Distribution of Ambler Class A Β-lactamase Genes and Evaluation of Resistance Patterns in Multi-Drug and Extensively-Drug Resistant *P. aeruginosa* Clinical Isolates

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ABSTRACT

Background and Objectives: Emergence and spread of multidrug-resistant (MDR) and extensively-drug resistant (XDR) *Pseudomonas aeruginosa* strains could complicate antipseudomonal chemotherapy. Dissemination of resistance genes, such as β-lactamases encoding genes by horizontal gene transfer can lead to development of multi-drug resistance in *P. aeruginosa*. The purpose of this study was to investigate the latest resistance patterns in MDR and XDR strains and evaluate Ambler class A β-lactamase gene distribution in *P. aeruginosa* clinical isolates.

Methods: One hundred molecularly and biochemically identified *P. aeruginosa* strains isolated from different clinical specimens were tested for sensitivity to 17 antibiotics using the Kirby-Bauer disk diffusion method. PCR was performed to detect *bla* TEM-1, *bla* SHV-1, *bla* REP-1 and *bla* VEB-1 genes. Results were analyzed using SPSS and NTSYSpc softwares.

Results: Based on the results of antibiogram, the highest rate of resistance was observed against amikacin (100%), aztreonam (83%), ceftazidime (55%), cefepime (55%) and netilmicin (48%). In addition, the frequency of MDR and XDR isolates was 95% and 5%, respectively. The *bla*SHV-1, *bla* TEM-1, *bla* PER-1 and *bla* VEB-1 genes were detected in 31%, 24%, 13% and 10% of the isolates, respectively.

Conclusion: Antibiotic resistance to β-lactam antibiotics and frequency of β-lactamase genes were relatively high in the study area. We also found that a significant proportion of XDR strains with different antibiotic resistance profile is isolated from tracheal specimens.

KEYWORDS: *Pseudomonas aeruginosa*, Beta-Lactamase, Multidrug Resistant, Extensively Drug Resistant.

INTRODUCTION

Distribution of multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogenic bacteria is an important public health threat due to the limited availability of effective antimicrobial agents (1). *Pseudomonas aeruginosa* is responsible for 9-10% of all hospital-acquired infections, especially in immunocompromised patients, which increases the risk of morbidity and mortality (2, 3).

The latter can be attributed to the spread of MDR and XDR *P. aeruginosa* strains in healthcare settings (4).

MDR strains are not susceptible to at least one agent in three or more antimicrobial categories(1), while XDR strains are not susceptible to at least one agent in all but two or fewer antimicrobial categories. Pan-drug resistant (PDR) strains are not susceptible to all agents in all antimicrobial categories (1). Intrinsic resistance of *Pseudomonas* spp. to common antibiotics, including penicillin and related β-lactams is mainly due to restricted outer-membrane permeability and secondary resistance mechanisms, such as energy-dependent efflux and β-lactamase production (5).

With the advent of β-lactamase enzymes among *P. aeruginosa* strains, treatment of infections caused by these microorganisms has become a serious challenge. Bacterial resistance to β-lactams is related to at least three mechanisms: A) mutations in genes encoding penicillin-binding proteins (PBPs) and acquisition of alternative PBPs with reduced affinity for β-lactams, B) change in the permeability of the cell wall due to altered expression of porins or active efflux, and C) inactivation of the drug by β-lactamases which is by far the most common mechanism (6, 7). Four molecular classes of β-lactamases have been reported in *P. aeruginosa* including metal-dependent class B and the metal-independent classes with active-site serine residue: classes A, C, and D (8). Class A extended spectrum β-lactamases (ESBLs) in *P. aeruginosa* causes resistance not only to carboxypenicillins and ureidopenicillins, but also to extended-spectrum cephalosporins (e.g. ceftazidime, cefepime, cefpirome) and aztreonam (9).

TEM-type β-lactamases are derivatives of TEM-1 or TEM-2 (10) and can act against penicillins, cephalosporins and related antibiotics (11). SHV-1 is the origin of the SHV-type enzymes that causes resistance to broad-spectrum penicillins (12). Apart from TEM and SHV and other identified enzymes in *P. aeruginosa*, *Pseudomonas* extended-resistant (PER), Vietnam ESBL (VEB), GES/IBC and BEL types show similar hydrolysis profiles (12, 13).

