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RESEARCH

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Molecular characterization of *NDM-1*-producing *Pseudomonas aeruginosa* isolates from hospitalized patients in Iran

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Abstract

Background: The emergence of carbapenem-resistant *Pseudomonas aeruginosa* is one of the most important challenges in a healthcare setting. The aim of this study is double-locus sequence typing (DLST) typing of bla_{NDM-1} positive *P. aeruginosa* isolates.

Methods: Twenty-nine bla_{NDM-1} positive isolates were collected during three years of study from different cities in Iran. Modified hodge test (MHT), double-disk synergy test (DDST) and double-disk potentiation test (DDPT) was performed for detection of carbapenemase and metallo-beta-lactamase (MBL) producing bla_{NDM-1} positive *P. aeruginosa* isolates. The antibiotic resistance genes were considered by PCR method. Clonal relationship of bla_{NDM-1} positive was also characterized using DLST method.

Results: Antibiotic susceptibility pattern showed that all isolates were resistant to imipenem and ertapenem. DDST and DDPT revealed that 15/29 (51.8%) and 26 (89.7%) of bla_{NDM-1} positive isolates were MBL producing isolates, respectively. The presence of bla_{OXA-10}, bla_{VIM-2}, bla_{IMP-1} and bla_{SPM} genes were detected in 86.2%, 41.4%, 34.5% and 3.5% isolates, respectively. DLST typing results revealed the main cluster were DLST 25-11 with 13 infected or colonized patients.

Conclusions: The presence of bla_{NDM-1} gene with other MBLs encoding genes in *P. aeruginosa* is a potential challenge in the treatment of microorganism infections. DLST showed partial diversity among 29 bla_{NDM-1} positive isolates.

Keywords: Pseudomonas aeruginosa, bla_{NDM-1}, DLST, MHT, MBL

Background

Pseudomonas aeruginosa is one of the most important hospital-acquired pathogens that causes miscellaneous opportunistic infections [1]. The emergence of multidrug-resistant (MDR: was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories) and extremely drug resistant (XDR: was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories) *P. aeruginosa* isolates has been considered as a major concern for the treatment of infections caused by these isolates [2]. Carbapenemases are a wide spectrum group of beta-lactamase which hydrolyzes carbapenems to other b-lactams including monobactams, penicillins, and cephalosporins. Although carbapenems are a commonly last resort treatment used for MDR *P. aeruginosa* infection, the emergence of carbapenem-resistant *P. aeruginosa* is becoming a main public health concern and is associated with high rates of mortality and morbidity among hospitalized patients [3, 4].

Resistance to carbapenems can be related to producing carbapenemase enzymes such as serine carbapenemases and the MBLs encoding genes such as IMP, VIM, and

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NDM enzymes [5]. The MBLs encoding genes such as bla_{VIM} and bla_{IMP} are one of the most clinically important classes of beta-lactamases; but, discovered transmissible New Delhi metallo-beta-lactamase-1 (*NDM-1*) is becoming the most threatening carbapenemase, recently [6–8]. The bla_{NDM-1} producing strains are resistant to a wide-ranging of other antibiotic groups and transport numerous additional resistance genes such as genes encodings resistance to fluoroquinolones, aminoglycosides, sulfonamides, and macrolides. Furthermore, the *NDM-1* enzyme is surfacing, resulting in almost whole resistance to antibiotics [8–10].

Molecular typing of *P. aeruginosa* is important to understand the local epidemiology, but it remains a challenging issue. The epidemiology of *P. aeruginosa* has been analyzed by an array of different typing methods such as Pulsed-field gel electrophoresis (PFGE) and Multilocus sequence typing (MLST) that are costly, required specific technical abilities and time to consume [11]. The newly described double-locus sequence typing (DLST) methods based on the partial sequencing of two highly variable loci to typing *P. aeruginosa* isolates which allowed us to obtain an unambiguous and standardized definition of types [12]. DLST has remarkable discriminatory power, reproducibility and is able to recognize high-risk epidemic clones [12]. Although bla_{NDM-1} positive isolates are rare, knowledge of its occurrence is considered as a serious menace, however, this study is the first report of DLST typing of bla_{NDM-1} positive *P. aeruginosa* isolates obtained from different part of Iran.

