

RESEARCH

Open Access



# Prevalence and molecular characterization of colistin resistance in *Pseudomonas aeruginosa* isolates: insights from a study in Ardabil hospitals

Saghar Jafari-Ramedani<sup>1</sup>, Maryam Nazari<sup>1</sup>, Mohsen Arzanlou<sup>1</sup>, Hadi Peeri-Dogaheh<sup>1</sup>, Amirhossein Sahebkar<sup>2,3</sup> and Farzad Khademi<sup>1,4\*</sup>

## Abstract

**Background** *Pseudomonas aeruginosa* is a common cause of nosocomial infections. However, the emergence of multidrug-resistant strains has complicated the treatment of *P. aeruginosa* infections. While polymyxins have been the mainstay for treatment, there is a global increase in resistance to these antibiotics. Therefore, our study aimed to determine the prevalence and molecular details of colistin resistance in *P. aeruginosa* clinical isolates collected between June 2019 and May 2023, as well as the genetic linkage of colistin-resistant *P. aeruginosa* isolates.

**Results** The resistance rate to colistin was 9% ( $n = 18$ ) among *P. aeruginosa* isolates. All 18 colistin-resistant isolates were biofilm producers and carried genes associated with biofilm formation. Furthermore, the presence of genes encoding efflux pumps, TCSs, and outer membrane porin was observed in all colistin-resistant *P. aeruginosa* strains, while the *mcr-1* gene was not detected. Amino acid substitutions were identified only in the PmrB protein of multi-drug- and colistin-resistant strains. The expression levels of *mexA*, *mexC*, *mexE*, *mexY*, *phoP*, and *pmrA* genes in the 18 colistin-resistant *P. aeruginosa* strains were as follows: 88.8%, 94.4%, 11.1%, 83.3%, 83.3%, and 38.8%, respectively. Additionally, down-regulation of the *oprD* gene was observed in 44.4% of colistin-resistant *P. aeruginosa* strains.

**Conclusion** This study reports the emergence of colistin resistance with various mechanisms among *P. aeruginosa* strains in Ardabil hospitals. We recommend avoiding unnecessary use of colistin to prevent potential future increases in colistin resistance.

**Keywords** *Pseudomonas aeruginosa*, Colistin, Multidrug-resistant

## Background

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen commonly found in hospital environments, particularly in intensive care units (ICUs). It is responsible for various nosocomial infections, including pulmonary, bloodstream, urinary tract, surgical site, and skin and soft tissue infections [1–4]. The treatment of *P. aeruginosa* infections typically involves the use of  $\beta$ -lactam, fluoroquinolone, and aminoglycoside antibiotics either alone or in combination [5, 6]. However, the misuse and

\*Correspondence:

Farzad Khademi

k\_farzad@yahoo.com; f.khademi@arums.ac.ir

<sup>1</sup> Department of Microbiology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran

<sup>2</sup> Biotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>3</sup> Applied Biomedical Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

<sup>4</sup> Arthropod-Borne Diseases Research Center, Ardabil University of Medical Sciences, Ardabil, Iran



© The Author(s) 2024. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

overuse of antibiotics have led to the emergence of antibiotic resistance in *P. aeruginosa* strains, making it difficult to effectively treat these infections. The rise in antibiotic resistance among Gram-negative bacteria, which began in the 1970s, continues to be a significant challenge [7–9]. In 2017, the World Health Organization (WHO) declared antimicrobial resistance a global crisis. The organization listed 12 bacterial families as the greatest threats to human health, with carbapenem-resistant *P. aeruginosa* strains being one of the most important [10]. Carbapenem antibiotics are commonly recommended for the treatment of multidrug-resistant (MDR) *P. aeruginosa* strains [8]. However, in line with the WHO report, our previous studies have confirmed the high prevalence of carbapenem-resistant *P. aeruginosa* isolates in various cities of Iran, particularly in Ardabil in the northwest of the country [3, 11]. This challenging situation has increased the reliance on polymyxins, such as polymyxin B and colistin, which are cationic lipopeptide antibiotics and considered as the last resort for treating Gram-negative bacteria, including carbapenem-resistant *P. aeruginosa* strains that are resistant to all other available antibiotics [12]. While colistin (also known as polymyxin E) resistance has not been observed in clinical isolates of *P. aeruginosa* in Ardabil city thus far, colistin-resistant *P. aeruginosa* has been reported in other cities in Iran and worldwide. Therefore, one of the aims of this study was to investigate the resistance of *P. aeruginosa* clinical strains to colistin in Ardabil, as well as their genetic linkage. The mechanisms of polymyxin resistance in Gram-negative bacteria are not fully understood. However, they can be mediated through plasmid-encoded genes, such as the acquisition of the mobilized colistin resistance-1 (*mcr-1*) gene, or through chromosomally encoded genes, including 1) cationic modification of lipid A in lipopolysaccharides (LPS) via two-component systems (TCSs: PhoPQ and PmrAB), 2) loss of LPS, 3) overexpression of efflux pump systems and capsular polysaccharides, 4) down-regulation of porin (OprD), and 5) enzymatic inactivation of colistin [7]. Therefore, another objective of this study was to determine the most common mechanisms of colistin resistance among drug-resistant *P. aeruginosa* clinical isolates collected from patients referred to hospitals in Ardabil, Iran.

## Materials and methods

### *P. aeruginosa* clinical isolates, materials, and equipment

In this cross-sectional study conducted in Iran, a country located southwest of the Asian continent, a total of 200 *P. aeruginosa* clinical isolates were utilized. These strains were collected from various specimens, including urine ( $n=90$ ), sputum ( $n=55$ ), wound ( $n=28$ ), blood ( $n=26$ ), and cerebrospinal fluid (CSF) ( $n=1$ ), obtained from patients referred to hospitals in Ardabil city, northwest

of Iran. The hospitals included Imam Khomeini ( $n=105$ ), Alavi ( $n=55$ ), Imam Reza ( $n=25$ ), Bu-Ali ( $n=6$ ), Sabalan ( $n=6$ ), Fatemi ( $n=2$ ), and Ghaem ( $n=1$ ). The data collection period spanned from June 2019 to May 2023.

The initial identification of *P. aeruginosa* clinical isolates was performed using phenotypic standard laboratory tests, which included assessments of pigment production, colony morphology, oxidase, catalase, IMViC pattern, and Gram staining. Confirmation of the species was subsequently achieved using the polymerase chain reaction (PCR) with a specific species primer [3].

The most important materials used in our study were the Master Mix for PCR/ERIC-PCR and real-time PCR (Ampliqon, Denmark), primers (Metabion, Germany), TRIzol™ Reagent (Bio Basic, Ontario, Canada), cDNA synthesis kit (Yekta Tajhiz Azma, Tehran, Iran), Mueller Hinton agar (Conda, Pronasida, Spain), colistin sulfate salt powder (Sigma-Aldrich co, St. Louis, MO,  $\geq 15,000$  U/mg), antibiotic disks (Padtan Teb, Iran), Cetrimide agar (Conda, Pronasida, Spain), and Trypticase Soy Broth (TSB) (QUELAB/UK). Additionally, the following equipment was used: Eppendorf thermal cycler (Germany), LightCycler® System (Roche Diagnostics), NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA), Agarose Gel Electrophoresis (Padideh Nojen Pars, Iran), and ELISA microplate reader (BioTek, USA).

