





Distribution of Class A Extended-Spectrum β -Lactamases Among *Pseudomonas aeruginosa* Clinical Strains Isolated from Ardabil Hospitals

Fereshteh Hasanpour¹, Nima Ataei¹, Amirhossein Sahebkar ^{2,3,4,5} and Farzad Khademi ^{1,6,*}

¹Department of Microbiology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran

²Biotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran

³Applied Biomedical Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

⁴School of Medicine, The University of Western Australia, Perth, Australia

⁵Department of Biotechnology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

⁶Arthropod-Borne Diseases Research Center, Ardabil University of Medical Sciences, Ardabil, Iran

*Corresponding author: Department of Microbiology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran. Email: k_farzad@yahoo.com

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Abstract

Background: Currently, the emergence of extended-spectrum β -lactamase (ESBL)-producing bacteria is becoming a major threat to patients in the hospital and community. Such enzymes have been recently detected in *Pseudomonas aeruginosa*, but there is no epidemiological data on the prevalence of ESBL-producing clinical isolates in the hospitals of Ardabil City (Iran).

Objectives: This study aimed to determine the phenotypic and genotypic prevalence of class A ESBL-producing *P. aeruginosa* strains in Ardabil City.

Methods: A total of 120 clinical isolates of *P. aeruginosa* collected from Ardabil hospitals were used in this study. Phenotypic detection of class A ESBL-producing *P. aeruginosa* isolates was performed using a double-disk synergy test. In addition, the detection of class A ESBL-encoding genes, including *Pseudomonas* extended resistant (*PER*), Vietnamese extended-spectrum β -lactamase (*VEB*), temoniera (*TEM*), sulfhydryl variable (*SHV*), cefotaximase (*CTX-M*), guyana extended-spectrum β -lactamase (*GES*), and *Pseudomonas*-specific enzyme (*PSE*), was performed using the polymerase chain reaction (PCR).

Results: The prevalence of class A ESBL-producing *P. aeruginosa* strains was 8.3% (10 out of 120) based on the double-disk synergy test. However, 40% (48 out of 120) of these isolates were found to carry genes encoding class A ESBLs based on PCR. Among 48 class A ESBL-positive strains, the prevalence of *PSE*, *TEM*, *VEB*, *CTX-M*, and *PER* genes were 64.6% (31/48), 25% (12/48), 4.2% (2/48), 4.2% (2/48), and 2% (1/48), respectively. However, the frequency of other class A ESBL genes (*SHV* and *GES* genes) was 0%.

Conclusions: Our results confirmed the presence of class A ESBL-producing *P. aeruginosa* strains in the hospital environment of Ardabil. On the other hand, the use of molecular tests can be a more precise and reliable method than phenotypic ones to identify these resistant strains and prevent the emergence of antibiotic resistance and ensuing treatment failure.

Keywords: *Pseudomonas aeruginosa*, Antibiotic Resistance, ESBL

1. Background

Pseudomonas aeruginosa (a non-fermentative gram-negative bacillus) is an important opportunistic pathogen that causes severe infections in hospitalized, immunocompromised, and cystic fibrosis patients (1, 2). This organism is responsible for 8% of all nosocomial infections, especially in intensive care units (ICUs) (3). Of note, the emergence of antibiotic resistance has limited the treatment of *P. aeruginosa* infections (4). One of the most common antibiotics used for the treatment of *P. aeruginosa* infections is β -lactams, especially carbapenems (5). Nevertheless, the US Centers for Disease Control and Preven-

tion (CDC) and WHO stated that carbapenem-resistant and multidrug-resistant (MDR) *P. aeruginosa* strains are becoming public health problems worldwide (6, 7). The prevalence of imipenem-resistant and MDR *P. aeruginosa* strains in Iran is 54% and 58%, respectively (8, 9).

Four mechanisms of resistance to β -lactam antibiotics have been identified in gram-negative bacteria, including (1) low affinity of penicillin-binding proteins (PBPs) to β -lactam antibiotics; (2) modification of the outer membrane porins; (3) drug efflux pump systems (Mex); and (4) production of β -lactamases (10). β -Lactamases, which were identified for the first time in *Escherichia coli*, are bac-

terial enzymes hydrolyzing β -lactam antibiotics and are considered the most common factor accounting for the resistance of gram-negative bacteria to β -lactams (10). β -Lactamase enzymes are divided into four classes according to the Ambler classification scheme (β -lactamases A to D) (5).