Methods

Study design, sampling, and bacterial isolates

A cross-sectional study was conducted at three major teaching Hospitals (Ahvaz, Tehran, and Isfahan) in Iran during three-year period. In total, 369 non-duplicate *P. aeruginosa* isolates were collected from different clinical sources such as trachea (84/369), wound (51/369), urine (79/369), punch biopsy (62/369), blood (34/369), sputum (35/369) and other (24/369). These samples were obtained from patient hospitalized in intensive care (ICU) and neonatal ICU (174/369), internal (149/369), emergency (11/369), other (15/369) and 20 samples from outpatients referred to laboratory center [13].

A total of 29 non-duplicate bla_{NDM-1} positive *P. aeruginosa* were collected from different clinical samples. The identification of *P. aeruginosa* was done by the conventional microbiology tests and confirmed by PCR with specific primers for *gyrB* gene [14].

PCR amplification of resistance genes

PCR amplification was performed for detection of bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{KPC}, bla_{GES}, bla_{SPM} and bla_{OXA-10} using a set of specific primers on a thermal cycler (Eppendorf AG, Germany) as described previously [15–17]. Sequencing of the amplicons was performed by the Bioneer Company (Bioneer, Daejeon, South Korea) and the nucleotide sequences were analyzed using GenBank nucleotide database at http://www.ncbi.nlm.nih.gov/blast/.

Antimicrobial susceptibility testing

Antibiotic susceptibility of the bla_{NDM-1} positive isolates was determined by the Kirby–Bauer method as recommended by the CLSI. The 11 standard antibiotic disks used include: imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), gentamicin (10 µg), piperacillin/tazobactam (100/10 µg), amikacin (30 µg), ciprofloxacin (5 µg) and aztreonam (30 µg) (Mast Group Ltd, UK). The ESBL phenotype was identified using combined disk method by disks of ceftazidime (30 mg) with (10 mg) and without clavulanic acid (Mast Group Ltd, UK), applied to all bla_{NDM-1} positive isolates (15). Moreover, the minimum inhibitory concentrations (MICs) of imipenem (10 µg/ml) [$\leq$ 2 mg/L (susceptible), 4 mg/L (intermediate), and $\geq$ 8 mg/L (resistant)] (Liofilchem, Roseto degli Abruzzi, Italy) were applied by gradient test strips to bla_{NDM-1} positive *P. aeruginosa* isolates [18].

Carbapenemase screening

The double-disk potentiation tests (DDPT) and double disk synergy test (DDST) was performed phenotypically for all bla_{NDM-1} positive described by Yong et al. [19].

Double-locus sequence typing method

DLST typing was carried out using amplification of ms172 and ms217 loci using specific primers as previously described (Basset and Blanc, 2014) and according to DLST website (http://www.dlst.org/). PCR products were purified and were sequenced by Bioneer Corporation (Bioneer, Daejeon, South Korea).

In the cases of any results of allele assignment, the allele was considered as a null allele. The allele profiles were compared and clustered by the UPGMA and Dice methods (Fig. 1), using an online data analysis service (nslico.ehu.es).

Results

During the three years of study, twenty-nine of bla_{NDM-1} positive isolates were collected, of them, 6 (20.7%) and 23 (79.3%) isolates were from burn patients and non-burn patients, respectively. The male to the female proportion in bla_{NDM-1} isolates was 3.22 (n= 20:9). The most bla_{NDM-1} positive strains were isolated from wound/punch (n = 11; 37.9%) followed by urine (n = 11; 37.9%)