Considering the importance of nosocomial infections caused by MDR and XDR *P. aeruginosa* and the hydrolyzing activity of TEM-1, SHV-1, VEB-1 and PER-1 enzymes against penicillins and broad-spectrum cephalosporins, we aimed to determine recent pattern of multi-drug and extensively-drug resistance in *P. aeruginosa* clinical isolates and evaluate distribution of *bla* $_{TEM-1}$, *bla* $_{SHV-1}$, *bla* $_{VEB-1}$ and *bla* $_{PER-1}$ genes among these isolates.

By consistent reporting of antimicrobial resistance profile and mechanisms to clinical and reference microbiology laboratories, there will be comparable data to be tracked and promote the prudent use of antibiotics.

MATERIAL AND METHODS

In this cross-sectional study, 100 clinical specimens from urine, trachea, catheter, wound and blood were collected from hospitalized patients in the Milad hospital in Tehran (Iran) from December 2015 to December 2016.

The study protocol was approved by the ethics committee of the Islamic Azad University, Tehran Medical Branch, Iran (Code: IR.IAU.TMU.REC.1396.277) and all volunteer were assured of the confidentiality of their data. Bacterial strains were isolated using direct culture of the specimens on MacConkey agar (Merck, Germany) and 5% sheep blood (Merck, Germany), followed by colony morphology and standard biochemical testing and numbered according to sampling time.

Total DNA was extracted from the isolated bacteria using a commercial kit (Bioneer, Daejeon, Korea). To confirm *P. aeruginosa* isolates, a PCR experiment targeting the 16S rDNA gene (a species-specific gene found in all *P. aeruginosa* strains) was performed.

Target sequence of the 16S rDNA gene was amplified by PCR using primers as described

by Spilker et al (14).

All *P. aeruginosa* isolates were subjected to antimicrobial susceptibility testing via disk diffusion assay according to the CLSI guidelines (2016). The following antibiotic disks (purchased from MAST, England) were used in the assay: gentamicin (10μg), tobramycin (30μg), netilmicin (10μg), amikacin (30μg), imipenem (10μg), meropenem (10μg), doripenem (10μg), cefepime (30μg), ceftazidime (30μg), ciprofloxacin (5μg), levofloxacin (5μg), colistin (25μg), polymyxin b (300μg), aztreonam, fosfomycin (200μg), ticarcillin (75μg) and piperacillin (30μg). MDR, XDR and PDR strains were determined according to the criteria described by Magiorakos et al. (1). The target sequence of β-lactamase gene was amplified by PCR using primers previously introduced by Lee et al. (15) (Table 1). Total reaction volume (25 μl) contained 2.5 μl 10X

PCR buffer (500 mM, KCL and Tris HCL, pH 8.4), 0.5 μl (12.5 mM) $MgCl_2$, 0.5 μl (200 mM dNTPs), 0.5 μl (25 pmol) of each primer, 2.5 μl (150 ng) of extracted DNA, 15 μl distilled water, and 1 U of Taq DNA polymerase (SinaClon, Iran). The PCR amplification for each gene was performed under the following conditions: initial denaturation at 96 °C for 5 min, followed by denaturation at 95 °C for 60 sec, annealing at 52 °C (TEM-1), 58 °C (SHV-1), 55 °C (VEB-1) and 50°C (PER-1) for 50 sec, extension at 72 °C for 70 sec (32 cycles), and final extension at 72 °C for 10 min (Table 1). After the amplification process, PCR products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide (0.5 μg/ml). Nuclease-free water was used as negative control and identified *K. pneumoniae* ESBL-positive isolates harboring the bla_{TEM-1}, bla_{SHV-1}, bla_{VEB-1} and bla_{PER-1} genes were used as positive control.

Table 1- Sequence of the primers used for amplification of the β-lactamase and 16S rDNA genes.