Class A β -lactamases hydrolyze penicillins and cephalosporins, class B β -lactamases hydrolyze carbapenems, class C β -lactamases hydrolyze cephalosporins and cephamycins, and class D β -lactamases hydrolyze penicillins, cephalosporins, and carbapenems (10). The frequency of different classes of β -lactamases has been assessed in *P. aeruginosa* strains isolated from clinical specimens in Ardabil, except for class A β -lactamases. Among class A β -lactamases, extended-spectrum β -lactamases (ESBLs) are clinically most important resistance enzymes, which can hydrolyze penicillins and the first-, second-, third-, and fourth-generations of cephalosporins and monobactam (11). ESBLs are inhibited by β -lactamase inhibitors and do not affect cephamycins or carbapenems (11).

2. Objectives

This study aimed to determine the frequency of genes encoding class A ESBLs, including *Pseudomonas* extended resistant (*PER*), Vietnamese extended-spectrum β -lactamase (*VEB*), temoniera (*TEM*), sulfhydryl variable (*SHV*), cefotaximase (*CTX-M*), guyana extended-spectrum β -lactamase (*GES*), and *Pseudomonas*-specific enzyme (*PSE*), among *P. aeruginosa* clinical isolates obtained from patients referred to the hospitals in Ardabil City, Iran.

3. Methods

3.1. *Pseudomonas aeruginosa* Strains and Antimicrobial Susceptibility Testing

A total of 120 non-duplicate clinical isolates of *P. aeruginosa* were used in this study. These clinical isolates were collected between June 2019 and January 2022 from different wards of Imam Reza, Imam Khomeini, Bu-Ali, Alavi, Sabalan, Fatemi, and Ghaem hospitals in Ardabil City, northwest of Iran. *Pseudomonas aeruginosa* strains were confirmed using phenotypic tests, including pigment (blue/green), Gram stain, oxidase, catalase, triple sugar iron agar (red/red), oxidative-fermentative (OF), indole (-), methyl red (-), Voges-Proskauer (-), and citrate (+), as well as molecular identification of the *ITS* gene (6). Antimicrobial susceptibility testing of *P. aeruginosa* strains against different antibiotics was previously determined using the Kirby-Bauer disk diffusion method according

to the Clinical and Laboratory Standards Institute (CLSI) guideline (12).

3.2. Phenotypic Detection of ESBLs

A combined double-disk synergy test was used for phenotypic detection of class A ESBL-positive *P. aeruginosa* strains. Phenotypic detection of ESBLs in *P. aeruginosa* strains is difficult due to the presence of inducible AmpC β -lactamase, impermeability, and efflux-mediated resistance (13). For this purpose, a colony suspension of *P. aeruginosa*, equivalent to a 0.5 McFarland standard, was prepared in normal saline and inoculated on the Mueller-Hinton agar surface (Conda, Pronasida, Spain) (14). Antibiotic-containing disks (Padtan Teb, Iran) and phenylboronic acid (Sigma-Aldrich, Germany, i.e., ceftazidime (CAZ, 30 μ g) vs. ceftazidime/phenylboronic acid and ceftazidime-clavulanate (CZA, 30/10 μ g) vs. ceftazidime-clavulanate/phenylboronic acid) were placed at 2 cm apart (center to center; Figure 1A). The plate was incubated at 37°C for 16 - 18 hours. A 5 mm or larger increase in the diameter of the growth inhibition zone around the combination disks compared with the disks alone was considered ESBL-positive *P. aeruginosa*.

3.3. Detection of ESBL-Encoding Genes

All *P. aeruginosa* strains were investigated for detection of class A ESBL genes, including *PER*, *VEB*, *TEM*, *SHV*, *CTX-M*, *GES*, and *PSE*, using the polymerase chain reaction (PCR; Eppendorf Thermal Cycler, Germany) (15). Briefly, DNA extraction was performed using the boiling method, and the quality was confirmed by a spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA) (6). The total volume of each PCR was 25 μ L containing PCR Master Mix (20 μ L; Ampliqon, Denmark), DNA template (3 μ L), and forward and reverse primers (2 μ L and 10 μ mol/L). Gene amplification programs, along with specific primer sequences, are listed in Table 1. Further, 5 μ L of each PCR product was electrophoresed on 1% agarose gel and observed under UV light (Figure 1B). The results were confirmed through DNA sequencing (Pishgam, Iran) (6). Clinical isolates of *Klebsiella pneumoniae* and *P. aeruginosa* ATCC 27853 carrying class A ESBL-encoding genes were used as positive control strains. Additionally, PCR Master Mix plus nuclease-free water was used as negative controls.

