



# Antibiotic resistance, biofilm production ability and genetic diversity of carbapenem-resistant *Pseudomonas aeruginosa* strains isolated from nosocomial infections in southwestern Iran

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## Abstract

**Background** This study was aimed to evaluate the antibiotic resistance, biofilm formation, and genetic diversity of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) strains isolated from four types of nosocomial infections (NIs) including urinary tract infection (UTI), ventilator-associated pneumonia (VAP), surgical site infection (SSI), and bloodstream infection (BSI).

**Methods and results** In total, 115 isolates of NIs-causing *P. aeruginosa* were collected from NIs. Antibiotic susceptibility testing (AST) was performed using disk diffusion method and minimum inhibitory concentrations. Biofilm formation was tested on 96-well polystyrene microtiter plates (MTP). CRPA isolates were genotyped using multiple-locus variable number of tandem repeat analysis (MLVA). The most resistance and susceptibility rates were observed to amikacin (70.6%) and colistin (96.1%), respectively. Colistin and meropenem were the most active antimicrobial agents in VAP, SSI, and BSI. While, colistin and cefepime were the most active in UTIs. In total, 52.2% ( $n=60/115$ ) of *P. aeruginosa* isolates were carbapenem resistant, of which 95.0%, 55.0%, and 5.0% were multidrug-resistant, extensively drug-resistant, and pandrug-resistant, respectively. There was a significant association between resistance to carbapenem and resistance to other antibiotics except for piperacillin/tazobactam. The biofilm production of CRPA isolates was 95.0%, of which 23.3% were strong biofilm producers. Based on MLVA, there were 34 different types of CRPA isolates classified into three main clusters and 5 sub clusters.

**Conclusion** The association of CRPA with other antibiotic resistance, the high rates of biofilm production, and the high genetic diversity of the isolates may be a warning of the need for a careful surveillance program.

**Keywords** Antibiotic resistance · Biofilm · MLVA · Nosocomial infections · *Pseudomonas aeruginosa*

## Introduction

Nosocomial infections (NIs) acquired in hospitals and also known as healthcare associated infections (HCAs), occur at 48 h after hospital admission, 3 days after discharge, or

30 days after surgery [1]. NIs associated problems with a global impact in hospital care are considered serious complications that worsen the prognosis of the underlying disease, cause a higher mortality rate, prolong the length of hospital stay, reduce the quality of patients' life, and lead to an increase in the cost of treatment. Hence, NIs require special attention to be managed and controlled [2]. The prevalence of NIs in different regions of Iran has been reported to be 0.4–15.6% [3]. In addition, urinary tract infection (UTI) with 26.8%, ventilator-associated pneumonia (VAP) with 20.3%, surgical site infection (SSI) with 19.7%, and bloodstream infection (BSI) with 13.5% are the most common NIs in Iran [4].

*Pseudomonas aeruginosa*, a Gram-negative aerobic rod, is still considered one of the most antibiotic resistance pathogens of NIs [2]. *P. aeruginosa* accounts for 10–11%

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of all NIs [2]. This bacterium with intrinsic resistance to various antimicrobial agents can easily tolerate many antibiotics including carbapenems (imipenem and meropenem) [2, 5–7]. Carbapenems have been reported to be the most effective antimicrobial agents against severe *P. aeruginosa* NIs and are often used as a last resort antibiotics in the treatment of bacterial infections [5, 6]. Nevertheless, overuse of antibiotics leads to the emergence of carbapenem-resistant *P. aeruginosa* (CRPA) strains with severe and fatal infections and increases the cost of therapy [7].

An important virulence factor for this bacterium is biofilm which is described as a set of bacterial cells coated by a polysaccharide layer [8]. Nowadays, the important role of biofilm-producing bacteria in medicine is well-recognized due to the rise of HCAs. Also, the biofilms play a main role in antibiotic resistance owing to microorganism protection, stop or slow down the antibiotic penetration, and altered microenvironment [9, 10]. The significant association of biofilm formation with CRPA was demonstrated in a previous study [11].

Considering the important role of *P. aeruginosa* as a causative agent of NIs, there is a need to use molecular typing methods for infection control and epidemiological studies. Various typing methods have been used to assess the genetic diversity and distribution of *P. aeruginosa* in different niches [12]. One of the most efficient methods to determine and study the genotypes of different bacteria is multiple-locus variable number of tandem repeats analysis (MLVA). Effective discrimination of different *P. aeruginosa* genotypes using MLVA has been reported previously [13]. Since most of the loci on the agarose gel are determined by polymerase chain reaction (PCR), this technique is considered to be repeatable, economical and least time consuming [14]. The popularity of MLVA as a clonality genotyping method for hospital-based epidemiological surveys has increased in recent years [12].

To the best of our knowledge, this research is one of the few studies on molecular typing of *P. aeruginosa* using MLVA method in several southern cities of Iran. This study was aimed to evaluate the antibiotic resistance patterns, biofilm formation ability, and genetic diversity of CRPA strains isolated from NIs in three cities of Khuzestan province, southwest Iran.

## Materials and methods

### Ethical clearance

Ethical approval was obtained from the Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1399.530). Written informed consent was obtained from all patients.