Primer	Target	Sequence 5′- 3′	Annealing Temperature (ºC)	PCR Product size (bp)	Accession number
PA-SS-F PA-SS-R	16S rDNA	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	58	956	AY486350-AY486387
TEM-F TEM-R	TEM-1 and derivatives	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATCA	52	1079	V00613
SHV-F SHV-R	SHV-1 and derivatives	TGGTTATGCGTTATATTCGCC GGTTAGCGTTGCCAGTGCT	58	870	M59181
VEB-F VEB -R	VEB-1	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	55	650	AF010416
PER-F PER-R	PER-1	AATTTGGGCTTAGGGCAGAA ATGAATGTCATTATAAAAGC	50	920	Z21957

The antimicrobial resistance patterns were analyzed using the NTSYSpc software (version 2.1, USA). The resistance and susceptibility of each isolate was scored 1 and 0, respectively, and cluster analysis and generation of dendrogram were carried out by unweighted pair group method with arithmetic averages (16). Finally, phenotypic and genotypic association of resistance to antibiotics were analyzed with SPSS (version 19) using the Spearman correlation coefficient and Chi-square test at significance level of 0.05.
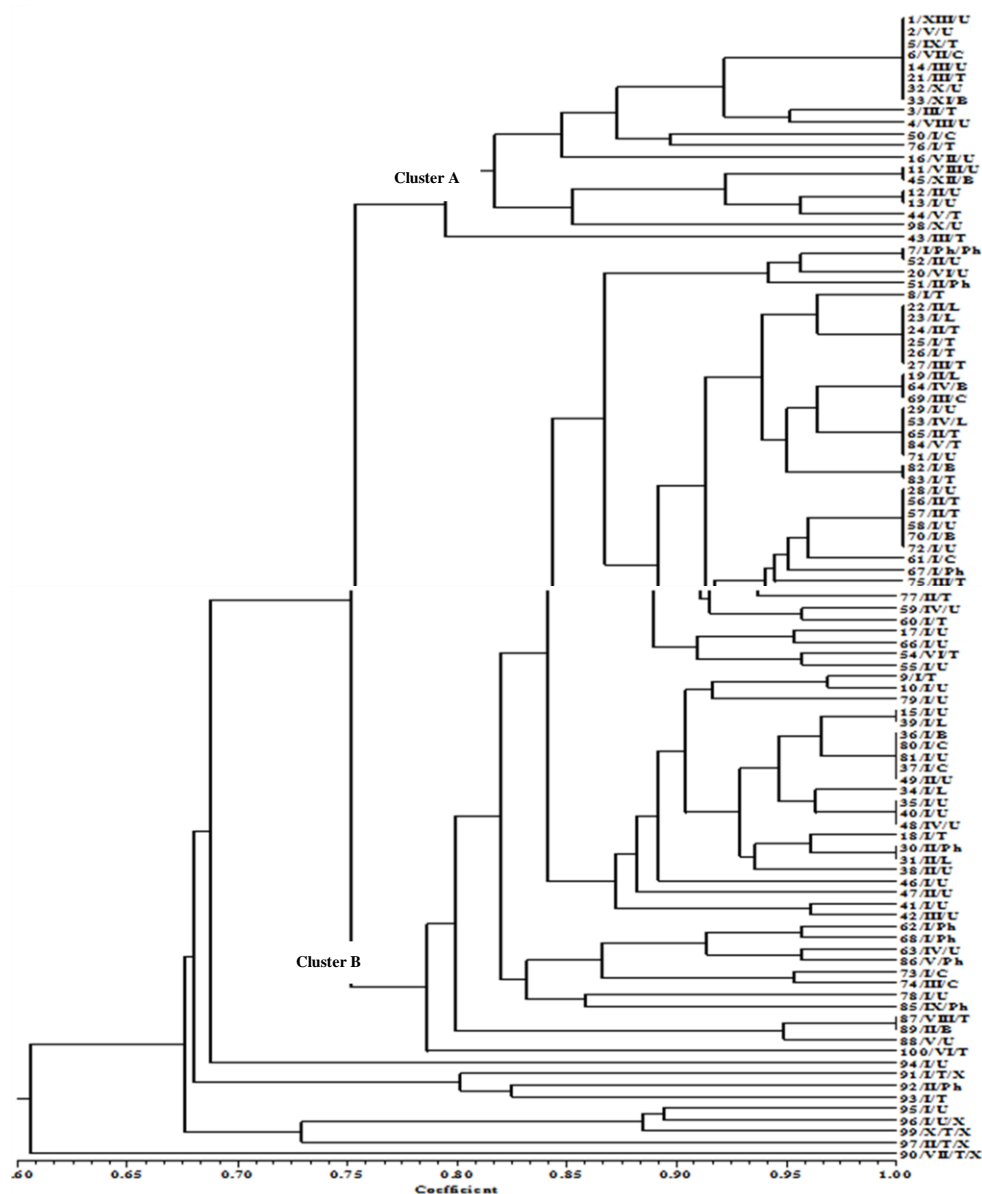
RESULTS