4. Results

In this study, 120 clinical isolates of *P. aeruginosa* were obtained from 59 males and 61 females. The age of the patients was between 6 and 89 years. Strains were collected from Imam Khomeini (47.5%), Alavi (35.9%), Imam

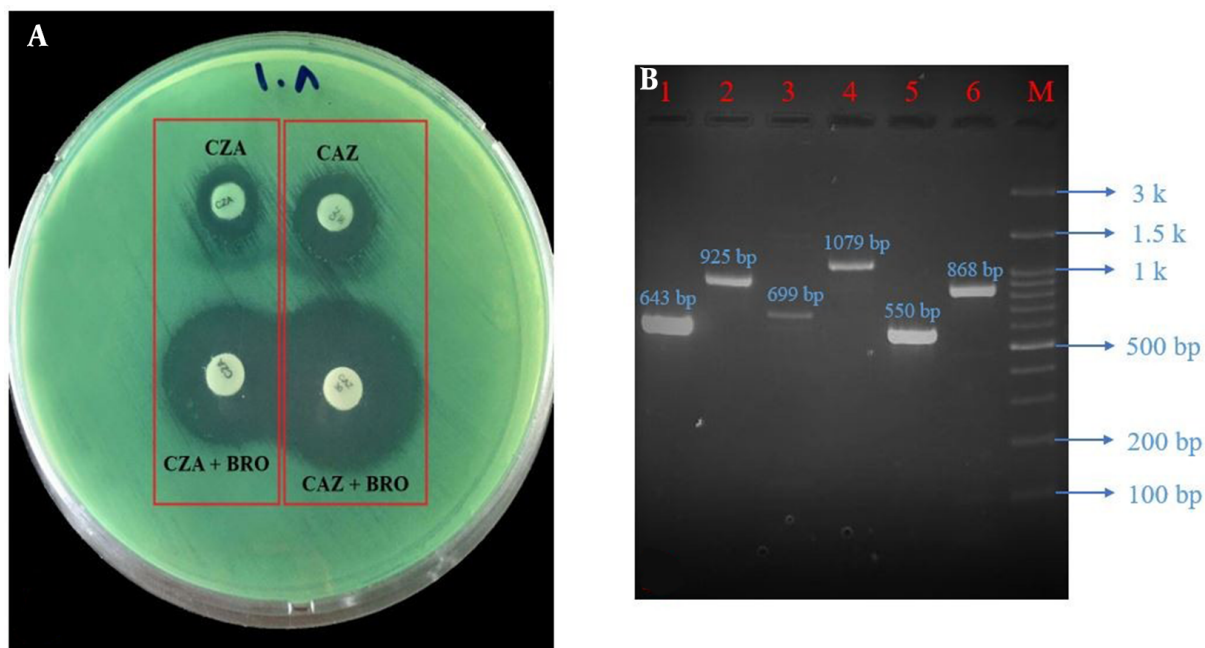


Figure 1. A, Phenotypic; and B, Genotypic detection of ESBL-producing *Pseudomonas aeruginosa*; Lane 1, Vietnamese extended-spectrum β -lactamase (VEB; 643 bp); lane 2, *Pseudomonas* extended resistant (PER; 925 bp); lane 3, *Pseudomonas*-specific enzyme (PSE; 699 bp); lane 4, temoniera (TEM; 1079 bp); lane 5, cefotaximase (CTX-M; 550 bp); lane 6, sulfhydryl variable (SHV; 868 bp); lane M, ladder (100 bp). Note that the SHV gene band is related to the positive control strain of *Klebsiella pneumoniae*. Abbreviations: CAZ, ceftazidime; CAZ/BRO, ceftazidime/phenylboronic acid; CZA, ceftazidime-clavulanate; CZA/BRO, ceftazidime-clavulanate/phenylboronic acid.

