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ORIGINAL RESEARCH

Molecular Epidemiology of Carbapenemase-Producing *Pseudomonas aeruginosa* Isolated from an Iranian University Hospital: Evidence for Spread of High-Risk Clones

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Purpose: Given the importance of treatment failure due to multidrug-resistant (MDR) strains, studies on population structure of these organisms are necessary to improve control strategies. Accordingly, the current study aimed to determine the prevalence of carbapenem-resistant *P. aeruginosa* (CRPA) at a teaching referral hospital in Iran and to analyze their molecular clonality by multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) for epidemiological purposes.

Methods: In this study, modified Hodge test (MHT) and double-disk synergy test (DDST) were used for carbapenemase production and metallo-β-lactamases (MBLs) screening, respectively. All *P. aeruginosa* isolates were tested for antimicrobial resistance. Moreover, MBL genes (*bla*IMP, *bla*VIM, *bla*SPM, *bla*NDM) were detected by multiplex PCR assay.

Results: Among 68 *P. aeruginosa* clinical isolates, 38 (55.88%) isolates were CRPA. Antibiotic susceptibility testing revealed that most of these isolates were MDR. PFGE analyses showed 5 common types and 27 single types among CRPA isolates. MLST analysis revealed three major clusters (MLST-sequence types (STs): 235, 357, and 861) among them. The 30 non-CRPA isolates corresponded mainly to MLST-STs 253, 360, and 446.

Conclusion: Our results showed that internationally distributed MLST-STs with widely genomic diversity have spread in our hospital, and clonal expansion of MDR strains of *P. aeruginosa* was described as well.

Keywords: carbapenem-resistant *P. aeruginosa*, CRPA, carbapenemase, metallo-β-lactamases, MBLs, multidrug-resistant, MDR, *Pseudomonas aeruginosa*, multilocus sequence typing, MLST, pulsed-field gel electrophoresis, PFGE

Introduction

Pseudomonas aeruginosa, as one of the major cause of nosocomial infections, is associated with significantly higher mortality and morbidity rates compared to the other members of Pseudomonadaceae family. It is responsible for numerous types of infections, including surgical site infections, urinary tract infections, pneumonia and bloodstream infections.[1] Currently, multidrug-resistant (MDR) and extensively drug-resistant (XDR) *P. aeruginosa* strains have become a global concern.[2] Due to intrinsic and acquired resistance of *P. aeruginosa* to a wide range of commonly

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used antimicrobial agents, effective treatment of infections caused by this bacterium is often challenging.[3] Currently, the most effective drugs for clinical management of severe *P. aeruginosa* infections are carbapenems.[3,4] However, due to the emergence of resistant strains producing carbapenemase which can hydrolyze carbapenems, penicillins, and cephalosporins, their effectiveness has been endangered.[4,5] According to the World Health Organization in 2017, new antibiotics were promptly required against carbapenem-resistant *P. aeruginosa* (CRPA) strains.[6]

Carbapenem resistance in *P. aeruginosa* can occur due to combination of mechanisms including decreased activity of efflux pump, decreased permeability of outer membrane as a result of porin alterations following chromosomal mutations, and carbapenemases production as the main resistance mechanism.[4] Carbapenemases are a kind of β-lactamase being assigned to the classes of A, D (referred to as serine carbapenemases), and B (referred to as metallo-β-lactamases).[5] Carbapenemase genes are commonly harbored on mobile genetic elements and have the potential for rapid dissemination. As a class of beta-lactams, carbapenems can inhibit the synthesis of the bacterial cell wall by binding to penicillin-binding proteins (PBPs). Clinically available carbapenems include imipenem, meropenem, ertapenem, and doripenem.[4] Metallo-β-lactamases (MBLs) confer resistance to all β-lactams, except the monobactams.[7] The most frequent MBLs comprise New Delhi metallo-β-lactamases (NDMs), Verona integron-encoded metallo-β-lactamase (VIM), and IMP (so-called for its imipenem-resistant phenotype) enzymes.[8] In addition, the most prevalent MBL genes in *P. aeruginosa* are the VIM and IMP types; particularly, VIM-2 has become the dominant MBL type worldwide.[9] However, in a previous study conducted in Iran *bla*IMP was reported as the most prevalent carbapenemase gene in *P. aeruginosa* isolates.[10]