### Study design and bacterial isolates

Over a 6-month period (October 2020–March 2021), 115 NIs-causing *P. aeruginosa* isolates were collected from hospitalized patients referred to teaching hospitals of three cities of Khuzestan province (Ahvaz, Abadan and Khorramshahr), southwest Iran. NIs were defined according to the diagnostic criteria of the Centers for Disease Control and Prevention (CDC) [15]. The isolates were related to four types of NIs including UTI, VAP, SSI and BSI, which were collected from different wards including intensive care unit (ICU), burn, infectious diseases, nephrology, emergency, pediatrics, gastroenterology, skin diseases, dialysis, internal medicine, and critical care unit (CCU). Initial identification of the isolates was based on typical growth on a selective medium Cetrimide agar (Merck, Darmstadt, Germany) and different biochemical tests including colony characteristics, sulphur indole and motility (SIM), triple sugar iron (TSI), urease, citrate, oxidase, and growth at 42 °C [16]. PCR of the *gyrB* gene was used for final identification [17]. *P. aeruginosa* ATCC® 27,853™ was used as a positive control. A stock of all bacterial isolates was prepared in a microtube containing tryptic soy broth (TSB) with 20% glycerol and stored at –80 °C until further investigation.

### Antimicrobial susceptibility testing (AST)

For antimicrobial susceptibility testing of the following antibiotics, the disk diffusion method was used according to the Clinical and Laboratory Standards Institute (CLSI) protocol: piperacillin (100 µg), piperacillin/tazobactam (100/10 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), tobramycin (10 µg), gentamicin (10 µg), amikacin (30 µg), and ciprofloxacin (5 µg) (MAST, Berkshire, UK) [18]. Moreover, minimum inhibitory concentrations (MICs) of imipenem, meropenem, and colistin were assayed by broth microdilution method according to CLSI instructions (18). *P. aeruginosa* isolates with MICs of  $\geq 8$  µg/ml for imipenem or/and meropenem and  $\geq 4$  µg/ml for colistin were described as carbapenem and colistin resistant, respectively [18]. To categorize multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) isolates, criteria defined by Magiorakos et al. [19] were employed. *P. aeruginosa* ATCC® 27,853™ [Iranian Research Organization for Science and Technology (IROST), Tehran, Iran] was used as a quality control strain according to the CLSI protocol.

## In vitro biofilm assay

To assess the biofilm formation ability of *P. aeruginosa* isolates on 96-well polystyrene microtiter plates (MTP), the crystal violet staining (CVS) method was applied as previously mentioned [20]. Uninoculated Muller-Hinton broth (MHB) and *P. aeruginosa* ATCC® 27,853™ were used sequentially as negative and positive controls. The biofilm forming ability of the isolates was classified into four categories: non biofilm producer:  $OD_t < OD_c$ , weak biofilm producer:  $OD_c < OD_t < 2 \times OD_c$ , moderate biofilm producer:  $2 \times OD_c < OD_t < 4 \times OD_c$  and strong biofilm producer:  $OD_t \geq 4 \times OD_c$ .  $OD_t$  demonstrates the mean OD of the three wells for each isolate and  $OD_c$  shows the mean OD of the three wells for the control [20].

## MLVA analysis

Bacterial DNA was extracted by boiling method as described previously [21]. Genotyping of CRPA isolates was carried out by MLVA using primers for seven loci, including ms142, ms172, ms213, ms214, ms215, and ms217 (Table 1) [22]. First, a mixture of PCR components was prepared containing 12.5  $\mu$ l of 2 $\times$  Taq Master Mix (Ampliqon, Odense, Denmark), 1  $\mu$ l of 10 pM of each primer (Metabion, Steinkirchen, Germany), 5  $\mu$ l of template DNA (50 ng), and double-distilled H<sub>2</sub>O to the total volume of 25  $\mu$ l per reaction. The thermal reactions were produced using BIO-RAD C1000 thermal cycler (Applied Biosystems, USA) as follows: the primary denaturation for 4 min at 94 °C, followed by 32 cycles (30 s at 93 °C, 30 s at 60 °C, and 60 s at 72 °C), and the final extension phase for 4 min at 72 °C. *P. aeruginosa* strain PAO1 was used as a control for all the experiments. PCR products were loaded in 2% agarose gel and stained with safe stain (Sinaclon Co., Tehran, Iran). Then the

ultraviolet gel documentation device (Protein Simple, San Jose, CA, USA) was used to observe the bands.

To determine the repeat units of each locus, the size of the flanking regions (offset size) was subtracted from the size of the PCR product to obtain the number of TRs, which is then divided to the distinction of the repeat size. The Bionumerics 7.6 software (Applied Maths, St-Martens-Latem, Belgium) was employed to cluster the MLVA profiles using the UPGMA (Unweighted Pair Group Method with Arithmetic) method. Furthermore, BioNumerics was utilized to create similarity coefficient of Pearson's correlation and the minimum spanning tree (MST). The dendrogram displayed the dissimilarity of the strain in accordance with the UPGMA algorithm. The Hunter-Gaston diversity index was used to determine the polymorphism index of the individual or combined VNTR loci. The strains with 80% or more similarity [based on 2 distinct VNTR loci (DLV)] were assigned to a single strain.

## Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 23.0 (Armonk, NY, USA) was used for statistical analysis of the data. To provide a comparison between groups, the Chi-square test or Fisher's exact test was used. Results with  $p < 0.05$  were considered significant.