### Colistin agar test

The susceptibility pattern of *P. aeruginosa* clinical isolates to colistin was determined based on the agar dilution MIC (minimum inhibitory concentration) method on Mueller–Hinton agar as suggested by the Clinical and Laboratory Standards Institute (CLSI) [13]. For this purpose, 3–5 fresh *P. aeruginosa* colonies from Mueller Hinton agar plates were picked and transferred to 4–5 mL sterile saline to prepare 0.5 McFarland turbidity standards. Bacterial standard suspensions were diluted in saline (1:10). A 10  $\mu$ L of each diluted bacterial suspension was poured onto a colistin agar plate. Colistin agar plates were prepared in required dilutions, *i.e.*, 0.5–16  $\mu$ g/mL. Incubation condition and length were maintained at 37 °C for 16–18 h. *P. aeruginosa* clinical isolates with MIC values  $\geq 4$   $\mu$ g/mL were considered as resistant strains. A colistin-resistant *Acinetobacter baumannii* clinical isolate was used as the positive control (MIC = 16  $\mu$ g/mL) (Ethics ID: IR.ARUMS.REC.1400.071).

The disk diffusion method was used to determine multiple drug resistance patterns (multidrug-resistant (MDR), extremely drug-resistant (XDR), and pandrug-resistant (PDR) strains) among colistin-resistant *P. aeruginosa* clinical isolates [14]. Furthermore, this method was employed to assess the resistance rates of colistin-resistant *P. aeruginosa* isolates against various antibiotics,

including piperacillin (100 µg), piperacillin-tazobactam (100/10 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), norfloxacin (10 µg), and ofloxacin (5 µg), as per our previous study [3]. *P. aeruginosa* ATCC 27853 was used as a reference strain.

#### Biofilm formation assay

Evaluation of biofilm production among colistin-resistant *P. aeruginosa* isolates was performed by a colorimetric assay [15]. For this aim, of the 1:100 diluted suspensions of *P. aeruginosa* isolates which had grown in TSB mediums, 150 µL were inoculated into a sterile 96-well flat bottom plate and incubated at 37 °C for 24 h. The plate was washed with 200 µL of phosphate-buffered saline (PBS) (pH ~7.4) three times. Biofilm fixation was done with 100 µL of methanol (99%) for 15 min and then the wells were air-dried. 150 µL of crystal violet stain (1%) was added to wells for 20 min, unbound stain washed with water, and then bound stain released through 150 µL of acetic acid (33%). ELISA microplate reader was used to measure the optical density (OD) of wells at 590 nm. Colistin-resistant *P. aeruginosa* isolates were divided into four categories including no biofilm producer if the OD of a strain (ODs) was less or equal to the OD negative control (ODc), weak biofilm producer if  $ODc < ODs < 2 \times ODc$ , moderate biofilm producer if  $2 \times ODc < ODs < 4 \times ODc$ , and strong biofilm producer if  $4 \times ODc < ODs$ . *P. aeruginosa* ATCC 27853 and sterile TSB medium were used as positive and negative controls, respectively. All experiments are performed in triplicate.

#### Detection of colistin resistance genes

Molecular identification of the genes encoding efflux pumps (i.e., *mexA*, *mexC*, *mexE*, and *mexY* genes), TCSs (i.e., *phoP*, *phoQ*, *pmrA*, and *pmrB* genes), outer membrane porin (*oprD* gene), and *mcr-1* gene, as well as genes involved in biofilm formation of *P. aeruginosa* (i.e., *algD*, *pslD*, *pelF*, *Pppl*, and *PAPI-1* genes) were performed by the PCR method. Used primers along with the PCR program for the detection of each gene were listed in Table 1. In brief, genomic DNA was extracted from the 200 *P. aeruginosa* clinical isolates by the boiling method [3] and confirmed by a spectrophotometer. Amplification of the genes was performed in a final volume of 15 µL (10 µL of Master Mix, with 3 µL of template DNA (50 ng/µL), and 2 µL of primers (10 µmol/L)) and then their presence was confirmed using the agarose gel electrophoresis and sequencing (Sanger method, Pishgam, Iran) techniques. It is worth mentioning that for some genes with non-specific bands on agarose gel,

the values mentioned above along with the PCR conditions were changed. Finally, we employed the enterobacterial repetitive intergenic consensus (ERIC)-PCR method to assess the genetic relatedness among colistin-resistant *P. aeruginosa* isolates. In pursuit of this, amplification reactions were conducted in a final volume of 50 µL using the primers and ERIC-PCR program outlined in Table 1. Subsequently, the ERIC-PCR products were electrophoresed on a 2% agarose gel, and the resulting band patterns were analyzed using the Dice distance method for similarity and the UPGMA analysis method for clustering (GelQuest software version 3.3.5.0). ERIC-PCR band patterns exhibiting >80% similarity were categorized as belonging to the same cluster [5].

#### Mutational analysis of the PhoPQ and PmrAB

Detection of colistin resistance-associated mutations among *P. aeruginosa* clinical isolates with multiple drug resistance was performed using the sequencing method. The PCR products of the *phoQ* and *pmrB* genes from *P. aeruginosa* clinical isolates MDR, XDR, and resistant to colistin were sent for sequencing. The nucleotide sequences were compared with colistin-susceptible *P. aeruginosa* reference strain ATCC 27853 using the BioEdit software (version 7.2.5). Additionally, an online data analysis service (available at <https://web.expasy.org/translate/>) was utilized to assess amino acid alterations.

#### Expression of the genes encoding efflux pumps, TCSs, and outer membrane porin

Expression levels of the *mexA*, *mexC*, *mexE*, *mexY*, *phoP*, *pmrA*, and *oprD* genes were determined among resistant isolates using the quantitative reverse transcription PCR (qRT-PCR) and specific sets of primers (Table 1). In brief, the total RNA of colistin-resistant *P. aeruginosa* isolates was extracted using the TRIzol™ Reagent. After confirming the quality and quantity of extracted RNAs (1 µg), cDNA synthesis was done according to the manufacturer's instructions. The qRT-PCR of the genes was carried out under conditions presented in Table 1 and in a final volume of 15 µL (SYBR Green PCR Master Mix (7 µL), primers (2 µL, 10 µmol/L), cDNA (1 µg/µL), and DEPC-treated water (5 µL)). The 30S ribosomal *rpsL* gene was used as the normalizing gene. The expression levels of genes in colistin-resistant *P. aeruginosa* isolates were determined relative to their expression levels in *P. aeruginosa* ATCC 27853 using the  $2^{-\Delta\Delta C_t}$  method. Expression for each gene was assessed in duplicate.