The potential of high-risk clones of *P. aeruginosa* for being selected and disseminating antimicrobial resistance has made them a serious issue for public health worldwide.[2] There are controversies about population structure and epidemic clonality of *P. aeruginosa*.[11] Multilocus sequence typing (MLST) is a typing method based on conserved housekeeping genes and is capable of tracking the global clonal history of the species with the highest accuracy.[12]

Hence, the aim of this study was to determine the prevalence of CRPA isolates at a teaching referral hospital in Iran and analyzing their molecular clonality by MLST as well as studying the clonal relationship among the isolates by pulsed-field gel electrophoresis (PFGE), in order to gain a better insight into the dynamics of MDR *P. aeruginosa* strains as it might help in the global comparisons.

Materials and Methods

Bacterial Isolation

From January 2018 to January of 2020, a total of 157 urine samples were collected from pathologically confirmed prostate cancer and bladder cancer patients hospitalized in the urology ward of Imam Khomeini hospital affiliated to Tehran University of Medical Sciences. The sampling was a part of routine hospital laboratory procedure and at the time of admission an informed consent was obtained from patients. All urine samples received in the microbiology laboratory were processed for identification of *P. aeruginosa*. Isolated bacteria were recovered on blood agar after an overnight incubation at 37°C. Sub-cultures were done on MacConkey agar for single colonies. Then the isolates were identified based upon standard microbiological procedures (Gram staining, colony morphology, and biochemical tests such as catalase, oxidase, lactose fermentation, Methyl red/Voges–Proskauer, Simmons' citrate, triple sugar iron, oxidation/fermentation, urease, nitrate reduction, H2S production, and motility tests).[13] Subsequently, strains were confirmed by polymerase chain reaction (PCR) for *oprL* gene.[12]

Antimicrobial Susceptibility Testing

Susceptibility testing against 10 antibiotics including piperacillin (100μg), piperacillin-tazobactam (100/10 μg), ceftazidime (30 μg), cefepime (30 μg), imipenem, meropenem (10 mg), aztreonam (30 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg), and ciprofloxacin (10 μg) (Oxoid; Basingstoke, UK) was performed for all non-duplicate *P. aeruginosa* isolates by Kirby-Bauer disk method on Mueller-Hinton agar (MHA) according to the Clinical & Laboratory Standards Institute (CLSI) guidelines.[14] The reference strain was *P. aeruginosa* PAO1.[12] In addition, E-test (AB Biodisk, Sweden) was used for determination of minimum inhibitory concentration (MIC) for ceftazidime, imipenem and meropenem according to the manufacturer's instructions and CLSI guidelines. Carbapenem-resistant isolates were defined as imipenem or/and meropenem MIC ≥8mg/L.[14] In addition, MDR was defined as resistance to at least one agent in more than three antimicrobial categories.[15]

Carbapenemase Assay

Carbapenemase assay was performed with the modified Hodge test (MHT) to identify carbapenemase activity in carbapenem-resistant isolates as described previously.[16] Briefly, a turbidity of 0.5 McFarland standard of the *E.coli* ATCC 25922 as an indicator organism was prepared in 5 mL of saline and was subsequently diluted by sterile saline (1:10). This was streaked to a MHA plate. Then, a 10-µg IPM disk (Becton Dickinson) was located in the center of the MHA plate. The study isolate was heavily streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 37°C. Interpretation was performed according to the CLSI guidelines. Briefly, observation of clover leaf-like indentation inhibition zone was defined as positive result (carbapenemase production by the test isolate). In addition, lack of *E. coli* 25922 growth along the test isolate growth streak within the disc diffusion zone was defined as MHT negative.

