



Research article

Molecular characterization of polymorphisms among *Pseudomonas aeruginosa* strains isolated from burn patients' woundsParastoo Parsa^a, Nour Amirmozafari^b, Bahareh Nowruzi^a, Mohammad Ali Bahar^{c,*}^a Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran^b Microbiology Department, School of Medicine, Iran University of Medical Sciences, Tehran, Iran^c Burn Research Center, Iran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Pseudomonas aeruginosa is one of the most common reasons for nosocomial infections. Given the high morbidity and mortality, as well as the cost of management, particularly in developing countries, burn injuries are considered important health concerns. Owing to the increased rate of resistance against antibiotics, this study aimed to isolate *Pseudomonas aeruginosa* strains from burn patient's wounds by analyzing antibiotic susceptibility and genetic profiling. In this regard, we explored the relationship between the nucleotide sequence and antibiotic susceptibility. In this cross-sectional study, 107 isolates of *P. aeruginosa* were collected from a major burn center in Tehran, Iran. The isolates were characterized with standard biochemical tests and examined by applying the Disk Diffusion method to find the patterns of sensitivity, and their genetic relationship was revealed by RAPD-PCR method. According to the antibiogram results, most of the isolates were resistant to 3 or more antibiotics tested and the most sensitivity was related to the Colistin antibiotic. RAPD-PCR method revealed a high polymorphism among *P. aeruginosa* isolates in Tehran. There was no significant association between the genotype groups and antibiotic susceptibility profiles. We evaluated the pattern of resistance to pathogenic organisms and identified multi-drug resistant organisms. Currently, Colistin antibiotic is the most suitable treatment option for burned patients. RAPD-PCR is a genotyping method with high efficiency for typing and categorizing different isolates of MDR-*P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is a life-threatening opportunistic bacterium found in hospitalized patients (Sorkh et al., 2017). In fact, *P. aeruginosa* causes nosocomial infections in immunosuppressed patients because of some diseases such as cystic fibrosis, neutropenia, especially burns (Diken Gur and Aksoz, 2016). This pathogen is the main reason for infections in burn patients owing to the destruction of skin as the first line of the innate immunity. In addition, it constitutes approximately 77% of mortality in burn patients during the past 25 years (Mahmudi et al., 2016). Tissue injury due to burn that is caused by thermal energy is one of the most painful events suffered by people worldwide. Numerous subjects experience burn injuries and thousands of which ultimately demise annually (Mehta et al., 2017). Although therapeutic approaches for burns have greatly developed, infections remain one of the major causes of death in these patients, particularly critically-ill burn patients. Compared with other hospitalized individuals, burn patients are

characterized by skin shortage, long hospital stays, and multiple invasive operations; therefore, they are more susceptible to infection (Dou et al., 2017). Multidrug-resistant (MDR) *P. aeruginosa* causes 4–60% of nosocomial infections, generating high mortality and morbidity in burn patients. Owing to its intrinsic resistance to a wide range of antibiotics, practically its capability of resisting to all effective antibiotics, *P. aeruginosa* is considered one of the major concerns for nosocomial infections in hospitals in recent years (Sorkh et al., 2017). The reasons for the emergence of MDR strains may be the use of antibiotics having high anti-pseudomonal activity and long-term antibiotic administration (Diken Gur and Aksoz, 2016).

Molecular methods, which are less influenced by environmental factors in comparison to phenotypic methods, play pivotal roles in probing the ways of pathogen transmission (Mahmudi et al., 2016). The diversity of *P. aeruginosa* strains has been broadly investigated by molecular typing methods, including ribotyping, repetitive-element-based Polymerase Chain Reaction (rep-PCR),

* Corresponding author.

E-mail address: bahar.ma@iums.ac.ir (M.A. Bahar).

