Original Article

Genetic characterization of *Pseudomonas aeruginosa*-resistant isolates at the university teaching hospital in Iran

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Abstract

Background: *Pseudomonas aeruginosa* is an opportunistic pathogen that is commonly responsible for nosocomial infections. The aim of this study was to perform a genotyping analysis of the *Pseudomonas aeruginosa*-resistant isolates by the multilocus sequence typing (MLST) method at the university teaching hospital in Iran.

Materials and Methods: Antimicrobial susceptibility was analyzed for *P. aeruginosa* isolates. Ceftazidime-resistant (CAZ_{res}) isolates with a positive double-disc synergy test were screened for the presence of extended-spectrum β-lactamase-encoding genes. Phenotypic tests to detect the metallo-β-lactamase strains of *P. aeruginosa* were performed on imipenem-resistant (IMP_{res}) isolates. Selected strains were characterized by MLST.

Results: Of 35 *P. aeruginosa* isolates, 71%, 45% and 45% of isolates were CAZ_{res}, IMP_{res} and multidrug resistant (MDR), respectively. Fifty-seven percent of the isolates carried the $bla_{OXAgroup\text{-}1}$. All the five typed isolates were ST235. Isolates of ST235 that were MDR showed a unique resistance pattern.

Conclusion: This study shows a high rate of MDR *P. aeruginosa* isolates at the university teaching hospital in Iran. It seems MDR isolates of *P. aeruginosa* ST235 with unique resistance pattern disseminated in this hospital.

Key Words: MDR, MLST, *Pseudomonas aeruginosa*, ST235

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Received: 17.04.2014, **Accepted:** 19.08.2014

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that is commonly responsible for nosocomial infections, including pneumonia, bloodstream infection, urine infection and ocular infection.[1] Antibiotic surveillance studies are necessary for the design of control strategies for preventing bacterial resistance and establishing therapeutic guidelines as well as for a better understanding of bacterial epidemiology.

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.161583

Multilocus sequence typing (MLST) is a strain-typing system that focuses strictly on conserved housekeeping genes.[2] The choice of seven loci ensures adequate variability in distinguishing the most closely related strains and still be able to track the global clonal history of the species with the highest possible accuracy. Because of these advantages, we used an MLST scheme to characterize the strains

How to cite this article: Fazeli H, Sadighian H, Esfahani BN, Pourmand MR. Genetic characterization of *Pseudomonas aeruginosa*-resistant isolates at the university teaching hospital in Iran. Adv Biomed Res 2015;4:156.

of *P. aeruginosa* isolated from the clinical isolates, which enabled us to compare our data with those in the database generated from various clinical and environmental *P. aeruginosa* strains obtained from various countries.

P. aeruginosa has intrinsic resistance to many antibiotics and produces a variety of virulence factors. The intrinsic resistance of *P. aeruginosa* to various antibiotics is generally due to its low outer membrane permeability, production of the inducible AmpC chromosomal β-lactamase and multidrug efflux pumps.[3,4] Thus, suitable drugs against *P. aeruginosa* infections are limited to aminoglycosides (e.g., amikacin), fluoroquinolones (ciprofloxacin remains the most active), selected β-lactams (e.g., ceftazidime and carbapenems) and one β-lactam/β-lactamase inhibitor combination (piperacillin/tazobactam).[5]

Like other Gram negative pathogens, *P. aeruginosa* is known to acquire resistance by producing various β-lactamases. Among plasmid-mediated β-lactamases, extended-spectrum β-lactamases (ESBLs) are generally known to hydrolyze cephamycins and metallo-β-lactamases (MBLs) can hydrolyze carbapenems, which cannot be easily hydrolyzed by traditional β-lactamases.[3,4]

MBLs comprise one of the most clinically important families of β-lactamases in Gram negative bacilli, largely due to their association with mobile genetic elements that often carry other resistance genes, resulting in multidrug resistance (MDR).[6] The acquired MBLs include the VIM and IMP families, SPM-1, GIM-1, SIM-1, AIM-1, KHM-1, NDM-1 and DIM-1.[3,4,7,8]

The IMP- and VIM-type enzymes, which are encoded by integron-borne genes, are currently the most widespread, being reported from several continents, and several allelic variants are known for each type.[3,4]

MLST has identified international clonal complexes (CCs) responsible for the dissemination of MBL-producing and MDR isolates of *P. aeruginosa*, particularly in European countries,[9,10] Japan,[11] Singapore and Brazil (http://pubmlst.org/paeruginosa/).