Of 100 *P. aeruginosa* clinical isolates, 40% were isolated from urine, 29% from trachea, 9% from pharynx, 8% from catheter, 7% from lesion, and 7% from blood specimens. All isolates were confirmed as *P. aeruginosa* through standard microbiological and biochemical tests. In addition, amplification of the 16S rDNA gene in the PCR method produced a DNA band of the expected size (956 bp) for all isolates.

All isolates were resistant to amikacin, while the lowest rate of resistance was observed against doripenem, levofloxacin and polymycin B (1% for all cases).

Of 100 isolates, 95% were MDR and 5% were XDR. Of XDR isolates, four strains were isolated from tracheal specimens and one was from urine specimen. No strain was identified as PDR. According to results of electrophoresis of the PCR products, the frequency of bla_{SHV-1}, bla_{TEM-1}, bla_{PER-1} and bla_{VEB-1} was 31%, 24%, 13% and 10%, respectively.

Figure 1. Dendrogram of *P. aeruginosa* isolates based on the antibiotic resistance profiles. Number of isolates (1-100) based on sampling time, Type (I-XIII), Sampling organ (U = Urinary, T = Trachea, Ph = pharynx, C = Catheter, B = Blood, L = Lesion), X (XDR strain).

Moreover, 19% of the isolates harbored at least two genes simultaneously, while 46% of the isolates did not carry any of these genes. Constructed dendrogram was drawn (NTSYSpc software) based on the antibiogram results to show probable similarity in resistance patterns between the isolates. For this purpose, the isolates were numbered according to the time of sampling. According to the dendrogram, all examined *P. aeruginosa*

strains (isolates number 1 to 90 and 100) can be divided into two clusters (A and B) at 75% similarity in resistance profile, while isolates 90 to 99 (except 98) did not show any similarity to isolates in clusters A and B (Figure 1).

There were positive and significant associations between presence of the β-lactamase genes and antibiotic resistance (Table 2).

Table 2- Significant relationships between the β-lactamase genes and antibiotic resistance

Genes	Meropenem	Imipenem	Ceftazidime	Cefepime	Piperacillin
SHV	0.032	-	-	-	-
TEM	0.00	0.00	0.041	0.006	-
VEB	-	0.01		0.001	0.035
PER	-	0.019	0.033	0.025	-

DISCUSSION

In the past decade, MDR *P. aeruginosa* strains emerged as an important threat to healthcare systems worldwide (17, 18). In Iran, numerous investigations have been recently conducted to determine the antibiotic resistance profile of *P. aeruginosa* (17-19).

In our study, the highest rate of resistance was observed against amikacin (100%), followed by aztreonam (83%), cefepime and ceftazidime (55%) and netilmicin (48%). Fortunately, only 1% of the MDR/XDR bacteria were resistant to polymyxin B, doripenem and levofloxacin. Based on these results, amikacin is not suitable for treatment of *P. aeruginosa*, while doripenem, levofloxacin and polymycin B may be antibiotics of choice for the treatment of infections caused by *P. aeruginosa*. In a study in South Africa, 94% of *P. aeruginosa* isolates were resistant to aztreonam and piperacillin, and the frequency of resistance to imipenem, ticarcillin, meropenem and ceftazidime was 88%, 88%, 82% and 76%, respectively (20). In a study in Thailand, the rate of resistance to ceftazidime, cefepime, amikacin, gentamicin, ciprofloxacin, levofloxacin, imipenem, meropenem and ciprofloxacin was 44.91%, 28.81%, 18.64%, 25.42%, 43%, 49.15%, 31.36%, 33% and 48.3%, respectively (21). In Iran, most studies have reported high resistance rates to amikacin and aztreonam and low resistance rate to levofloxacin and polymexin B (22, 23), which might indicate the imprudent use of amikacin and aztreonam in Iran.

