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Original article

Antibiotyping and Genotyping of *Pseudomonas aeruginosa* Strains Isolated from Mottahari Hospital in Tehran, Iran by ERIC-PCR

Hossein Fazeli[1], Bahram Nasr Esfahani[1], Mahboubeh Sattarzadeh[2], Hajar Mohammadi Barzelighi[1]*

[1]Department of Bacteriology, Faculty of Medicine, Isfahan University of Medical sciences, Isfahan, IR Iran
[2] Motahhari Burn Hospital, Iran University of Medical Sciences, Tehran, IR Iran

Corresponding author: Hajar Mohammadi Barzelighi, department of Bacteriology, Faculty of Medicine, Isfahan University of Medical sciences, HezarJerib Ave., Isfahan, IR Iran, E-mail: Hajar_mohamadi@yahoo.com, Tel: +982166522467

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Abstract

Background: *Pseudomonas aeruginosa* has become the most common cause of infections in burn patients. The aim of this study was to investigate the antibiotyping and genotyping of *P. aeruginosa* strains isolated from burn patients in Mottahari hospital during June-October 2016.
Materials and Methods: A total of 78 *P. aeruginosa* strains were collected from wound infected patients. Identification of the isolates was performed by biochemical tests and confirmed by specific 16srDNA PCR. Antimicrobial susceptibility testing was done by disk diffusion method according to the CLSI guidelines. The isolates were then evaluated for genotyping by ERIC-PCR.
Results: From a total of 78 collected isolates, 77 isolates (98.7%) were confirmed as *P. aeruginosa* by specific PCR. We found 4 antibiotypes. The highest resistance was observed to imipenem and gentamicin (~100%), and the most sensitivity was shown to colistin (100%). Overall, MDR phenotype was observed in most of the isolates (98.7%). The PCR of ERIC box produced 52 different patterns and 3 main clusters. Also, 59 (83%), 2 (3%), and 9 (13%) isolates were included in Cluster A, B, and C, respectively, and Cluster A was the predominant ERIC profile.
Conclusion: The high resistance to antibiotics in our study may be due to their abundant use as the prophylactic or treatment regimen in wound infections. So appropriate use of antibiotics seems necessary, and colistin is a proper choice for treatment of burn infection. In genotyping, 3 main clusters and 52 different patterns were shown. The majority of the *P. aeruginosa* strains isolated from burn patients were related and belonged to Cluster A.

Key words: P. aeruginosa, Burns, Genotyping technique

1. Background

Pseudomonas aeruginosa is one of the most common causes of nosocomial and healthcare-associated infections and responsible for lung, urinary tract, surgical site infections and sepsis (1-2). This bacterium is characterized by inherent resistance to a wide variety of antimicrobials; the inherent resistance is always mediated by antibiotic resistance genes (3). The increasing use of antibiotics rises the numbers of invasive procedures and immunocompromised individuals in healthcare settings, and develops intrinsic and acquired resistance to antimicrobial agents in this bacteria, leading to frequent Multi Drug Resistant *P. aeruginosa* (4). Infections caused by MDR isolates are associated with increased prices, duration of hospitalization, and particularly, morbidity and mortality rates (5).

Antimicrobial resistance is caused by two mechanisms including: genetic mutations occurred with a low frequency, and acquisition of various resistance genes. Consequently, latter way is regarded as a major contributor to the wide distribution and spread of antimicrobial resistance by mobile genetic elements such as plasmids and transposons, via either vertical or horizontal transfer as a result of selection pressure (6). Typing of this nosocomial *P. aeruginosa* is very important for tracking the source of outbreaks and performing effective control methods in order to prevent from pathogen distribution (7). Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR is an appropriate assay for DNA typing associated with high type ability, stability, and rapid turnaround reproducibility with low complexity and cost, which can be used to screen, discriminate, and determine genetic relatedness among the strains with the same accuracy of PFGE (7-8). This method is a common technique in which the regions between target repetitive non-coding sequences in the bacterial genome are exhibited (7).

2. Objectives

The aims of this study was to investigate the antibiotyping and ERIC-PCR genotyping of the *P. aeruginosa* strains isolated from burn patients of Mottahari hospital in Tehran during June-October 2016.

3. Materials and Methods

3.1. Bacterial strains

In this cross-sectional study, 78 isolates of *P. aeruginosa* were collected from 63 patients (more than 1 sample from a patient by an interval) admitted to Mottahari hospital in Tehran. The isolates were collected from wound, blood, and catheter of the patients with wound infection and identified in the hospital clinical laboratory by the biochemical tests. The study samples were collected in a 5-month period (from June 2016 to October 2016) and transferred to the bacteriology laboratory of Tarbiat Modares University of Medical Sciences and reidentified by phenotypic tests including Gramstaining, citrate, catalase, oxidase, growth on MacConkey agar, TSI, oxidative-fermentative test at 42°C, Methyl Red/ Voges Proskauer (MRVP)(9) and then confirmed by specific PCR for 16srDNA of *P. aeruginosa* (10)(Table 1).

