added and the mixture was incubated for 10 min at 65°C. The solution was extracted with 750 µL of chloroform/isoamylalcohol (24:1), spun and the aqueous phase was re-extracted with phenol/chloroform/isoamylalcohol (25:24:1). DNA was precipitated with 500 µL of isopropanol from the aqueous phase; the DNA pellet was washed with 70% ethanol, dried briefly and re-suspended in 100 µL of TE buffer and the DNA concentration has been determined by measuring absorbance of the sample at 260 nm on a UV spectrophotometer[18].

**RAPD analysis:** Typing was performed by RAPD analysis. RAPD-PCR mixtures were set up and incubated as previously described[16]. Reactions mixtures (25 µL) were made optimum for *P. aeruginosa* and contained 40 ng of genomic DNA, 40 p mol of primer 272 (MWG Biotech, Germany, AGCGGGCCAA)[19], 1 unit of Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 250 mM of each deoxynucleoside triphosphate (MBI Fermentas), 1x reaction buffer supplied by manufacturer and 3 mM $MgCl_2$ (MBI Fermentas). DNA was amplified in ASTEC PCR Thermal Cycler PC 707-02 (ASTEC, Fukuoka, Japan) with the cycling conditions as follows: (i) 94°C for 5 min, 36°C for 5 min and 72°C 5 min, for 4 cycles and (ii) 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, for 30 cycles, followed by a final extension step at 72°C for 10 min. RAPD products (one-third of each reaction mixture) were separated by horizontal gel electrophoresis in 1.5% (wt/vol) agarose gels (20 wells; 11 by 14 cm) with tris-borate-EDTA (TBE) running buffer at 9 V $cm^{-1}$ for 4 h. DNA molecular size markers were included in all gels (GeneRuler 100 bp DNA ladder, MBI Fermentas). After the gels were stained with ethidium bromide, they were observed in Gel-Documentation system (UVItec, Cambridge, UK). In the pattern resulting from RAPD assays, only the major reproducible bands that were detected by UV fluorescence and comprised 500-1,500 bp were taken into account in the analysis, as previously described. Isolates with RAPD patterns that different by one or more discrete bands were considered different; otherwise, the isolates were considered identical[16].

## RESULTS

**Bacterial Isolates:** As 127 *P. aeruginosa* isolates were obtained from clinical specimens and two environmental isolates were obtained from inanimate samples. All isolates were identified as *P. aeruginosa* by API 20NE. Environmental isolates were recovered from a sink (n = 1) and a floor (n = 1). All cultures from the hands of the medical personnel were negative for *P. aeruginosa*.

**Drug susceptibility testing**: Drug susceptibility tests have showed high resistance to ceftizoxime (86.8%), aztreonam (80.6%), kanamycin (79.8%), tetracycline (78.3%) and ceftazidime (75.2%) and more susceptibility to imipenem (69.8%), piperacillin/tazobactam (65.9%) and amikacin (58.9%). 42 Multidrug-Resistant (MDR) *P. aeruginosa* isolates were recovered from clinical and one isolate was recovered from environment. The results of drug susceptibility tests are showed in Table 1.

**RAPD fingerprinting of *P. aeruginosa*:** All collected *P. aeruginosa* isolates from burn wounds and environmental samples were typed by RAPD fingerprinting by primer 272 to determine the genotype variation. The RAPD fingerprints that possessed similarity coefficients of greater than 0.8 when examined

Table 1: *In vitro* susceptibilities of 129 *P. aeruginosa* isolates to 13 antimicrobial agents

| | No. (%) | | |
|---|---|---|---|
| Antibiotic | Sensitive | Intermediate | Resistant |
| AMK | 76 (58.9) | 8 (6.2) | 45 (34.9) |
| ATM | 18 (14.0) | 7 (5.4) | 104 (80.6) |
| CTX | 54 (41.9) | 10 (7.7) | 65 (50.4) |
| CAZ | 31 (24.0) | 1 (0.8) | 97 (75.2) |
| ZOX | 5 (3.9) | 12 (9.3) | 112 (86.8) |
| CIP | 49 (38.0) | 15 (11.6) | 65 (50.4) |
| GEN | 29 (22.5) | 2 (1.5) | 98 (76.0) |
| IPM | 90 (69.8) | 19 (14.7) | 20 (15.5) |
| KAN | 24 (18.6) | 2 (1.5) | 103 (79.8) |
| MEM | 79 (61.2) | 2 (1.6) | 48 (37.2) |
| PIP | 27 (20.9) | 13 (10.1) | 89 (69.0) |
| TZP | 85 (65.9) | 19 (14.7) | 25 (19.4) |
| TET | 19 (14.7) | 9 (7.0) | 101 (78.3) |

