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RESEARCH ARTICLE

Characteristics of multiresistant *Pseudomonas aeruginosa* isolates from burn patients in Iran

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ABSTRACT

Infections caused by multidrug resistant (MDR) *Pseudomonas aeruginosa* isolates in burn patients restrict therapeutic strategies. The current study aimed to analyze antibiotic resistance genes and multilocus sequence typing (MLST) of *P. aeruginosa* strains isolated from burn patients in Shahid Motahari hospital in Tehran, Iran.

Altogether 63 *P. aeruginosa* isolates were characterized in this study. Antibiotic susceptibility testing was performed by disc diffusion method. PCR was performed to determine the frequency of resistance genes. The expression rates of *mexB*, *mexY* genes were evaluated by Real-Time PCR. Genotyping of isolates was performed by MLST analysis. All isolates were MDR in this study. The highest resistance was detected against gentamicin, tobramycin, and cefoxitin (100%), while all isolates were susceptible to colistin. Altogether 14 resistance profiles were determined, and profile 1 included more than 50% of the isolates with the highest resistance. In this study bla_{ampC}, bla_{VIM-2}, bla_{OXA-10}, and *aac(6′)-Ib* resistance genes were detected in all isolates. The expression levels of *mexB* and *mexY* genes were upregulated in 66.6 and 88.8% of MDR isolates, respectively. Overexpression of both genes was detected in 55.5% of the isolates.

MLST analysis revealed five sequence types (STs), including ST235, ST664, ST532, ST2637, and ST230, which showed a significant relationship with antibiotic resistance profiles. The present study indicates an increase in antibiotic resistance against different antibiotic families among *P. aeruginosa* isolates. We describe the circulation of globally distributed STs among hospitalized patients, and we report ST235 as the most common MDR clone in our study.

KEYWORDS

Pseudomonas aeruginosa, antibiotic resistance, extended-spectrum β-lactamase, metallo-β-lactamase, aminoglycosides, MLST

INTRODUCTION

Annually, about 265,000 deaths due to burn injuries are reported by the World Health Organization (WHO) [1]. Immunosuppression is one of the consequences of burn injuries, which in turn increases the susceptibility of burn patients to infections [1]. The most important reason is the loss of the skin's natural defense barrier. Also, the complex interaction of anti-inflammatory signals results in dysregulation of immune responses [2]. Hence, due to the improper functioning of the immune system and the presence of antibiotic-resistant microorganisms, infection is the main cause of death in burn patients (42–65%) [3]. The prevalence of multidrug-resistant (MDR) bacteria in burn treatment centers declines the

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ability of physicians to administrate appropriate antibiotic treatments against infectious agents [4]. Despite preventive measures, burn wounds are usually colonized by bacteria such as *Pseudomonas aeruginosa* after the second week of hospitalization [5].

P. aeruginosa is a Gram-negative, non-fermentative, aerobic bacterium that is widely present in nature. This organism is an important opportunistic pathogen in burn patients, which is responsible for 10–20% of nosocomial infections in different parts of the world [6]. *P. aeruginosa* is characterized by innate and acquired resistance to different families of antibiotics, such as beta-lactams (penicillins, cephalosporins, monobactams, carbapenems), aminoglycosides, fluoroquinolones, and lipopeptides [7]. The mechanisms by which *P. aeruginosa* gains resistance against β-lactam antibiotics include genetic mutations, transmission of lactamase genes, increased expression of efflux pump genes, and decreased cell membrane permeability [8].

According to Ambler classification (1980), β-lactamases are classified into four categories: extended-spectrum β-lactamases (ESBL) (class A), metallo-β-lactamases (MBL) (class B), cephalosporinases (AmpC) (class C), and oxacillinases (OXA) (class D) [9].

Another common antibiotic resistance mechanism is the enzymatic modification of aminoglycosides. These enzymes include aminoglycoside phosphoryl transferase (*aph*), aminoglycoside acetyltransferase (*aac*), and aminoglycoside nucleotidyltransferase (*ant*) [10].