Interpretation of the results of qRT-PCR was performed as follows: for the *mexA* and *mexC* genes; two-fold, for the *mexE* gene; tenfold, and for the *mexY* gene; fourfold expression rates compared with the reference strain of *P. aeruginosa* ATCC 27853 were considered as

**Table 1** Used primers along with PCR/ERIC-PCR and qRT-PCR programs

Gene	Oligonucleotide sequence (5' to 3')	PCR/ERIC-PCR condition	Amplicon size (bp)	qRT-PCR condition	Reference
<i>mexA</i>	F: CCTGCTGGTCGCGAATTTGGG R: CCAGCAGCTTGAGCGCTGG	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 64 °C for 30 sec Extension at 72 °C for 1 min 34 cycles	332	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 64 °C for 20 sec (40 cycles) 72 °C for 30 sec	[16]
<i>mexC</i>	F: TTGGCTATGGCCATCGGTT R: ATCGAAGTCTGTGGCTGA	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 59 °C for 30 sec Extension at 72 °C for 1 min 34 cycles	390	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 59 °C for 20 sec (40 cycles) 72 °C for 30 sec	[16]
<i>mexE</i>	F: ATCCCACTTCTCTGGCGCT R: GGTGGCCTTTCTTCAACAGT	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 59 °C for 30 sec Extension at 72 °C for 1 min 34 cycles	260	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 59 °C for 20 sec (40 cycles) 72 °C for 30 sec	[16]
<i>mexY</i>	F: CCGCTACAACGGGCTATCCCT R: AGCGGATGACCCAGCTTTC	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 62 °C for 30 sec Extension at 72 °C for 1 min 34 cycles	246	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 62 °C for 20 sec (40 cycles) 72 °C for 30 sec	[16]
<i>oprD</i>	F: CGACCTGTGCTCCGCAACTA R: TTGCATCTGCCCCACTTCAG	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 57 °C for 30 sec Extension at 72 °C for 1 min 34 cycles	301	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 64 °C for 20 sec (40 cycles) 72 °C for 30 sec	[17]
<i>rpsL</i>	F: GCTGCAAAACTGCCCGCAAG R: ACCGCAGGTGCCAGGGAAC	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 72 °C for 1 min 34 cycles	250	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 64 °C for 20 sec (40 cycles) 72 °C for 30 sec	[16]
<i>PAP1-1</i>	F: CATCAACCGGATCGACGAAGT R: GTCAACCTCTGATCCAAAAGTT	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 60 °C for 1 min Extension at 72 °C for 1 min 34 cycles	462	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 64 °C for 20 sec (40 cycles) 72 °C for 30 sec	[18]
<i>pelF</i>	F: GAGGTACAGTACATCCGTGG R: TCATGAATCTCCGTGGCTT	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 58 °C for 1 min Extension at 72 °C for 1 min 34 cycles	789	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 64 °C for 20 sec (40 cycles) 72 °C for 30 sec	[18]
<i>pslD</i>	F: TGTAGACCGTGCTCAACGAC R: CTTCCGGCCGATCTTCATC	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min Annealing at 60 °C for 1 min Extension at 72 °C for 1 min 34 cycles	369	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 64 °C for 20 sec (40 cycles) 72 °C for 30 sec	[18]

**Table 1** (continued)

Gene	Oligonucleotide sequence (5' to 3')	PCR/ERIC-PCR condition	Amplicon size (bp)	qRT-PCR condition	Reference
<i>PpgI</i>	F: GTGGTGGGGACTATACGAA R: GTAGTTGGCGACGAACAGGTA	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min } Annealing at 59 °C for 1 min } 34 cycles Extension at 72 °C for 1 min }	327		[18]
<i>algD</i>	F: CGTCTGCCCGCAGATCGGCT R: GACCTCGACGGTCTTGCGGA	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min } Annealing at 63 °C for 1 min } 34 cycles Extension at 72 °C for 1 min }	313		[3]
<i>phoP</i>	F: TTGGCCACCACCTCTATAC R: GAAGTGAACGGCTTGACC	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 59 °C for 20 sec (40 cycles) 72 °C for 30 sec }	282	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min } Annealing at 58 °C for 1 min } 34 cycles Extension at 72 °C for 1 min }	(This study)
<i>phoQ</i>	F: GCAACGAATTCACACCAC R: GAATGTCACAGGCTCAGTTC	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min } Annealing at 60 °C for 1 min } 34 cycles Extension at 72 °C for 1 min }	964		(This study)
<i>pmrA</i>	F: GACCAAGCCCTTCGATCTC R: AGGTGGTGGACGTGGACTT	Pre-incubation at 95 °C for 600 sec (1 cycle) Amplification { 95 °C for 20 sec 60 °C for 20 sec (40 cycles) 72 °C for 30 sec }	294	Initial denaturation at 94 °C for 5 min (1 cycle) Denaturation at 94 °C for 30 sec } Annealing at 65 °C for 30 sec } 30 cycles Extension at 72 °C for 30 sec }	(This study)
<i>pmrB</i>	F: CCTACCACCTTCGCTGAAG R: GAAGTGCAGTTCGACGATGC	Initial denaturation at 94 °C for 5 min (1 cycle) Denaturation at 94 °C for 30 sec } Annealing at 65 °C for 30 sec } 22 cycles Extension at 72 °C for 30 sec }	1211		[19]
<i>mcr-1</i>	F: CGGTGAGTCGTTTGTTTC R: CTGTGGTCTGTAGGG	Initial denaturation at 95 °C for 5 min (1 cycle) Denaturation at 94 °C for 1 min } Annealing at 54 °C for 1 min } 34 cycles Extension at 72 °C for 1 min }	309		[19]
<i>ERIC</i>	F: ATGTAAGCTCCTGGGGATTAC R: AAGTAAGTACTGGGGTGAGCG	Initial denaturation at 94 °C for 5 min (1 cycle) Denaturation at 91 °C for 1 min } Annealing at 25 °C for 2 min } 40 cycles Extension at 72 °C for 2 min }	100–1700		[20]

overexpression [16]. For the *oprD* gene, the expression rate  $\leq 30\%$  relative to the reference strain was considered as down-regulation [17]. In addition, for the *phoP* and *pmrA* genes, expression levels higher than those of *P. aeruginosa* ATCC 27853 were considered as increased gene expression [12].

## Result

Among 200 *P. aeruginosa* clinical isolates obtained from hospitalized patients, 18 isolates (9%) were resistant to colistin antibiotic according to the agar dilution method. Characteristics of these 18 colistin-resistant *P. aeruginosa* isolates were presented in Table 2. In addition, MIC values of colistin for clinical isolates of *P. aeruginosa* are reported in Fig. 1. Among 200 *P. aeruginosa* strains, MIC values of 0.5, 1, 2, and 4  $\mu\text{g}/\text{mL}$  were found in 2 (1%), 125 (62.5%), 55 (27.5%), and 18 (9%) isolates, respectively. All colistin-resistant isolates showed MIC = 4  $\mu\text{g}/\text{mL}$ .

Of the 18 colistin-resistant *P. aeruginosa* strains, 6 strains showed multiple drug resistance patterns (4 MDR and 2 XDR) in the disk diffusion method. The susceptibility patterns of colistin-resistant *P. aeruginosa* strains to various antibiotics along with their virulence gene profiles are presented in Table 3. The resistance rates of 18 colistin-resistant *P. aeruginosa* isolates to different antibiotics were as follows: piperacillin 11.1%, piperacillin-tazobactam 5.5%, ceftazidime 11.1%, cefepime 11.1%, aztreonam 0%, imipenem 38.9%, meropenem 11.1%, gentamicin 11.1%, tobramycin 11.1%, amikacin 5.5%, ciprofloxacin 50%, levofloxacin 50%, norfloxacin 50%, and ofloxacin 55.5%.

A high genetic diversity was observed among the 18 colistin-resistant *P. aeruginosa* clinical isolates by detecting 15 different ERIC-PCR band patterns (Fig. 2). Among tested strains, 15 colistin-resistant *P. aeruginosa* exhibited unique genotypes (subgroup), while genotype subgroup 6 comprised three isolates. Details of band patterns for each species are depicted in Supplementary Figure S1.

