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Prevalence and Characteristics of Metallo-beta-Lactamase-positive and High-risk Clone ST235 *Pseudomonas aeruginosa* at Ardabil Hospitals

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Abstract

Background: Carbapenems are the most commonly administered drugs for the treatment of multidrug-resistant *Pseudomonas aeruginosa* (MDR *P. aeruginosa*) infections. However, carbapenem-resistant *P. aeruginosa* is spreading rapidly and has led to a new threat to human health worldwide.

Objectives: The current study aimed to determine the prevalence of imipenem-resistant *P. aeruginosa*, detect metallo-β-lactamase (MBL)-producer isolates, and evaluate their clonal relationships in strains isolated from patients referring to the hospitals of Ardabil city, Iran.

Methods: The resistance rate to imipenem was evaluated using the disk diffusion method. Double-disk synergy test and PCR technique were used for phenotypic and genotypic screening of MBL-positive *P. aeruginosa*, respectively. Ultimately, enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and multilocus sequence typing (MLST) methods were used for assessing clonal relatedness among the isolates.

Results: The prevalence of imipenem-resistant *P. aeruginosa* strains was estimated at 57.1% (48 out of 84 isolates). In addition, 45 (93.7%) out of 48 imipenem-resistant *P. aeruginosa* isolates were phenotypically screened as MBL-positive, among which 16 (35.5%) and three (6.6%) isolates harbored *blaIMP* and *blaVIM-1* genes, respectively. However, *blaNDM*, *blaSIM-2*, *blaSPM*, and *blaGIM-1* genes were not detected in this study. MBL-producing *P. aeruginosa* strains were divided into 42 ERIC-PCR types. Based on the results of MLST, *P. aeruginosa* ST235 was the only identified sequence type.

Conclusions: Our results revealed a high and alarming prevalence of imipenem-resistant and *blaIMP*-positive *P. aeruginosa* ST235 at Ardabil hospitals. Continuous monitoring is essential to control the further spread of this highly virulent and drug-resistant clone.

Keywords: *Pseudomonas aeruginosa*, Imipenem, Drug Resistance, Metallo-β-Lactamase, Clonal Relation

1. Background

β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems, are good choices for the treatment of many infectious diseases caused by clinically important *Pseudomonas aeruginosa*. This Gram-negative opportunistic pathogen is frequently found in hospital environments, particularly intensive care units, and patients that use devices such as ventilators and blood catheters (1-3). Today, the emergence of highly resistant *P. aeruginosa*, especially multidrug resistant (MDR) strains, which are associated with 13% of all hospital-acquired infections, is a global public health issue (4). The Infectious Diseases Society of America (IDSA) (2016) recommends dual empiric therapy for the treatment of MDR *P. aeruginosa* strains (4). However, there is also a notion that necessitates antibiotic selection based on the data of local resistance patterns (4).

Carbapenems and third-generation cephalosporins are the best available drugs against MDR bacteria (3). However, the production of β-lactamase enzymes, especially class B (zinc-dependent), in *P. aeruginosa* isolates can cause serious challenges for treating MDR *P. aeruginosa* infections (1, 3). In 2017, the World Health Organiza-

tion (WHO) reported that carbapenem-resistant *P. aeruginosa* isolates have posed a new threat to human health (3). The total prevalence of *P. aeruginosa* strains resistant to imipenem and meropenem (two carbapenems) in Iran is 31.6% and 40%, respectively (5). However, there is no report on the rate of carbapenem-resistant *P. aeruginosa* strains and their resistance mechanisms in Ardabil, a capital city in the northwest of Iran with 578,000 inhabitants (2021). Hence, the continuous monitoring of carbapenem-resistant *P. aeruginosa* strains and resistance mechanisms in local strains is a necessity and urgent task. An equally important task is to find new ways of infection control and new antibiotics development (3).