The ability of *P. aeruginosa* to pump unwanted chemicals such as anionic detergents and various antibiotics through membrane multidrug efflux pumps has been investigated in several studies [11]. So far, the properties of four efflux pumps have been studied, including MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN, and MexX-MexY-OprM. These pumps operate on a variety of dedicated substrates, which in turn contribute to increasing resistance to antibiotics [11].

The dissemination potential of MDR *P. aeruginosa* strains makes it necessary to investigate their population structure genetically. Multilocus sequence typing (MLST) is an unequivocal method used to type bacterial species and characterize diverse clinical and environmental isolates based on conserved housekeeping gene sequences. This method could be used to track global clonal evolutionary relationships [12].

Therefore, the objective of this study was to determine the antibiotic resistance patterns of MDR *P. aeruginosa* strains isolated from burn patients and to investigate the clonal relationships between them.

MATERIALS AND METHODS

Sample collection and characterization

From June to October 2016, a total of 63 *P. aeruginosa* isolates were collected from wound, blood, and catheter samples of burn patients hospitalized in Motahari Burn Centre in Tehran. Demographic data of these patients such as gender and hospitalization ward were collected. All the isolates were characterized by biochemical tests, including Gram staining, citrate, catalase, oxidase, growth on MacConkey agar, triple sugar iron agar (TSI) test, oxidative-fermentative (OF) test, growth at 42 °C, and methyl red Voges Proskauer (MRVP) test. All culture media were purchased from Merck Company distributor (Merck; Germany). Phenotypic characterization was confirmed by PCR amplification using 16srRNA specific primers (16srDNA-F, 5′-GGGGGATCTTCGGACCTCA-3′; and 16srDNA-R, 5′- TCCTTAGAGTGCCCACCCG-3′) [13]. Genomic DNA was extracted by boiling method and subjected to PCR amplification under the following thermal cycling program: an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 3 min.

Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of the isolates were determined by Kirby-Bauer disc diffusion assay on Mueller-Hinton agar (MHA; Merck; Germany) according to Clinical Laboratory Standards Institute (CLSI; 2017, 27th Edition) guidelines [14]. *P. aeruginosa* ATCC 27853 was used as a quality control. The isolates were classified as susceptible, intermediate, and resistant based on CLSI breakpoints. The antibiotic discs used in this study were purchased from Padtan Teb Company (Padtan Teb, Iran) and these included ciprofloxacin (CIP: 5 μg), amikacin (AK: 30 μg), gentamicin (GEN: 10 μg), tobramycin (TOB: 10 μg), colistin (CST: 10 μg), ceftazidime (CAZ: 30 μg), ceftazidime-clavulanic acid (30/10 μg), aztreonam (AZT: 30 μg), amoxicillin-clavulanic acid (20/10 μg), imipenem (IMP: 10 μg), cefotaxime (30 μg), cefotaxime-clavulanic acid (30/10 μg), cefoxitin (30 μg), ticarcillin (TIC: 75 μg), piperacillin (PIP: 100 μg), and piperacillin/tazobactam (PTZ: 100/10 μg).

Double-disc synergy test (DDST) was used to detect metallo-β-lactamase (MBL) producing strains as follows: two imipenem discs were placed at a defined distance on Mueller-Hinton agar plates which were swabbed by *P. aeruginosa* isolates, one disc was impregnated by EDTA (0.5 M), an inhibition zone diameter of more than 7 mm was considered as MBL producing [15]. Extended-spectrum β-lactamase (ESBL) producing isolates were also detected by DDST using 3rd generation cephalosporins such as ceftazidime (30 μg) and cefotaxime (30 μg) alone and in combination with clavulanic acid (10 μg). Also, ESBL-producing isolates were determined with an inhibition zone diameter of ≥5 mm around the discs of ceftazidime-clavulanic acid and cefotaxime-clavulanic acid [16]. MDR isolates were determined by WHONET 2017 software (O'Brienand Stelling Co).