In the present study, 95% of the *P. aeruginosa* isolates were MDR, which is similar to the results of studies performed by Safaei et al. (24) and Mirsalehian et al. (25). However, this rate is lower than the rates reported by Katvoravutthichai et al. in Thailand (43.22%), Walkty et al. in Canada (14.3%) and Vaez et al. in Iran (51.8%) (21, 26, 27). The prevalence of XDR strains (5%) in our study was lower than that reported by Palavutitotai et al. (2018) in Thailand (22%) (28).

We found no significant association between source of isolation and antibiotic resistance, but the frequency of MDR strains was highest in blood specimens. In this regard, Rajat et al. reported that the frequency of resistant *P. aeruginosa* strains was highest in pus/swab samples (29). In our study, 80% of the XDR strains were isolated from tracheal specimens,

which can make treatment of *P. aeruginosa* respiratory infections (such as cystic fibrosis) more challenging. In the present study, we profiled the isolates based on the antibiotic resistance profile. At 75% similarity, the generated dendrogram based on the antibiogram showed the presence of two main cluster of *P. aeruginosa* (clusters A and B). Regarding the sampling time, nine isolates (no 90-97 and 99) with similar sampling time had no similarity in resistance profile with other isolates in clusters A and B and were therefore categorized under a separate antibiotic resistance profile. This indicates emergence of new isolates with different antibiotic resistance profiles over time. Interestingly, all five XDR isolates were placed in a single group, which suggests that these isolates could be emerging strains and might expand in the future.

In the present study, 31%, 24%, 13% and 10% of the *P. aeruginosa* isolates carried the bla_{SHV-1}, bla_{TEM-1}, bla_{PER-1} and bla_{VEB-1} genes, respectively. In some countries such as Korea, plasmids encoding class A β-lactamases (TEM, SHV, PER and VEB) were not found in *P. aeruginosa* (15). However, these plasmids are reportedly prevalent in other Asian countries, Southeastern Europe and Africa (Kosovo, Algeria, and Tunisia) (18, 30-32).

In a study in Thailand, from 118 non-duplicate clinical isolates of *P. aeruginosa*, 6.7% carried the bla_{VEB-1}, bla_{PER-1} and bla_{TEM-1} (and derivatives) genes, and bla_{SHV-1} was not observed (21). The notably high prevalence of bla_{PER-1}, bla_{VEB-1}, bla_{TEM} and bla_{SHV} observed in this study indicates the wide-spread dissemination of ESBL-encoding β-lactamase genes in Iran, which is in accordance with previous reports (22, 25, 33). In Qazvin (Iran), Amirkamali et al. reported that 13.3% of *P. aeruginosa* isolates contain the bla_{VEB-1} gene (22). Mirsalehian et al. found that 74.6% and 49.2% of *P. aeruginosa* are positive for presence of the bla_{PER-1}, and bla_{VEB-1} genes, respectively (25).

In another study in Tehran (Iran), 21.6% of *P. aeruginosa* isolates carried the bla_{PER-1} gene (33). The results of this study showed that both antibiogram and genotypic methods are necessary for identification of β-lactam resistant isolates, since a number of isolates harboring the β-lactamase genes were susceptible to β-lactam antibiotics and vice versa. However, in most studies, molecular

methods such as PCR have been suggested to be more sensitive (34).

Furthermore, we detected significant associations between presence of the β-lactamase genes and resistance to β-lactam antibiotics.

CONCLUSION

In this study, 95% of the *P. aeruginosa* clinical isolates were MDR strains, which can have devastating effects on community health. A number of single clones, especially XDR isolates, can be considered as emerging strains and are epidemiologically noteworthy. We

found a significant, positive association between presence of the β-lactamase genes, especially the *bla* TEM-1 and *bla* SHV-1 and resistance to β-lactam antibiotics, which signifies the need for new treatment strategies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding publication of this study.

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