Arbitrarily Primed-PCR (AP-PCR), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphic (RFLP), Random Amplified Polymorphic DNA (RAPD) assay, and Pulsed-Field Gel Electrophoresis (PFGE) (Wolska et al., 2012). Nowadays, PCR-based fingerprinting methods have gained attraction in epidemiological studies because of their rapid nature, power of discrimination, and successful typing (Diken Gur and Aksoz, 2016). The major benefit of RAPD-PCR is that it does not require DNA pre-sequencing. The wide range of useful primers makes the technique as a great diagnostic approach. Reproducible RAPD bands are detectable by a precise selection of primers and optimization of PCR circumstances for target species and replication to confirm that only reproducible bands are scored (Kumari and Thakur, 2014). Among these methods, RAPD-PCR method is useful in long-term studies as it can show the differences in the whole genome, rather than a specific region in the bacterial genome (Sorkh et al., 2017). Indeed, RAPD method has the advantage that it can be performed cost-effectively and will totally amplify a range of fragments of most DNA and show polymorphisms (Kumari and Thakur, 2014).

2. Materials and methods

2.1. Isolation of *P. aeruginosa*

In this cross-sectional study, 107 *P. aeruginosa* isolates were collected from burn patients hospitalized in Shahid Motahhari Hospital in Tehran, Iran from March 2017 to August 2018. The sampling procedure from wounds consisted of swabs taken from clinically deep areas of the burn wounds when clinical signs of wound infection were evident. The ethics committee of Iran University of Medical Sciences approved the study before collecting the samples. The samples were immediately transferred to the laboratory. The isolates were identified as *P. aeruginosa* by the application of culture and standard biochemical tests for identification of *P. aeruginosa* as follows: Gram-negative, non-lactose fermenting, oxidase-positive colonies, which oxidized glucose and maltose, grew on cetrimide agar at 42 °C, and pigment production in Mueller Hinton Agar (HiMedia, India). Then *P. aeruginosa* isolates were preserved at -80 °C in Trypticase Soy Broth medium (Merck, 1.05459, Germany) containing 18% glycerol until further processing.

2.2. Antibiotic susceptibility

Drug susceptibility was determined by the Kirby-Bauer disk diffusion method (Padtan Teb, Iran) on Mueller Hinton agar (HiMedia, India), in accordance with Clinical and Laboratory Standards Institute (CLSI, 2017) guidelines. A total of six antibiotics were selected for assessing the drug resistance of *P. aeruginosa* including, Amikacin (AN, 30 mg), Ceftazidime (CAZ, 30 mg), Ciprofloxacin (CP, 5 mg), Colistin (CO, 10 mg), Gentamicin (GM, 10 mg), Imipenem (IPM, 10 mg). *Pseudomonas aeruginosa* ATCC 27853 was used as the laboratory standard for these tests.

2.3. Isolation of *P. aeruginosa* genomic DNA

Genomic DNA was extracted from overnight cultures of *P. aeruginosa* by boiling method. Template DNA was prepared and a cell pellet obtained from 1.5 ml of overnight culture was resuspended in 500 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0). After centrifugation and boiling for 10 min the supernatant was used for PCR (Galli et al., 1992; Sabath, 1980). The DNA quality was evaluated by calculating the absorbance of the specimen at 260 nm using a spectrophotometer.

2.4. RAPD analysis

DNA amplification was performed on a thermocycler (Bio-Rad, USA) in a final volume of 15 ml containing Taq DNA Polymerase Master Mix (7.5 ml), double distilled water (4.5 ml), Template DNA (2ml), primer 272 (3'-AGCGGGCCAA-5') and primer 208 (3'-ACGGCCGACC-5') (1 ml)

[2,6], according to the following protocol: initial denaturation (94 °C for 5 min) followed by 35 cycles of denaturation (94 °C for 1min), annealing (39 °C for 1 min), extension (72 °C for 2 min), and a final cycle of extension at 72 °C for 10 min. The RAPD-PCR products were loaded on a 1.5% (w/v) agarose gel with 0.5 mg/ml of Green Viewer and were analyzed by gel electrophoresis and banding patterns were observed in Gel-Documentation system (Uvitec, UK). We used a one-kilobase DNA ladder (Fermentas, Canada) as a molecular size standard.