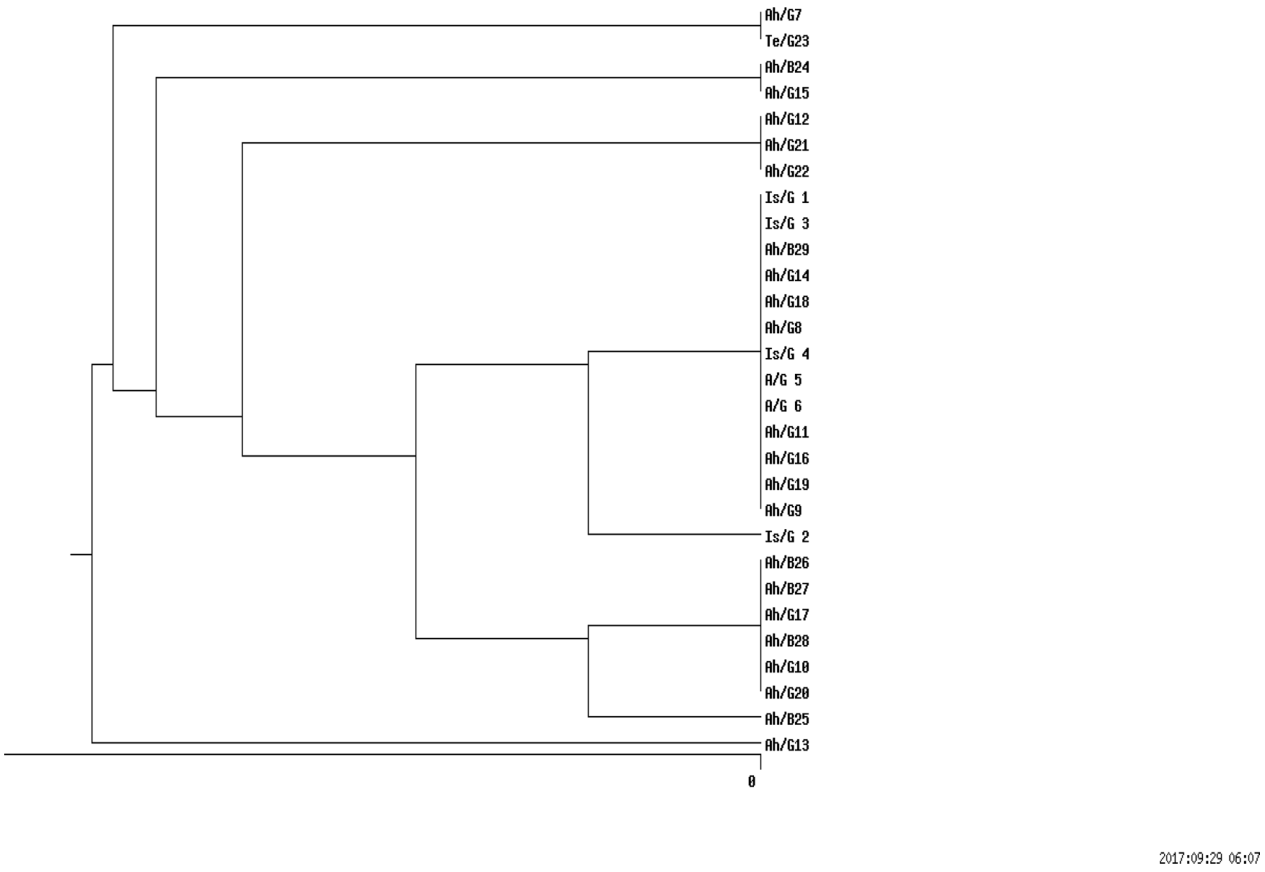

Fig. 1 Dendrogram of DLST result analysis of 29 $bla_{\text{NDM-1}}$ *P. aeruginosa* clinical strains isolated from hospitals in three different cities (Tehran, Ahvaz, Isfahan) of Iran. Results were clustered by UPGMA and compared by Dice Methods; Is; Isfahan, Ah; Ahvaz, Te; Tehran, G; General hospital, B; Burn hospital

samples, whereas, the majority of the $bla_{\text{NDM-1}}$ isolates were obtained from ICU ward (n = 21: 72.4%), followed by internal ward (n = 6; 20.7%) and burn ward (n = 6; 20.7%) (Table 1). Antibiotic susceptibility pattern showed that all isolates were resistance to imipenem and ertapenem, moreover, the resistance rate of meropenem was 96.5%. In contrast, the highest sensitivity was against to piperacillin/tazobactam and amikacin (41.4%). All $bla_{\text{NDM-1}}$ positive isolates were defined as MDR. The full results of antibiotic resistance pattern of $bla_{\text{NDM-1}}$ positive *P. aeruginosa* isolates showed in Table 2.