Detection of antibiotic resistance genes by PCR

Genomic DNA was extracted by boiling method and subjected to PCR reaction to amplify the following genes:

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bla$_{IMP-1}$, *bla*$_{VIM-2}$, *bla*$_{KPC}$, *bla*$_{ampC}$, *bla*$_{OXA-2}$, *bla*$_{OXA-10}$, *aph(3′)-V1, ant(2″)- Ia, aac(6′)-Ib,* and *aac(6′)-II*. The list of primers and sequences is presented in Table 1. The PCR mixture included 12.5 µL of master mix and 1 µL of primer. PCR reaction was performed by SensoQuest LabCycler (Germany) under the following conditions: an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 20 s, annealing (at specific temperature for each gene shown in Table 1) for 20 s, extension at 72 °C for 40 s, and a final extension step at 72 °C for 2 min. Amplified samples were electrophoresed on 1% agarose gel. Ethidium bromide was used to stain the gel. The bands were documented under UV light (Uvitec Cambridge; France).

Evaluation of *mexB* and *mexY* gene expression by real-time PCR

Total RNA of nine isolates and *P. aeruginosa* ATCC 27853 as a control were extracted by Favor prepTM blood/cultured cell total RNA mini kit (Favorgen, Taiwan) according to the manufacturer's protocol. Afterwards, the RNAs were reverse transcribed by cDNA synthesis kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's protocol. Quantification of extracted RNA and synthesized cDNA was done by nanodrop (Westberg, Netherland). The expression level of *mexB* and *mexY* genes was quantified by a LightCycler device (Roche, Germany) with SYBR Green 2X master mix (Ampliqon, Denmark). To normalize the expression level of the targeted genes, the housekeeping *rpsl* gene (30S ribosomal protein S12) was used as an internal control [17]. *P. aeruginosa* ATCC 27853 strain was used as a control. The primer sequences are shown in Table 1. Real-time PCR amplification was performed under the following conditions: initial denaturation at 95° for 10 min, followed by 35 cycles of denaturation at 95 °C for 15 s, 61 °C for 20 s, and 72 °C for 20 s. The final extension step was performed at 72 °C for 1 min. All amplifications were done in triplicate. The relative expression level of *mexB* and *mexY* genes was measured by $E^{-\Delta\Delta CT}$ (E = 1 + primer efficiency) [18].

Multilocus sequence typing

Totally, 10 strains were selected for molecular typing based on the results of antibiogram and the presence of antibiotic resistance genes. According to the MLST scheme created by Curran et al. (2004), seven housekeeping genes including *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA,* and *trpE* were chosen [19]. PCR primers were in accordance with a public database for molecular typing (pubmlst.org database) and synthesized by Macrogen Company, Korea. For *trpE*, *ppsA*, *guaA*, *nuoD*, *mutL*, and *acsA*, PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 20s, annealing (at specific temperature for each gene) for 25 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 2 min. For the *aroE* gene, PCR amplification was performed with 30 cycles of denaturation at 94 °C for 50 s, annealing at 62.5 °C for 50 s, and

Table 1. Primer sequences, amplification lengths, and annealing temperatures

Gene Name	Primers (5'……3')	Product Length (bp)	Annealing (°C)	Reference
ampC (real-time PCR)	F: GCTCCACCAACGGCTTC R: CTGAGGATGGCGTAGGC	124	61	[40]
bla$_{IMP}$	F: CTACCGCAGCAGAGTCTTTG R: AACCAGTTTTGCCTTACCAT	587	57	[41]
bla$_{VIM}$	F: ATGGTGTTTGGTCGCATATC R: TGGGCCATTCAGCCAGATC	510	59	[42]
bla$_{OXA-10}$	F: TCAACAAATCGCCAGAGAAG R: TCCCACACCAGAAAAACCAG	227	61	[43]
bla$_{OXA-2}$	F: AAGAAACGCTACTCGCCTGC R: CCACTCAACCCATCCTACCC	486	61.5	[44]
bla$_{KPC}$	F: AGTTCTGCTGTCTTGTCTC R: CTGTGCTTGTCATCCTTG	798	58	[40]
aph (3′)-V1	F: CGGAAACAGCGTTTTAGA R: TTCCTTTTGTCAGGTC	717	52	[45]
ant (2″)- Ia	F: GCTCACGCAACTGGTCCAGA R: GGCACGCAAGACCTCAACCT	719	61	[46]
aac(6')-Ib	F: CATGACCTTGCGATGCTCTA R: GCTCGAATGCCTGGCGTCTT	490	61	[47]
aac(6')-II	F: GCTCGAATGCCTGGCGTCTT R: GACTCTTCCGCCATCGCTCT	179	66.2	[47]
rpsL (real-time PCR)	F: GCAAGCGCATGGTCGACAAGA R: CGCTGTGCTCTTGCAGGTTGTGA	201	60	[48]
mexB (real-time PCR)	F: GTGTTCGGCTCGCAGTACTC R: AACCGTCGGGATTGACCTTG	244	60	[49]
mexY (real-time PCR)	F: CCGCTACAACGGCTATCCCT R: AGCGGGATCGACCAGCTTTC	246	60	[49]