Among different antibiotic resistance mechanisms in *P. aeruginosa*, e.g., efflux pumps, loss of porins, and production of β-lactamase enzymes (6), none has yet been investigated in clinical isolates of *P. aeruginosa* in Ardabil. Therefore, the current study aimed to perform phenotypic and genotypic screening for metallo-β-lactamase (MBL)-producing *P. aeruginosa* isolates. According to the Ambler classification scheme, four classes of β-lactamases are identified, including serine β-lactamases A, C, and D, and MBL (class B) (7). Serine β-lactamases A and D and MBL are able to hydrolyze carbapenems (carbapenemase) (8). The MBL-producing *P. aeruginosa* strains are associated with high morbidity and mortality rates because these organisms are resistant to all β-lactam antibiotics, except for monobactams (9). The most common MBL enzymes are Imipenemase (IMP), Verona Integrin-encoded MBL (VIM), New Delhi MBL (NDM), German Imipenemase (GIM), and Seoul Imipenemase (SIM). The MBL-coding genes are located on the plasmid and bacterial chromosomes and can disseminate among bacteria through highly mobile genetic elements (9). To impede the spread of imipenem-resistant isolates of *P. aeruginosa*, the investigation of the clonal relatedness of MBL-producing *P. aeruginosa* strains is of paramount importance.

2. Objectives

Given the importance of MBL-producing (imipenem-resistant) strains, we aimed to determine the prevalence of imipenem resistance and evaluate the genetic relatedness among MBL-positive *P. aeruginosa* strains using enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and multilocus sequence typing (MLST) methods.

3. Methods

3.1. Pseudomonas aeruginosa Isolates

A total of 84 clinical isolates of *P. aeruginosa* were used in the present study. Bacterial isolates were collected from different specimens of patients who were referred to Ardabil hospitals from June 2019 to February 2021. The collected isolates were confirmed by standard laboratory tests, including pigment production, Gram-staining, catalase, and oxidase, as well as species-specific PCR (10). Confirmed bacteria were tested to identify imipenem resistance and MBL-positive isolates using phenotypic and genotypic methods.

3.2. Phenotypic Screening for MBL Enzymes

The Kirby-Bauer's disk diffusion method was used to evaluate *P. aeruginosa* resistance to the imipenem antibiotic (10 μg, Padtan Teb, Iran). Imipenem-resistant isolates were tested by double-disk synergy test, including imipenem plus imipenem-ethylenediaminetetraacetic (IPM-EDTA) (10 μg/750 μg) (Cypress Diagnostics, Belgium) disks, for phenotypic screening of MBL-producing *P. aeruginosa* isolates. Briefly, a 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL) of *P. aeruginosa* was prepared and spread on Mueller-Hinton Agar (Conda, Pronasida, Spain) as a lawn culture. Imipenem and IPM-EDTA disks were placed at a 20 mm distance from each other. A growth inhibition zone of $\geq$ 7 mm around the IPM-EDTA disk was considered as MBL-producing *P. aeruginosa*. Interpretation of disk diffusion results was done according to the Clinical and Laboratory Standards Institute (CLSI) guidelines 2018 (11). For the quality control of antimicrobial susceptibility testing disks, *P. aeruginosa* (ATCC 27853) standard strain was used.

3.3. Genotypic Screening for MBL Enzymes

Total genomic DNA of imipenem-resistant and MBL-producer *P. aeruginosa* isolates was extracted using the boiling method. The PCR details, including specific primer sequences and specific annealing temperatures are presented in Table 1, and used for the detection of MBL genes, including *blaIMP*, *blaVIM-1*, *blaNDM*, *blaSIM-2*, *blaSPM*, and *blaGIM-1* genes. The PCR was carried out in a 25 μL volume. Each PCR mixture contained 20 μL of PCR master mix (Ampliqon, Denmark), 2 μL of primers (100 pmol, 1 μL), and 3 μL of genomic DNA. Amplified products were visualized through 1% agarose gel electrophoresis and confirmed by sequencing.