Infections with MBL-producing and MDR isolates of *P. aeruginosa* have been shown to be associated with mortality rates higher than infections with MBL-negative *P. aeruginosa* isolates.[12,13]

Ceftazidime, cefepime and carbapenems (including imipenem), first described in Enterobacteriacae and as extended-spectrum AmpC have been referred and reported in clinical isolates of *P. aeruginosa*.[14]

OXA enzymes are mostly narrow-spectrum β-lactamases that confer resistance to aminopenicillins and carboxypenicillins and narrow-spectrum cephalosporins although several OXA-type enzymes are ESBLs like OXA-45 (Poole, *et al*., 2011). OXA group-1 β-lactamases, especially OXA-10, has the highest frequency among all OXA-type β-lactamases in Iran.[15,16]

Carbapenems (e.g., meropenem, imipenem) are an important class of anti-pseudomonal β-lactams owing to their stability to most β-lactamases, and are of particular use in treating infections associated with ESBL- and AmpC-producers. β-lactamases capable of hydrolyzing carbapenems are known and include class A and class D carbapenemases (the latter also referred to as carbapenem-hydrolyzing class D β-lactamases, CHDLs) and class B MBLs.[3]

The objectives of the present study were to analyze the genetic characters of *P. aeruginosa* by the MLST method for MDR isolates of *P. aeruginosa* recovered from the first 3 months of 2012 at the university teaching hospital in Iran. As a future plan, it will be continued as a nationwide surveillance program in Iran.

MATERIALS AND METHODS

Sample collection and clinical data

During the first 3 months of 2012, a total of 35 consecutive and nonduplicate *P. aeruginosa* isolates were collected from patients at the university teaching hospital in Iran. The isolates were collected from various infections in different wards of the hospital. Various specimens included urine, blood, catheter, tracheal aspirate and wound. To avoid duplicates, only one isolate was selected from each patient, unless isolates showed different resistance profiles. The isolates were stored at -76°C in 20% skimmed milk until used in this study. Over the study period, all the isolates were identified using biochemical tests and then strains were confirmed by polymerase chain reaction (PCR) for *oprL* gene.

Antimicrobial susceptibility tests and detection of ESBLs by phenotypic tests

Antimicrobial susceptibility tests were performed on Mueller-Hinton agar (Oxoid, Basingstoke, UK) plates with commercially available discs (MAST, Bootle, Merseyside, UK) by the Kirby Bauer disc diffusion method. Susceptibilities were defined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[17] Susceptibilities to 10 antimicrobial agents, which are primarily effective against *P. aeruginosa* strains, were determined. The following β-lactam antibiotics were used:

Piperacillin 100 μg, piperacillin/tazobactam 100/10 μg, ceftazidime 30 μg, ceftazidime/clavulanic acid 30/10 μg, cefepime 30 μg, meropenem 10 μg, imipenem 10 μg, aztreonam 30 μg, amikacin 30 μg and ciprofloxacin 5 μg discs. *P. aeruginosa* PAO1 was used as the reference strain in the susceptibility tests.