Table 1. A List of the Primers and Polymerase Chain Reaction Programs Used in the Present Study

Gene	Oligonucleotide Sequence (5' to 3')	Thermal Cycling Condition for Amplification	Amplicon Size (bp)	Reference
VEB	F: CGACTTCATTCCCGATGC;	1 cycle: Initial denaturation at 95°C for 5 min; 34 cycles: Denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min	643	(15)
	R: GGACTCTGCAACAAATACGC			
PER	F: AATTGGGCTTAGGGCAGAA	1 cycle: Initial denaturation at 95°C for 5 min; 34 cycles: Denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min	925	(15)
	R: ATGAATGTCATTATAAAAGC			
PSE	F: AATGGCAATCAGCGCTTC;	1 cycle: Initial denaturation at 95°C for 5 min; 34 cycles: Denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min	699	(15)
	R: GCGCGACTGTGATGATA			
GES	F: ATGCGCTTCATTACGCAC	1 cycle: Initial denaturation at 95°C for 5 min; 34 cycles: Denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min	864	(15)
	R: CIATTGTCCGTGCTCAGG			
SHV	F: TGGTTATGCGTTATATCGCC	1 cycle: Initial denaturation at 95°C for 5 min; 34 cycles: Denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min	868	(15)
	R: GGTIAGCGTTGCCAGTGCT			
TEM	F: ATAAAATCTTGAAGACGAAA;	1 cycle: Initial denaturation at 94°C for 5 min; 34 cycles: Denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min	1079	(15)
	R: GACAGTTACCAATGCTTAATCA			
CTX-M	F: CGCTTTGGCATGTGCAG	1 cycle: Initial denaturation at 94°C for 5 min; 34 cycles: Denaturation at 94°C for 45 s, annealing at 51°C for 40 s, and extension at 72°C for 1 min	550	(15)
	R: ACCGCGATATCGTTGGT			

Abbreviations: PER, *Pseudomonas* extended resistant; VEB, Vietnamese extended-spectrum β -lactamase; TEM, temoniera; SHV, sulfhydryl variable; CTX-M, cefotaximase; GES, guyana extended-spectrum β -lactamase; PSE, *Pseudomonas*-specific enzyme

Reza (7.5%), Bu-Ali (3.3%), Sabalan (3.3%), Fatemi (1.6%), and Ghaem (0.9%) hospitals. In addition, these clinical specimens were isolated from urine (48.3%), sputum (25%), blood (15.9%), wound (10%), and cerebrospinal fluid (0.8%). However, the infection type of patients referred to hospitals was not clear. According to the results of the double-disk synergy test, 8.3% (10 out of 120) of clinical isolates of *P. aeruginosa* were selected as class A ESBL-producing strains. The class A ESBL-positive *P. aeruginosa* strains were found in 5 male and 5 female patients. These bacteria were isolated from urine (n = 5), sputum (n = 4), and wound (n = 1) specimens. In addition, *P. aeruginosa* strains carrying class A ESBLs in phenotypic tests were obtained from patients referred to Imam Reza (n = 2), Imam Khomeini (n = 4), and Alavi (n = 4) hospitals. The characteristics of 10 ESBL-positive *P. aeruginosa* strains are listed in Table 2.

On the other hand, of the 120 *P. aeruginosa* strains, 48 (40%) carried genes encoding class A ESBLs based on the PCR. Among 48 class A ESBL-positive strains, the prevalence of *PSE*, *TEM*, *VEB*, *CTX-M*, and *PER* genes were 64.6% (31/48), 25% (12/48), 4.2% (2/48), 4.2% (2/48), and 2% (1/48), respectively. However, the frequency of genes encoding other class A ESBLs (*SHV* and *GES* genes) was 0%. The accession numbers for amplified genes (i.e., OQ024009 to OQ024015) were retrieved from the GenBank database.

5. Discussion

The following antibiotic-resistant microorganisms are considered serious health concerns worldwide: *Enterobacteriaceae*, *Acinetobacter*, *Campylobacter*, *Candida*, *Enterococcus*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Mycobacterium*, *Clostridium*, *Neisseria*, and *Pseudomonas* (7, 16). Furthermore, concerns regarding the spread of ESBL-producing bacteria are significantly increasing because treatment outcome in patients infected with such pathogens is less satisfactory (11). ESBLs have been abundantly reported in *E. coli* and *K. pneumonia* and have recently been detected in *P. aeruginosa* (11). Therefore, it is necessary to prevent the spread of ESBL-producing bacteria, especially in hospital environments.