2.5. Statistical analysis

Statistical analyses were carried out with SPSS version 23.0 (SPSS Inc., Chicago, Illinois, USA). The normality of the data was evaluated by Kolmogorov–Smirnov test. In descriptive analysis, central indicators such as mean and percentage of frequency and coefficient of dispersion such as Standard Deviation (SD) were used. The one-sample Chi-square and Fisher's exact tests were used to assess significant intergroup variations. A value of P less than 0.05 was considered statistically significant. The RAPD fingerprints were analyzed both by an observer and also by computer with GelCompar II software.

3. Results

In the current study, 107 *P. aeruginosa* isolates were collected from the clinical wounds of the hospitalized burn patients, with different burn degrees from different wards of Motahhari Hospital. A total of 87.1% (n = 14) and 12.9% (n = 61) of *P. aeruginosa* strains were isolated from male and female patients with different burn percentages, respectively. The patients' ages ranged from 1 to 79 years, the majority (42.3%) of whom were between the ages of 20–40 years. The demographic and clinical characteristics of the burn patients (Table 1). In the following, 107 *P. aeruginosa* strains were typed phenotypically using antibiotic susceptibility test, and genotypically using the RAPD-PCR method. In this study, the strains were most frequently isolated from men's ward. According to the dendrogram obtained from GelCompar II software, the similarity percentage for primer 208 was considered to be 65% and accordingly, they were divided into 7 genotype groups (A-G) and for primer 272 (Figure 1), 60% was considered and accordingly, they were divided into 5 genotype groups (A-E) (Figure 2). No significant relationship was found between the genetic groups and the degree of burn and antibiotic resistance.

3.1. Antibiotic resistance

The findings of this study revealed that *P. aeruginosa* strains had the highest resistance to Gentamicin and Imipenem antibiotics (97.1%) followed by Ciprofloxacin (96.2%), Amikacin (95.3%), and Ceftazidime (66.3%), with the lowest resistance to Colistin (0.0 %) (Table 2). In other words, all isolates were sensitive to Colistin. There was no significant association between the genotype groups and antibiotic susceptibility profiles.

3.2. RAPD-PCR

The profiles were compared and clustered by the GelCompar II software. Analysis of genetic linkage showed 55%–100% similarity for primer 272 (Figure 1) and 50%–100% similarity for primer 208 (Figure 2) among *P. aeruginosa* isolates. The length sizes of RAPD fragments ranged from 100 bp to 3000 bp. Totally, 2 to 12 bands (primer 208) and 6 to 13 bands (primer 272) were detected on the gel electrophoresis of RAPD-PCR products. The RAPD-PCR results revealed a high polymorphism among *P. aeruginosa* isolates.

4. Discussion

In the current study, 107 isolates of *P. aeruginosa* were investigated using disk diffusion and RAPD-PCR methods for a better understanding

Table 1. The demographic and clinical characteristics of the burn patients.

Gender	Age	Cause of Burn	Burn Percentage	Burn Degree					
Male	87.1%	Up to 10 years	11.53%	Gas	28.20%	<10%	21.79%	Degree 1	-
		10–20 years	6.41%	Gasoline	23.07%	10–30%	19.23%		
Female	12.9%	20–40 years	42.30%	Electricity	16.66%	30–50%	34.61%	Degree 2	34.54%
		40–60 years	28.20%	Boiling water	5.12%	50–70%	16.66%		
		Over 60 years old	11.53%	Alcohol	3.84%	>70%	7.69%	Degree 3	65.45%
				Other	23.07%				