As shown in Table 3, colistin-resistant *P. aeruginosa* strains were carrying the genes encoding resistance to other antibiotics such as metallo- $\beta$ -lactamase (*IMP* gene), AmpC cephalosporinase, extended-spectrum  $\beta$ -lactamase (*TEM* and *PSE* genes), oxacillinase (*oxa-2* and *oxa-23* genes), and efflux pumps (*qacE $\Delta$ 1*, *qacE*, *qacG*, and *cepA* genes). These genes are involved in the emergence of MDR and XDR *P. aeruginosa* strains.

Biofilm formation was identified in all 18 colistin-resistant *P. aeruginosa* isolates in the colorimetric assay. Among them, 7 (38.9%) isolates were weak biofilm producers, 8 (44.4%) isolates were moderate biofilm producers, and 3 (16.7%) isolates were strong biofilm producers.

In addition, the presence of genes encoding biofilm (*i.e.*, *algD*, *pslD*, *pelF*, *Ppgl*, and *PAPI-1* genes) was detected in all 18 colistin-resistant *P. aeruginosa* isolates in the PCR and confirmed by sequencing. The GenBank accession numbers for our nucleotide sequences of detected genes in this study are OR855380 to OR855387.

The role of the *phoQ* and *pmrB* gene mutations in the emergence of colistin-resistant *P. aeruginosa* was evaluated in 6 MDR and XDR clinical isolates. Analysis of the *phoQ* gene revealed the following nucleotide substitutions: at positions 473 C  $\rightarrow$  T, 495 G  $\rightarrow$  A, 552 T  $\rightarrow$  C, 583 T  $\rightarrow$  C, 675 T  $\rightarrow$  G, 702 A  $\rightarrow$  G, 1110 C  $\rightarrow$  T, 1146 C  $\rightarrow$  T, and 1155 C  $\rightarrow$  T. None of these nucleotide substitutions led to amino acid alterations. Furthermore, similar results were observed for the *pmrB* gene and nucleotide changes did not result in amino acid substitutions except for Tyr345His. Nucleotide substitutions of the *pmrB* gene were as follows: G at position 507 to A, G at position 639 to A, A at position 645 to G, G at position 690 to C, T at position 696 to C, A at position 750 to G, A at position 762 to G, C at position 891 to T, and T at position 1033 to C.

Expression levels of the *mexA*, *mexC*, *mexE*, *mexY*, *phoP*, and *pmrA* genes in 18 colistin-resistant *P. aeruginosa* strains were as follows: 88.8% ( $n=16$ ), 94.4% ( $n=17$ ), 11.1% ( $n=2$ ), 83.3% ( $n=15$ ), 83.3% ( $n=15$ ), and 38.8% ( $n=7$ ), respectively. In addition, down-regulation of the *oprD* gene was observed in 44.4% ( $n=8$ ) of colistin-resistant *P. aeruginosa* strains. The presence of a plasmid-borne *mcr-1* gene and its association with colistin resistance in *P. aeruginosa* strains was not confirmed in this study.

## Discussion

In recent years, there has been a significant reduction in the susceptibility of *P. aeruginosa* to polymyxins, despite its inherent sensitivity to these antibiotics [21]. The current study reported, for the first time, a prevalence of 9% for colistin-resistant *P. aeruginosa* in Ardabil city. Other studies conducted in different regions of Iran and abroad have reported varying rates of colistin resistance among *P. aeruginosa* isolates. These include studies by Abd El-Baky et al. in Egypt (21.3%) [22], Rossi et al. in Brazil (6.3%) [23], Wi et al. in South Korea (7.4%) [24], Zarate et al. in Peru (7.2%) [25], Farajzadeh Sheikh et al. in Iran (Ahvaz, Tehran, and Isfahan) (1.3%) [26], Goli et al. in Iran (Tabriz) (2%) [27], Heidari et al. in Iran (Isfahan and Shiraz) (7%) [28], Malekzadegan et al. in Iran (Shiraz) (0%) [29], Tahmasebi et al. in Iran (Hamadan) (3.9%) [30], and Talebi et al. in Iran (Tehran) (0%) [19].

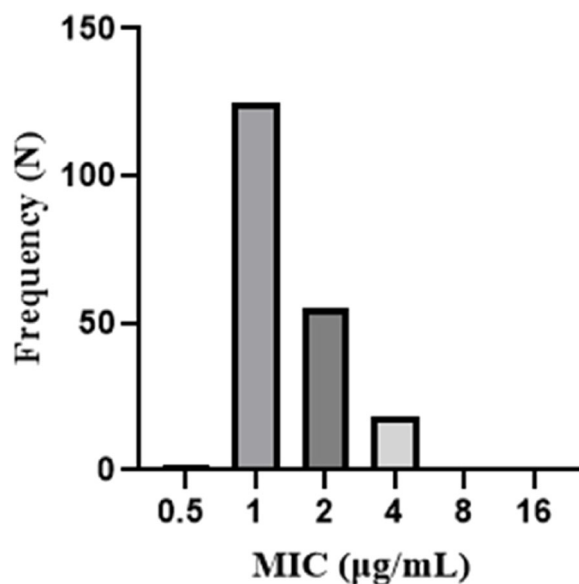
The variations in colistin resistance rates among *P. aeruginosa* isolates in different regions can be attributed

**Table 2** Mechanisms of colistin resistance among 18 colistin-resistant *P. aeruginosa* clinical isolates

Isolate number	Type of specimen	Hospital	Resistance type			Resistance mechanisms			Efflux pumps overproduction			
			Resistance type	Biofilm production	Amino acid alteration	Two-component systems overproduction		Porin down-regulation	<i>mexA</i>	<i>mexC</i>	<i>mexE</i>	
						<i>phoP</i>	<i>pmrA</i>					<i>phoQ</i>
8	Blood	Imam Khomeini	Moderate		No change	4.4	45.5	2.2	14.5	88.6	6.7	15.7
24	Urine	Alavi	Weak	XDR	Tyr345His	1.8	0.0	0.0	4.1	41.3	0.0	6.8
45	Urine	Bu-Ali	Weak			5.7	0.0	0.2	6.4	55.7	0.0	9.3
46	Blood	Bu-Ali	Weak			4.1	0.0	0.4	6.9	71.5	0.0	12.7
51	Urine	Alavi	Weak			3.5	0.0	0.3	2.8	15.7	0.0	6.1
71	Urine	Alavi	Strong			14.6	64.8	5.5	14.7	136.2	10	19
72	Urine	Imam Reza	Weak	MDR	Tyr345His	1	0.0	0.0	0.7	5.2	0.0	1
76	Urine	Imam Reza	Weak			1.5	0.0	0.6	5	67.1	0.0	9.9
79	Urine	Imam Reza	Moderate	MDR	Tyr345His	6.6	0.0	0.0	9	55.7	0.0	23.9
80	Wound	Imam Khomeini	Strong	MDR	Tyr345His	9.6	0.0	0.5	68.1	119.4	0.0	10.3
81	Urine	Imam Khomeini	Moderate	MDR	Tyr345His	4.7	0.0	0.9	63.1	51.9	0.0	3.9
82	Urine	Imam Reza	Moderate			14.5	18.7	60.5	35.7	34.2	8	30.9
89	Urine	Imam Khomeini	Strong			8.8	45.8	0.4	36.2	89.8	4	21.4
91	Urine	Imam Khomeini	Moderate			3.5	11.5	0.0	10.7	29.8	0.0	6.7
92	Urine	Imam Khomeini	Moderate			4.3	0.0	6.1	5.3	28.4	0.0	11.1
117	Urine	Sabalan	Moderate			1	33.3	0.0	1.2	0.4	0.0	1.6
141	Sputum	Imam Reza	Moderate	XDR	Tyr345His	1	6.4	0.1	2.2	24.2	3	3.4
143	Wound	Imam Khomeini	Weak			12	0.0	0.7	22.1	151.1	11.7	40.7