## Results

### Clinical isolates

Sixty out of 115 (52.2%) *P. aeruginosa* isolates in our study were carbapenem resistant. Of the 115 isolates, 54.8% and 45.2% were collected from males and females, respectively. The highest and lowest rates of *P.*

**Table 1** Primer characteristics of multiple VNTR loci used in the polymerase chain reactions

VNTR locus	Primer sequence (5–3)	Tandem repeat size (bp)	Reference
ms172	F: GGATTCTCTCGCACGAGGT R: TACGTGACCTGACGTTGGTG	54	[22]
ms142	F: AGCAGTGCCAGTTGATGTTG R: GTGGGGCGAAGGAGTGAG	115	[22]
ms223	F: TTGGCAATATGCCGGTTTCGC R: TGAGCTGATCGCCTACTGG	106	[22]
ms213	F: CTGGGCAAGTGTGGTGGATC R: TGGCGTACTCCGAGCTGATG	103	[22]
ms214	F: AAACGCTGTTTCGCCAACCTCTA R: CCATCATCCTCTACTGGGTT	115	[22]
ms215	F: GACGAAACCCGTCGCGAACA R: CTGTACAACGCCGAGCCGTA	129	[22]
ms217	F: TTCTGGCTGTCGCGACTGAT R: GAACAGCGTCTTTCTCTCGC	109	[22]

*aeruginosa* isolates were found in ICU (53.0%,  $n = 61$ ) and CCU (1.7%,  $n = 2$ ), respectively. The frequency of isolates in other wards was as follows: internal medicine (9.6%,  $n = 11$ ), burn (9.6%,  $n = 11$ ), infectious diseases (7.8%,  $n = 9$ ), nephrology (3.5%,  $n = 4$ ), pediatrics (3.5%,  $n = 4$ ), dialysis (3.5%,  $n = 4$ ), emergency (2.6%,  $n = 3$ ), gastroenterology (2.6%,  $n = 3$ ), and skin diseases (2.6%,  $n = 3$ ). In total, 33.9% of isolates belonged to VAP type of NIs, followed by BSI (26.0%), SSI (22.6%), and UTI (17.3%). Of 60 CRPA isolates, 53.3% of them were related to women. Most CRPA isolates were obtained from ICU ward (55.0%) and the most percentage of them was related to VAP type of NIs (36.6%). The characteristics of the 60 CRPA isolates can be found in Table 2.

### AST patterns

Of the 115 isolates, 60 (52.1%) and 57 (49.5%) were resistant to imipenem and meropenem, respectively. The most resistance rate was observed to amikacin (70.6%) and ciprofloxacin (65.7%). The resistance rates of other tested antibiotics were as follows: piperacillin (59.8%), ceftazidime (59.8%), aztreonam (54.9%), tobramycin (52%), gentamicin (51%), cefepime (37.9%), and piperacillin/tazobactam (42.2%). On the other hand, the most susceptibility rate was observed to colistin (96.1%). Regarding the type of NIs, colistin and meropenem were the most active antimicrobial agents in VAP, SSI, and BSI. While, colistin and cefepime were the most active in UTIs (Table 3). There was a significant association between resistance to carbapenem and resistance to other antibiotics except for piperacillin/tazobactam ( $p = 0.0001$ ) (Table 4). Also, the results revealed that 95.0%, 55.0%, and 5.0% of carbapenem resistant isolates were MDR, XDR, and PDR, respectively.

### In vitro biofilm assay

Of 115 *P. aeruginosa* isolates, 21 (18.3%), 21 (18.3%), and 61 (53.0%) isolates were strong biofilm producers, moderate biofilm producers, and weak biofilm producers, respectively. While, 12 (10.4%) of isolates were not biofilm producers. The rate of biofilm production in CRPA isolates was 95.0%, while this rate in carbapenem-susceptible *P. aeruginosa* (CSPA) isolates was 83.6%. Furthermore, the rates of strong and moderate biofilm formation were higher in CRPA isolates than in CSPA isolates (Table 3). Most biofilm production in CRPA isolates was related to VAP (36.6%) followed by BSI (25.0%), SSI (25.0%), and UTI (8.3%). There was a significant connection between biofilm formation and resistance to piperacillin and ceftazidime ( $p < 0.05$ ).

### MLVA genotyping

With regard to the amplification of seven distinct VNTR loci, the CRPA isolates were assessed by MLVA method. In general, there were 34 various MLVA types of CRPA isolates in our study which were assigned to seven main clusters, 11 subclusters, 25 singlone and nine multitone: 13 types in cluster I, three types in cluster II, six types in cluster III, eight types in cluster IV, two types in cluster V, one type in cluster VI and one type in cluster VII. The most frequent types include type 2, 6 and 28, each with three isolates (Fig. 1). According to the diversity index (hunter-gaston), the highest diversity was at VNTR loci ms142 and the lowest at VNTR loci ms223. Most of the samples belonging to cluster I showed moderate and strong biofilm formation ability. Figure 2 shows the MST patterns derived from the MLVA genotyping for the CRPA isolates. In this figure, the desired strains classified into 34 types are represented as clones based on the number of strains that were categorized. This concept is based on the association between the numbers of repetitions that were considered as comparisons in each category [46].

### Discussion

*P. aeruginosa* is known as one of the main causes of NIs. To hinder the spread of this resistant microorganism and to take sufficient measures to prevent infections, it is necessary to monitor the susceptibility to antimicrobial agents among isolates [23]. In the present study, colistin (96.1%) showed the most susceptibility which was in line with previous studies [11, 24]. Its effectiveness can be explained by factors such as the considerable cost of colistin, its nephrotoxicity and its limited use outside hospitals [25]. Cephalosporins, carbapenems, aminoglycosides and fluoroquinolones are considered four important groups of anti-pseudomonal agents [26]. In this research, the high resistance to amikacin (70.6%) and ciprofloxacin (65.7%) was in agreement with previous studies [24, 27]. However, there were reports recommending amikacin and ciprofloxacin as effective antibiotics against nosocomial *P. aeruginosa* isolates [28, 29].