Table 1. Primer Sequences Used for Amplifying Metallo-β-lactamase Genes in the Present Study

Gene	Primer Sequence (5′ to 3′)	Annealing Temperature	Amplicon Size (Bp)	Reference
blaIMP	F: GAAGGCGTTTATGTTCATAC	58°C	587	(12)
	R: GTACGTTTCAAGAGTGATGC			
blaVIM-1	F: AGTGGTGAGTATCCGACAG	52°C	261	(13)
	R: ATGAAAGTGCGTGGAGAC			
blaNDM	F:GCAGCTTGTCGGCCATGCGGGC	59°C	782	(12)
	R:GGTCGCGAAGCTGAGCACCGCAT			
blaSIM-2	F: TACAAGGGATTCGGCATCG	52°C	570	(14)
	R: TAATGGCCTGTTCCCATGTG			
blaSPM	F: GCGTTTTGTTTGCTC	52°C	786	(15)
	R:TTGGGGATGTGAGACTAC			
blaGIM-1	F: TCGACACACCTTGGTCTGAA	52°C	477	(16)
	R: AACTTCCAACTTTGCCATGC			
ERIC-R	R: ATGTAAGCTCCTGGGGATTCAC	48°C	-	(17)

3.4. Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction

Clonal relatedness between MBL-producing *P. aeruginosa* strains was assessed using the ERIC-PCR method. The ERIC-PCR profile of each MBL-producing *P. aeruginosa* isolate was determined through the oligonucleotide primers and specific annealing temperature presented in Table 1. Each PCR mixture contained master mix (20 μL), template DNA (1 μL), and ERIC1-R primer (1.5 μL) (total volume of 22.5 μL). The ERIC-PCR patterns were visualized using 2% agarose gel electrophoresis. All analyses were done by an online data analysis service (inslico.ehu.es), followed by the depiction of a phylogenetic tree.

3.5. Multilocus Sequence Typing

Molecular typing of *blaIMP*-positive *P. aeruginosa* isolates was done using the MLST technique, a DNA sequence-based typing method using seven housekeeping genes. The seven housekeeping genes were amplified by PCR method, and PCR products were analyzed using 1% agarose gel electrophoresis. Amplified products were sent for sequencing, and each *P. aeruginosa* isolate received a sequence type number via an online data analysis service (pubmlst.org). The primer sequences and specific annealing temperatures are indicated in Table 2.

3.6. Statistical Analysis

The GraphPad InStat statistical software (version 3) was used for data analysis. The Student's *t*-test and chi-square (χ^2) were used to interpret the data. A p value of < 0.05 was considered statistically significant.

4. Results

Eighty-four *P. aeruginosa* strains were collected from five hospitals (Alavi, Bu-Ali, Imam Reza, Imam Khomeini, and Sabalan) in Ardabil city. Based on the disk diffusion results, 48 (57.1%) out of 84 isolates were imipenem-resistant *P. aeruginosa*. Imipenem-resistant isolates were collected from 22 (45.9%) male (mean age 58 $\pm$ 22.6 years) and 26 (54.1%) female (mean age 55.1 $\pm$ 17.2 years) patients (P = 0.61). In addition, the distribution of imipenem-resistant *P. aeruginosa* strains according to the specimen type, and hospital ward were as follows: Urine 27 (56.2%), sputum 12 (25%), wound 6 (12.5%), blood 3 (6.25%), Intensive Care Unit (ICU) 13 (27%), neurology ward 13 (27%), emergency ward 7 (14.6%), internal ward 12 (25%), and pediatrics ward 3 (6.25%). Based on the double-disk synergy test, 45 (93.7%) out of 48 imipenem-resistant *P. aeruginosa* isolates were phenotypically screened as MBL-producing strains (Figure 1A). Additionally, among MBL-producing strains, 16 (35.5%) and three (6.6%) isolates harbored *blaIMP* and *blaVIM-1* genes, respectively (Figure 1B). Other MBL encoding genes, i.e., *blaNDM*, *blaSIM-2*, *blaSPM*, and *blaGIM-1* genes, were not detected in this study. The ERIC sequences of 45 MBL-producing *P. aeruginosa* isolates were amplified using ERIC 1R primer (Table 1) and isolates with similarities higher than 80% were considered clonally related strains. As