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extension at 72 °C for 60 s. Amplification reactions were carried out in a final volume of 25 µL, containing 2 µL of template DNA (500 ng μL^{-1}), 12.5 µL of PCR Master-mix (Ampliqon, Denmark), and 1 µL of each primer (10 pM). Amplified products were purified, and both DNA strands were sequenced by ABI 3730X capillary sequencer (Macrogen, Korea).

The genetic similarity and evolutionary distance between the selected isolates were determined via a public database (https://pubmlst.org/organisms/pseudomonas-aeruginosa). The genetic relationship between *P. aeruginosa* isolates was investigated based on the minimum spanning tree obtained by PHYLOVIZ and the neighbor-joining tree obtained by iTOL (interactive tree of life) and UPGMA (Fig. 1).

RESULTS

Sample collection and characterization

In this study, 63 *P. aeruginosa* isolates were collected from burn patients with wound infection in Motahari hospital in Tehran during June to October 2016. These isolates were recovered from wound (53, 84.1%), blood (4, 6.3%), catheter (4, 6.3%), and tissue (2, 3%) samples. Also, 79% of burn patients were male, and 21% were female. Among them, 73, 16, and 11% were hospitalized in male, female, and pediatric wards, respectively. All isolates were identified as *P. aeruginosa* by microbiological and biochemical tests as well as by 16srRNA PCR assay.

Antimicrobial susceptibility testing

Antibiotic resistance patterns of the isolates are presented in Table 2. The highest resistance was observed against gentamicin (100%), tobramycin (100%), amoxicillin-clavulanic acid (100%), cefoxitin (100%), ciprofloxacin (98.5%), amikacin (98.5%), and imipenem (98.5%), whereas the highest sensitivity was against colistin (100%). As shown in Table 2, 14 resistance profiles were determined, and 52.3% of the isolates belonged to resistance profile 1. All isolates were MBL-producing bacteria according to DDST, whereas only 29 isolates (46%) were ESBL producers.

Detection of antibiotic resistance genes by PCR

The bla_{KPC} and bla_{IMP-1} genes were not detected in any of the isolates, whereas bla_{ampC}, bla_{VIM-2}, bla_{OXA-10}, bla_{OXA-2}, *ant(2″)- Ia, aac(6′)-Ib*, *aac(6′)-II*, and *aph(3′)-V1* were detected in 100, 100, 100, 3.1, 69.8, 100, 71.4, and 19% of the isolates. The isolates were classified into nine genotypes according to the presence of ESBL and MBL genes (bla_{KPC}, bla_{ampC}, bla_{IMP-1}, and bla_{VIM-2}), oxa-type β-lactamase genes (bla_{OXA-2}, and bla_{OXA-10}), and aminoglycoside resistance genes (*ant (2″)-Ib, ant(6′)-Ib, aac(6′)-II,* and *aph(3′)-V1*) (Table 3).

Evaluation of *mexB* and *mexY* gene expression by real-time PCR

The expression levels of *mexB* and *mexY* genes in the selected isolates and *P. aeruginosa* ATCC 27853 were measured in triplicate and presented as mean ± SD.