In this study, more than 50.0% of *P. aeruginosa* isolates were resistant to piperacillin (59.8%), ceftazidime (59.8%), aztreonam (54.9%), tobramycin (52.0%), and gentamicin (51.0%). These results were in line with previous reports from Nepal [8], Iran [27], and Ethiopia [28]. However, Lila et al. [30] from Kosovo and Hemmati et al. [33] from Iran reported lower resistance rates for ceftazidime and ciprofloxacin compared to our findings. These discrepancies may be due to differences in the type and race of the population studied, the source of isolates, the methods used to measure the antibiotic susceptibility, the presence or absence of

**Table 2** Characteristics of 60 carbapenem-resistant *Pseudomonas aeruginosa* isolates

Isolates	Sex	Hospital name	Hospital sector	Infection site	Biofilm formation ability	Resistance profile	Antimicrobial resistance classification	NIs type
1	Male	Emam	ICU	Urine	Moderate	PIP TZP FEP ATM IPM MEM—GEN	XDR	UTI
10	Male	Emam	ICU	Tracheal	Strong	PIP FEP ATM IPM MEM—GEN	XDR	VAP
14	Female	Emam	ICU	Blood	Strong	PIP TZP FEP ATM IPM MEM—GEN	XDR	BSI
100	Female	17 sh	Internal medicine	Blood	Negative	PIP TZP FEP IPM MEM—GEN	MDR	BSI
108	Female	Golestan	Surgery	Tracheal	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
109	Female	Golestan	ICU	Tracheal	Moderate	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
11	Male	Emam	ICU	Catheter	Moderate	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
110	Female	Golestan	Surgery	Blood	Weak	PIP TZP FEP IPM MEM—GEN	MDR	BSI
111	Male	Golestan	CCU	Drainage	Moderate	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
112	Male	Golestan	Surgery	Blood	Weak	PIP FEP ATM IPM MEM—GEN	MDR	BSI
113	Male	Golestan	ICU	Blood	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	BSI
114	Female	Golestan	surgery	Blood	Strong	PIP TZP FEP ATM IPM MEM—GEN	PDR	BSI
12	Female	Emam	ICU	Tracheal	Moderate	PIP TZP FEP ATM IPM MEM—GEN	PDR	VAP
13	Female	Emam	Infectious diseases	Wound	Weak	PIP FEP ATM IPM MEM—GEN	MDR	SSI
15	Female	Emam	Surgery	Wound	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	SSI
18	Female	Emam	ICU	Tracheal	Weak	PIP FEP ATM IPM MEM—GEN	MDR	VAP
2	Male	Emam	ICU	Blood	Weak	TZP ATM IPM MEM—GEN	XDR	BSI
20	Female	Emam	ICU	Tracheal	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
21	Female	Emam	ICU	Tracheal	Weak	PIP FEP ATM IPM MEM—GEN	MDR	VAP
23	Female	Emam	Internal medicine	Blood	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	BSI
25	Female	Emam	Nephrology	Wound	Strong	PIP ATM IPM MEM—GEN	MDR	SSI
26	Female	Emam	Internal medicine	Tracheal	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
31	Male	Emam	CCU	Wound	Weak	PIP FEP ATM IPM MEM—GEN	MDR	SSI
33	Female	Emam	Infectious diseases	Wound	Strong	PIP TZP FEP ATM IPM MEM—GEN	XDR	SSI
35	Male	Emam	ICU	Urine	Moderate	PIP FEP ATM IPM MEM—GEN	MDR	UTI
37	Female	Emam	Infectious diseases	Tracheal	Strong	PIP TZP ATM IPM MEM—GEN	XDR	VAP

**Table 2** (continued)

Isolates	Sex	Hospital name	Hospital sector	Infection site	Biofilm formation ability	Resistance profile	Antimicrobial resistance classification	NIs type
40	Female	Emam	Infectious diseases	Wound	Weak	PIP FEP ATM IPM MEM—GEN	MDR	SSI
45	Male	Emam	ICU	Tracheal	Strong	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
34	Female	Emam	ICU	Blood	Weak	ATM IPM MEM—GEN	MDR	BSI
61	Male	Taleghani	ICU	Blood	Moderate	PIP TZP FEP ATM IPM MEM—GEN	XDR	BSI
62	Female	Taleghani	ICU	Wound	Weak	PIP FEP ATM IPM MEM—GEN	MDR	SSI
63	Male	Taleghani	Pediatric	Blood	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	BSI
64	Female	Taleghani	ICU	Blood	Weak	PIP FEP ATM IPM MEM—	MDR	BSI
67	Male	Taleghani	ICU	Blood	Moderate	PIP TZP FEP ATM IPM MEM—GEN	XDR	BSI
68	Female	Taleghani	Surgery	Wound	Weak	PIP ATM IPM MEM—GEN	MDR	SSI
69	Female	Taleghani	Pediatric	Abscess	Moderate	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
70	Male	Taleghani	ICU	Blood	Negative	PIP —ATM IPM MEM—GEN	MDR	BSI
73	Male	Taleghani	Surgery	Biopsy	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	SSI
74	Female	Taleghani	Emergency	Biopsy	Weak	PIP FEP ATM IPM MEM—GEN	MDR	SSI
75	Male	Taleghani	ICU	Blood	Weak	PIP FEP ATM IPM MEM—GEN	MDR	BSI
76	Female	Taleghani	ICU	Blood	Weak	PIP TZP FEP IPM MEM—GEN	MDR	BSI
77	Male	Taleghani	ICU	Blood	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	BSI
78	Female	Taleghani	Infectious diseases	Biopsy	Moderate	PIP FEP ATM IPM MEM—GEN	MDR	SSI
79	Female	Taleghani	Surgery	Biopsy	Weak	PIP FEP ATM IPM MEM—GEN	MDR	SSI
83	Female	Valasr	Internal medicine	Wound	Moderate	PIP TZP FEP ATM IPM MEM—GEN	XDR	SSI
84	Female	Valasr	ICU	Broncho alveolar lavage	Strong	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
85	Female	Valasr	ICU	Broncho alveolar lavage	Weak	ATM IPM MEM—GEN	MDR	VAP
87	Male	Naft	ICU	Urine	Weak	PIP FEP ATM IPM MEM—GEN	MDR	UTI
88	Male	Naft	ICU	Tracheal	Moderate	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
9	Male	Emam	ICU	Urine	Strong	PIP TZP FEP ATM IPM MEM—GEN	XDR	UTI
92	Male	Taleghani	Burn	Biopsy	Negative	PIP TZP FEP ATM IPM MEM—GEN	XDR	SSI
94	Male	Taleghani	Burn	Urine	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	UTI