Table 2. Primer Sequences Used for Multilocus Sequence Typing of *blaIMP*-producing *Pseudomonas aeruginosa* Strains

Protein	Gene	Primer Sequence (5′ to 3′)	Annealing Temperature	Amplicon Size (Bp)	Reference
Shikimate dehydrogenase	*aroE*	F: ACGATTTCCCCGGGTTC	57.4°C	642	pubmlst.org
		R: CGCGCCAGAGGAAGAAT			
Acetyl coenzyme A synthetase	*acsA*	F: CTGGTGTACGCCTCGCTGAC	67°C	836	pubmlst.org
		R: TAGATGCCCTGCCCCTTGAT			
GMP synthase	*guaA*	F: CGGCCTCGACGTGTGGATGA	71°C	940	pubmlst.org
		R: GAACGCCTGGCTGGTCTTGTGGTA			
DNA mismatch repair protein	*mutL*	F: AGCCTGGCAGGTGGAAAC	66°C	619	pubmlst.org
		R: CAGGGTGCCATAGAGGAAGTC			
NADH dehydrogenase I chain C, D	*nuoD*	F: ACCGCCACCCGTATCTG	63°C	1042	pubmlst.org
		R: TCTCGCCCATCTTGACCA			
Phosphoenolpyruvate synthase	*ppsA*	F: GGGTAGCAAGGCGATCAAGATG	66.4°C	1034	pubmlst.org
		R: GGTTCTCTTCTTCCGGCTCGTAG			
Anthralite synthetase component I	*trpE*	F: GCCGATCCCTCCGAGGAAAATG	63°C	919	pubmlst.org
		R: CCCGGCGCTTGTTGATGGTT			

shown in Figure 2, 42 different ERIC-PCR types were identified in MBL-producing *P. aeruginosa* strains. Based on MLST, seven housekeeping genes were detected using PCR (Figure 3), and ST235 was the only identified clone (Table 3). As shown in Table 3, the highest prevalence of *P. aeruginosa* ST235 isolation was from urine specimens (50%), Alavi hospital (68.7%), and ICU ward (37.5%).

5. Discussion

Antibiotic resistance is growing rapidly worldwide. Therefore, antimicrobial resistance surveillance programs are required, especially in developing countries such as Iran, to monitor and predict the trend of drug resistance and assess the overuse and misuse of antimicrobial agents and associated consequences (18). The emergence of imipenem-resistant *P. aeruginosa* strains is a concerning issue because these strains show simultaneous resistance to multiple antibiotics (19). In the present study, the prevalence of imipenem-resistant *P. aeruginosa* strains was 57.1%, which is higher than reports from Tabriz (49%), Ahvaz (42.9%), Urmia (30.8%), Zanjan (29.2%), Guilan (23.3%), Zahedan (17.2%), and Hamadan (7.5%) and lower than in Tehran (70.4%) (5). Carbapenem-resistant *P. aeruginosa* prevalence in other countries has been reported as follows: Pakistan 49.5%, Philippines 31.1%, Thailand 28.7%, Japan 28.5%, Singapore 23.3%, Korea 22%, and Romania and Greece > 45%, and the Netherlands, Germany, Austria, Belgium, Denmark, Finland, French, Iceland, Luxemburg, and Malta < 20% (19).

Patients contaminated with imipenem-resistant *P. aeruginosa* strains experience worse treatment outcomes than those infected with imipenem-susceptible strains, causing long-term hospitalization, high costs, and substantial mortality (19). Although *P. aeruginosa* resistance to carbapenems is multifactorial, the production of MBL enzymes is one of the most common mechanisms (19, 20). According to the results of a meta-analysis, the total prevalence of MBL-producing *P. aeruginosa* in Iran was 32.4% (21). In addition, the most prevalent genes encoding MBL enzymes in Iran were *blaVIM* (19%) and *blaIMP* (11%) (21). In the current study, 53.5% (45 out of 84) of *P. aeruginosa* isolates were phenotypically screened as MBL-producing strains, which were divided into 42 different ERIC-PCR types based on the ERIC-PCR technique (Figure 2). On the other hand, our findings revealed that the prevalence of IMP-producing strains among imipenem-resistant *P. aeruginosa* clinical isolates was 35.5% in Ardabil city.