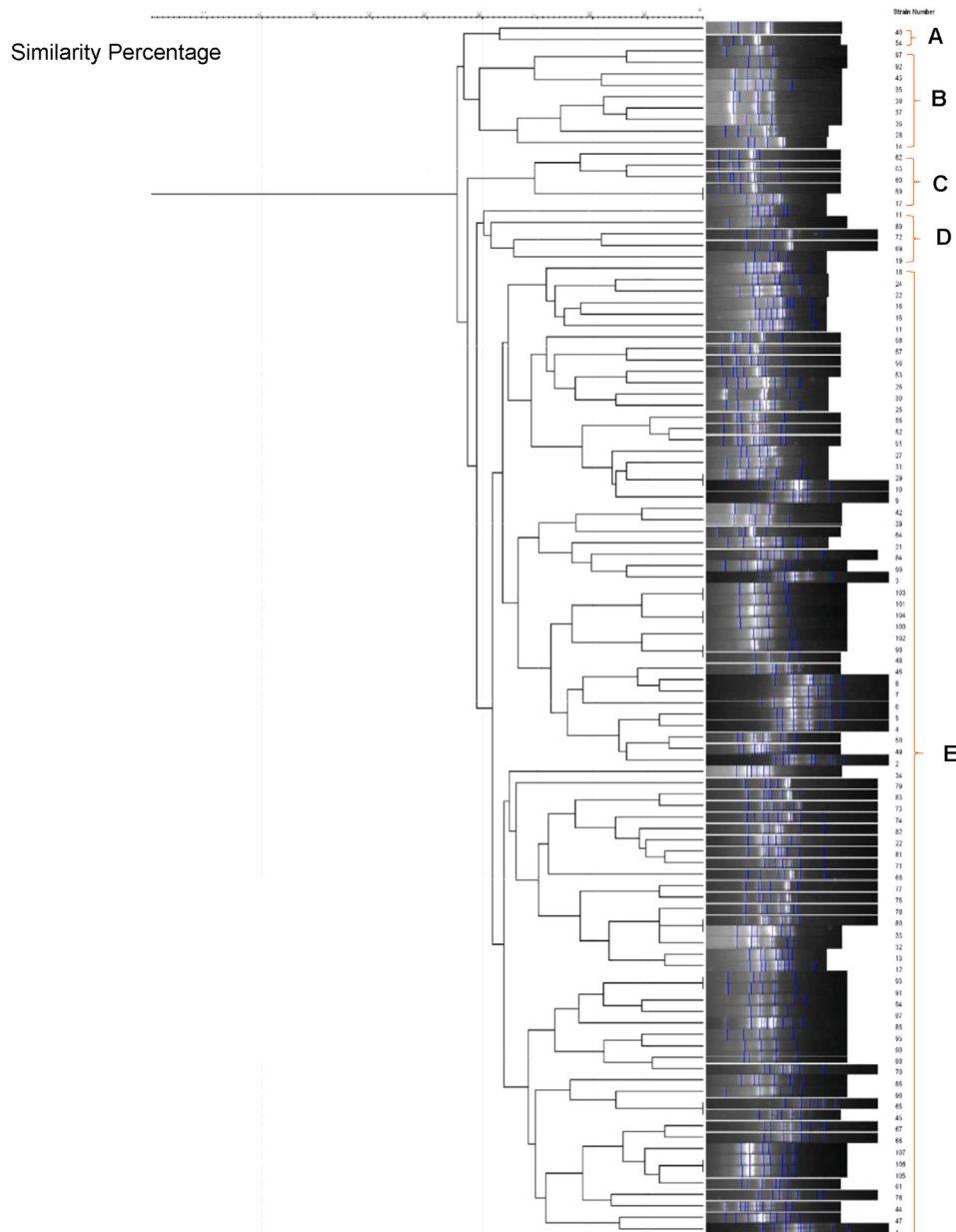


Figure 1. Dendrogram of *P. aeruginosa* isolates based on RAPD-PCR Analysis (Primer 272).

of antibiotic resistance rates and *P. aeruginosa* clone diversity. RAPD is a method that does not essentially evaluate the whole genome. Nowadays, there are controversial findings whether such typing methods can confirm the broad diversity of bacterial populations in the environment (Lanotte et al., 2004). Our findings showed the most resistance to Ciprofloxacin, Imipenem, and Amikacin antibiotics. Considering that

the Quinolones, Carbapenems, and Aminoglycosides are still the drug of choice for MDR *P. aeruginosa*, this high rate of resistance is very alarming.