*P. aeruginosa* ATCC 27853

<sup>a</sup> Overexpressed values *phoP*, *pmrA*, *mexA*, *mexC*, *mexE*, *mexY*, and down-regulated values of *oprD* were indicated in bold



**Fig. 1** MIC values of colistin for 200 clinical isolates of *P. aeruginosa*

to several factors. These include differences in sample size, methods used for antimicrobial susceptibility testing, and specific local factors. In the case of Ardabil, the higher rate of colistin resistance compared to other cities in Iran may be attributed to three main factors. Firstly, the use of colistin in veterinary medicine for the growth promotion of food-producing animals, as Ardabil is an agricultural and animal husbandry province [31]. This practice can contribute to the selection and spread of colistin-resistant strains. Secondly, the misuse of antibiotics in intensive care units, which can lead to the emergence of resistant strains [22]. It's worth noting that Iran has been reported to have the second-highest antibiotic consumption rate in the world, with antibiotic consumption 16 times higher than the global standard (mehrnews.com/xYQDx), as stated by the secretary of the Infectious Diseases Association of Iran. Thirdly, while the transmission of colistin-resistant *P. aeruginosa* within hospitals or across the entire health-care system has been a concern [32], our investigation revealed no genetic correlation among colistin-resistant strains collected from different hospitals in Ardabil city. However, we did observe three strains in genotype subgroup 6 that were shared between two distinct hospitals (Fig. 2). A similar finding was reported by Khosravi et al. for MDR *P. aeruginosa* isolates in Ahvaz [33]. In contrast, Zarei et al. identified clonal relatedness between clinical and environmental *P. aeruginosa* isolates [20].

Regarding the emergence of colistin-resistant *P. aeruginosa* strains in Ardabil hospitals, various bacterial factors

may be involved. One such factor is biofilm formation. A biofilm is a bacterial population encased in an outer polymer layer, consisting of host immune system products or bacterial secreted polymers like exopolysaccharides (EPS), extracellular DNA, and proteins. Biofilms play a role in acute burn wound infections [1, 34]. Studies have shown that extracellular DNA within the biofilm, along with factors such as magnesium or calcium starvation, low pH, and antimicrobial peptides (including colistin), can contribute to polymyxin resistance through modifications in LPS via the activation of TCSs like PmrAB and PhoPQ [34, 35]. Our study's findings are consistent with the previous research discussed above. We observed that all colistin-resistant strains of *P. aeruginosa* in our study were capable of producing biofilms, as indicated in Table 2. This ability poses a significant challenge in the treatment of *P. aeruginosa* infections, particularly those affecting wounds. Furthermore, the presence and excessive production of alginate EPS within the biofilm provide *P. aeruginosa* with protection against phagocytic cells and antibiotic treatments [34]. Apart from the *algD* gene, which encodes alginate, we identified the presence of other genes associated with biofilm formation (*pslD*, *pelF*, *PpgI*, and *PAPI-1*) in all colistin-resistant *P. aeruginosa* isolates. However, the prevalence of these chromosomal genes differed from a study conducted by Rajabi in Iran, where the respective prevalence rates were as follows: *algD* 78.6%, *pelF* 70.5%, *pslD* 36.6%, *PpgI* 0%, and *PAPI-1* 77.6% [18]. Notably, the presence of the *PAPI-1* gene in all *P. aeruginosa* isolates in our study supports the findings of Qiu et al., which suggest that this large pathogenicity island can be transmitted between *P. aeruginosa* strains [36].

Modifications of the negatively charged phosphate groups of lipid A through adding phosphoethanolamine mediated by the *mcr* gene can lead to polymyxins resistance [35]. However, our study did not find evidence of the involvement of this plasmid-borne gene in the emergence of colistin-resistant *P. aeruginosa* strains. Similar results were obtained in Tabriz, Iran, where all colistin-resistant Gram-negative isolates, including *P. aeruginosa* strains, tested negative for *mcr* genes [37]. Additionally, another study conducted in Ardabil on clinical isolates of colistin-resistant *A. baumannii* also demonstrated the absence of this gene (data not published). One possible explanation for the 0% prevalence of the *mcr-1* gene in our study is its association with *Enterobacteriaceae*, particularly *Escherichia coli*, which are resistant to colistin and commonly isolated from animal sources [37].

Multiple studies have confirmed that mutations in the components of TCSs, namely PmrB and PhoQ proteins, play a significant role in the development of polymyxin resistance in *P. aeruginosa* strains. These mutations result

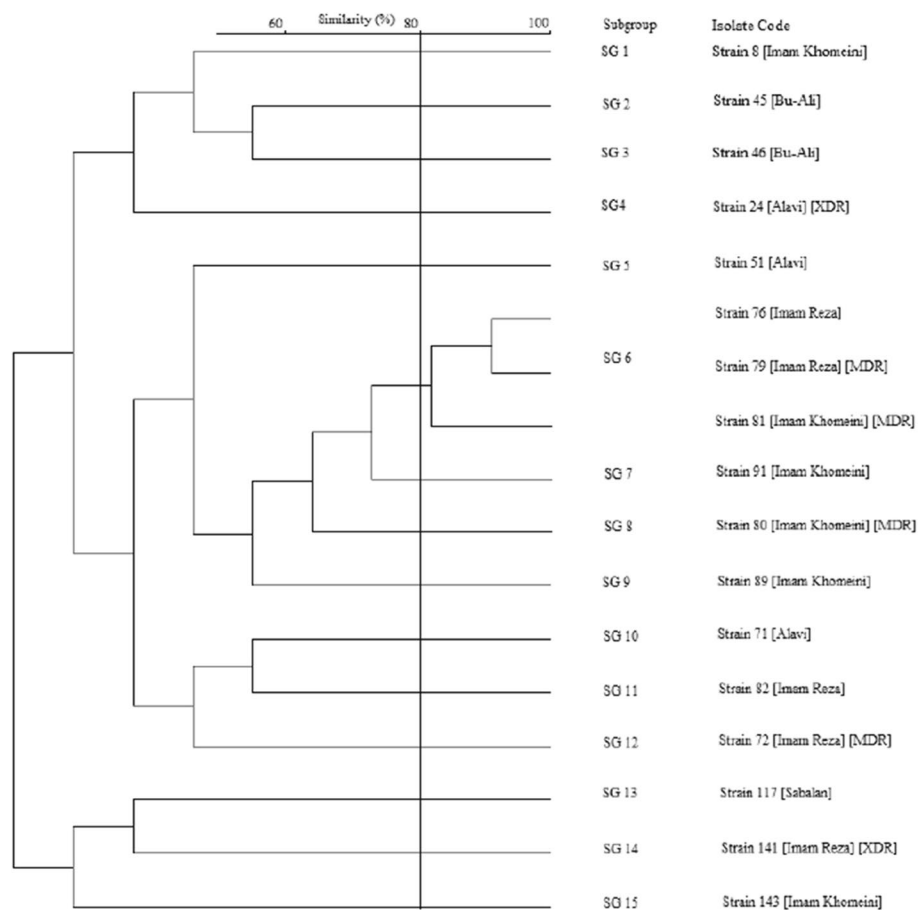


**Table 3** Profiles of antibiotic resistance and virulence genes of 18 colistin-resistant *P. aeruginosa* clinical isolates