There are various phenotypic and genotypic tests to detect ESBLs in *Enterobacteriaceae* clinical isolates with little or no chromosomal β -lactamase activity (11). Based on the ESBL screening phenotypic test, 8.3% of *P. aeruginosa* strains isolated from Ardabil hospitals were identified as ESBL-producing strains. Similar results to our study have been reported by Nazari Alam et al. (12.7%) (17), Tavajjohi and Moniri (9.2%) (18), and Laudy et al. (12.2%) (19). However, other studies from Iran and other countries have reported higher prevalence rates, including Vahdani et al. (18%) (20),

Akhi et al. (39.3%) (21), Mirsalehian et al. (39.4%) (22), Jabalameli et al. (42.8%) (23), Tawfik et al. (16%) (24), Shaikh et al. (25.1%) (25), and Chen et al. (87.5%) (26).

Cefotaximase, TEM, and SHV enzymes are considered the most prevalent ESBLs in bacterial pathogens globally (11). Various studies have reported the prevalence of these enzymes in clinical isolates of *P. aeruginosa* worldwide. In this regard, Bokaeian et al. found that TEM was present in 100% of isolates and SHV in 6.6% of them (27). Imani Foolad et al. reported SHV in 37.5% and TEM in 12.5% of isolates (28), while Nazari Alam et al. found SHV in 52.4% and TEM in 33.3% of isolates (17). Dong et al. did not detect any SHV or TEM in their isolates (29), and Jiang et al. found TEM in 17% of their isolates (13).

In the current study, the prevalence of TEM, CTX-M, and SHV genes were 10% (12/120), 1.6% (2/120), and 0%, respectively. Among *P. aeruginosa* and *Acinetobacter* species, PER- and oxacillinase (OXA)-type enzymes are more prevalent (11). In studies conducted by Lee et al. in Korea (15), Tawfik et al. in Saudi Arabia (24), and Chen et al. in China (26), as well as other studies in Iran, including Akhi et al. in Tabriz (21), Jabalameli et al. in Tehran (23), Alikhani et al. in Hamadan (30), and Amirkamali et al. in Qazvin (31), the prevalence of PER-type ESBLs were 0%, 0%, 13.8%, 27.5%, 50%, 26.6%, 0%, respectively. Nevertheless, the frequency of the PER gene was 0.8% (1/120) in the present study. Another most common class A ESBLs among *P. aeruginosa* strains is PSE-type enzymes (15). In this study, the most prevalent gene encoding class A ESBLs was PSE (25.8%; 31/120). Lee et al. reported the prevalence of PSE-type ESBLs at 6.3% in *P. aeruginosa* strains (15).

The frequency of VEB-type (1.6%; 2/120) and GES-type (0%) ESBLs in *P. aeruginosa* strains was lower in the current study than that reported by Tawfik et al. in Saudi Arabia (68% and 20%, respectively) (24). However, Lee et al. in Korea reported that none of the *P. aeruginosa* strains harbored genes encoding VEB- and GES-type ESBLs (15). Differences between the results of phenotypic and genotypic tests for ESBL screening in this study can be attributed to the presence of inducible chromosomal β -lactamases, efflux pumps, and higher impermeability in *P. aeruginosa* compared with *Enterobacteriaceae* (13).

In studies conducted by Khademi et al. on the same clinical isolates of *P. aeruginosa* in Ardabil, the prevalence and upregulation/downregulation of AmpC β -lactamase enzyme, efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM), and OprD porin among carbapenem-resistant and MDR strains were evaluated (data unpublished). The prevalence of inducible AmpC β -lactamase enzyme in phenotypic and genotypic tests was 52.5% and 100%, respectively. Among them, 33.3% of isolates showed a high expression level of the *ampC* gene

Table 2. Characteristics of Extended-Spectrum β -Lactamase-Positive *Pseudomonas aeruginosa* Strains Isolated from Ardabil Hospitals