This result is higher than data from Ahvaz (0.5%, 11.7%, and 28.4%) (22-24), Zanjan (23.3% and 14.3%) (25, 26), Isfahan (31.3%) (27), Markazi (2.8%) (28), Zahedan (0.3%) (29), Kermanshah (15.1%) (30), Shiraz (3.3%) (31), and Tehran (13 %) (32). Additionally, among imipenem-resistant isolates, the rates of VIM-producing *P. aeruginosa* strains were 6.6% in our study, which is lower than that reported from Mashhad (11.4% and 50%), Zanjan (32%), Markazi (38%), studies from

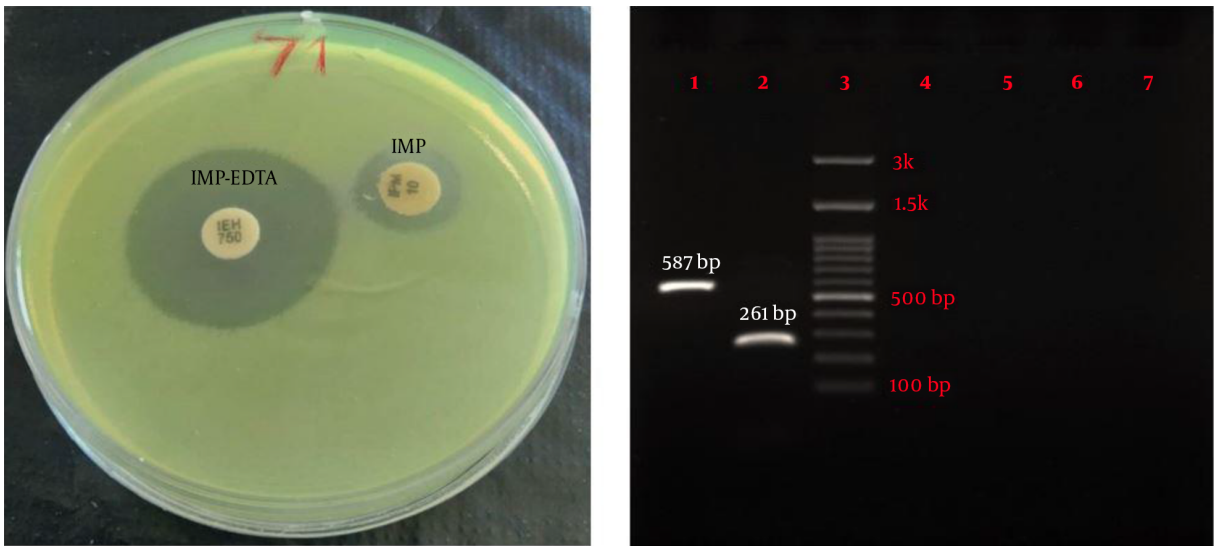

Figure 1. Phenotypic and genotypic screening for MBL-producer *Pseudomonas aeruginosa*. (A) Double disk synergy test for phenotypic detection of MBL enzymes and (B) Agarose gel electrophoresis of amplified products of MBL genes. lane 1: *IMP* (587 bp), lane 2: *VIM* (261 bp), lane 3: Ladder (100 bp), lane 4: *NDM* (782 bp), lane 5: *SIM-2* (570 bp), lane 6: *SPM* (786 bp), and lane 7: *GIM* (477 bp).