To detect possible ESBL production, the double disk synergy test (DDST) was performed with disks containing ceftazidime (30 mg) alone and in the presence of clavulanate (10 mg). Ceftazidime-resistant isolates were suspected to be ESBL producers when a positive DDST was observed (increase in the ceftazidime inhibition zone of >5 mm in the presence of clavulanic acid as compared with when tested alone) (CLSI guidelines).[17]

To screen for MBL-producing strains, an initial screen test and a phenotypic confirmatory test were performed. This was done to determine resistance to meropenem,[17] followed by the phenotypic MBL detection in all isolates by the DDST with disks containing imipenem alone and with EDTA.[18] MDR isolates were determined according to the MDR definition by Lang *et al*.[19]

PCR amplification for the detection of P. areruginosa and β-lactamese genes

The total DNA from *P. aeruginosa* isolates was extracted using a DNA extraction kit (DNeasy blood and tissue, QIAGEN, Hilden, Germany). PCR was carried out at a 50 μL volume by using 25 μl of the EmeraldAmp MAX HS PCR Master Mix (Takara Shuzo, Shiga, Japan), 50 ng of genomic DNA of the test strain and 0.2 μM of each primer. PCR with the following cycling parameters was performed: Initial denaturation at 94˚C for 240 s; 30 cycles of denaturation at 94˚C for 30 s, annealing for 30 s and extension at 72˚C for 60 s; and a final extension at 72˚C for 300 s. Published primers[20-24] or sets of primers that were designed in this study were used [Table 1].

MLST

Based on resistance pattern, β-lactamase gene and source of infection, MLST was performed on 10 representative isolates according to the protocol published by Curran *et al*.[2] Seven internal fragments of the genes *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE* were amplified and the resulting PCR products were purified with the QIAquick PCR purification kit (QIAGEN). PCRs were performed using the same amplification mixture and reaction conditions as those described in the published protocol. Sequencing was carried out with primers as corrected at the http://pubmlst.org website [Table 2].

The nucleotide sequences were determined using both strands. The database at http://pubmlst.org/paeruginosa was used to assign numbers to particular alleles and to identify sequence types (STs). Isolates that have five or more identical alleles (single-or double-locus variants) were considered part of the same CC.[2,25]

RESULTS

Bacterial isolates

During the first 3 months of 2012, a total of 35 nonduplicate *P. aeruginosa* isolates were collected from adult patients (19 male and 16 female) suffering from different *P. aeruginosa* infections. Specimens included 14 urine, nine blood, six tracheal aspirates, four catheters and two wound swabs. Sixteen individuals harbored MDR isolates of *P. aeruginosa*.

Table 1: Oligonucleotides used as primers for PCR amplification of the β-lactamase and oprL genes

Name	Target	Sequence (5′>>>3′)	Annealing temperature (˚C)	Amplicon size (bp)	References
OXA1-F	$bla_{OXA\text{-}group\text{-}1}$	TCAACAAATCGCCAGAGAAG	61	276	20
OXA1-R		TCCCACACCAGAAAAACCAG			
PER-F	bla_{PER}	CCTGACGATCTGGAACCTT	56	641	21
PER-R		CCGTCCATCAGGCAACA			
KPC-F	bla_{KPC}	AGTTCTGCTGTCTTGTCTC	55	798	Present study
KPC-R		CTGTGCTTGTCATCCTTG			
BEL-F	bla_{BEL}	TGCTGTTCCTTGTCATTC	55	782	Present study
BEL-R		TAATAACGCCCTTTCCTC			
VIM-F	bla_{VIM}	GTCCGTGATGGTGATGAGT	58	437	22
VIM-R		ATTCAGCCAGATCAGCATC			
IMP-F	bla_{IMP}	CATGGTTTGGTGGTTCTTGT	56	448	23
IMP-R		ATAATTTGGCGGACTTTGGC			
AmpC-F	bla_{AmpC}	GCTCCACCAACGGCTTC	58	124	Present study
AmpC-R		CTGAGGATGGCGTAGGC			
oprL-F	*oprL*	ATGGAAATGCTGAAATTCGGC	57	504	24
oprL-R		CTTCTTCAGCTCGACGCGACG			

Table 2: Oligonucleotide primers used for *P. aeruginosa* multilocus sequence typing