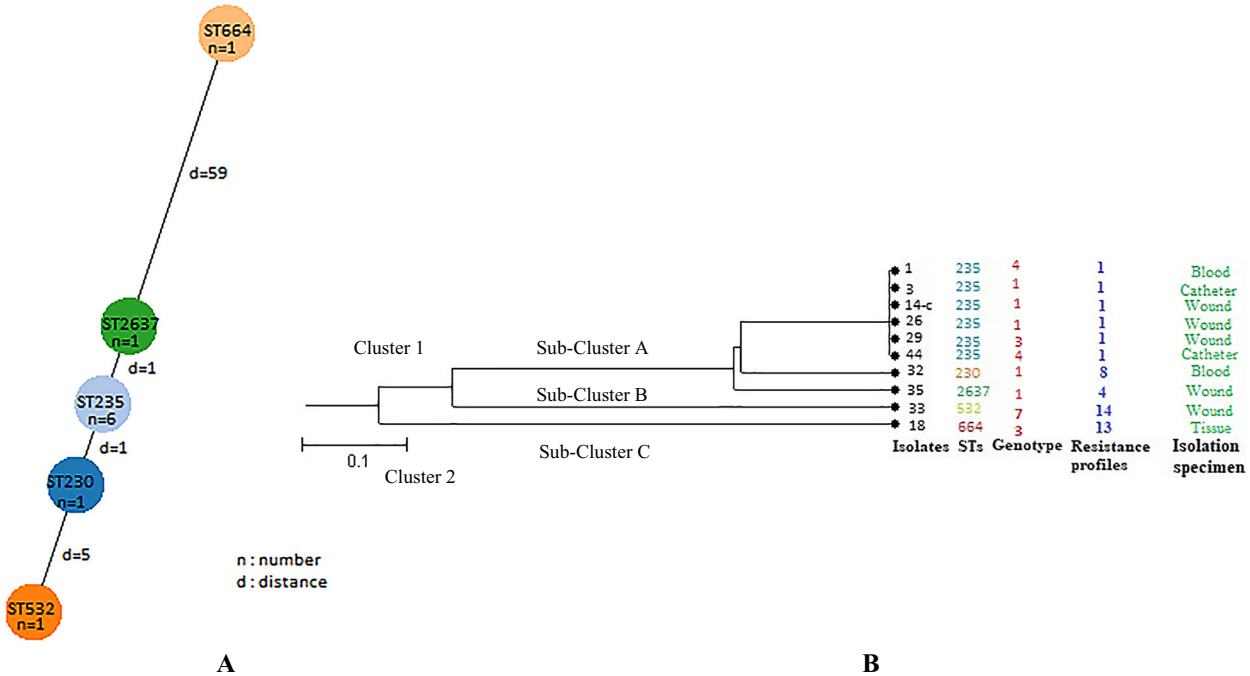

Fig. 1. A) Minimum spanning tree based on STs of *P. aeruginosa* isolates. The nudes indicate the number of STs, and the distance between the nudes represent the number of locus differences. d: distance, n: number. B) Dendrogram of *P. aeruginosa* isolates based on UPGMA method along with phenotypic and genotypic resistance profile and isolation specimen

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Table 2. Antibiotic resistance profile of *P. aeruginosa* isolates

Resistance Profile	Antibiotic resistance pattern											Percent
	PIP	PTZ	CAZ	AZT	IMP	GEN	TOB	AK	CIP	CST	TIC	
1	R	R	R	R	R	R	R	R	R	S	R	52.5%
2	R	R	R	I	R	R	R	R	R	S	R	12.8%
3	R	R	S	I	R	R	R	R	R	S	R	9.7%
4	R	R	S	R	R	R	R	R	R	S	R	8.1%
5	I	R	S	R	R	R	R	R	R	S	R	3.4%
6	I	R	R	R	R	R	R	R	S	S	R	1.5%
7	I	S	S	I	R	R	R	R	R	S	R	1.5%
8	R	R	R	R	I	R	R	S	R	S	R	1.5%
9	I	I	S	I	R	R	R	R	R	S	R	1.5%
10	I	R	R	I	R	R	R	R	R	S	I	1.5%
11	I	I	R	I	R	R	R	R	R	S	I	1.5%
12	I	I	R	S	R	R	R	R	R	S	I	1.5%
13	R	I	S	S	R	R	R	R	R	S	I	1.5%
14	R	R	R	I	R	R	R	R	R	S	I	1.5%