3.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by disc diffusion methods; the break points for antibiotic susceptibility

were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (11). Antibiotic discs used in this study contained ciprofloxacin (5µg), ceftazidime (30µg), amikacin (30µg), gentamicin (10µg), imipenem (10µg), trimethoprim/sulfamethoxazole (75µg) and colistin (10µg) (MAST, England). The results were validated using *P. aeruginosa* ATCC 27853 (11).

3.3. DNA extraction and PCR methods for 16srDNA and ERIC-PCR genotyping

The genomic DNA was extracted using boiling method as follow, one loopful of fresh overnight bacteria on Brain Heart Infusion agar plates was picked up and suspended in 200µL of sterile water and boiled for10 min, after centrifugation in 12000 rpm, the supernatant was subjected to PCR by 16srDNA and ERIC primers (Table1). Amplification was performed using Bio Rad Thermal Cycler, Germany, in 12.5 µL volume containing 1µL of purified DNA, 6.5 µL of PCR Master-mix (bioneer, USA), and 0.5 µL 10pM of each primer. The PCR conditions were as follow: an initial denaturation at 94°C for 5 min; 25 cycles of denaturation at 94°C for 45 s; annealing at 61 and 55°C, respectively, for 45 s; and an extension at 72°C for 60 s; followed by an final extension at 72°C for 2 min. PCR products were separated by electrophoresis on a 1% agarose gel and detected by comparison with a 100bp DNA ladder as a size marker under UV doc apparatus.

4. Results

4.1. Bacterial strains

Among 63 patients diagnosed with wound infection episode in Motahhari hospital, a referral burn hospital in Tehran, 78 isolates were collected in a 5-months period. Symptoms of infection include pus, swelling of the wound margins, ulcers color changes, bleeding, and fever. Totally, 77 isolates (99%) were reidentified as *P. aeruginosa* by Gram staining, conventional biochemical tests, and 16srDNA PCR analysis (Fig. 1), and 1 isolate was excluded.

4.2. Antimicrobial susceptibility test

Antibiotic susceptibility patterns of isolates obtained from wound, blood, and catather specimens and their antibiotyping by disc diffusion test are shown in Table 2. The highest resistance among the isolates was observed against gentamicin (100%), imipenem (98.7%), ciprofloxacin (98.7%), amikacin (97%), and ceftazidim (75%), respectively. In contrast, the most sensitivity was observed to colistin (100%). Overall, MDR phenotype was observed in most of the isolates (98.7%).

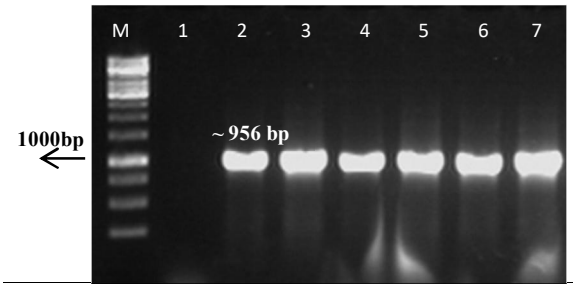


Figure 1. PCR amplification of 16srDNA F-R, Lanes 1, 2 representatives of negative and positive controls, Lanes 3-7 *P. aeruginosa* isolates, M: 100 bp DNA size marker.