Locus and function	Primer sequence (5′to 3′)		Amplicon size
	Forward	Reverse	
acsA			
Amplification	ACCTGGTGTACGCCTCGCTGAC	GACATAGATGCCCTGCCCCTTGAT	842
Sequencing	GCCACACCTACATCGTCTAT	GTGGACAACCTCGGCAACCT	390
aroE			
Amplification	TGGGGCTATGACTGGAAACC	TAACCCGGTTTTGTGATTCCTACA	825
Sequencing	ATGTCACCGTGCCGTTCAAG	TGAAGGCAGTCGGTTCCTTG	495
guaA			
Amplification	CGGCCTCGACGTGTGGATGA	GAACGCCTGGCTGGTCTTGTGGTA	940
Sequencing	AGGTCGGTTCCTCCAAGGTC	TCAAGTCGCACCACAACGTC	372
mutL			
Amplification	CCAGATCGCCGCCGGTGAGGTG	CAGGGTGCCATAGAGGAAGTC	940
Sequencing	AGAAGACCGAGTTCGACCAT	ATGACTTCCTCTATGGCACC	441
nuoD			
Amplification	ACCGCCACCCGTACTG	TCTCGCCCATCTTGACCA	1,042
Sequencing	ACGGCGAGAACGAGGACTAC	TTCACCTTCACCGACCGCCA	366
ppsA			
Amplification	GGTCGCTCGGTCAAGGTAGTGG	GGGTTCTCTTCTTCCGGCTCGTAG	989
Sequencing	GGTGACGACGGCAAGCTGTA	TCCTGTGCCGAAGGCGATAC	369
trpE			
Amplification	GCGGCCCAGGGTCGTGAG	CCCGGCGCTTGTTGATGGTT	811
Sequencing	TTCAACTTCGGCGACTTCCA	GGTGTCCATGTTGCCGTTCC	441

Antimicrobial susceptibility profiles and identification of β-lactamase-producing isolates

Of the 35 *P. aeruginosa* isolates, 25 (71%) isolates showed resistance to ceftazidime. The ceftazidime-resistant strains were subjects for the DDST. Eleven ESBL-producing isolates were detected according to the phenotypic test. In order to confirm this, the presence of genes for various ESBLs was investigated by PCR. Thus, all of the isolates were evaluated for the presence of ESBL-encoding genes (bla_{PER}, bla_{BEL} and bla_{KPC}) by PCR amplification. Also, all the isolates were evaluated for $bla_{OXA\text{-}group\text{-}1}$ and $bla_{AmpC.}$ As a result, none of the isolates had bla_{PER}, bla_{BEL} and bla_{KPC}. Twenty (57%) isolates were related to OXA group-1 positive refer to different samples included urine, blood, tracheal aspirate and catheter. Also, all of the isolates harbored the bla_{AmpC} gene.

Forty-five percent and 51% of all clinical isolates were IMP_{res} and MER_{res}, respectively, and phenotypic tests for MBL detection were performed for the MER_{res} isolates. Nine isolates were positive and they were suspected for MBLs. In order to confirm it, PCR for two MBL genes (bla_{VIM}, bla_{IMP}) were performed for these 11 isolates. Results showed there were no bla_{VIM}, bla_{IMP} genes in these isolates. Resistance to ciprofloxacin and amikacin were observed in 54% of the isolates.

Most of the *P. aeruginosa* strains isolated from patients at the university teaching hospital in Iran had a high level of resistance to ceftazidime [Table 3]. Of all the isolates, 16 (45%) clinical isolates were identified to be MDR. These 16 MDR isolates represented resistance to all examined antibiotics that were included — penicillins (piperacillin), cephalosporins (ceftazidime and cefepime), carbapenems (imipenem and meropenem), monobactams (aztreonam), fluoroquinolones (ciprofloxacin) and aminoglycosides (amikacin).

Molecular epidemiology based on MLST

Based on resistance pattern, β-lactamase gene and the source of infection, five of the most frequent isolates that showed resistance to all tested antibiotics were chosen for the MLST. These selected isolates were positive for $bla_{OXAgroup\text{-}1}$. All of them included bla_{AmpC}. All selected strains were successfully typed. MLST showed that the typed isolates belonged to one ST, all of which were original (www.pubmlst.org/paeruginosa/), and they were designated as ST235 [Table 4].