The mortality rate as a result of infections caused by this bacterium is approximately 51%. Intrinsic and acquired resistance of this bacterium to antimicrobial factors increases the mortality rate of patients

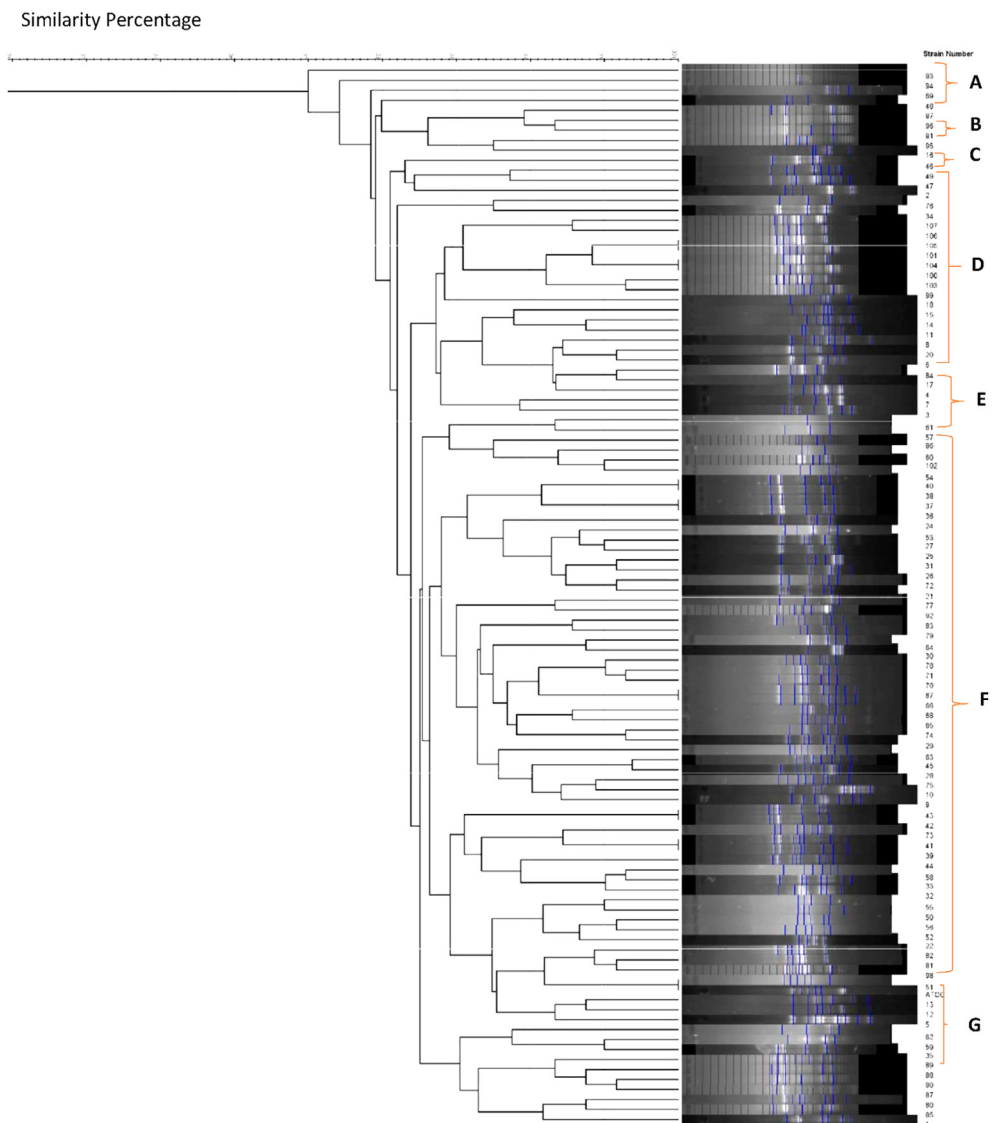


Figure 2. Dendrogram of *P. aeruginosa* isolates based on RAPD-PCR Analysis (Primer 208).