The MICs of imipenem against $bla_{\text{NDM}-1}$ positive *P. aeruginosa* isolates are presented in Table 1. Overall, the results of MICs of imipenem showed 82.7% (24/29) isolates were high-level imipenem-resistant isolates (MIC ≥32). DDST and DDPT revealed that 15/29 (51.8%) and 26 (89.7%) of $bla_{\text{NDM-1}}$ positive isolates were MBL producing isolates, respectively. In addition, the results of MHT showed that 27/29 (89.6%) of $bla_{\text{NDM}-1}$ positive *P. aeruginosa* isolates were carbapenemase-producing isolates.

The distribution of carbapenemase genes and other antibiotic resistance genes among $bla_{\text{NDM-1}}$ positive *P. aeruginosa* isolates are presented in Table 2. However, PCR analysis showed none of the $bla_{\text{NDM-1}}$ positive *P. aeruginosa* isolates contained bla_{KPC} and bla_{GES} genes. The presence of $bla_{\text{VIM-2}}$, $bla_{\text{IMP-1}}$ and bla_{SPM} genes were detected in 41.4%, 34.5% and 3.5% isolates, respectively. Among $bla_{\text{NDM-1}}$ positive *P. aeruginosa* isolates, the $bla_{\text{OXA-10}}$ beta-lactamase was the most frequently gene recognized in 86.2% (25/29).

The combination of $bla_{\text{VIM-2/}}$ $bla_{\text{OXA-10}}$ and $bla_{\text{IMP-1/}}bla_{\text{OXA-10}}$ beta-lactamase was found in 8 (27.6%) and 10 (34.5%) isolates, respectively. Moreover, among $bla_{\text{NDM-1}}$ positive *P. aeruginosa* isolates the co-harboring of three genes, $bla_{\text{OXA-10}}$, $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-2}}$ was found in two isolates and only one isolate contained bla_{SPM} that in combination with $bla_{\text{VIM-2}}$ and $bla_{\text{OXA-10}}$.

In the current study, the DLST method was tested for *all* $bla_{\text{NDM-1}}$ isolates recovered over a period of four years from various hospital wards. DLST results revealed partial diversity among 29 $bla_{\text{NDM-1}}$ positive isolates. Totally,

Table 1 The detailed results of *P. aeruginosa* with and without bla_{NDM-1}

Variable	*P. aeruginosa* (without bla_{NDM-1}) N=340 (%)	P. aeruginosa (with bla_{NDM-1}) N=29 (%)	p-value
Sex			
Male	199 (58.5)	20 (69)	0.2
Female	141 (41.5)	9 (31)	
Type of sample			
Trachea	80 (23.5)	4 (13.8)	0.2
Urine	68 (20)	11 (37.9)	197
Punch	60 (17.6)	2 (6.9)	0.1
Wound	42 (12.3)	9 (31)	0.005
Sputum	34 (10)	1 (3.5)	0.2
Blood	32 (9.4)	2 (6.9)	0.6
Other	24 (70.5)	–	–
Type of patients			
Burn	45 (13.2295)	6 (20.7)	
ICU	153 (45)	21 (72.4)	0.09
Internal	143 (42.1)	6 (20.7)	0.02
Emergency	11 (3.2)	–	–
Outpatients	18 (5.3)	2 (6.9)	0.7
Other	15 (4.4)	–	–

ICU Intensive care unit

8 different DLST profile (DL type) (four different common types and three single type) were detected (Table 2). The most common type including 13 isolates (45%) from different hospitals (in Ahvaz and Isfahan). A total of 29, 27 sequences were ms172 and ms217 whereas, 2 strains carried null alleles for these loci. The DLST profile 5–91 were detected in 3 (50%) burn isolates. The details of information about DL type of *P. aeruginosa* isolates are presented in Table 2.

Discussion

P. aeruginosa, one of the most common opportunistic pathogen associated with nosocomial infections, including pneumonia, urinary tract infections, and wound infections [1, 20]. Although carbapenems are often used as a therapeutic agent for treating infections caused by *P. aeruginosa*, the high emergence of carbapenem resistance significantly decreases their usefulness [21, 22]. The presence of bla_{NDM-1} producing isolates which may increase resistance to carbapenems is increasing among patients in healthcare systems [23]. In the current study, we described molecular characterization of bla_{NDM-1} producing of *P.aeruginosa* isolates with phenotypic and genotypic methods.