The MLST results are summarized in Table 4. There was a correlation between resistance patterns and STs. International ST, ST235, was represented by all typed isolates that were MDR and OXA group-1 positive. All isolates of ST235 showed resistance to all tested antibiotics.

DISCUSSION

Resistance to antimicrobial agents is an increasing public health threat. It limits therapeutic options and

leads to increased mortality and morbidity.[26] Given the increasing resistance rates in *P. aeruginosa*, MDR can be expected to become more prevalent in many hospitals.

The present study was aimed to determine the genetic characterization of the MDR isolates of *P. aeruginosa* isolated from various infections and wards at the university teaching hospital in Iran.

According to our data, 45% of the isolates were MDR. The results showed that 71% of *P. aeruginosa* isolated from different infections and wards at the university teaching hospital in Iran were ceftazidime resistant. The rate of CAZ_{res} isolates of *P. aeruginosa* in Iran was 66-100%, and our result was in this range.[15,27,28]

The results of current study confirmed the potency of ESBL in 31% of the *P. aeruginosa* isolates based on the DDST.

In a study conducted in two hospitals in Tehran, Iran, 12.5% of the *P. aeruginosa* isolates were resistance to imipenem.[29] Yosefi *et al*. showed that 38.1% of the IMP_{res} isolates were belonged to *P. aeruginosa* at the Imam Hospital in Orumieh, Iran.[28] As discussed by Ranjbar *et al*., 97.5% of imipenem-resistant isolates were found among the burned patients in Tehran.[30] Mirsalehian *et al*. showed similar results in a burn hospital (100% IMP_{res}) in Tehran.[15] In the present study, 45% and 51% of the clinical isolates were resistance to imipenem and meropenem, respectively. This rate was significantly lower than the IMP_{res} rates of other studies in burn hospitals in Iran. However, this is a high level of resistance to carbapenems, and it caused problems in the treatment of infections by *P. aeruginosa*, especially ESBL strains. Phenotypic MBL examination confirmed the potent of MBL in 25% of the clinical isolates of *P. aeruginosa*. According to our investigation by PCR, bla_{VIM}, bla_{IMP} were not detected in our isolates. It seems that there is another type of MBL in these isolates.

Surveillance for four types of β-lactamases included $bla_{OXA\ group\text{-}1}$, bla_{PER}, bla_{BEL} and bla_{KPC} by the PCR method, showing that 57% of the isolates were OXA group-1 positive and other β-lactamase genes were not found.

As shown in different studies, the varied range of resistance to ceftazidime and imipenem among *P. aeruginosa* clinical isolates may be due to some factors including hospital wards (ICU or burn units), the antibiotics administered among patients and acquired β-lactamase genes.

MLST experiments have shown that *P. aeruginosa* strains of two international CCs, CC111 (previously described as CC4) and CC235 (previously described as CC11), are responsible for the dissemination of MBL genes in European countries.[31,32]

Meanwhile, ST235 (belongs to CC235) is an internationally widespread clone that has been previously associated with PER, OXA and VIM enzymes.[10] ST235 has also been detected in Spain, linked to the production of GES ESBLs.[33]

In the current study, ST235 was detected among five isolates typed. All typed MDR isolates (resistance to all tested antibiotics, [Tables 3 and 4]) belonged to one clonal type associated with ST235. According to the results, it seems that there is a relation between the susceptibility profiles and STs of the examined isolates. In this study, MDR pattern and OXA group-1 gene were exclusively detected in the *P. aeruginosa* isolates of ST235, suggesting that clonal spreading of the strain ST235 played a key role in dissemination of the MDR pattern and OXA group-1 gene in *P. aeruginosa* at the studied hospital in Tehran.

