

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

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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 important agent of nosocomial and healthcare-associated infections and responsible for lung, urinary tract, surgical site infections and sepsis (1-2). This bacterium is described by inherent resistance to different antimicrobial agents; this type of resistance is always intermediated 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 rate, and acquisition of different resistance genes. Consequently, latter way is considered as a major participant to the extensive dissemination 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 proper techniqu for DNA typing characterized as high type ability, stability, and rapid reversal reproducibility with low complication and price, 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 intergenic regions of target repetitive non-coding sequences in the genome of bacteria are represented (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, were collected 78 isolates of *P. aeruginosa* 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 carried out 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 for 10 min, after centrifugation in 12000 rpm, the supernatant was subjected to PCR by 16srDNA and ERIC primers (Table 1). 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 a 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 catheter 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%).

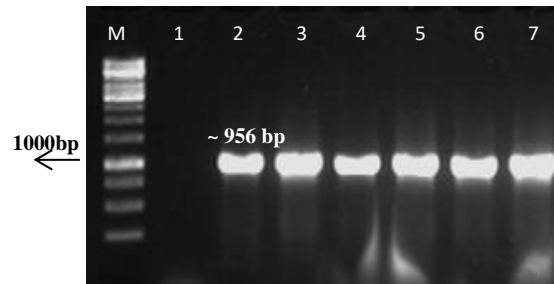


Figure 1. PCR amplification of 16srDNA F-R, Lanes 1, 2 representatives of negative and positive controls, Lanes 3-7 *P. aeruginosa* isolates, M: 100 bp DNA size marker.

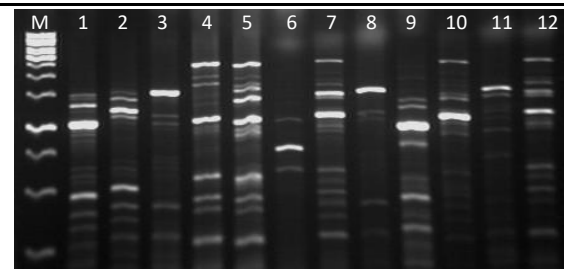


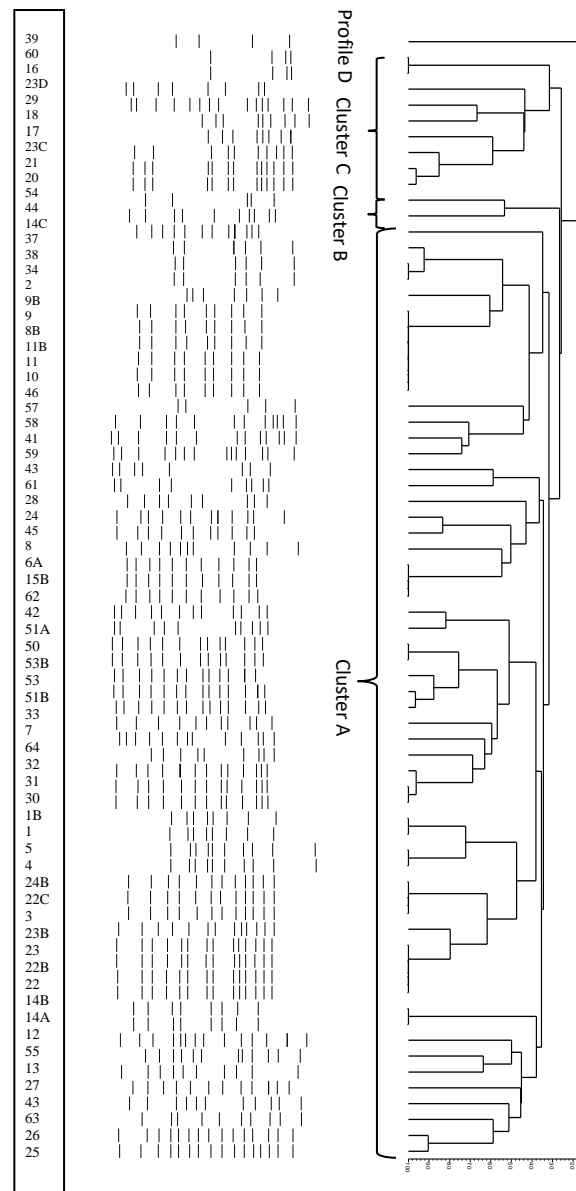
Figure 2. Lane 1-12: ERIC-PCR patterns of *P. aeruginosa* isolates; M: 1kb DNA size marker.

Table 1: Oligonucleotide primers used in this study.

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

Table 2: Antibiotyping patterns and antibiotic resistant and susceptibility rates in *P. aeruginosa* isolates.

Antibiotypes	Antibiogram pattern						Percent
	Caz	Cip	Ak	Gen	Ipm	Cst	
Antibiotype I	R	R	R	R	R	S	72.7%
Antibiotype II	S	R	R	R	R	S	24.6%
Antibiotype III	R	R	S	R	R	S	1.29%
Antibiotype IV	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 ERIC fingerprinting of 71 *P. aeruginosa* isolates from burn patients, Tehran. Column (at the right) displays 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 innovation of a large diversity of antimicrobial agents, MDR *P. aeruginosa* continues to be a significant agent 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 general antibiotic resistance genes and the high speed spread of environmental resistance genes have made it problematic 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 among the resistance of bacteria 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 therapeutic agent. In a study performed by Ranjbar et al. (2011) in Baqiyatallah hospital in Tehran, the frequency of antibiotic resistance was shown as follow: imipenem 97.5%, ceftazidime 57.5%, ciprofloxacin 65%, amikacin 90% and gentamicin 67.5 (15). In another study by Rajaie et al. (2015), resistance to ceftazidime, ciprofloxacin, amikacin, tobramycin and gentamicin was reported as 96, 88, 96, 88, and 92%, respectively (16). In a study performed by Fazeli et al. (2013), the resistance rate to ciprofloxacin, gentamicin, and amikacin, was reported as 65, 60, and 50%, respectively (17). In another study by Hosseini et al. (2016), resistance to ceftazidime, ciprofloxacin, amikacin, tobramycin, and gentamicin, was reported as 85, 93, 87, 83, and 83%, respectively. As it is shown, the results of these four above-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 possessed only one real clone that was made by two isolates. A total of 5 clones contained isolates collected from the same patients (more than 1 sample from a patient by an interval), and 6 clones possessed isolates collected from different patients. On the other hand, it was verified that the same patient could have been infected either by the different isolates that belonged to the same cluster (23A, B and 23C, D) or by the isolates located to different clones (8A, 8B and 23A, 23D). As formerly reported by Sener et al. (2001), these findings supposed that some of the patients were colonized and infected by either the similar or different isolates along the hospitalized duration(18).

According to the data, it was supposed that ERIC-PCR was a discriminatory method for typing of *P.aeruginosa* isolates, and a combination scheme consist of genotyping and phenotyping methods is the best system 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* isolates collected from cystic fibrosis (CF) patients, PFGE and rep-PCR were capable to identify 58 distinct clonal groups. They proposed that ERIC-PCR is appropriate, low-priced, reproducible, rapid, and discriminatory typing method for efficient epidemiological surveillance of *P.aeruginosa* isolates in CF patients (8). Khosravi et al. (2016) determined the genetic diversity of MDR *P.aeruginosa* isolates collected from wound infection of burn patients in Ahvaz, Iran, by ERIC-PCR. Unlike to our study, they found high level of

heterogeneity in genotypes of *P.aeruginosa* isolates and revealed no genetic relatedness 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* isolates collected 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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