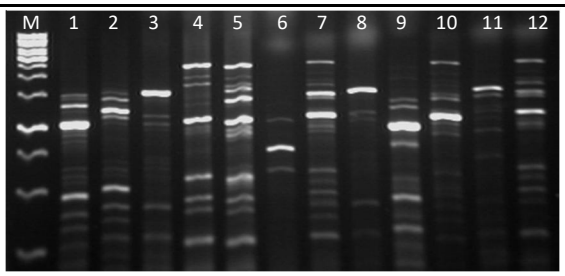


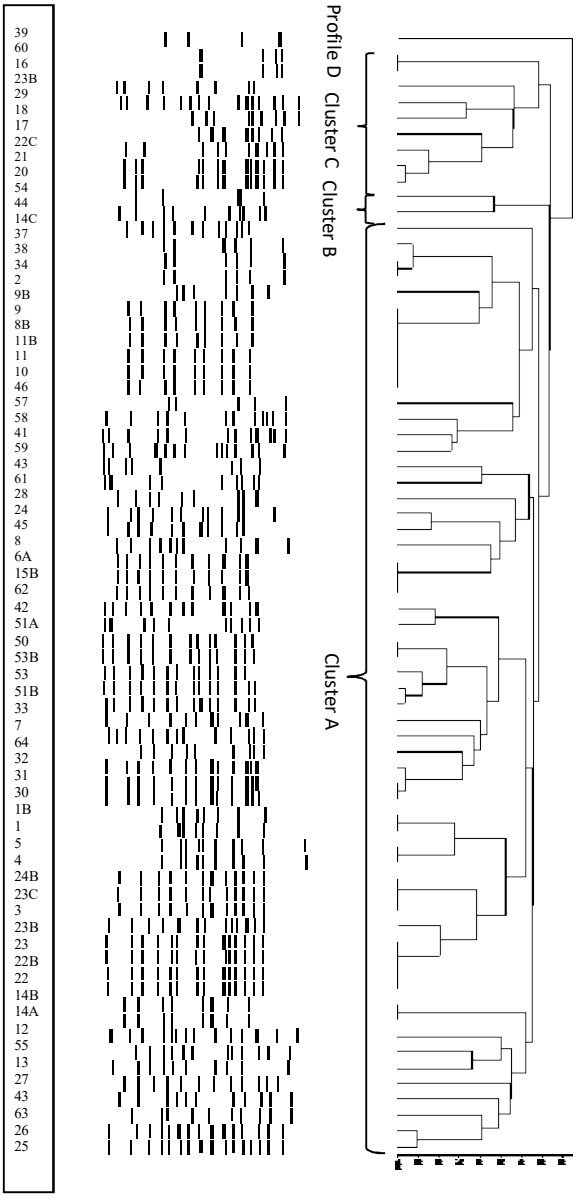
Figure 2: Lane 1-12: ERIC-PCR patterns of *P. aeruginosa* isolates; M: 1kb DNA size marker.

Table 1: Oligonucleotide primers used in this study.

Primer designation	Primer sequence (5' →3')	PCR product size (bp)	Reference
16srDNA	F: GGGGGATCTTCGGACCTCA R: TCCTTAGAGTGCCCACCCG	956	(10)
ERIC-1 ERIC-2	F: ATGTAAGCTCCTGGGGATTCA R: AAGTAAGTGACTGGGGTGAGCG	Variable	(21)

Table2: Antibiotyping patterns and antibiotic resistant and susceptibility rates in *P. aeruginosa* isolates.

Antibiotypes	Antibiogram pattern						Percent
	Caz	Cip	Ak	Gen	Ipm	Cst	
AntibiotypeI	R	R	R	R	R	S	72.7%
AntibiotypeII	S	R	R	R	R	S	24.6%
AntibiotypeIII	R	R	S	R	R	S	1.29%
AntibiotypeIV	R	S	I	R	S	S	1.29%
Total	R: 58/77(75%) S: 19/77 (25%)	R: 76/77 (~99%) S: 1/76 (1%)	R: 75/77 (97%) S: 1/77 (~1%) I: 1/77 (~1%)	R: 77/77 (100%)	R: 76/77 (~99%)	S: 77/77 (100%)	~100%



Dendrogram demonstrates Cluster analysis by enterobacterial repetitive intergenic consensus fingerprinting of 71 *P. aeruginosa* isolates from burn patients, Tehran. The scale indicates the percentage of genetic similarity. Columns (at the right) indicate list of isolates. They show 52 different patterns and 3 main clusters.

4.3. PCR results for 16srDNA and ERIC genotyping

Among 78 samples isolated from 63 patients, 77 isolates (98.7%) were confirmed as *P. aeruginosa* by 16srDNA specific primer. The detection of ERIC box by PCR produced 52 different patterns with 4-14 bands ranging from 250-950 bp in 71 isolates. The dendrogram of similarity was obtained using the UPGMA method, and the existence of 3 main clusters was demonstrated (A-C), 59 isolates (83%) were included in Cluster A which was the predominant ERIC profile; 2 (3%) and 9 (13%) isolates were included in Cluster B and C, respectively. Only 1 isolate showed different profile with genetic diversity not found in other isolates (Profile D).

5. Discussion

Despite the advances in hospital surveillance and the introduction of a wide variety of antimicrobial agents, MDR *P. aeruginosa* continues to be an important cause of nosocomial infections. This species is one of the most important microorganisms causing various clinical problems as a result of high resistance to antimicrobial agents (12). The presence of widespread antibiotic resistance genes and the high speed dissemination of environmental resistance genes have made it difficult to control and eradicate this pathogen (12).