**Table 2** (continued)

Isolates	Sex	Hospital name	Hospital sector	Infection site	Biofilm formation ability	Resistance profile	Antimicrobial resistance classification	NIs type
96	Male	Taleghani	ICU	Burn	Moderate	PIP TZP FEP ATM IPM MEM—GEN	PDR	SSI
98	Male	Taleghani	ICU	Burn	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	SSI
52	Male	Emam	Infectious diseases	Tracheal	Strong	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
55	Male	Emam	ICU	Tracheal	Strong	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
59	Female	Beheshti	ICU	Broncho alveolar lavage	Strong	PIP FEP ATM IPM MEM—	MDR	VAP
106	Female	Golestan	ICU	Catheter	Strong	PIP —ATM IPM MEM—GEN	MDR	VAP
49	Male	Golestan	Internal medicine	Tracheal	Strong	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP
99	Male	17 sh	ICU	Catheter	Weak	PIP TZP FEP ATM IPM MEM—GEN	XDR	VAP

PIP piperacillin, TZP piperacillin/tazobactam, FEP cefepime, ATM aztreonam, IPM imipenem, MEM meropenem, GEN gentamicin, AMK amikacin, CIP ciprofloxacin, TOB tobramycin, CAZ ceftazidime, COL colistin, UTI urinary tract infection, VAP ventilator-associated pneumonia, SSI surgical site infection, BSI bloodstream infection, MDR multidrug resistant, XDR extensive drug resistant, PDR pandrug resistant, ICU intensive care unit

**Table 3** Antibiotic sensitivity patterns of *Pseudomonas aeruginosa* isolates in four types of NIs (%)

Antibiotic	BSI			SSI			VAP			UTI		
	S	I	R	S	I	R	S	I	R	S	I	R
Piperacillin	24.1	17.2	58.6	26.5	17.6	55.9	22.2	11.1	66.7	40.0	10.0	50.0
Piperacillin/tazobactam	20.7	13.8	65.5	41.2	20.6	38.2	33.3	44.4	22.2	40.0	10.0	50.0
Cefepime	44.8	17.2	37.9	44.1	17.6	38.2	26.9	15.4	57.7	50.0	10.0	40.0
Ceftazidime	24.1	20.7	55.2	29.4	17.6	52.9	22.2	7.4	70.4	40.0	0.0	60.0
Aztreonam	20.7	27.6	51.7	29.4	23.5	47.1	18.5	11.1	70.4	40.0	10.0	50.0
Imipenem	41.4	20.7	37.9	32.4	14.7	52.9	25.9	7.4	66.7	30.0	10.0	60.0
Meropenem	55.2	13.8	31	47.1	0.0	52.9	33.3	3.7	63.0	40.0	0.0	60.0
Gentamicin	27.6	37.9	34.5	38.2	11.8	50	11.1	18.5	70.4	40.0	20.0	40.0
Amikacin	10.3	6.9	82.8	29.4	5.9	64.7	22.2	11.1	66.7	40.0	0.0	60.0
Tobramycin	34.5	34.5	31.0	44.1	2.9	52.9	22.2	7.4	70.4	40.0	10.0	50.0
Ciprofloxacin	6.9	10.3	82.8	32.4	8.8	55.8	18.5	14.8	66.7	30.0	30.0	40.0
Colistin	100	0.0	0.0	97.1	0.0	2.9	92.6	0.0	7.4	100.0	0.0	0.0

S susceptible, I intermediate, R resistant, UTI urinary tract infection, VAP ventilator-associated pneumonia, SSI surgical site infection, BSI bloodstream infection

antibiotic consumption surveillance programs, the presence or absence of antibiotic use surveillance in agriculture, livestock and other industries, and differences in the epidemiology of the regions.