Strain Number	Specimen	Virulence Gene Profiles ^a	Antibiotic Resistance Profiles ^a	
			Phenotypic Resistance	Genotypic Resistance
23	Wound	<i>algD, plcN, lasB, plcH, exoS, toxA, exoU</i>	MDR, MBL, AmpC, ESBL	<i>gyrA, parC, IMP, qacEΔ1, oprD, oxa-2, oxa-10, intI-1</i>
35	Urine	<i>algD, plcN, lasB, plcH, toxA, exoU</i>	MDR, MBL, AmpC, ESBL	<i>gyrA, parC, IMP, qacEΔ1, fabV, cepA, oprD, oxa-10</i>
50	Urine	<i>algD, plcN, lasB, plcH, toxA, exoU</i>	MDR, MBL, AmpC, ESBL	<i>IMP, qacEΔ1, oprD, oxa-2, oxa-10, intI-1</i>
59	Sputum	<i>algD, plcN, lasB, plcH, exoS, toxA, exoU</i>	MDR, MBL, AmpC, ESBL	<i>gyrA, parC, qacEΔ1, fabV, cepA, oprD, oxa-2, oxa-10, oxa-23, intI-1</i>
61	Sputum	<i>plcN, lasB, plcH, exoS, toxA</i>	MDR, AmpC, ESBL	<i>gyrA, parC, qacEΔ1, qacE, qacG, fabV, cepA, oprD, intI-1</i>
65	Sputum	<i>algD, plcN, lasB, plcH, exoS, toxA, exoU</i>	MDR, MBL, AmpC, ESBL	<i>gyrA, parC, IMP, qacEΔ1, fabV, cepA, oprD, oxa-2, oxa-10, oxa-23, intI-1, PSE</i>
73	Urine	<i>algD, plcN, lasB, plcH, toxA, exoU, pilB</i>	AmpC, ESBL	<i>qacEΔ1, qacE, fabV, cepA, oprD, oxa-23, intI-1, PSE</i>
79	Urine	<i>algD, plcN, lasB, plcH, toxA, exoU, pilB</i>	MDR, MBL, AmpC, ESBL	<i>qacEΔ1, qacE, qacG, fabV, cepA, oprD, oxa-2, oxa-23, intI-1</i>
100	Urine	<i>algD, plcN, lasB, plcH, exoS, toxA, exoU, pilB</i>	MDR, ESBL	<i>oprD, oxa-2, oxa-10, oxa-23, intI-1</i>
108	Sputum	<i>algD, plcN, lasB, plcH, exoS, toxA, pilB</i>	AmpC, ESBL	<i>oxa-2, oxa-10, PSE</i>

Abbreviations: *algD*, GDP-mannose 6-dehydrogenase; *plcN*, non-hemolytic phospholipase C; *plcH*, hemolytic phospholipase C; *lasB*, elastase; *exoS*, exoenzyme S; *exoU*, exoenzyme U; *toxA*, exotoxin A; *pilB*, type 4 fimbrial biogenesis protein; *gyrA*, DNA gyrase subunit A; *parC*, topoisomerase IV subunit A; *intI1*, class 1 integrase; MBL, metallo- β -lactamase; AmpC, AmpC cephalosporinase; MDR, multidrug-resistant; ESBL, extended-spectrum β -lactamase; IMP, imipenemase; OXA, oxacillinase; *qacE Δ 1*, *qacE*, *qacG* and *cepA*, efflux pumps genes; *fabV*, triclosan resistance gene; *oprD*, imipenem outer membrane porin; PSE, *Pseudomonas*-specific enzyme.

^a Virulence gene and antibiotic resistance profiles are based on previous studies.

based on real-time PCR. In addition, the role of 4 efflux pumps of *P. aeruginosa* in the emergence of drug-resistant clinical isolates was obvious. Overexpression of the *mexA*, *mexC*, *mexE*, and *mexY* genes was seen in 36 (75%), 40 (83.3%), 5 (10.4%), and 20 (41.6%) carbapenem-resistant and MDR *P. aeruginosa* clinical isolates. Furthermore, the *oprD* gene downregulation was observed in 79.1% of these *P. aeruginosa* isolates. The sequencing method was used to identify many TEM and SHV family variants. Temoniera and SHV family variants were not determined in our study, and this was the main limitation of the current study.

5.1. Conclusions

Our results confirmed that PSE, TEM, VEB, CTX-M, and PER were the predominant genes encoding class A ESBLs in *P. aeruginosa* strains in Ardabil City. On the other hand, we concluded that the use of molecular tests could be a more precise and reliable method than phenotypic ones to identify these resistant strains and prevent the emergence of antibiotic resistance and ensuing treatment failure.

Footnotes

Authors' Contribution: Study concept and design: F. K.; acquisition of data: F. H. and N. A.; analysis and interpretation of data: F. K.; drafting of the manuscript: F. K.; revision

of the manuscript: A. S.; all authors read and approved the final manuscript.

Conflict of Interests: The authors declare that there are no conflicts of interest.

Ethical Approval: IR.ARUMS.MEDICINE.REC.1401.035

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