Imipenem (IPM)-EDTA Double-Disk Synergy Test (DDST)

To identify MBL activity, DDST was performed. Overnight liquid cultures of the test isolates were adjusted to the McFarland 0.5 standard turbidity and were inoculated to MHA plates. Two discs with 10 µg imipenem were placed on the plate. Then, 10 µL of 0.5 M solution of EDTA (as chelating agent) was added to one of the imipenem disc. After overnight incubation at 37 °C, the presence of increased inhibition zone (≥5 mm) in the EDTA-supplemented disc was considered as positive for MBL production. Additionally, carbapenem-susceptible isolates (disc inhibition zone diameters of ≤25 mm) were tested for carbapenemase activity as well. MBL production by the DDST-positive isolates were confirmed by PCR for carbapenemase genes.[17]

Detection of Resistance Genes

Chromosomal DNA was extracted using a DNAasy kit (Qiagen, Tokyo, Japan) as recommended by the manufacturer. Purified genomic DNA was used as the template for PCR. All carbapenem-resistant isolates were tested for MBL genes (*bla*IMP, *bla*VIM, *bla*SPM, *bla*NDM) using multiplex PCR assay described previously.[18]

MLST

MLST was accomplished for both CRPA and non-CRPA isolates, as described by Curran et al.[19] Briefly, seven genes including *acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A, and *trp*E were amplified, and the PCR products were purified with the purification kit (QIAGEN). Sequencing was performed with the primers as corrected at the http://pubmlst.org website using the Applied Biosystems (ABI) 3730XL DNA analyzer. Then their allelic profile and MLST-STs were determined via the *P. aeruginosa* MLST website (http://pubmlst.org/paeruginosa/).

Genotyping with PFGE

The *P. aeruginosa* isolates were genotyped by PFGE as described previously.[20] DNA digestion was performed using *Spe*I restriction enzyme (New England BioLabs-IZASA, Barcelona, Spain) according to the manufacturer instructions (18 hours at 36 °C). The DNA macrorestriction fragments were separated in the agarose gel by PFGE using a contour-clamped homogeneous electric field (CHEF) electrophoresis System (Bio-Rad, Hercules, California, USA) on a 1.2% agarose. The genomic DNA of *Salmonella enterica* serotype Braenderup H9812 (CDC), digested with 20 IU XbaI (Roche Diagnostics) for 20 h at 37 °C was used as the size marker. The images of stained gels with ethidium bromide were visualized by ultraviolet transillumination and photographed. Digital images were stored electronically as TIFF files and were analyzed by GelComparII software version 4 (Applied Maths, Sint-Martens-Latem, Belgium). The UPGMA method (unweighted pair group method using arithmetic mean Algorithm) was utilized as the clustering method (1% band tolerance, 1% optimization). Interpretation of results was performed according to the criteria of relatedness suggested by Tenover et al.[21] Clonal relatedness was defined according to a similarity (Dice) coefficient greater than 0.80; otherwise, the isolates were not considered to be related and were defined as single type or distinct PFGE profile.

Results

Clinical Isolates

In the current study, a total of 68 *P. aeruginosa* isolates were recovered from urine samples of urology cancer patients, among which 38 (55.88%) isolates were carbapenem resistant (Figure 1).

Antibiotics Susceptibility Pattern

The susceptibility of CRPA isolates to the different antimicrobial agents tested is shown in Table 1. According to Etest method, 38 (100%) and 36 (92.1%) isolates were resistant to imipenem (MIC≥16mg/mL) and ceftazidime (MIC≥32 mg/mL), respectively (Table 2).