Another important finding of the current research was the high resistance rate of 52.1% and 49.5% against imipenem and meropenem, respectively. In previous studies, the rate of carbapenem resistance differed greatly from 17.5% to 100.0% [11, 24, 30–33] In this study, *P. aeruginosa* isolates in southwestern Iran showed higher rates of resistance

to carbapenems than in several other countries, including China (37.2% for imipenem) [6] and Kosovo (12.5% for meropenem and 17.5% for imipenem) [30]. However, Kunwar et al. [8] from Nepal, Patel et al. [10] from India, and El-Mahdy et al. [11] from Egypt found high resistance rates (ranging from 42.5% to 100.0%) of *P. aeruginosa* to carbapenem antibiotics. In recent years, the emergence of carbapenem-resistant Gram-negative bacteria, including CRPA, has posed a new challenge in the medical field. Because the treatment of these species is associated with many problems.

**Table 4** Comparison of antibiotic resistance and biofilm production in CRPA and CSPA isolates

Variable	CRPA (60)	CSPA (55)	<i>p</i> value
Piperacillin	52 (86.6%)	15 (27.3%)	0.0001
Piperacillin/tazobactam	25 (41.6%)	23 (41.8%)	0.6652
Cefepime	48 (80.0%)	3 (5.5%)	0.0001
Ceftazidime	54 (90.0%)	15 (27.3%)	0.0001
Aztreonam	47 (78.3%)	12 (21.8%)	0.0001
Gentamicin	47 (78.3%)	10 (18.2%)	0.0001
Amikacin	54 (90.0%)	25 (45.5%)	0.0001
Tobramycin	46 (76.7%)	16 (29.1%)	0.0001
Ciprofloxacin	53 (88.3%)	21 (38.2%)	0.0001
Colistin	4 (6.7%)	0 (0.0%)	0.0001
<b>Biofilm</b>			
Biofilm production	57 (95.0%)	46 (83.6%)	0.49
Strong	14 (23.3%)	7 (12.7%)	
Moderate	13 (21.7%)	8 (14.6%)	
Weak	30 (50.0%)	31 (56.4%)	
Negative	3 (5.0%)	9 (16.4%)	

CRPA Carbapenem-resistant *Pseudomonas aeruginosa*, CSPA Carbapenem-susceptible *Pseudomonas aeruginosa*

The high resistance rates of *P. aeruginosa* isolates against carbapenems in our region indicate that the use of this group of antimicrobials need to be done with caution and as much as possible based on the results of the antibiogram test.

The assessment of resistance pattern in four types of NIs (VAP, SSI, BSI and UTI) was performed discretely and provided useful data on the effective antibiotics in each single type. Higher resistance to most antibiotics was observed in *P. aeruginosa* isolated from VAP compared to other types of NIs. In another study from Serbia, the susceptibility of VAP pathogens to common antibiotics was also low [34]. It has already been noted that the antibiotic susceptibility of *P. aeruginosa* isolates from SSI tends to be higher than that of hospital-acquired respiratory tract infections and UTIs [35]. In recent research, the antibiotic susceptibility of *P. aeruginosa* isolates from SSI to most antibiotics was higher than that of VAP and BSI except UTI. In general, studies have reported a remarkably variable frequency of drug resistant *P. aeruginosa*, which can be explained by the different study population, different site of isolation, different antibiotic used in the treatment, the severity of the disease, length of hospital stay, and methods used [1, 33].

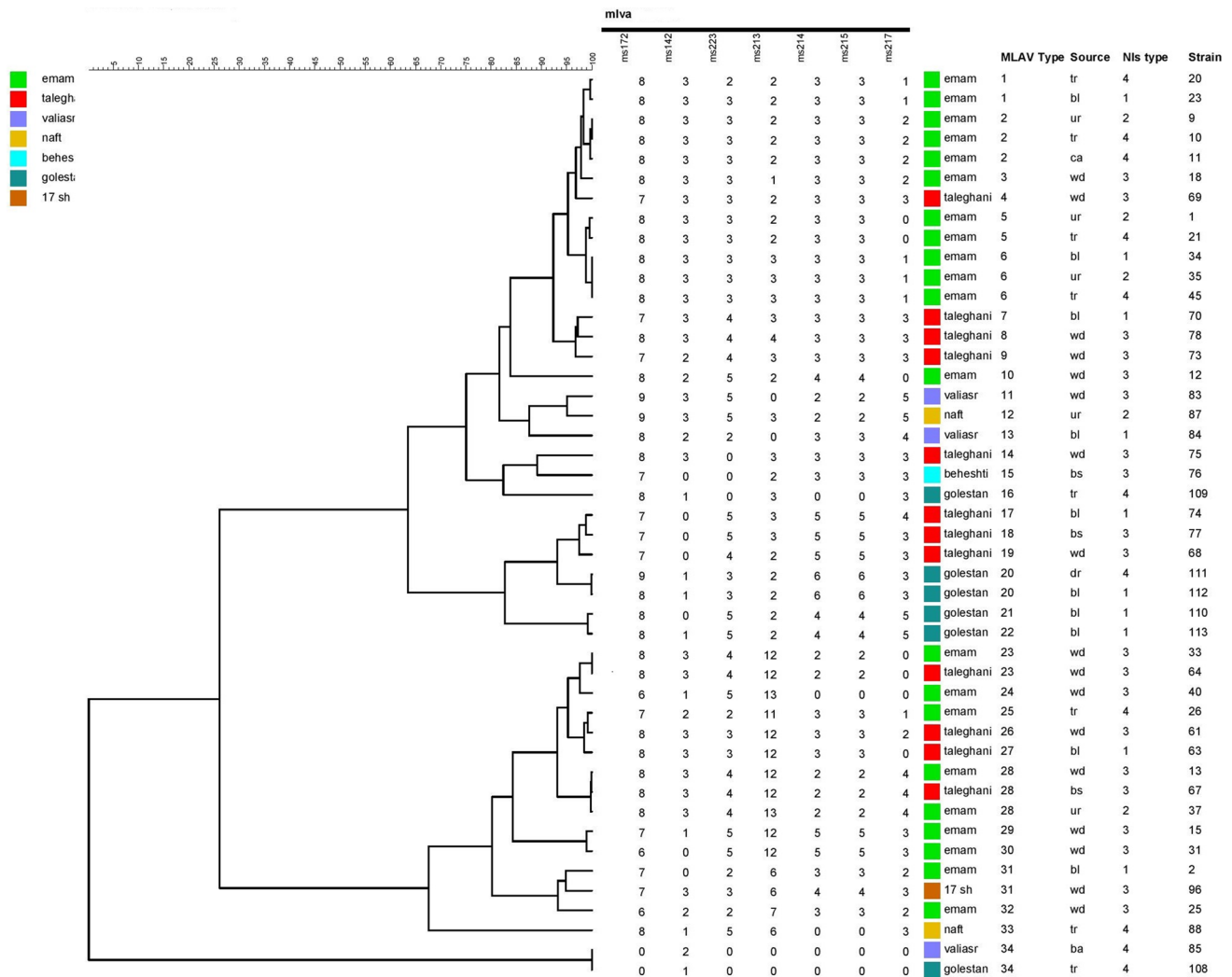
Among the different wards, ICU (55.0%) and among NI types, VAP (36.6%) had the highest rates of CRPA isolates, respectively. These results were is consistent with other studies that reported ICU admission and medical device as important risk factors for CRPA [6, 36]. In our research, 95.0% of CRPA isolates were MDR, 55.0% were XDR, and 5.0% were PDR which was noticeable and can be a threat for