Table 2. The Comparison of frequency of resistance and sensitivity to antibiotics studied in total isolates.

Antibiotic	Sensitive		Intermediate		Resistant		Total	
	Total	%	Total	%	Total	%	Total	%
Amikacin (30) (AN)	5	4.67	0	0	102	95.3	107	100
Ceftazidime (30) (CAZ)	34	31.7	2	1.86	71	66.3	107	100
Ciprofloxacin (5) (CP)	3	2.80	1	0.93	103	96.2	107	100
Gentamycin (10) (GM)	3	2.80	0	0	104	97.1	107	100
Imipenem (10) (IMP)	3	2.80	0	0	104	97.1	107	100
Colistin (10) (CI)	107	100%	0	0	0	0	0	0

affected. Although among available antibiotics, Carbapenems (Meropenem, Ertapenem, Doripenem, Imipenem) are commonly used to treat *P. aeruginosa* infections, their widespread application has increased the resistance of this organism to these antibiotics. Nevertheless, some resistant strains to Carbapenem have been detected,

which are defective in the expression of OprD (Li et al., 2012; Rossolini and Mantengoli, 2005).

By removing and disrupting the skin barrier as the first-line defense of innate immunity, burn injuries generate a suitable environment for different infections; thus microorganisms inevitably colonize even if

topical antibacterial agents are administered (Vindenes and Bjerknes, 1995). The results of our research were similar to some reports from Iran and other countries (Fazeli and Momtaz, 2014; Vaez et al., 2015). For example, according to a study conducted in Tehran by Adabi et al., *P. aeruginosa* has been reported as the most prevalent bacterium found in the wounds of burn patients (Lari et al., 2000). In another study carried out by Zolfaghari et al. in Qom city on burn samples, *P. aeruginosa* was determined as the most common cause of nosocomial infection. The emergence of increased resistance in this organism to antimicrobial agents has raised concerns such as decreased therapeutic strategies and increased mortality (Zolfaghari, 2011).

In a study on the resistance pattern of 94 strains of *P. aeruginosa* isolated from wounds of burn patients showed that these strains had a high resistance to the antibiotics prescribed (Adabi et al., 2015). Numerous studies have been also conducted in this regard, e.g. Niitsuma et al. In Japan in 2001, reported *P. aeruginosa* strains isolated from sputum were resistant to Ceftazidime (4.6%), Imipenem (15.7%), and Meropenem (8.8%) (Niitsuma et al., 2001). There are many studies in this area in Iran. In the study of Bavasheh and Karmostaji in the South of Iran, the highest resistance rate which was observed to sulfamethoxazole and ceftazidime, were (94.44%) and (61.11%), respectively (35). In another similar study by Ranjbar et al. at Baqiyatallah Hospital in Tehran, the antibiotic resistance were as follows: ceftazidime (57.5%), Amikacin (90%), Ciprofloxacin (65%), Gentamycin (67.5%), and Imipenem (97.5%) (Ranjbar et al., 2011). In addition, in a study by Kianpour et al. in Isfahan, it was shown that resistance to antibiotics Ceftriaxone, Amikacin, Ceftazidime, Ciprofloxacin, Tazocin, and imipenem were 82.14%, 57.14%, 53.57%, 42.85%, 39.28%, and 14.28%, respectively (Kianpour et al., 2010). Contrary to our findings, SinemDiken Gur and Nilufer Aksoz in 2016 acclaimed Amikacin and Ceftazidime were the most effective antibiotics (Diken Gur and Aksoz, 2016). Sharma showed that all (100%) of the *P. aeruginosa* isolates were sensitive to Imipenem and Meropenem (Khosravi et al., 2016).