PIP: piperacillin, PTZ: piperacillin tazobactam, CAZ: ceftazidime, AZT: azithromycin, IMP: imipenem, GEN: gentamicin, TOB: tobramycin, AK: amikacin, CIP: ciprofloxacin, CST: colistin, TIC: ticarcillin

Table 3. Genotyping of *P. aeruginosa* isolates according to the presence of resistance genes

Genotype	Pattern										N (%)
	bla_{KPC}	*ampC*	*ant*	*aac1*	*oxa10*	bla_{OXA-2}	*aph*	*aac2*	bla_{VIM}	bla_{IMP}	
Genotype 1	N	P	P	P	P	N	N	P	P	N	23 (36.5)
Genotype 2	N	P	N	P	P	N	N	P	P	N	11 (17.5)
Genotype 3	N	P	P	P	P	N	N	N	P	N	10 (15.9)
Genotype 4	N	P	P	P	P	N	P	P	P	N	7 (11.1)
Genotype 5	N	P	N	P	P	N	N	N	P	N	6 (9.5)
Genotype 6	N	P	N	P	P	N	P	P	P	N	2 (3.2)
Genotype 7	N	P	N	P	P	N	P	N	P	N	2 (3.2)
Genotype 8	N	P	P	P	P	P	N	P	P	N	1 (1.5)
Genotype 9	N	P	P	P	P	P	P	P	P	N	1 (1.5)

The expression levels of *mexB* and *mexY* genes were ≥6 and ≥1.8 times higher in the selected isolates than in the control, respectively. Overexpression of the *mexB* gene was observed in 66.6% of the isolates, whereas 88.8% of the isolates overexpressed the *mexY* gene. Overexpression of both *mexB* and *mexY* genes was observed in 55.55% of the isolates.

Multilocus sequence typing

Overall, 10 *P. aeruginosa* isolates were analyzed to determine their allelic profiles. The isolates were chosen according to their antibiotic resistance profiles: six isolates from resistance profile 1 and one isolate from each of profiles 4, 8, 13, and 14. Sequence analysis of housekeeping genes revealed five sequence types (ST235, ST664, ST532, ST2637, and ST230) in the isolates, among which six isolates belonged to ST235, and the other isolates revealed unique sequence types. Totally, three different alleles for *acsA, aroE, guaA, nuoD, ppsA,* and *trpE* and four different alleles for *mutL* were detected among the isolates.

In addition, all six isolates distributed in ST235 were of resistance profile 1. This sequence type is assumed as the founder ST. The characteristics of the seven sequenced housekeeping genes are presented in Table 4.

DISCUSSION

Resistance to a variety of antimicrobial agents is a threat to global health, limits the choice of medication, and leads to problems in infection control [20]. MDR *P. aeruginosa* strains are the causative agents of nosocomial infections, which cause numerous clinical problems, especially in people suffering from impaired immunity, such as burn patients [21].

In this study, a broad-spectrum resistance against antimicrobial agents from different antibiotic families was observed among the isolates, considering that all the isolates were multidrug-resistant. Also, based on the disc diffusion method results, 14 resistance profiles were achieved, while more than 50% of the isolates belonged to resistance profile 1 (Table 2). In profile 1, the isolates were resistant against all tested antibiotics (except colistin), such as PIP, PTZ, CAZ, AZT, IMP, GEN, TOB, AK, CIP, and TIC (Table 2). In profile 2 (12.6% of the isolates), intermediate and complete susceptibility was observed against AZT and CST, respectively. Susceptibility against CAZ and CST and intermediate resistance to AZT were detected in profile 3 (9.5%) (Table 2).

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Table 4. Sequence types according to the number of alleles

Sequence Type (ST)	Frequency	*acsA*	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*
ST235	6	38	11	66	13	1	2	4
ST664	1	9	5	70	54	4	40	10
ST230	1	38	11	5	5	1	2	4
ST532	1	5	4	5	5	5	20	4
ST2637	1	38	11	66	3	1	2	10

Totally, the phenotypic findings derived from the antibiogram determined an increase in antibiotic resistance among *P. aeruginosa* strains isolated from burn patients in Motahari hospital in Tehran during 2016. Corehtash et al. (2015) reported antibiotic resistance rates similar to the results obtained in this study [22]. In contrast, some studies have reported lower antibiotic resistance rates compared to the present study results [23], this may be due to the high antibiotic consumption in the burn centre and the effect of antibiotic selective pressure on increasing antibiotic resistance. According to the results, all the isolates were sensitive to colistin, which is consistent with the results of another study by Akhi et al. (2015) [24]. It was assumed that colistin could be responsive as a therapeutic agent.