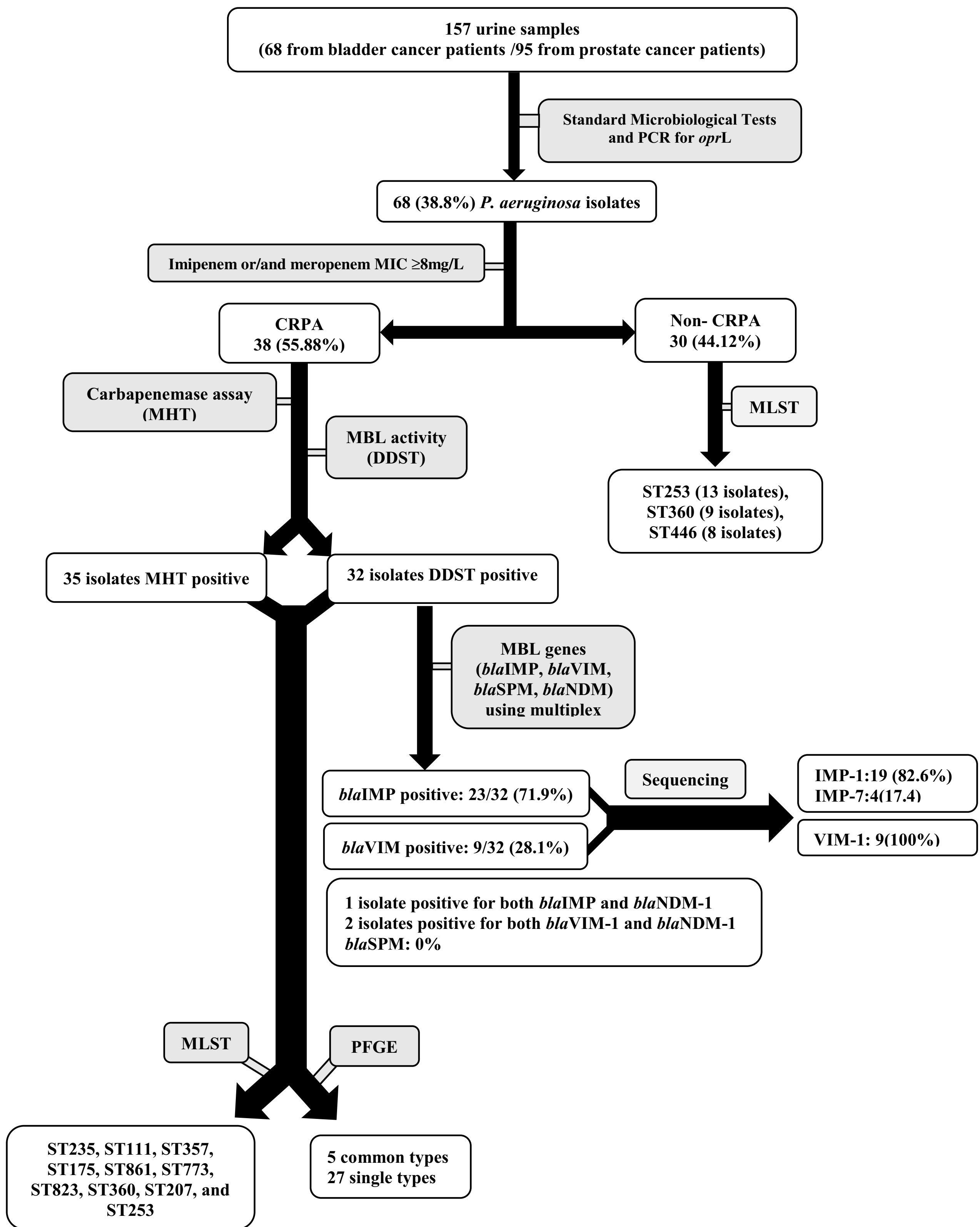

Figure 1 Flow of sample collection and study design through the study and detection of carbapenemase-producing *P. aeruginosa* isolate.