Table 3. Results of Multilocus Sequence Typing Technique for *blaIMP*-producing *Pseudomonas aeruginosa* Strains

Isolate	Specimen	Hospital	Ward	Seven Housekeeping Genes							Sequence Type (ST)
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*	
18	Urine	Alavi	ICU	38	11	3	13	1	2	4	235
23	Wound	Imam Khomeini	Internal	38	11	3	13	1	2	4	235
24	Urine	Alavi	ICU	38	11	3	13	1	2	4	235
25	Urine	Alavi	ICU	38	11	3	13	1	2	4	235
33	Sputum	Alavi	ICU	38	11	3	13	1	2	4	235
34	Sputum	Alavi	ICU	38	11	3	13	1	2	4	235
37	Urine	Bu-Ali	Pediatrics	38	11	3	13	1	2	4	235
39	Urine	Imam Khomeini	Internal	38	11	3	13	1	2	4	235
43	Urine	Alavi	ICU	38	11	3	13	1	2	4	235
50	Wound	Imam Khomeini	Emergency	38	11	3	13	1	2	4	235
55	Urine	Imam Reza	Internal	38	11	3	13	1	2	4	235
57	Sputum	Alavi	Neurology	38	11	3	13	1	2	4	235
58	Sputum	Alavi	Neurology	38	11	3	13	1	2	4	235
62	Sputum	Alavi	Neurology	38	11	3	13	1	2	4	235
65	Sputum	Alavi	Neurology	38	11	3	13	1	2	4	235
66	Urine	Alavi	Neurology	38	11	3	13	1	2	4	235

Ahvaz (8%), Kermanshah (8.3%), Tehran (33% and 13%) and Isfahan (43%, 21% and 14.6%) and higher than in Zahedan (3.7%), Shiraz (4.2%), studies from Ahvaz (0.4% and 0.8%), Kermanshah (0.9%), Tehran (0%, 0.1%, 0.5%, and 2%), and Isfahan (0.5%) (21). The *blaIMP* and *blaVIM* types are the most prevalent MBLs in the world (33). Imipenem-resistant *P. aeruginosa* did not contain *blaNDM*, *blaSIM-2*, *blaSPM*, and *blaGIM-1* genes. Similar results in line with our study were reported from other cities of Iran such as Tehran, Ahvaz, Isfahan, Markazi, Zahedan, and Shiraz (21).