Isolate number	Antibiotic resistance profiles <sup>a</sup>														Virulence gene profiles <sup>a</sup>			
	Phenotypic																	
	Combined test																	
Colistin agar test MIC (µg/mL)	PIP	TZP	CAZ	FEP	ATM	IMP	MEM	GEN	TOB	AMK	CIP	LXV	NOR	OFX	MDR/XDR/TDR	Genotypic		
8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	AmpC	<i>qacED1, fabV, cepA</i>	<i>algD, plcN, lasB, plcH, exoS, toxA, pilB</i>
24	R	I	R	R	I	R	R	R	R	R	R	R	R	R	R	MBL	<i>gyrA, gyrB, parE, parC, qacED1, IMP, TEM, oxa-2, oxa-10, int1-1</i>	<i>algD, plcN, lasB, plcH, toxA, exoU</i>
45	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	AmpC	<i>qacED1, fabV, cepA, oxa-2, TEM</i>	<i>algD, plcN, lasB, plcH, toxA, exoU</i>
46	S	S	S	S	S	S	S	S	S	I	S	S	S	S	S	AmpC	<i>fabV, cepA, oxa-2, TEM</i>	<i>algD, plcN, lasB, plcH, exoS, toxA, exoU</i>
51	I	S	S	S	S	I	S	S	S	I	S	S	S	S	S	AmpC	<i>qacED1, fabV, cepA</i>	<i>algD, plcN, lasB, plcH, exoS, toxA, exoU</i>
71	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	MBL	<i>oxa-23</i>	<i>algD, plcN, lasB, plcH, exoS, toxA, exoU, pilB</i>
72	S	S	S	S	S	R	S	S	S	S	S	R	R	R	MDR	MBL	<i>qacED1, qacE, fabV, cepA, oxa-2, PSE, int1-1</i>	<i>algD, plcN, lasB, plcH, toxA, exoU, pilB</i>
76	I	S	S	S	S	I	S	S	S	S	S	R	R	R	R	AmpC	<i>oxa-23, PSE, int1-1</i>	<i>algD, plcN, lasB, plcH, toxA, exoU, pilB</i>
79	I	I	I	S	S	R	S	S	S	S	S	R	R	R	MDR	MBL AmpC ESBL	<i>qacED1, qacE, qacG, fabV, cepA, oxa-2, oxa-23, int1-1</i>	<i>algD, plcN, lasB, plcH, toxA, exoU, pilB</i>
80	S	S	S	S	S	R	S	S	S	S	S	R	R	R	MDR	MBL AmpC	<i>fabV, cepA, oxa-2, oxa-23, PSE, int1-1</i>	<i>algD, plcN, lasB, plcH, toxA, exoU, pilB</i>
81	S	S	S	S	I	R	S	S	S	I	R	R	R	R	MDR	MBL AmpC	<i>oxa-2, oxa-23, PSE, int1-1</i>	<i>algD, plcN, lasB, plcH, toxA, pilB</i>
82	I	S	S	S	I	S	S	S	S	S	S	S	S	S	S	AmpC	<i>oxa-23, PSE</i>	<i>algD, plcN, lasB, plcH, exoS, toxA, pilB</i>
89	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S		<i>oxa-2, oxa-23</i>	<i>algD</i>
91	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R		<i>oxa-2, oxa-10, oxa-23, PSE, int1-1</i>	<i>algD</i>
92	S	S	S	S	S	I	S	S	S	S	S	R	R	R	R	AmpC	<i>oxa-2, oxa-10, oxa-23, PSE, int1-1</i>	<i>algD</i>
117	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S	AmpC	<i>oxa-2, PSE</i>	<i>algD</i>
141	I	I	R	R	I	R	R	R	R	S	R	R	R	R	R	ND	ND	<i>algD</i>
143	R	R	S	I	S	I	I	S	S	S	S	S	S	S	I	ND	ND	<i>algD</i>

Abbreviations: PIP Piperacillin, TZP Piperacillin-Tazobactam, CAZ Ceftazidime, FEP Cefepime, ATM Aztreonam, IMP Imipenem, MEM Meropenem, GEN Gentamicin, TOB Tobramycin, AMK Amikacin, CIP Ciprofloxacin, LXV: Levofloxacin, NOR: Norfloxacin, OFX Ofloxacin, R: Resistant, S Susceptible, I Intermediate, ESBL Extended-spectrum β-lactamase, AmpC AmpC cephalosporinase, ND Not determined

<sup>a</sup> Presented data on profiles of antibiotic resistance and virulence genes are based on previous studies



**Fig. 2** Dendrogram of colistin-resistant *P. aeruginosa* clinical isolates based on the ERIC-PCR band patterns

in the upregulation of the *arnBCADTEF* operon, leading to the substitution of phosphate groups of lipid A with the cationic 4-amino-4-deoxy-L-arabinose in the LPS structure [38]. Several different mutations have been reported in various studies; however, in our study, we observed only the amino acid alteration Tyr345His in the sensor kinase protein PmrB, a component of the PmrAB TCS. This amino acid alteration has also been reported in other studies conducted by Barrow et al. [39], Sellera et al. [40], Lee et al. [41], and Schurek et al. [42]. It appears that the Tyr345His substitution in the PmrB protein is not involved in the activation of the response regulator PmrA through phosphorylation in colistin-resistant *P. aeruginosa* strains isolated from hospitals in Ardabil. As shown in Table 2, except for strain 141, colistin-resistant *P. aeruginosa* strains containing the Tyr345His mutation did not exhibit overproduction of the *pmrA* gene. In our study, nucleotide substitutions in the *phoQ* gene did not result in amino acid alterations. Therefore, similar to the *pmrB* gene, there is no association between mutations in the *phoQ* gene and subsequent overproduction of the *phoP* gene in the emergence of colistin-resistant

*P. aeruginosa* strains. The overproduction of *PmrB* and *PhoP* genes among colistin-resistant *P. aeruginosa* strains may be attributed to factors other than mutations, such as low levels of magnesium or calcium, low pH, and antimicrobial peptides [34, 35].

Table 2 provides evidence that the development of colistin resistance in *P. aeruginosa* strains in Ardabil hospitals is a result of multiple factors. Previous reports have suggested that the ParRS TCS in *P. aeruginosa* is also involved in the emergence of polymyxin-resistant strains by down-regulating the expression of the porin protein OprD [43]. In this study, this resistance mechanism was confirmed in 44.4% of colistin-resistant *P. aeruginosa* strains. Furthermore, mutations in the ParRS TCS lead to low to moderate levels of resistance to polymyxins by enhancing the production of the MexXY/OprM efflux pump [44]. It is noteworthy that a significant production of efflux pumps, compared to other resistance mechanisms, was observed in 18 colistin-resistant *P. aeruginosa* strains in the current study: MexAB-OprM 88.8%, MexCD-OprJ 94.4%, MexEF-OprN 11.1%, and MexXY-OprM 83.3%. Goli et al. also demonstrated increased

expression of genes encoding the MexAB-OprM and MexXY-OprM efflux pumps in two colistin-resistant *P. aeruginosa* strains [27].