The overall data show that the frequency of bla_{NDM-1} producing *P. aeruginosa* isolates was 7.8% (29/369). In addition, there are variable reports of bla_{NDM-1} from different countries in Europe and Asian. The bla_{NDM-1} producing *P. aeruginosa* isolates has been also detected in Iran, recently. Shokri et al. from Isfahan (Center of Iran) in 2017 reported 6% of *P. aeruginosa* isolates were bla_{NDM-1} positive which is slightly lower than the result obtained in the present study [24]. In another study, Dogonchi et al. reported one isolate of *P. aeruginosa* harboring bla_{NDM-1} in the north of Iran [25]. Recently, Azimi et al. described a lower frequency rate for bla_{NDM-1} (7%) among carbapenem-resistant *P. aeruginosa* [26]. Moreover, the bla_{NDM-1} gene was also revealed by Riahi Rad et al. in Iran (21.4%) [27] and Takahashi et al. in Nepal (7%) [28].

With regard to the fact that our isolates were collected from hospitals of different cities in Iran and also regarding the results of previous studies, an increasing trend of bla_{NDM-1} producing *P. aeruginosa* strains can be observed in Iranian hospitals where it could be an endemic and serious concern in future. One of the important reason of possible increase of this phenomenon among Gram-negative isolates is inappropriate and excessive prescription and use of carbapenems in our hospitals, which leads to selective pressure.

According to our results, the most bla_{NDM-1} positive isolates (72.4%) were collected from the ICU ward that these findings are broadly consistent with the previous studies conducted in Iran [24, 25]. This finding suggests that the ICU ward is can be a risk factor and major source for the dissemination of resistant genes in the Iranian hospitals. Our results presented that bla_{NDM-1} positive isolates had highly resistant to all antibiotics commonly used in the clinic which is in agreement with to the results of other studies [24, 29, 30].

In spite of the fact that the bla_{NDM-1} gene demonstrating the sensitivity of bacteria to aztreonam, 62% of bla_{NDM-1} positive isolates were resistant to this agent that could be related to the presence of other beta-lactamase genes. Based on screening of other carbapenemase and metallo β-lactamase genes, bla_{OXA-10} was the most frequently detected beta-lactamase among bla_{NDM-1} positive strain and bla_{IMP-1} was second, which is in contrast to other reports where bla_{VIM} was significantly associated with bla_{NDM-1} [31, 32].

One of the important findings in this study was the emergence of the co-harboring of bla_{NDM-1} positive *P. aeruginosa* isolates with more than one carbapenemase gene and metallo β-lactamase determinants, simultaneously. Accordingly, we report the first isolate of *P. aeruginosa* producing four carbapenemases co-existence $bla_{NDM-1,}$ bla_{VIM--2}, bla_{IMP-1} and bla_{OXA-10} from Iran. One of them that was obtained from urine sample was resistant to all antibiotics used except to TZP that were intermediate. Furthermore, we demonstrated the

Table 2 The detailed results of bla_{NDM-1} isolates

No.	Gender	City/hospital	Sample/ward	MBLs genes Non-MBLs Genes	Phenotypic tests			Antibiogram Pattern										DLST type
					Hodge test	MIC IPM	ESBL	IMP	MEM	ETP	TZP	CEP	AN	CIP	GEN	CAZ	CTX	AZT	
1	M	I/general	Wound/ICU	NDM-1, IMP-1, OXA-10	−	≥ 32	−	R	R	R	S	S	S	R	R	R	R	S	25–11
3	M	I/general	Urine/ICU	NDM-1, OXA-10	+	≥ 32	−	R	R	R	R	S	S	R	R	R	R	R	25–11
4	M	I/general	Urine/ICU	NDM-1, IMP-1, OXA-10	+	≥ 32	+	R	R	R	I	R	R	R	R	R	R	R	25–11
5	F	A/general	Urine/ICU	NDM-1, OXA-10	+	≥ 32	−	R	R	R	I	R	R	R	R	R	R	S	25–11
6	F	A/general	Urine/ Internal	NDM-1, VIM-2, OXA-10	+	≥ 32	−	R	I	R	S	S	S	S	S	S	I	R	25–11
8	F	A/general	Blood/ICU	NDM-1, VIM-2, IMP-1, OXA-10	+	≥ 32	−	R	R	R	R	R	S	S	I	R	R	R	25–11
9	M	A/general	Urine/internal	NDM-1, OXA-10	+	≥ 32	−	R	R	R	R	R	S	I	R	R	R	R	25–11
11	M	A/general	Wound/internal	NDM-1, VIM-	+	8	−	R	R	R	S	R	S	R	R	R	R	S	25–11