Reviewing previous studies indicates that the resistance to different antibiotics is relatively high in *P. aeruginosa* species, which is dependent on the time and location of the species. On the other hand, these resistance patterns are constantly changing, which should be taken into account. The results of this study showed that *P. aeruginosa* is highly resistant to beta-lactam antibiotics, including anti-pseudomonas penicillins, and this broad group of antibiotics is relatively ineffective on this organism. According to this study, the only antibiotic that has 100% effect on this bacterium is Colistin (polymyxin E), which is an antibiotic with partial neurotoxic and nephrotoxic properties and it is considered to be the last line of treatment in infections caused by multidrug-resistant organisms, including *P. aeruginosa*, *Acinetobacter baumannii* and carbapenamase-producing *Enterobacters* (Montero et al., 2009).

Another finding of the present study is the high percentage of MDR strains. The occurrence of MDR in *Pseudomonas* species is important and its upward trend has always been reported. For example, in a study by Mir Salehiian et al. on burn patients, multiple resistance was observed in 87% of cases (Mirsalehian et al., 2010). In another study conducted by Fazeli et al. in Isfahan, all isolates from a burn hospital were resistant to more than three antibiotics (Fazeli et al., 2012). A similar study was done by Mardaneh et al. that the highest antibiotic resistance of Imipenem was reported at 85% (Mardaneh et al., 2013), which the highest resistance to Imipenem and Gentamicin was reported at 97.0% in the present study.

Long-term use of antibiotics alone leads to the association of developing resistance and the appearance of cross-resistance among antibiotics, which results in the emergence of MDR *P. aeruginosa* strains, which can be transmitted from patient to patient and lead to nosocomial infections (Diken Gur and Aksoz, 2016). Active monitoring is mandatory to detect resistant strains of nosocomial infections, especially in burn patients. Therefore, quick and proper solutions should be considered to prevent the spread of pseudomonas species infection. Plasmid-mediated resistance is possible in many ways. This transmission will result in resistance to 3 or 5 antibiotics or even more. Multiple antibiotic

resistance to effective antibiotic classes such as beta-lactams, aminoglycosides, and quinolones is generally prevalent and is widely seen in Gram-negative bacteria (Jafari et al., 2013; Mardaneh et al., 2013; Morita et al., 2012).

Genetic techniques along with phenotypic tests can now identify strains isolated from different sites in different parts of the hospital, patient samples and finally, identify the source of the infection. In order to find this link between clinical and environmental strains, some techniques such as endonuclease cleavage analysis, phenotypic testing, pulse-field gel electrophoresis or as in the present study, RAPD-PCR can be used.

The results showed that the isolates obtained in the present study had a different banding pattern in the two types of primers used 208 and 272. This variation in the number of bands can be related to the primer sequence used, access to multiple primer binding sites in the genome, or the genetic pattern quality. It seems that some random primers may work better than other primers and may produce repeatable results (Tyler et al., 1997). It seems that primers with relatively high C + G content perform best for RAPD-PCR (Oladunmoye et al., 2009). The two types of primers used in this study were previously used in other studies to compare the results with PFGE methods. The presence of polymorphic banding pattern using RAPD-PCR technique indicates that there is a high rate of polymorphism in the *P. aeruginosa* genome. This predicts true compatibility in this bacterium with a genome of 5,570 reads (Stover et al., 2000).