Table 1 The Susceptibility of Carbapenem-Resistant *P. Aeruginosa* Isolates to the Different Antimicrobial Agents

Isolate N(%)	ST	No (%)	Selected Antimicrobial Agents								
			Piperacillin N(%)	Ceftazidime N(%)	Cefepime N(%)	Aztreonam N(%)	Piperacillin/Tazobactam N(%)	Gentamicin N(%)	Tobramycin N(%)	Amikacin N(%)	Ciprofloxacin N(%)
CRPA 38 (55.88)	235	14 (37.0)	14 (100)	14 (100)	14 (100)	14 (100)	14 (100)	14 (100)	11 (78.5)	6 (42.8)	14 (100)
	357	8 (21.0)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	5 (62.5)	5 (62.5)	8 (100)
	175	4 (10.5)	4 (100)	4 (100)	3 (75)	4 (100)	3 (75)	4 (100)	1 (25)	1 (25)	3 (75)
	111	4 (10.5)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	1 (25)	1 (25)	4 (100)
	253	2 (5.3)	2 (100)	1 (50)	0	0	0	2 (100)	0	0	0
	360	2 (5.3)	0	0	0	0	0	2 (100)	0	0	0
	773	1 (2.6)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0	0
	207	1 (2.6)	0	1 (100)	1 (100)	0	0	1 (100)	0	0	0
	823	1 (2.6)	1 (100)	1 (100)	1 (100)	0	1 (100)	1 (100)	0	0	1 (100)
	861	1 (2.6)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0	1 (100)
Total N(%)			**35 (92.1)**	**36 (92.1)**	**33 (86.8)**	**32 (84.2)**	**32 (84.2)**	**38 (100)**	**18 (47.3)**	**13 (34.2)**	**31 (81.5)**
Non-CRPA 30 (44.12)	253	13 (43.3)	8 (61.5)	8 (61.5)	4 (30.7)	4 (30.7)	4 (30.7)	11 (84.6)	4 (30.7)	3 (23.0)	4 (30.7)
	360	9 (30.0)	0	0	0	0	0	6 (66.6)	0	0	0
	446	8 (26.7)	3 (33.3)	3 (33.3)	1 (12.5)	1 (12.5)	1 (12.5)	5 (62.5)	1 (12.5)	1 (12.5)	1 (12.5)
Total N(%)			**11 (36.6)**	**11 (36.6)**	**5 (16.6)**	**5 (16.6)**	**5 (16.6)**	**22 (73.3)**	**5 (16.6)**	**4 (13.3)**	**5 (16.6)**

Abbreviations: CRPA, carbapenem-resistant *P. aeruginosa*; CSPA, carbapenem-susceptible *P. aeruginosa*; ST, sequence type based on MLST analysis.

Table 2 MIC90 and MIC50 Values for Meropenem, Ceftazidime, and Cefepime Among CRPA and CSPS Isolates

Antimicrobial Agent				CRPA	CSPA
Meropenem	Resistance Breakpoint (µg/mL)		≥8		
	Resistant N(%)			38(100)	0
	Range (µg/mL)	MIC50 MIC90		8 to >32 8 to >32	– –
Cefepime	Resistance Breakpoint (µg/mL)		≥32		
	Resistant N(%)			33 (86.8)	5 (16.6)
	Range (µg/mL)	MIC50 MIC90		16 to >1024 16 to >1024	2–32 2–32
Ceftazidime	Resistance Breakpoint (µg/mL)		≥32		
	Resistant N(%)			36 (92.1)	11 (36.6)
	Range (µg/mL)	MIC50 MIC90		32 to >1024 64 to >1024	2–32 2–32

Abbreviations: CRPA, carbapenem-resistant *P. aeruginosa*; CSPA, carbapenem-susceptible *P. aeruginosa*.

Carbapenemase-Producing and Carbapenemase Genes

MHT and DDST were positive in 35 and 32 isolates, indicated carbapenemase and MBL production, respectively. PCR assay indicated that 23 and 9 of the isolates were positive for *bla*IMP and *bla*VIM genes, respectively. Sequencing of PCR products showed that 19 (82.60%) IMP products belonged to the IMP-1 group and 4 (17.40%) IMP products belonged to the IMP-7 group. All VIM products belonged to the VIM-1 group. Four isolates were positive for both *bla*IMP and *bla*NDM-1 genes and 2 isolates were found to be harboring with both *bla*VIM and *bla*NDM-1 genes. No *bla*SPM isolates were found. Three (7.9 %, 3/38) carbapenem-resistant isolates were found to be negative for both phenotypically carbapenemase tests and for the carbapenemase genes studied.