MBL production was detected in all isolates (100%), whereas ESBLs production was positive in 46% of the isolates based on DDST. Among ESBL and MBL genes, bla_{KPC} and $bla_{\text{IMP-1}}$ were negative in all isolates, whereas bla_{ampC} and $bla_{\text{VIM-2}}$ were all positive (100%) (Table 3), these results were not consistent with the phenotypic findings. Oxa-type β-lactamase genes including $bla_{\text{OXA-2}}$ and $bla_{\text{OXA-10}}$ were positive in 3% and 100% of the isolates, respectively (Table 3). Oxa-type β-lactamases belong to Ambler class D and usually have narrow-spectrum activity; however, broad-spectrum resistance due to point mutations has been reported in some studies (reference: OXA-type beta-lactamases). The aminoglycoside resistance genes (*ant (2″)-Ib, ant(6′)-Ib, aac(6′)-II,* and *aph(3′)-V1*) identified in the isolates are shown in Table 3, which were consistent with the phenotypic results and resistance to AK and TOB. Generally, the existence and prevalence of antibiotic resistance genes were partly similar to the phenotypic results of the disc diffusion test. The results of this study are mostly inconsistent with the results of other studies [25]. Some studies have also shown an increase in the prevalence of genes belonging to different classes of Ambler classification over the years [26]. Since all the resistance genes investigated in this study are part of mobile genetic elements (transposons and integrons) [27], horizontal gene transfer could be responsible for increasing the prevalence of resistance genes and subsequently increasing antibiotic resistance in most cases.

It was determined that the expression levels of the *mexB* gene from MexA-MexB-OprM efflux pump and the *mexY* gene from MexX-MexY-OprM efflux pump were upregulated in six (66.6%) and eight (88.8%) isolates, respectively. Overexpression of both genes was observed in five (55.5%) isolates. These results are relatively similar to the results of another study by Goli et al. (2018) [28]. Efflux pumps have been identified as one of the remarkable structures involved in resistance to different classes of antibiotics [29], and the high rate of antibiotic resistance obtained in this study is probably related to the overexpression of efflux pump genes.

According to the antibiotic resistance patterns, 52.3% of the isolates with different genotypes belonged to resistance profile 1, indicating that the high resistance of these isolates was not mainly due to the presence of antibiotic resistance genes, and other resistance mechanisms may also be involved.

Overexpression of efflux pump genes in clinical strains of *P. aeruginosa* has been reported to be 14–75% [30], which is consistent with this study results. There are many reports about the overexpression of efflux pump genes in our country [31] and other countries. The expression level of efflux pump genes in this study was relatively higher than those reported by Dumas et al. (2006) in Switzerland [29], Mesaros et al. (1999) in Belgium [32], and other studies [33].

The fold change in *mexB* and *mexY* gene expression in the present study was probably due to the higher antibiotic selective pressure in burn patients. There was a significant correlation between the resistance against all the examined antibiotics except colistin and the activity of efflux pumps (P-value < 0.05).

Overall, this study results indicate that the emergence of resistance phenotype and MDR isolates is probably due to different mechanisms.

MLST was performed on 10 isolates to determine their evolutionary relationships. The results showed high genetic diversity among the isolates with five STs belonging to two clusters (1 and 2) and three sub-clusters (A–C), among which ST235 was the predominant ST (n = 6) (Fig. 1). ST235 is considered as a high-risk clone [34]. The other isolates belonged to ST230, ST532, ST664, and ST2637. ST2637 has not been previously reported in any other study. All six isolates distributed in ST235 belonged to resistance profile 1. All the isolates distributed in MLST-STs harbored *blaampC, blavim-2, blaoxa10,* and *aac(6′)-Ib* genes, and ST235 isolates also harbored *acc(6′)-II* gene.