Table 3: Antimicrobial susceptibility of 35 *P. aeruginosa* by the disc diffusion method

Antibiotic	Con. (μg)	Sensitive No. (%)	Resistance No. (%)
Piperacillin	100	8 (23)	27 (77)
Piperacillin/tazobactam	100/10	11 (32)	24 (68)
Ceftazidime	30	10 (29)	25 (71)
Ceftazidime/clavulanate	30/10	14 (40)	21 (60)
Cefepime	30	15 (43)	20 (57)
Imipenem	10	19 (55)	16 (45)
Meropenem	10	17 (49)	18 (51)
Aztreonam	30	9 (26)	26 (74)
Ciprofloxacin	5	16 (46)	19 (54)
Amikacin	30	16 (46)	19 (54)

Table 4: Multilocus sequence typing (MLST) in selected *P. aeruginosa* isolates

Source of infection	OXA group-1 gene	ampC gene	No. of isolates	MDR[a]	Antibiotic susceptibility[b]				MLST.	Clonal complex
					CAZ	IMP	CIP	ST	Allelic profile (acs, aro, gua, mut, nuo, pps, trp)	
Urine	+	+	2	+	R	R	R	235	(38-11-3-13-1-2-4)	CC235
Blood	+	+	2	+	R	R	R	235	(38-11-3-13-1-2-4)	CC235
Catheter	+	+	1	+	R	R	R	235	(38-11-3-13-1-2-4)	CC235

[a]Multidrug resistant (MDR); [b]Resistant (R) or susceptible (S) according to the CLSI breakpoints; CAZ: Ceftazidime; IMP: Imipenem; CIP: Ciprofloxacin

Reports of epidemiological characteristics of MDR *P. aeruginosa* (MDR-PA) isolates from Asian countries are rare. In Japan, IMP-1-producing *P. aeruginosa* isolates of ST357 and ST235 were reported.[11] In Korea, IMP-6-producing *P. aeruginosa* isolates of ST235 were detected.[33] MDR-PA isolates of ST235 and isolates of ST235 with more susceptibility were reported in Czech.[31] In most of the studies, isolates of ST235 showed a high level of resistance to antipseudomonas antibiotics. In Orumieh, Iran, Yousefi *et al*.[34] described ST773 as a MDR clone of *P. aeruginosa* at the burn ward. More than 90% of these isolates were resistant to piperacillin-tazobactam, ceftazidime, cefepim, imipenem, meropenem, aztereonam, amikacin and ciprofloxacin. In the above-mentioned study by Yousefi *et al*.,[34] most of the isolates were identified to be MDR. Also, ST235, ST207, ST623, ST967, ST970 and ST972 were reported in Orumieh, Iran (http://pubmlst.org/paeruginosa/).

The MLST approach was useful in revealing clonal relatedness between isolates. There was an identical ST in the current study (in Tehran, center of Iran) with another study in Orumieh (north-west of Iran).[34] *P. aeruginosa* isolates of ST235 were identified in both studies. In both studies, isolates of ST235 were recognized as MDR.

As a result of our study, all the isolates of ST235 showed identical resistance patterns and they harbored a unique susceptibility profile.

In conclusion, the results showed a high level of resistance to antipseudomonas antibiotics among the *P. aeruginosa* isolates at the university teaching hospital in Iran. Our investigation showed 45% MDR isolates, which could pose a serious clinical threat. As a result of this, isolates of ST235 were MDR, although in this study most of the *P. aeruginosa* clinical isolates harbored antibiotic resistance profiles like the resistance patterns of typed isolates belonging to ST235. According to studies in other countries,[31,33,35] and as discussed by Yousefi *et al*. in Iran[34] and shown in the present study, it was suggested that this clone (ST235), which showed MDR, spreads in Iran. Obviously, further investigations are needed to confirm the same. Spread of MDR isolates in hospitals can pose a serious problem for the treatment of infections caused by this clone of *P. aeruginosa*.

ACKNOWLEDGMENT

The authors acknowledge Dr. Kyungwon Lee from the Department of Laboratory Medicine, Yonsei University College of Medicine Seoul, Korea for kindly providing the bla_{IMP}-positive *P. aeruginosa* isolate as a gift. Thanks are also due to Dr. Fereshteh Shahcheraghi from the Department of Bacteriology, Pasteur Institute of Iran, who provided the plasmids containing the bla_{VIM} and bla_{PER} genes.

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Source of Support: Nil, **Conflict of Interest:** None declared.