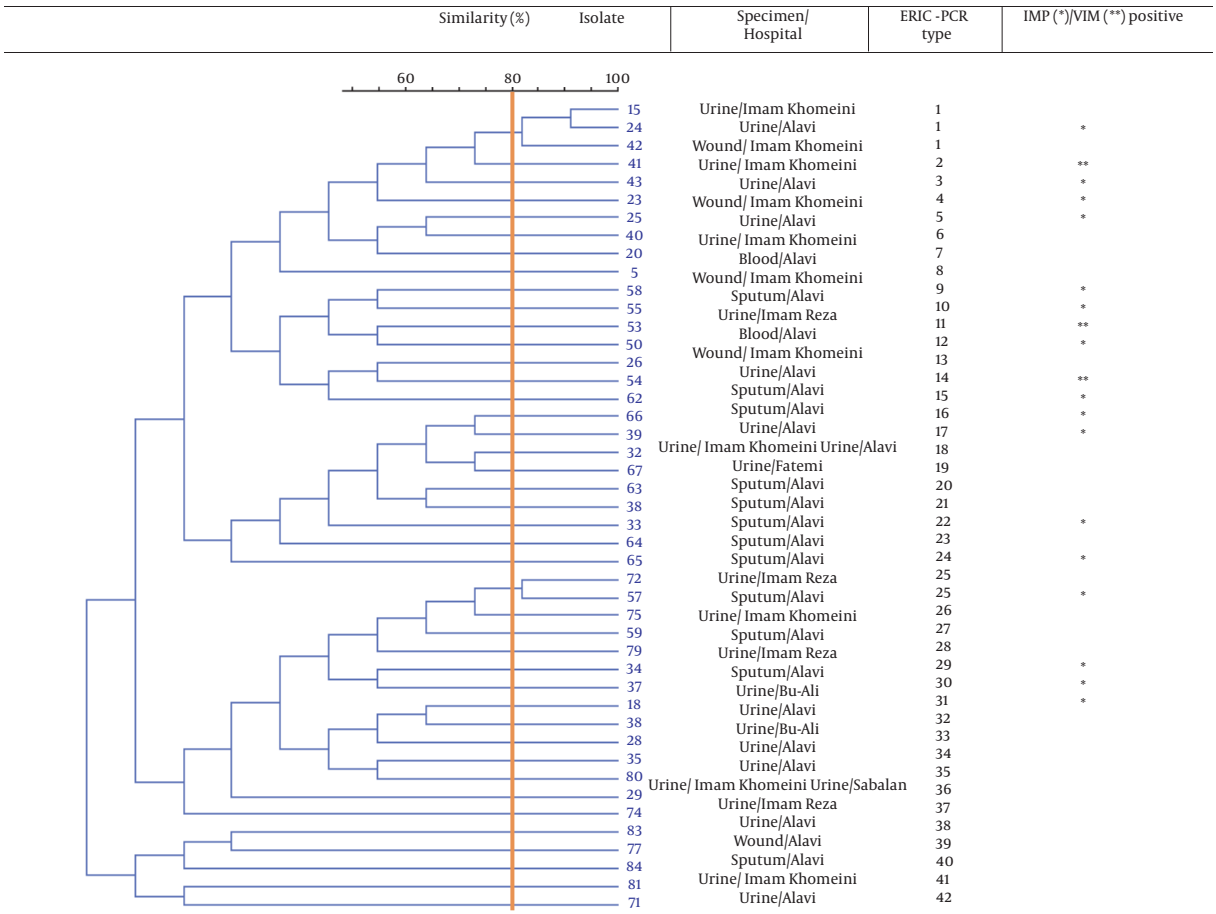

Figure 2. Dendrogram of ERIC-PCR patterns showing clonal relationships between MBL-producing *Pseudomonas aeruginosa* strains. Isolates with similarities >80% were considered clonally related strains.

As known, MLST is a powerful molecular typing method for epidemiological studies of *P. aeruginosa* infections. According to MLST typing, *P. aeruginosa* sequence types (ST), ST111, ST175, ST235, ST244, and ST395, have a global distribution and are associated with outbreaks (34, 35). Among these sequence types, *P. aeruginosa* ST235 is the most prevalent one and is associated with highly virulent infections and multidrug resistance to carbapenems, aminoglycosides, and fluoroquinolones (34-36). Molecular typing of *P. aeruginosa* strains in the current study indicated that all IMP-producing isolates belonged to ST235 (Table 3). The isolation of *P. aeruginosa* ST235 in different hospitals in Ardabil city warns that this high-risk clone is disseminated in the investigated hospitals.

5.1. Conclusions

The high prevalence of imipenem-resistant *P. aeruginosa* strains in our region calls for combination therapy using synergistic antibiotics to achieve proper treatment of *P. aeruginosa* infections. On the other hand, considering that all IMP-producing isolates belonged to *P. aeruginosa* ST235, the emerging and spreading of this predominant clone in the investigated hospitals is an important challenge owing to the highly virulent and drug-resistant nature of this clone. Therefore, the continuous monitoring of drug resistance trends, resistance mechanisms, and virulence genes in local strains is essential.