the future. This high rate of resistance among our isolates may be explained by the fact that they were isolated from NIs. In El-Mahdy et al. [11] study, 91.2% of CRPA isolates were MDR and there was a significant association between MDR and carbapenem resistance. In the study by Rasool et al. [37], 92.3% of CRPA isolates was XDR. According to our findings, there was statistically significant difference in resistance rates between carbapenem-resistant and carbapenem-susceptible isolates for all tested antibiotics except piperacillin/tazobactam, as mentioned in other studies [6, 11].

In a recent study, *P. aeruginosa* isolates were evaluated in terms of biofilm production. Biofilm formation in carbapenem-susceptible and carbapenem-resistant isolates was 83.6% and 95.0%, sequentially. In the study by El-Mahdy et al. [11], biofilm production in CRPA isolates (94.0%) was higher than CSPA (65.2%) isolates. In another study by Cho et al. [38], 92.7% of CRPA isolates were biofilm producers. In the present study, *P. aeruginosa* strains isolated from VAP indicated higher biofilm production in comparison to other types of NIs. Furthermore, the rate of strong biofilm producer isolates in this type was also higher. Our results were in agreement with the study by Cepas et al. [29] in which *P. aeruginosa* isolates from respiratory samples were found to have greater biofilm formation ability than the other types [39]. There was also a close positive correlation between biofilm formation and the occurrence of VAP in the study by Uppe et al. [40]. However, the results obtained from studies by Davarzani et al. [20] and Bahador et al. [21] indicated a higher rate of strong biofilm production in urine samples than other samples. Although a higher rate of antibiotic resistance was found in biofilm-forming isolates compared to non-biofilm-forming isolates, there was only a significant association between biofilm production and resistance to piperacillin and ceftazidime ( $p < 0.05$ ). These findings were in line with some studies that reported a significant connection between biofilm production and resistance to various antibiotics [41, 42]. Moreover, a significant association between MDR and biofilm production was observed in some studies [43, 44]. However, in some studies, no significant connection was observed between biofilm formation and resistance to individual antibiotics or MDR, suggesting that other resistance mechanisms including efflux pumps, altered outer membrane permeability, toxin/antitoxin systems, and expression of the  $\beta$ -lactamase resistance genes are involved [20, 26, 45].

In the present study, the MLVA typing technique with seven distinct VNTR loci was used to genetically characterize the isolated CRPA strains, resulting in the identification of 34 types. Although the MLVA method is less accurate than techniques such as pulsed-field gel electrophoresis (PFGE), because the strains are encoded based on the number of repeats in the VNTR loci, it is easy to examine the