Table 2 (continued)

				2, IMP-1															
14	M	A/general	Wound/ICU	NDM-1, VIM-2, OXA-10	+	≥ 32	−	R	R	R	S	R	S	R	R	R	R	S	25–11
29	F	A/burn	Wound/ICU	NDM-1, VIM-2, OXA-10	+	≥ 32	−	R	R	R	R	R	R	R	R	I	R	R	25–11
18	M	A/general	Urine/internal	NDM-1, OXA-10	+	≥ 32	−	R	R	R	I	R	S	R	R	R	I	R	25–11
19	M	A/general	Urine/ICU	NDM-1, IMP-1, OXA-10	+	≥ 32	−	R	R	R	I	R	I	R	R	R	S	R	25–11
16	M	A/general	Urine/NICU	NDM-1, IMP-1, OXA-10	+	≥ 32	−	R	R	R	I	S	I	S	R	I	S	R	25–11
23	M	T/gener a	Urine/ICU	NDM-1, OXA-10	−	2	+	R	R	R	R	R	R	R	R	S	R	I	32–39
7	M	A/general	Sputum/internal	NDM-1, IMP-1, OXA-10	+	≥ 32	−	R	R	R	I	R	R	R	R	R	R	R	32–39
25	M	A/burn	Punch/ICU	NDM-1, OXA-10	+	16	−	R	R	R	R	R	R	R	R	S	R	R	5–6
2	F	I/general	Urine/ICU	NDM-1, IMP-1, OX	+	16	−	R	R	R	I	S	S	R	R	R	R	R	5–11

Table 2 (continued)

				A-10															
10	F	A/general	Urine/internal	NDM-1, VIM-2, IMP-1, OXA-10	+	≥ 32	−	R	R	R	I	R	R	R	R	R	R	R	5–91
17	M	A/general	Blood/OP	NDM-1, IMP-1, OXA-10	+	≥ 32	−	R	R	R	I	R	S	R	R	R	S	R	5–91
26	M	A/burn	Punch/ICU	NDM-1, OXA-10	+	16	−	R	R	R	R	R	R	R	R	S	R	I	5–91
27	M	A/burn	Wound/ICU	NDM-1, OXA-10	+	≥ 32	−	R	R	R	R	R	R	R	R	S	R	R	5–91
28	F	A/burn	Wound/ICU	NDM-1, VIM-2, OXA-10	+	≥ 32	−	R	R	R	I	R	R	R	R	S	R	I	5–91
20	M	A/general	Trachea/ICU	NDM-1, IMP-1, OXA-10	+	≥ 32	−	R	R	R	S	R	S	R	R	I	R	R	5–91
12	M	A/general	Trachea/ICU	NDM-1, VIM-2, OXA-10	+	≥ 32	−	R	R	R	S	R	S	R	R	R	R	S	20–68
21	M	A/general	Trachea/ICU	NDM-1, IMP-1	+	≥ 32	+	R	R	R	R	R	R	R	R	R	R	R	20–68
22	M	A/general	Trachea/ICU	NDM-1, OX	+	≥ 32	−	R	R	R	R	R	S	R	S	R	R	I	20–68

Table 2 (continued)

				A-10															
13	F	A/general	Wound/ICU	NDM-1, VIM-2	+	≥ 32	−	R	R	R	S	R	S	R	S	R	R	I	9–115
15	M	A/general	Wound/OP	NDM-1, IMP-1	+	≥ 32	−	R	R	R	R	R	S	R	R	R	I	S	Nul l
24	F	A/burn	Wound/ICU	NDM-1, VIM-2, OXA-10	+	≥ 32	−	R	R	R	R	R	R	R	R	I	R	R	Nul l