This study was designed with two main purposes of tracing the antibiotic resistance, and susceptibility patterns and ERIC-PCR genotyping of *P. aeruginosa* strains isolated from burn patients. In our study, *P. aeruginosa* isolates were included in 4 antibiotypes, and 76 isolates (98.7%) were resistant to 3 families of antibiotics and considered as Multi Drug Resistant (MDR) *P. aeruginosa*. Similar to many studies, the term MDRPA was used to refer to the isolates resistant to at least

three different classes of antimicrobial agents, mainly aminoglycosides, carbapenems, quinolones, and cephalosporins (13).

Our results showed that resistance rate to gentamicin, imipenem, ciprofloxacin, amikacin, and ceftazidime was very high, which may be due to the abundant use of them as the prophylactic or treatment regimen in wound infections in burn patients. It has been shown that there is a significant correlation between the bacteria resistance and antibiotic usage (14).

All of our isolates (100%) were susceptible to colistin, and similar to Memar et al. (2016) study, colistin was represented as a therapieutic agent. In a study performed by Ranjbar et al. (2011) in Baqiyatallah hospital in Tehran, the frequency of resistance to antibiotics was shown as follow: ceftazidime, 57.5%; amikacin 90%, ciprofloxacin 65%, gentamicin 67.5%, and imipenem 97.5% (15). In another study by Rajaie et al. (2015), resistance to amikacin, tobramycin, ciprofloxacin, gentamicin, and ceftazidime was reported as 96, 88, 88, 92, and 96%, respectively (16). In a study carried out by Fazeliet al. (2013), the resistance rate to amikacin, gentamicin, and ciprofloxacin was reported as 50, 60, and 65%, respectively (17). In another study by Hosseiniet al. (2016), resistance to amikacin, tobramycin, ciprofloxacin, gentamicin, and ceftazidime was reported as 87, 83, 93, 83, and 85%, respectively. As it is shown, the results of these four mentioned studies are in accordance with our study results and show an increase in antibiotic resistance in *P.aeruginosa* isolates in Iran. Genotyping of isolates by ERIC-PCR produced 52 different patterns and three main clusters (A-C) by UPGMA method. About 83% of the isolates were included in Cluster A and related together. According to the data, it is necessary to prevent and control the pathogen transmission in burn hospital. In aforementioned cluster, 28 isolates had 100% similarity, forming 10 real clones; Cluster C had only one real clone, which was formed by two isolates. A total of 5 clones contained strains isolated from the same patients (more than 1 sample from a patient by an interval), and 6 clones had strains isolated from different patients. On the other hand, it was verified that the same patient could have been infected either by the isolates located in the same cluster (23A, B and 23C, D) or by the isolates classified as belonging to different clones (8A, 8B and 23A, 23D). As previously described by Sener et al. (2001), these findings suggest that some of the patients were colonized by either the same or different isolates along the time(18).

According to the data, it was supposed that ERIC-PCR was a discriminatory method for typing of *P.eruginosa* isolates, and a combination of genotyping and phenotyping methods is the best way to characterize *P. aeruginosa* isolates. Wolska et al. (2008) obtained similar results. In their study, two typing methods (Ribotyping and ERIC-PCR) were evaluated for discriminating 62 clinical strains of *P. aeruginosa.* They detected 9 and 36 genotypes for each method, respectively. They suggested that a combination of these methods would achieve more discriminatory power (19). Syrmis et al. (2004) used Rep-PCR and PFGE methods for genotyping of 163 *P. aeruginosa* strains isolated from cystic fibrosis (CF) patients, PFGE and rep-PCR were capable to identify 58 distinct clonal groups. They proposed that ERIC- PCR is suitable , inexpensive, fast, reproducible, and discriminatory genotyping method for efficient epidemiological surveillance of *P. aeruginosa* isolates of patients with CF(8). Khosravi et al. (2016) determined the genetic diversity of MDR *P. aeruginosa* strains isolated from burn and wound infections in Ahvaz, Iran, by ERIC-PCR. Unlike to our study, they obtained high level of genotypic heterogeneity in *P. aeruginosa* strains and demonstrated no genetic correlation between them (20).

6. Conclusion

The high resistance rate to antibiotic in our study may be due to abundant use of them as prophylactic or treatment regimen in burn patients. Therefore, suitable use of antibiotics seems necessary, and colistin is a suitable choice for treatment of wound infections. A majority of *P. aeruginosa* strains isolated from burn patients were related and belong to Cluster A, and the prevention and control of this pathogen transmission is necessary.

Conflict of interests

The authors declare they have no conflict of interest**.**

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Authors' Contribution

All of authors contribute to this study.

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