In the present study, which was carried out on genotyping of 107 isolates of *P. aeruginosa*, in banding analysis created by RAPD-PCR method based on percentage and similarity bands, repeatability different patterns of *P. aeruginosa* from different patients with Gel Compare software were observed. Although molecular genotyping of *P. aeruginosa* has been performed in many studies, the molecular genotyping of *P. aeruginosa* species by RAPD-PCR method has been carried out by a few researchers. Nanvazadeh et al. performed molecular genotyping of *P. aeruginosa* species isolated from 71 burn wounds by RAPD-PCR method and identified the dominant genotype and showed that this method is suitable for assay of polymorphism among genes of *P. aeruginosa* (Nanvazadeh et al., 2013). In the study of Nanvazadeh et al., in 2013, 50 clinical samples were taken from Taleghani Hospital in Ahvaz and 50 samples were taken from *Pseudomonas aeruginosa* isolates in which RAPD-PCR was performed with only primer 272, even though in our study, in addition to primer 272, primer 208 was also used. In their study, genotypes related to type 1, 2, and 6 were determined in patients with severe burns that were sampled from their wounds and blood samples, but in our study there was no relationship between the genotype and burn degree or burn percentage, and so on. Also, their study did not examine the antibiotic resistance pattern, while we also tested the diffusion disc. The Hachem et al. study was performed on cancer patients who were infected with resistant *Pseudomonas aeruginosa* (MDR) and treated with Colistin and other drugs other than Colistin for three years, while our study was conducted on burn patients infected with resistant *Pseudomonas aeruginosa* (MDR). The Hachem et al. study used the MIC (E-Test Method) method, but we used antibiogram test. Mahmmudi et al., (2016) showed that the polymorphism of 200 hospital specimens in different areas of the hospital using RAPD-PCR varied based on their antibiotic resistance pattern (Mahmmudi et al., 2016). Salimi et al. in Tehran, identified 8 different genotypes with 6% polymorphism in *P. aeruginosa* species isolated from burn patients by RAPD-PCR. Previous studies have reported high levels of polymorphism in the genome of this organism (Salimi et al., 2010). Khosravi et al. also reported the high level of genotypic heterogeneity in *P. aeruginosa* strains isolated from Taleghani burn center in Ahvaz by ERIC-PCR analysis method (Khosravi et al., 2016). Several studies reported genetic diversity and heterogeneity among *P. aeruginosa* clinical isolates using ERICPCR, rep-PCR, PFGE, and RAPD-PCR methods in hospitals of Iran as well as other countries (Nanvazadeh et al., 2013; Selim et al., 2015).

The results of the RAPD-PCR method used in this study indicates that the isolated *P. aeruginosa* isolates have a high polymorphism, presumably due to their high rate of genetic variation. This high diversity may be one of the selective benefits for a strain to adapt to the environment and may be useful for propagating and domesticating wild strains. Similar to different studies, the results of this study support the hypothesis that genetic variation can be due to cross-establishment or contamination with the same source or acquisition of genes independent of environmental strains. Patient-to-patient transmission rate is somehow low, but not zero. Cross-contamination transmission confirmation is not accurate and possible since different factors are involved, so each banding pattern in *P. aeruginosa* can represent the dominant environmental strain in one place.

5. Conclusion

Antibiotic susceptibility/resistance is caused by a change in genotype or gene expression. Given the rapid growth of antibiotic resistance due to the overuse of these drugs, the evaluation of the resistance pattern of pathogenic organisms and in particular, strains associated with hospital-acquired infections in wounded patients burn and identify multidrug-resistant organisms seem necessary. The analysis of antibiotic resistance profiles of the strains show that Colistin is the most effective antibiotic, and the highest resistance rates were detected for Gentamicin and Imipenem. There was no association between the genotype groups that were determined using RAPD-PCR analysis, and the antibiotype patterns that were determined for the strains. According to the results of this study, RAPD-PCR is an effective method to determine the clonal relationship between MDR *P. aeruginosa* strains. Compared with different methods of infectious agent genotyping, this method is important. On the other hand, the greater the number of bands created in a technique, the greater the possibility of differentiation between strains. Therefore, RAPD-PCR is a suitable method for typing and classifying different isolates of *P. aeruginosa*, however, more patients, more isolates, and further investigation on its presumptive clinical and environmental sources are needed.

Declarations

Author contribution statement

Parastoo Parsa: Performed the experiments; Wrote the paper.
Nour Amirmozafari: Conceived and designed the experiments.
Bahareh Nowruzzi: Analyzed and interpreted the data.
Mohammad Ali Bahar: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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