PFGE Typing

All CRPA isolates were analyzed by PFGE. Among these isolates, 5 common types (CTs; pulsotypes with a minimum similarity of 80%) and 27 single types (dissimilar pulsotypes) were observed. The constructed dendrogram for relative relatedness among CRPA isolates is shown in Figure 2.

MLST

MLST analysis revealed different STs for *P. aeruginosa* isolates (ST235, ST111, ST357, ST175, ST861, ST773, ST823, ST360, ST207, and ST253). ST235 and ST253 were the most prevalent STs among the carbapenem-resistant and carbapenem-susceptible isolates, respectively. Molecular characteristics of *P. aeruginosa* isolates are summarized in Table 1. The 30 non-CRPA isolates corresponded mainly to ST253, ST360, and ST446 (Figure 1, Table 1).

Discussion

The hospital environment is the most important source of MDR bacterial infections, especially infections involving *P. aeruginosa*.[22] MDR *P. aeruginosa* strains are common causes of nosocomial infections worldwide[23] and this can be particularly severe in patients with impaired immune systems, such as cancer patients.[24]

There are limited information on the CRPA population structure in Iran. In the present study, we observed that 55.88% (n =38) isolated *P. aeruginosa* were carbapenem resistant. The potential source of colonization could be both hospital and community. Resistance to imipenem has been reported to vary from 12.5% to 100% in different previous studies in Iran.[25–27] Additionally, the prevalence of MDR and XDR *P. aeruginosa* strains has been reported to 15–30% in different geographical regions.[28–30] Antimicrobial susceptibility profiles of CRPA isolates indicated a high level of resistance to most antibiotics studied (Table 1). CRPA isolates were resistant to the majority of antibiotics studied. The non-CRPA isolates showed lower MICs of ceftazidime, cefepime, and meropenem than CRPA isolates (Table 2). In this study, MHT and DDST indicated that carbapenem-resistance among