AMK: Amikacin; ATM: Aztreonam; CTX: Cefotaxime; CAZ: Ceftazidime; ZOX: Ceftizoxime; CIP: Ciprofloxacin; GEN: Gentamicin; IPM: Imipenem; KAN Kanamycin; MEM: Meropenem; PIP: Piperacillin; TZP: Piperacillin-Tazobactam; TET: Tetracycline

Table 2: RAPD types classification and MDR isolates

| RAPD type | No. of isolates (patient's No.) | No. of MDR isolates |
|---|---|---|
| RAPD1 | 65 (1-3, 8-15, 17-19 28, 33-35, 40, 46-50, 54, 57, 59-63, 67-70, 76, 78, 79, 81, 85-90, 92, 95, 96, 99-102, 105-108, 114, 127, 129, E1) | 31 (1, 3, 9, 12-16, 19, 28, 33-35, 40, 49, 50, 59, 61, 67, 76, 81, 86-88, 95, 100, 102, 107, 114, 122, E1) 116, 117, 119, 120, 122, |
| RAPD2 | 29 (6, 7, 24-27, 30, 32, 36, 41, 44, 51, 58, 64, 65, 71-74, 84, 91, 93, 94, 98, 103, 109-111, 123, 128) | 9 (7, 24, 26, 36, 41, 58, 91, 110, 128) |
| RAPD3 | 12 (4, 29, 31, 43, 53, 55, 56, 66, 75, 104, 113, 124) | 3 (31, 53, 75) |
| RAPD4 | 8 (22, 32, 45, 52, 66, 77, 82, 96, 118, 125, E2) | - |
| RAPD5 | 5 (23, 37, 80, 115, 121) | - |
| RAPD6 | 5 (21, 39, 42, 97 and 126) | - |
| RAPD7 | 3 (16, 20 and 83) | - |
| RAPD8 | 2 (5, 112) | |

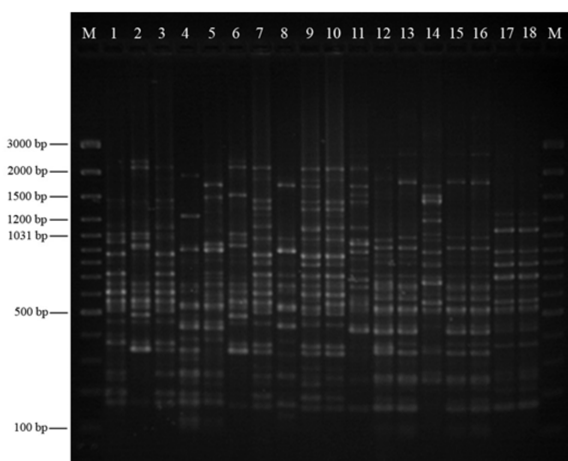


Fig. 1: The RAPD fingerprinting of *P. aeruginosa* strains; (Lanes 1, 3, 7, 9 and 10): (E1) are RAPD1, Lanes (8, 12, 13, 15 and 16): RAPD2; (Lanes 2 and 6) RAPD3; (Lanes 17 and 18): (E2) RAPD4; (Lane 1): RAPD5; (Lane 14): RAPD6; (Lane 4): RAPD7; (Lane 5): RAPD8 profile; (Lane M): GeneRuler 100 bp DNA ladder

side by side were considered identical and assigned a RAPD type. Based on this procedure we found eight unique RAPD types. The RAPD typing results are shown in Table 2.

All MDR *P. aeruginosa* isolates were categorized in three types of RAPD patterns, RAPD type1, 2 and 3. Sixty-four patients were colonized with isolates of RAPD type 1; twenty-nine patients with RAPD type 2 isolates and 12, 7, 5, 5, 3 and 2 patients were colonized with RAPD type 3, 4, 5, 6, 7 and 8, respectively. In addition two environmental isolates were found in RAPD type 1 and RAPD type 4. These RAPD profiles were discriminatory among BU isolated strains, with different numbers, sizes and intensities of amplified DNA bands. RAPD profiles for some *P. aeruginosa* isolates which obtained by primer 272 are showed in Fig. 1.