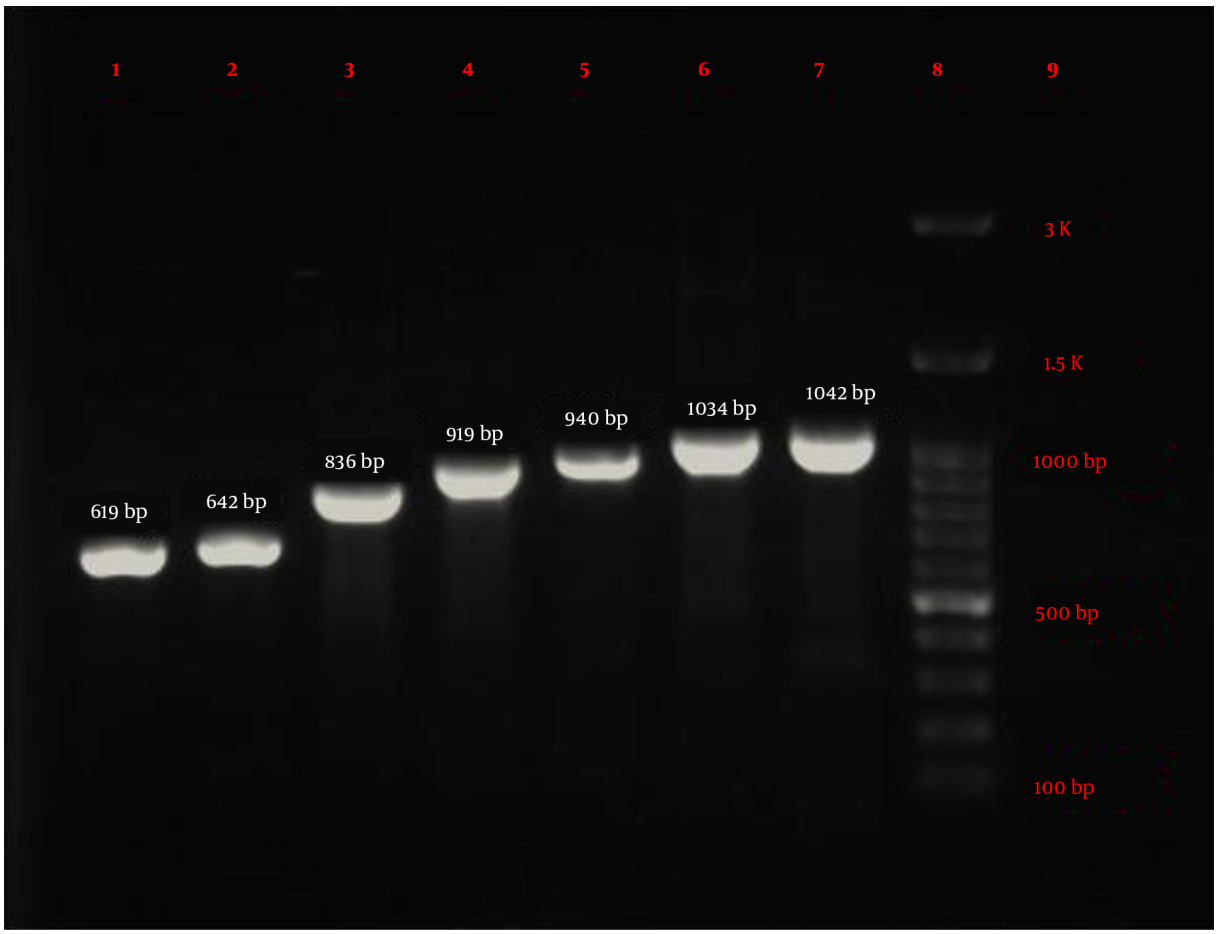

Figure 3. Electrophoresis results of seven housekeeping genes amplified by PCR in MLST technique. lane 1: *mutL* (619 bp), lane 2: *aroE* (642 bp), lane 3: *acsA* (836 bp), lane 4: *trpE* (919 bp), lane 5: *guaA* (940 bp), lane 6: *ppsA* (1034 bp), lane 7: *nuoD* (1042 bp), lane 8: Ladder (100 bp), and lane 9: negative control.

Footnotes

Authors' Contribution: Study concept and design: Farzad Khademi, Mohsen Arzanlou, and Jafar Mohammadshahi; Acquisition of data: Somayeh Safarirad and Farzad Khademi; Analysis and interpretation of data: Farzad Khademi and Hamid Vaez; Drafting of the manuscript: Farzad Khademi; Revision of the manuscript: Hamid Vaez and Amirhossein Sahebkar.

Conflict of Interests: The authors declare that there is no conflict of interest.

Ethical Approval: This research was approved by the Research Ethics Committee of Ardabil University of Medical Sciences (approval ID: IR.ARUMS.REC.1398.591).

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