### Limitation of the study

In the current research, there were the following limitations due to insufficient resources: 1) the mutations in the *pmrB* and *phoQ* genes were not assessed in all colistin-resistant *P. aeruginosa* isolates, 2) the role of other TCSs (such as ParRS) in the emergence of polymyxin-resistant strains was not studied. And, 3) all variants of *mcr* (including *mcr-2* to *-9*) gene were not investigated.

### Conclusion

The detection of colistin resistance among clinical isolates of *P. aeruginosa* in Ardabil hospitals, higher than in other cities in Iran, is a significant finding. Our study suggests that this resistance can be attributed to various mechanisms, including amino acid alterations in TCSs, overproduction of TCSs, down-regulation of porin, and overproduction of efflux pumps. These results indicate that there may be insufficient infection control measures in Ardabil hospitals and a potential issue with the indiscriminate use of colistin in both humans and animals, which can complicate the treatment of *P. aeruginosa* infections. Therefore, it is recommended to avoid the unnecessary use of this antibiotic to prevent the potential increase in colistin resistance in the future.

### Abbreviations

<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
ATCC	American Type Culture Collection
CLSI	Clinical and Laboratory Standards Institute
WHO	World Health Organization
MDR	Multidrug-Resistant
XDR	Extremely Drug-Resistant
PDR	Pandrug-Resistant
ICUs	Intensive Care Units
MIC	Minimum Inhibitory Concentration
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
LPS	Lipopolysaccharide
TCSs	Two-Component Systems
PBS	Phosphate-Buffered Saline
OD	Optical Density
ESBL	Extended-spectrum $\beta$ -lactamase
MBL	Metallo- $\beta$ -lactamase
AmpC	AmpC cephalosporinase
PIP	Piperacillin
TZP	Piperacillin-Tazobactam
CAZ	Ceftazidime
FEP	Cefepime
ATM	Aztreonam
IMP	Imipenem
MEM	Meropenem
GEN	Gentamicin
TOB	Tobramycin
AMK	Amikacin

CIP	Ciprofloxacin
LVX	Levofloxacin
NOR	Norfloxacin
OFX	Ofloxacin
R	Resistant
S	Susceptible
I	Intermediate
ND	Not determined

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12866-024-03309-1>.

#### Supplementary Material 1.

### Acknowledgements

The authors wish to thank Meysam Manouchehrifard for his help in ERIC-PCR analysis and Dr. Roghayeh Teimourpour for kindly providing the colistin-resistant *Acinetobacter baumannii* strain, our colleagues at Ardabil University of Medical Sciences, Iran.

### Authors' contributions

SJR: Methodology, Investigation, and Formal analysis. MN: Methodology, and Investigation. MA: Conceptualization, Methodology, Review, and Editing. HPD: Review, and Editing. AS: Review, and Editing. FK: Conceptualization, Supervision, Project administration, and Original draft preparation.

### Funding

This research has been financially supported by the Vice Chancellor for Research (grant number: 401000409), Ardabil University of Medical Sciences, Iran.

### Availability of data and materials

The datasets generated and analyzed during the current study are available in the NCBI GenBank repository, under the accession numbers: OR855380 to OR855387

### Declarations

#### Ethics approval and consent to participate

This research has been approved by the Regional Research Ethics Committee (approval ID: IR.ARUMS.MEDICINE.REC.1401.111). All methods were carried out according to relevant guidelines and regulations. Clinical isolates were collected from the hospital's bacterial repository solely for research purposes, and neither patient samples nor patient data were utilized in this study. Therefore, the requirement for informed consent from participants was waived by the Regional Research Ethics Committee of Ardabil University of Medical Sciences.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no competing interests.

Received: 20 December 2023 Accepted: 17 April 2024

Published online: 03 May 2024

### References

- Jabalameli F, Mirsalehian A, Khoramian B, Aligholi M, Khoramrooz SS, Asadollahi P, Taherikalani M, Emameini M. Evaluation of biofilm production and characterization of genes encoding type III secretion system among *Pseudomonas aeruginosa* isolated from burn patients. *Burns*. 2012;38(8):1192–7.
- Namaki M, Habibzadeh S, Vaez H, Arzanlou M, Safarirad S, Bazghandi SA, Sahebkar A, Khademi F. Prevalence of resistance genes to biocides in