## DISCUSSION

*P. aeruginosa* isolated from burn wounds have significant effect on the mortality and morbidity in hospitalized burn patients particularly in developing countries. Determination of relationship between genotype and drug resistance is an important factor for choosing efficient approach to managing these infections. In this report, we were investigated relationship between genotypes and drug susceptibility patterns of *P. aeruginosa* isolates, to find an appropriate approach to elimination of this infection. RAPD-PCR is a genotypic identification and characterization system that has shown great specificity and sensitivity to define bacterial isolates. It has been showed that RAPD typing can be used successfully for typing *P. aeruginosa* as highly effective method[16], so it was applied for fingerprinting all isolates obtained from patient population that refer to Shahied Motahhari BU. All 131 *P. aeruginosa* isolates described in this study were typeable by RAPD typing. *P. aeruginosa* isolates were analyzed by disc diffusion method for drug susceptibility identification and it was found some isolates resistance with many antibiotics. We were found 43 MDR isolates with three RAPD profiles (RAPD1, RAPD2 and RAPD3). These results reveal different potential sources for MDR isolates. Totally, eight different RAPD profiles were identified, which were analyzed for any relationship by environmental and MDR isolates. These results showed two environment sources for infection (RAPD1 and RAPD4) and it is possible that some important outsources agents for other RAPD patterns was missed in this study.

**Drug susceptibility:** Multidrug-Resistant (MDR) bacteria have commonly been reported as the cause of nosocomial outbreaks of infection in burn units or as colonizers of the wounds of burn patients[1,14,15,19,20]. Antimicrobial resistance has been reached to a troubling point in *P. aeruginosa* isolated from burn patients in Iran. Previous studies demonstrated resistance for many antibiotics usually used to treatment of burn injuries that infection by *P. aeruginosa* in Iranian hospitals[1,14-15,19]. For example in one study, *P. aeruginosa* isolates were resistance to

ceftizoxime (99%), ceftazidime (59.6%), ticarcilin (50%), ceftriaxone (44.3%) and cefoperazone (37.5%)[15] and in another study, resistances of 75% for imipenem and 39% for ciprofloxacin in *P. aeruginosa* isolated from nosocomial source were showed[7]. It was showed *P. aeruginosa* as the main infectious agents in the Tohid Burn Center in Tehran with the frequency of 73.9 and it was reveal that these isolates were resistance over 95% for gentamicin, carbenicillin, co-trimoxazole, ceftizoxime and tetracycline, 90% for amikacin and 82% for ciprofloxacin[8].

*P. aeruginosa* has been demonstrates as the leading cause of nosocomial infectious in Iranian BU[8]. It was shown 97.7% susceptibility to amikacin in 1988 isolated *P. aeroginosa* in Iran, 49% resistance in 1997 isolates[15] and 95% in 2003 isolates[19], in this study we showed 45% resistance for this antibiotic. Antibiotic resistance variation may in related to different new resources of infection. Several strains, including the epidemic MDR RAPD1 strains, acquired resistance to amikacin, aztreonam, ceftazidime, imipenem, meropenem and piperacillin, which are the first-line antibiotics that were used in BU. This may illustrates 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* clone in burn units have been reported[19,20].

**Acquisition routes:** *P. aeruginosa* colonization may originate from endogenous sources such as intestinal tract or from exogenous sources such as contaminated equipment or other patients colonized with *P. aeruginosa*. Understanding the routes of colonization is crucial to the development of effective 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[4], we isolated two *P. aeruginosa* strains from the inanimate hospital environment that were important source of patients' infections. The large number of unique genotypes observed in the patients, however, suggests that most of patients were colonized from an exogenous source. On the other hand, 64 patients were colonized with the RAPD1 strain, 29 patients were colonized with the RAPD2 strain and 12, 7, 5, 5, 3 and 2 patients were colonized with RPAD3 to RPAD8 isolates, respectively. In addition a thorough survey of the inanimate hospital environment successes to identify two ongoing reservoirs of RAPD1 and RAPD4 strains.

Several studies have demonstrated that cross-acquisition can play an important part in the epidemiology of nosocomial colonization and infection with *P. aeruginosa*[2,3]. In our BU, transmission of 74 patients (RAPD1 and RAPD4 profiles) were from the environmental resources and other isolates may originates from staff, equipment or other sources in the BU.

## CONCLUSION

In conclusion our findings highlighted the need for further attention to disinfection inanimate hospital environment to limit transfer of *P. aeruginosa* in this BU; moreover, use of some antimicrobial agents must be restricted due to existence of high resistance to them.

## ACKNOWLEDGMENT

We are grateful to Mrs. Sara Amiri for expert technical assistance and computer analysis and Dr. Mohammad Ali Bahar for his support to obtaining samples. This study was supported by National Institute of Genetic Engineering and Biotechnology and Shahed University.

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