No significant relationship was observed between STs and the presence of resistance genes; however, there was a significant association between STs and phenotypic resistance profiles. As reported earlier, all detected ST235 strains belonged to profile 1. This result is similar to the findings of other studies [23].

All ST235 strains were isolated from the wound, blood, and catheter samples of grade III burn patients in different

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Table 5. Demographic data, resistance profiles, and genotyping of *P. aeruginosa* isolates

Isolates	Isolation Specimen	Isolation Unit	Burn Degree	Resistance Profile	Genotype	Sequence Type
1	Blood	BICU	III	1	4	ST235
3	Catheter	Pediatric	III	1	1	ST235
14-C	Wound	BICU	III	1	1	ST235
18	Tissue	Male	III	13	3	ST664
26	Wound	Reconstructive	III	1	1	ST235
29	Wound	Emergency	II	1	3	ST235
32	Blood	BICU	III	8	1	ST230
33	Wound	Male	II	14	7	ST532
35	Wound	Pediatric	III	4	1	ST2637
44	Catheter	BICU	III	1	4	ST235

hospital wards including the burn intensive care unit (BICU) as well as pediatric and restorative wards. The findings indicated the circulation of ST235 across different wards of the hospital. Two isolates from the blood samples belonged to ST235 and ST230, and two other ST235 isolates were collected from catheters. This may be due to the invasiveness and pathogenicity of the isolates, which were not investigated in this study. The strains isolated from the wound samples belonged to different sequence types: ST235, ST664, ST532, and ST2637. The presence of ST235 in 75% of BICU isolates probably indicates the circulation of ST235 and the high risk of nosocomial infections in this ward. Similar results have been reported for the dissemination of ST235 in different geographical regions of Iran [35]. In addition, it is worth noting that ST235 is the predominant isolate of *P. aeruginosa* in Europe, Asia Pacific, and South Africa [36]. The typing results obtained in this study seem to exhibit a similar pattern to international research results. Isolate 32 belonging to ST230, genotype 1, and resistance profile 8 (Fig. 1B) was isolated from blood. ST230 strains have previously been reported in Europe as VIM-producing strains. Some studies tracking STs have determined a link between ST235 and ST230 and introduced ST230 as a single-locus variant of ST235 [37]. The genotyping results (presence pattern of resistance genes), minimum spanning trees, and dendrogram results indicated the similarity of ST235 and ST230 and the presence of a linkage between them (Fig. 1) (Tables 4 and 5). These findings are in accordance with the results of last study indicating that ST230 was one of the closest relatives of ST235 isolates [37].

The distance between ST235 and ST2637 seems to be similar to that between ST235 and ST230. Similarly, ST2637 strains belonged to genotype 1, whereas they phenotypically belonged to resistance profile 4 and covered 7.5% of the isolates in this study. As far as we know, this is the first report of the presence of ST2637 in *P. aeruginosa* isolates in Iran and the world.

ST532 was detected in one isolate in this study (Fig. 1). ST532 isolate did not carry *ant* and *acc(6′)-II* genes (genotype 7) and belonged to resistance profile 14. ST532 has been reported as a circulating carbapenemase producer in Italy and as a high-risk clone in Saudi Arabia and South Africa [38]. ST532 has not been reported in Iran so far.

Isolate 18 belonged to genotype 3, resistance profile 13, and ST664. ST664 has recently been reported in Iran, Pakistan, and France [39]. In the present study, this ST was resistant against the majority of antipseudomonal agents except colistin, CAZ, and AZT. The limitations of this study included the high cost of DNA sequencing and software limitations.

CONCLUSION

In the current study, MDR *P. aeruginosa* isolates collected from a burn hospital in Tehran were examined to determine their resistance patterns, resistance genetic backbone, circulation, typing, and sources. The findings suggest the increase in antibiotic resistance due to high consumption and selective pressure of antibiotics as a reason for the evolution of MDR *P. aeruginosa* isolates. In this study, 14 antibiotic resistance patterns, 9 genotypes, and 5 STs were detected among the isolates, of which two STs were reported for the first time in Iran.

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