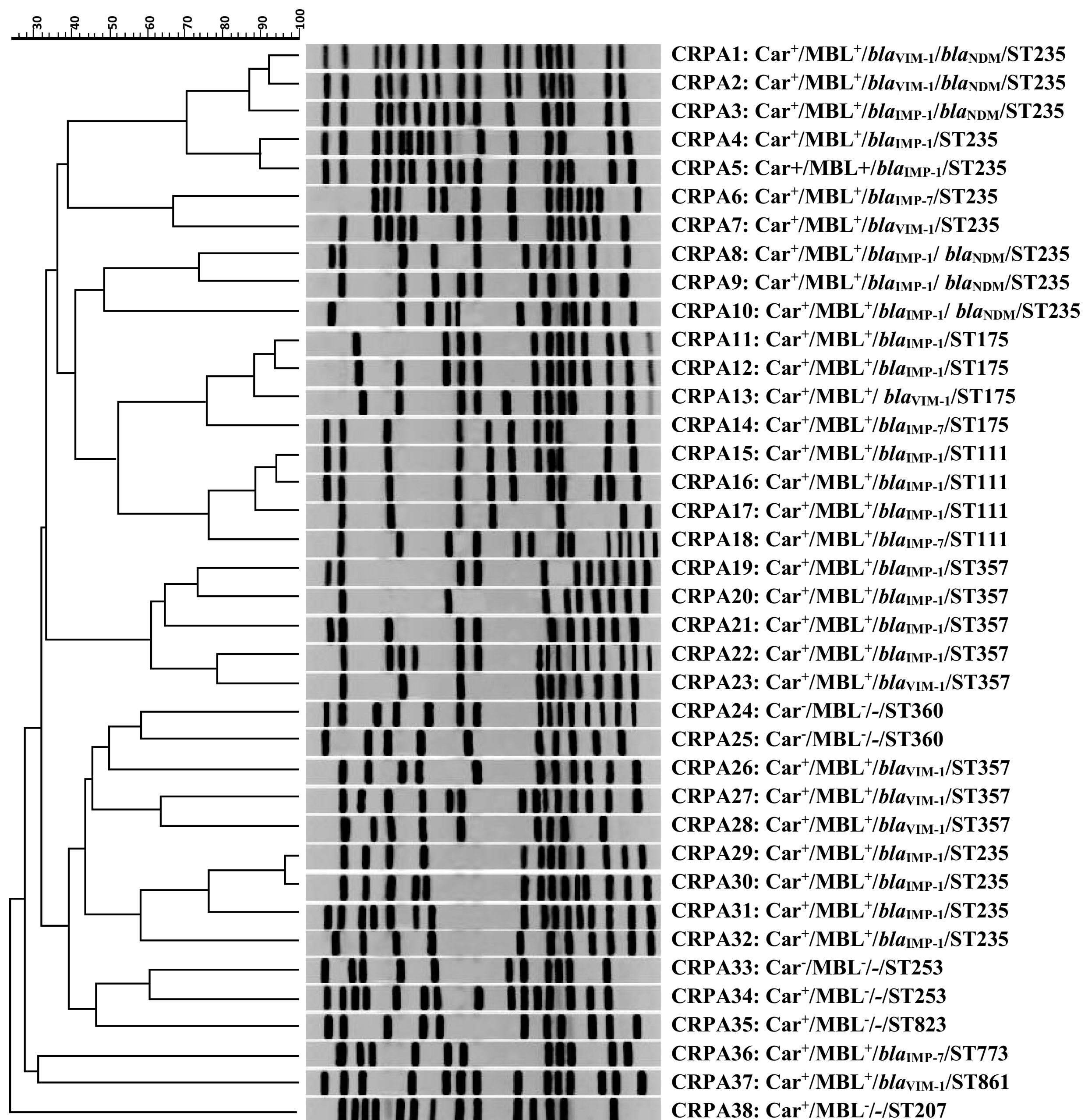

Figure 2 Genomic analysis of SpeI-digested DNA from *P. aeruginosa*isolates. Dendrogram based on the relationships among CRPA isolates derived from the UPGMA and Dice coefficients, using Gel CompanII software. Data about the results of MHT to identify carbapenemase activity, DDST for phenotypic detection of MBL activity, multiplex PCR assay for detection of MBL genes, and sequence type (ST) based on MLST analysis are presented, respectively.
Abbreviation: CRPA, carbapenem-resistant *P. aeruginosa*.

P. aeruginosa isolates studied was mainly mediated by MBLs (32/35 isolates).

Among several molecular typing methods proposed for *P. aeruginosa*, PFGE seems to be the most discriminatory method for short-term epidemiological studies.[31] However, due to its extensive genomic diversities, MLST is more suitable for evaluating long-term evolutionary relationships.[19] In the present study, two typing methods were used to determine epidemic clones of *P. aeruginosa* isolates. Our results have shown high genetic diversity among them. The carbapenem-resistant isolates comprised 11 STs. Totally 26 of the isolates were distributed in STs 235

(n =14), 357 (n=8), and 111 (n=4), which have been considered as high-risk clones.[32] However, the 30 non-resistant isolates comprised 3 MLST clones, with STs 360 (n=9), 446 (n=8) and 253 (n =13). Most of the STs have been reported previously.[25,33]

In this study, ST235 emerged as the most prevalent MLST-ST accounting for 14 CRPA isolates. This sequence type is widely disseminated in the world and has been reported from other countries including Hungary, Italy, Greece, Poland, Sweden, Japan, and Norway.[34–37] Moreover, ST235 is an international clone associated with poor clinical outcomes.[38] It has been reported as MBL producing strains responsible for outbreaks throughout Europe, Asia, and South America.[35,39-44]