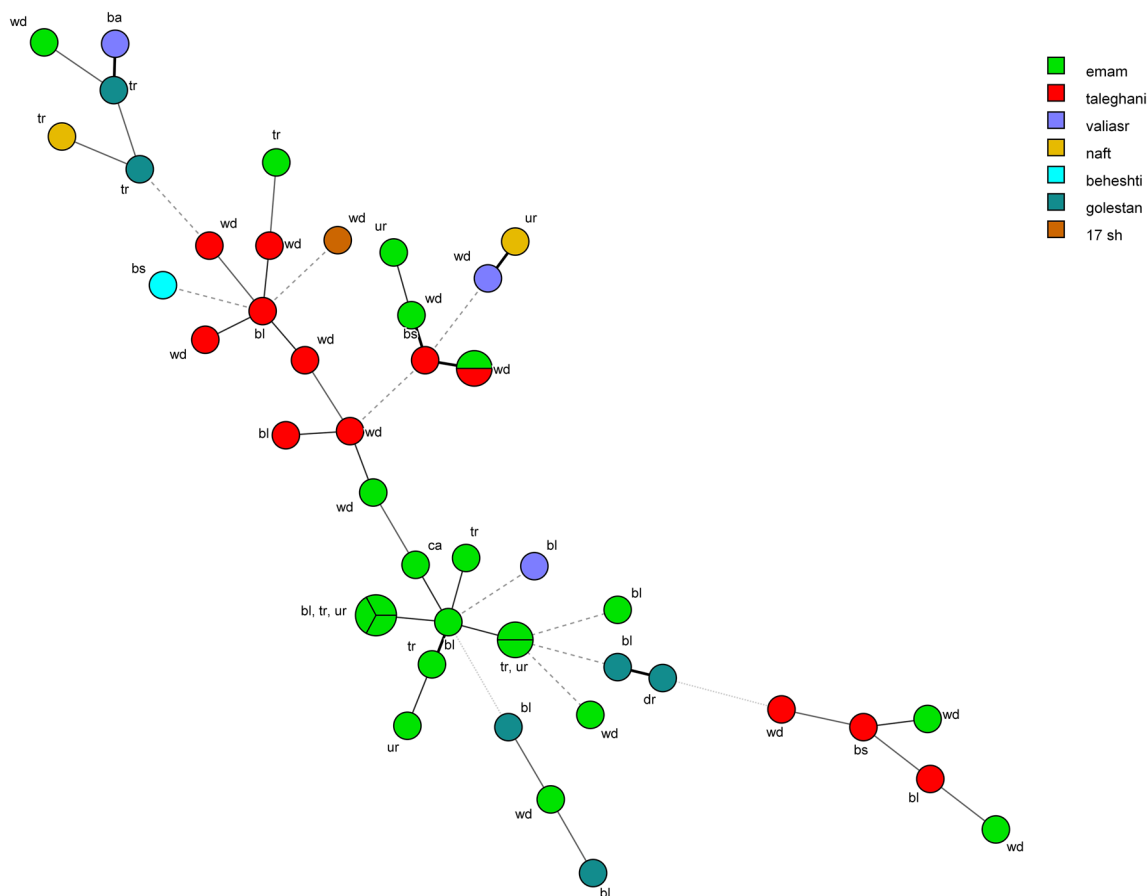




**Fig. 1** Clustering analysis of MLVA results of Carbapenem-resistant *Pseudomonas aeruginosa* isolates using UPGMA algorithm. *bl* blood, *ba* broncho alveolar lavage, *ca* catheter, *bs* burns, *wd* wound, *ur* urine, *tr* tracheal

clonal correlation of a large number of isolates [46]. In this study, most isolates belonged to cluster I followed by cluster IV and were mostly from one hospital but there was a lot of variety in sample type. According to our results, *P. aeruginosa* isolates from the same medical ward had remarkably high diversity. These findings indicated that these organisms vary in genotype over time as well as within the same host [47]. Considering the high genetic diversity of CRPA isolates, which are mainly found in ICU wards, it is difficult to properly implement infection control policies. In this research, the MLVA-7 showed good results in typing of *P. aeruginosa* isolates and all studied isolates were typeable. However, in a previous experiment by Lalancette et al. [48] from Canada, three not-typeable strains were detected using MLVA-7 as follows: (MS142, MS211, MS213, MS215, MS216, MS222, MS223). In another study by Johansson et al. [49] from Sweden, an agreement of 91.0% was

observed between MLVA and PFGE in clone identification of *P. aeruginosa* isolates from cystic fibrosis patients. Seven VNTR markers used in the study by Shokoozhadeh et al. [13] were the same as those used in our study and indicated 36 different genotypes among 72 isolates which offered a moderate discriminatory power by these VNTR markers. It is recommended that more VNTR markers be used in the future studies to evaluate their discriminatory power in determination of relatedness of circulating clones of *P. aeruginosa* isolates in our region. Also, due to the spread of antibiotic resistance among bacteria causing NIs, the effect of other newer antimicrobial and anti-biofilm agent (such as antimicrobial peptides) need to be evaluated in future studies. One of the limitations of the current study was the lack of detailed characteristics (such as race) of the patients due to the covid-19 pandemic and the absence of continuous connection with the hospital staff.



**Fig. 2** Minimum spanning tree of the MLVA results of Carbapenem-resistant *Pseudomonas aeruginosa* isolates. *bl* blood, *ba* broncho alveolar lavage, *ca* catheter, *bs* burns, *wd* wound, *ur* urine, *tr* tracheal

## Conclusion

Carbapenem resistance in *P. aeruginosa* is indicative of a problem in NIs. Our findings showed that resistance to carbapenem had significant association with resistance to other antibiotics, which deteriorates this problem and limits options for treatment. Furthermore, high genetic diversity was observed among CRPA isolates, which necessitates constant monitoring of the molecular epidemiology of *P. aeruginosa* to detect any minor changes in the epidemiology of *P. aeruginosa* infections during the time.

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writing- original draft preparation. ZF: conceptualization, data curation, formal analysis, writing-reviewing and editing. SS: conceptualization, supervision, writing-reviewing and editing. Writing- original draft preparation. MS: writing-reviewing and editing, formal analysis. All authors read and approved the final manuscript.

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**Data availability** All analyzed data within this study can be obtained from the corresponding author on request.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors have no conflict of interest.

**Ethical approval** Ethical approval was obtained from the Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1399.530). Written informed consent was obtained from all patients.

**Consent to participate** All patients provided written informed consent.

**Consent for publication** Not applicable.

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