Imipenem (IPM), meropenem (MEM), ertapenem (ETP), piperacillin-tazobactam (TZP), cefepime (CFP), amikacin (AN), ciprofloxacin (CIP), gentamicin (GEN), ceftazidime (CAZ), cefexime (CTX), azteronam (AZT). A, Ahvaz; I, Isfahan; T, Tehran; OP, Outpatient; ICU

co-harboring of $bla_{\text{NDM-1}}$ with metallo-β-lactamases genes such as $bla_{\text{OXA-10}}$, $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-2}}$ in *P. aeruginosa.* The coexistence of carbapenemases encoding genes with $bla_{\text{NDM-1}}$ positive *P. aeruginosa* isolates has been reported in several Asian and European countries including in India ($bla_{\text{NDM-1}}$ + bla_{IMP}+ bla_{VIM}+ $bla_{\text{SPM)}}$, Denmark ($bla_{\text{NDM-1}}$ + $bla_{\text{VIM-5}}$+ $bla_{\text{VIM-6}}$) [33], Bangladesh ($bla_{\text{NDM-1}}$, $bla_{\text{VIM-1}}$, $bla_{\text{VIM-2}}$, $bla_{\text{IMP-1}}$) [34] and Turkey ($bla_{\text{VIM-1}}$+ $bla_{\text{VIM-2}}$+ $bla_{\text{GES-5}}$ [35].

The previous studies revealed that the acquisition of MBL determinants such as $bla_{\text{NDM-1}}$, bla_{VIM}, *bla* IMP and bla_{SPM} led to the emergence of MDR or XDR *P. aeruginosa* [36, 37].

To the extent of our knowledge, this study is the first to report of Molecular typing of $bla_{\text{NDM-1}}$ positive isolates in *P. aeruginosa* by DLST method.

Among various genotyping technique, DLST as a reliable genotyping method, provide a new, rapid, typability, stability and low-cost of epidemiological surveillance of *P. aeruginosa* isolates [11, 38]. Analysis of DLST types revealed that the majority (19/29) of the isolates belonged to DLST type 25-11 and 5-91.

In addition, 8 $bla_{\text{NDM-1}}$ positive isolates were clustered into two DLST types and 3 singletons. Accordingly, $bla_{\text{NDM-1}}$ positive isolates were relatively heterogeneous, however, the route of transmission is not clear. These results highlight the importance of investigating carbapenem-resistant *P. aeruginosa* isolates in health care settings in our region.

Conclusions

The occurrence of $bla_{\text{NDM-1}}$ isolates in *P. aeruginosa* is a large challenge in the treatment and worrying for global health. DLST type 25-11 is a significant cluster because a large number of $bla_{\text{NDM-1}}$ isolates showed this genotype and also DLST type 5-91 known as an alarming type in burn patients. This work suggests that the DLST as an appreciated method in typing of $bla_{\text{NDM}-1}$ strains; this technique reducing considerably the time and the cost of the molecular analysis and providing a reliable phylogenetic study. This information can help to generate the proper strategies for accurate and specific use of this antibacterial which can help to control of $bla_{\text{NDM-1}}$ isolates.

Abbreviations

ESBL: Extended-spectrum b-lactamases; CLSI: Clinical and Laboratory Standards Institute; CCs: Clonal complex; DLV: Double-locus variants; DDST: Double-disk synergy test; DDPT: Double-disk potentiation test; KTP: Kidney transplant patients; ST: Sequence types; SLV: Single-locus variants; SD: Standard deviation; UPEC: Uropathogenic *Escherichia coli*; UTI: Urinary tract infections; MLST: Multilocus sequence typing; VFs: Virulence factors.

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Authors' contributions

AA designed the study and reviewed the manuscript, and edited the final version. MSH contributed to design the study, collected the data, and drafted the manuscript. AA analyzed the data, reviewed the manuscript, and edited the final version. All authors read and approved the final manuscript

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Declarations

Ethics approval and consent to participate
This study was also confirmed and permitted by the Ethics Committee of Clinical Research Development Unit of Baqiyatallah Hospital" IR.BMSU.REC.1398.241".

Consent for publication
Not applicable.

Competing interests
The authors report no conflicts of interest in this work.

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