- antibiotic-resistant *Pseudomonas aeruginosa* clinical isolates. *Mol Biol Rep*. 2022;49(3):2149–55.
3. Bazghandi SA, Arzanlou M, Peeridogaheh H, Vaez H, Sahebkar A, Khademi F. Prevalence of virulence genes and drug resistance profiles of *Pseudomonas aeruginosa* isolated from clinical specimens. *Jundishapur J Microbiol*. 2021;14(8):e118452.
  4. Hasanpour F, Ataei N, Sahebkar A, Khademi F. Distribution of Class A Extended-Spectrum  $\beta$ -Lactamases Among *Pseudomonas aeruginosa* Clinical Strains Isolated from Ardabil Hospitals. *Jundishapur J Microbiol*. 2023;16(4):e135726.
  5. Khademi F, Maarofi K, Arzanlou M, Peeri-Dogaheh H, Sahebkar A. Which missense mutations associated with DNA gyrase and topoisomerase IV are involved in *Pseudomonas aeruginosa* clinical isolates resistance to ciprofloxacin in Ardabil? *Gene Rep*. 2021;24:101211.
  6. Safarirad S, Arzanlou M, Mohammadshahi J, Vaez H, Sahebkar A, Khademi F. Prevalence and characteristics of metallo-beta-lactamase-positive and high-risk clone ST235 *Pseudomonas aeruginosa* at Ardabil hospitals. *Jundishapur J Microbiol*. 2021;14(3):e115819.
  7. El-Sayed Ahmed MA, Zhong LL, Shen C, Yang Y, Doi Y, Tian GB. Colistin and its role in the Era of antibiotic resistance: an extended review (2000–2019). *Emerg Microbes Infect*. 2020;9(1):868–85.
  8. Khademi F, Ashrafi SS, Neyestani Z, Vaez H, Sahebkar A. Prevalence of class I, II and III integrons in multidrug-resistant and carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates. *Gene Rep*. 2021;25:101407.
  9. Neyestani Z, Khademi F, Teimourpour R, Amani M, Arzanlou M. Prevalence and mechanisms of ciprofloxacin resistance in *Escherichia coli* isolated from hospitalized patients, healthy carriers, and wastewaters in Iran. *BMC Microbiol*. 2023;23(1):191.
  10. World Health Organization. WHO publishes list of bacteria for which new antibiotics are urgently needed. WHO; [Cited 24 October 2017]. Available from: <http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/>.
  11. Vaez H, Salehi-Abargouei A, Khademi F. Systematic review and meta-analysis of imipenem-resistant *Pseudomonas aeruginosa* prevalence in Iran. *Germs*. 2017;7(2):86–97.
  12. Lin J, Xu C, Fang R, Cao J, Zhang X, Zhao Y, Dong G, Sun Y, Zhou T. Resistance and heteroresistance to colistin in *Pseudomonas aeruginosa* isolates from Wenzhou China. *Antimicrob Agents Chemother*. 2019;63(10):10–128.
  13. CLSI. Performance standards for antimicrobial susceptibility testing. 31<sup>th</sup> ed. Wayne: Clinical and Laboratory Standards Institute; 2021.
  14. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect*. 2012;18(3):268–81.
  15. O'Toole GA. Microtiter dish biofilm formation assay. *Vis Exp*. 2011;47:e2437.
  16. Yousefi S, Nazari M, Ramazanzadeh R, Sahebkar A, Safarzadeh E, Khademi F. Association of carbapenem and multidrug resistance with the expression of efflux pump-encoding genes in *Pseudomonas aeruginosa* clinical isolates. *Acta Microbiol Immunol Hung*. 2023;70(2):161–6.
  17. Nazari M, Ahmadi H, Hosseinzadeh S, Sahebkar A, Khademi F. Imipenem resistance associated with amino acid alterations of the OprD porin in *Pseudomonas aeruginosa* clinical isolates. *Acta Microbiol Immunol Hung*. 2023;70(3):206–12.
  18. Rajabi H, Salimizand H, Khodabandehloo M, Fayyazi A, Ramazanzadeh R. Prevalence of *algD*, *pslD*, *pelF*, *PpgI*, and *PAP1-1* Genes involved in biofilm formation in clinical *Pseudomonas aeruginosa* strains. *BioMed Res Int*. 2022;2022:1–7.
  19. Talebi G, Hakemi-Vala M. Survey on some carbapenems and colistin resistance genes among *Pseudomonas aeruginosa* isolates from burn and cystic fibrosis patients, Tehran. *Iran Arch Clin Infect Dis*. 2019;14(5):e93651.
  20. Zarei O, Shokoozadeh L, Hossainpour H, Alikhani MY. Molecular analysis of *Pseudomonas aeruginosa* isolated from clinical, environmental and cockroach sources by ERIC-PCR. *BMC Res Notes*. 2018;11(1):1–7.
  21. Abraham N, Kwon DH. A single amino acid substitution in PmrB is associated with polymyxin B resistance in clinical isolate of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett*. 2009;298(2):249–54.
  22. Abd El-Baky RM, Masoud SM, Mohamed DS, Waly NG, Shafik EA, Mohareb DA, Elkady A, Elbadr MM, Hetta HF. Prevalence and some possible mechanisms of colistin resistance among multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa*. *Infect Drug Resist*. 2020:323–32.
  23. Rossi F, Girardello R, Cury AP, Di Gioia TS, Almeida JN, Duarte AJ. Emergence of colistin resistance in the largest university hospital complex of São Paulo, Brazil, over five years. *Braz J Infect Dis*. 2017;21:98–101.
  24. Wi YM, Choi JY, Lee JY, Kang CI, Chung DR, Peck KR, Song JH, Ko KS. Emergence of colistin resistance in *Pseudomonas aeruginosa* ST235 clone in South Korea. *Int J Antimicrob Agents*. 2017;49(6):767–9.
  25. Zarate M, Barrantes D, Cuicapuza D, Velasquez J, Fernández N, Salvatierra G, Tamariz J. Frequency of colistin resistance in *Pseudomonas aeruginosa*: first report from Peru. *Rev Peru Med Exp Salud Publica*. 2021;38:308–12.
  26. Farajzadeh Sheikh A, Shahin M, Shokoozadeh L, Halaji M, Shahcheraghi F, Ghanbari F. Molecular epidemiology of colistin-resistant *Pseudomonas aeruginosa* producing NDM-1 from hospitalized patients in Iran. *Iran J Basic Med Sci*. 2019;22:38–42.
  27. Goli HR, Nahaei MR, Rezaee MA, Hasani A, Kafil HS, Aghazadeh M. Emergence of colistin resistant *Pseudomonas aeruginosa* at Tabriz hospitals. *Iran Iran J Microbiol*. 2016;8(1):62–9.
  28. Heidari L, Sepahvand S, Darvishi M, Jafari R. Molecular analysis of PmrA and PmrB genes in colistin-resistant *Pseudomonas aeruginosa* strains via PCR method. *Pak J Pharm Sci*. 2019;32(3 (Supplementary)):1175–7.
  29. Malekzadegan Y, Abdi A, Heidari H, Moradi M, Rastegar E, Sedigh E-S. In vitro activities of colistin, imipenem and ceftazidime against drug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates in the south of Iran. *BMC Res Notes*. 2019;12:1–5.
  30. Tahmasebi H, Dehbashi S, Arabestani MR. Prevalence and molecular typing of colistin-resistant *Pseudomonas aeruginosa* (CRPA) among  $\beta$ -lactamase-producing isolates: a study based on high-resolution melting curve analysis method. *Infect Drug Resist*. 2020:2943–55.
  31. Mohammadzadeh M, Shabanali Fami H, Motiee N, Sanjabi M. Identification of organic milk production potentials and requirements in rural and tribal areas from the viewpoints of ardabil provincial animal husbandry experts. *J Rural Res*. 2020;10(4):698–713. [https://doi.org/10.22059/jrur.2019.275421.1328\[inPersian\]](https://doi.org/10.22059/jrur.2019.275421.1328[inPersian]).
  32. Brachaczek P, Lonc A, Kretzschmar ME, Mikolajczyk R, Horn J, Karch A, Sakowski K, Piotrowska MJ. Transmission of drug-resistant bacteria in a hospital-community model stratified by patient risk. *Sci Rep*. 2023;13(1):18593.
  33. Khosravi AD, Hoveizavi H, Mohammadian A, Farahani A, Jenabi A. Genotyping of multidrug-resistant strains of *Pseudomonas aeruginosa* isolated from burn and wound infections by ERIC-PCR. *Acta Cir Bras*. 2016;31:206–11.
  34. Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog*. 2008;4(11):e1000213.
  35. Huang J, Li C, Song J, Velkov T, Wang L, Zhu Y, Li J. Regulating polymyxin resistance in Gram-negative bacteria: roles of two-component systems PhoPQ and PmrAB. *Future Microbiol*. 2020;15(6):445–59.
  36. Qiu X, Gurkar AU, Lory S. Interstrain transfer of the large pathogenicity island (PAP1-1) of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci*. 2006;103(52):19830–5.
  37. Aghapour Z, Hasani A, Aghazadeh M, Rezaee MA, Ganbarov K, Pourlak T, Gholizadeh P, Asgharzadeh M, Tanomand A, Kafil HS. Genes involved in colistin resistance of gram-negative isolates in the northwest of Iran. *Gene Rep*. 2019;14:81–6.
  38. Bolard A, Schniederjans M, Haüssler S, Triponney P, Valot B, Plésiat P, Jeannot K. Production of norspermidine contributes to aminoglycoside resistance in *pmrAB* mutants of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2019;63(10):10–128.
  39. Barrow K, Kwon DH. Alterations in two-component regulatory systems of phoPQ and *pmrAB* are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2009;53(12):5150–4.
  40. Sellera FP, Fernandes MR, Moura Q, Souza TA, Nascimento CL, Cerdeira L, Lincopan N. Draft genome sequence of an extensively drug-resistant *Pseudomonas aeruginosa* isolate belonging to ST644 isolated from a footpad infection in a Magellanic penguin (*Spheniscus magellanicus*). *J Glob Antimicrob Resist*. 2018;12:88–9.

41. Lee JY, Ko KS. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn Microbiol Infect Dis.* 2014;78(3):271–6.
42. Schurek KN, Sampaio JL, Kiffer CR, Sinto S, Mendes CM, Hancock RE. Involvement of pmrAB and phoPQ in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 2009;53(10):4345–51.
43. Mlynarcik P, Kolar M. Molecular mechanisms of polymyxin resistance and detection of *mcr* genes. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* 2019;163(1):28–38.
44. Muller C, Plésiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and  $\beta$ -lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 2011;55(3):1211–21.

### **Publisher's Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.