In our study, all of the ST235 isolates were MBL positive and majority of them harbored *bla*IMP (n=11) while only 3 isolates produced VIM type MBLs. Four of carbapenem-resistant isolates belonged to ST235 were positive for both *bla*IMP and *bla*NDM genes. All were MDR as well, suggesting the key role of the strain ST235 in dissemination of the MDR strains at the studied hospital. Our results indicated that ST357 was the second most frequently isolated sequence type, with 8 isolates all were MDR. This ST was previously reported from both Iran and other Asian countries such as Japan and Korea.[42,45] In addition, ST111 and ST175 were the other sequence types reported in the current study. ST111 as well as ST175 have been also recognized as MBL-producing epidemic lineages.[46,47] ST175 is also a globally distributed clone and has been documented as a contaminant of the hospital environment.[48] The nine remaining carbapenem-resistant isolates belonged to different STs including ST861, ST773, ST823, ST360, ST207, and ST253.

Several studies reported *bla*VIM gene as the most common MBL found in CRPA.[49,50] However, in our study *bla*IMP gene was the most frequent detected carbapenemase gene in *P. aeruginosa* isolates, which is in consistent with a previous study in Iran.[10]

PFGE was used to investigate the epidemiological characteristics of *P. aeruginosa* isolates. This method is valuable for characterization of outbreaks and their sources.[51] In the present study, 5 CTs were found among carbapenem-resistant isolates, which may represent five outbreaks. All the isolates belonged to the same PFGE-CTs had both similar MLST-ST and similar antibiotic resistance pattern. The chromosomal digestion patterns revealed a high diversity among CRPA isolates. This could be explained by acquisition of these strains from various sources.

This study is subjected to some limitations. First, the lack of enough previous information about the genetic composition of strains for comparison purposes. Second, it was a single-center study; therefore, additional studies are required to reach a broader insight on the clonal dynamics of CRPA contaminating hospitals in Iran. In summary, the population structure of *P. aeruginosa* present in our hospital shows that the MDR isolates studied are grouped in the prevalent STs reported in other Iranian hospitals and at the international level. In addition, IMP-1, producing *P. aeruginosa* are the carbapenemase-producing strains mainly circulating at our hospital environment.

Conclusions

The present study shows the prevalence of CRPA with limited susceptibility to antibiotics in hospital environment. Therefore, proper antibiotic strategies should be formulated in order to overcome these problems. Moreover, our results indicated that internationally distributed MLST-STs with widely genomic diversity have spread in our hospital. The PFGE analysis also showed the possibility of localized outbreak during the period of study. In conclusion, clonal expansion of MDR strains of *P. aeruginosa* was described in a urology ward in Iran. The finding highlights the significance of continuingly following the epidemiological relatedness of MDR *P. aeruginosa* spreading in hospital environment to improve infection control and prevention strategies.

Abbreviations

CRPA, Carbapenem-resistant *P. aeruginosa*; CHEF, Contour-clamped homogeneous electric field; DDST, Double-disk synergy test; XDR, Extensively drug-resistant; MBLs, Metallo-β-lactamases; MIC, Minimum inhibitory concentration; MHT, Modified Hodge test; MHA, Mueller-Hinton agar; MDR, Multidrug-resistant; MLST, Multilocus sequence typing; NDMs, New Delhi metallo-β-lactamases; PCR, Polymerase chain reaction; PFGE, Pulsed-field gel electrophoresis; PFGE-CT, PFGE-Common type; PFGE-ST, PFGE-Single type; MLST-ST, MLST-Sequence type; UPGMA, Unweighted pair group method using arithmetic mean Algorithm; VIM, Verona integron-encoded metallo-β-lactamase.

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Author Contributions

All authors have contributed in the acquisition of data, data analysis and interpretation, drafting of the manuscript, and

critical revision of the manuscript for scientific and factual content. All authors have approved the final content of the manuscript and agree to be accountable for all aspects of the work.

Disclosure

The authors have no conflict of interest to declare.

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