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Original Article

Prevalence of Extended-Spectrum β-Lactamases Genes in Clinical Isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa* is an opportunistic pathogen resistant to various antibiotics. The aim of the present study was to study resistant patterns in clinical isolates of *P. aeruginosa*, classify them into pandrug resistance (PDR), extensive drug resistance (XDR) and multidrug resistance (MDR) groups, and identify extended-spectrum β-lactamase (ESBL)-positive isolates using the phenotypic and genotypic methods.

Methods: This cross-sectional study was conducted on 161 *P. aeruginosa* isolates collected from the city of Isfahan, Iran. Antibiotic susceptibility tests were performed using 11 antimicrobial agents. ESBL-positive strains were identified using the phenotypic and genotypic methods.

Results: The highest level of antibiotic resistance was observed against ceftazidime (77.64%). None of the isolates was resistant to polymyxin B. In the phenotypic method, 64 isolates (39.75%) were found as ESLB-positive, whereas 132 isolates (81.98%) were ESBL-positive in the genotypic method. The number of ESBL-positive isolates in the genotypic method was significantly higher than in the phenotypic method. The frequency of XDR and MDR isolates was 50.93% and 27.32%, respectively. None of the isolates was PDR. The frequency of the bla_{TEM} gene was significantly higher than other genes ($P<0.0001$).

Conclusion: It was revealed that the genotypic method was much more accurate in identifying ESBL-positive strains than the phenotypic method. Therefore, use of the molecular method may increase the chance of successful treatment with antibiotics of the β-lactam family.

Keywords: Drug Resistance, β-lactamases, *Pseudomonas aeruginosa*.

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INTRODUCTION

Pseudomonas aeruginosa is an obligate aerobic, gram-negative and non-spore forming bacillus, which is part of the normal flora of skin and intestines in humans. The bacterium is also found in water and soil. This opportunistic bacterium is one of the most important causes of hospital-acquired infections that can lead to bacteremia, meningitis, urinary tract infection, pulmonary infections, etc., especially in immunocompromised individuals (1, 2). Researchers have found that mortality rates of burn wound infections caused by this bacterium are 40-50% (3). *P. aeruginosa* is also the fourth most frequently isolated pathogen from surgical-site infections (4). Emergence of antibiotic resistance among pathogenic bacteria, especially in health centers and hospitals, has become a major health concern (2). Clinical findings indicate that infections caused by resistant pathogens increase mortality rates and treatment costs (5). It has been found that infections caused by antibiotic-resistant bacteria also increase hospitalization duration by 6.4 to 12.7 days (6). According to the Centers for Disease Control and Prevention (CDC), pattern of resistance of bacteria to multiple antimicrobial agents can be classified into pandrug resistant (PDR), extensive drug resistant (XDR) and multidrug resistant (MDR). Strains resistant to at least one agent from three or more groups of antibiotics were defined as MDR. Those resistant to at least one agent in all but two or fewer antibiotic groups were defined as XDR. Finally, strains resistant to all agents from all antibiotic groups were defined as PDR (7).

One of the important reasons of resistance of *P. aeruginosa* to a wide spectrum of antibiotics is the ability to encode β-lactamase, an enzyme able to destroy the β-lactam ring in the chemical structure of β-lactam antibiotics. Chromosome- and plasmid-borne extended-spectrum β-lactamases (ESBLs) are able to destroy the β-lactam ring in a broad spectrum of β-lactam drugs (4, 8-11). ESBL is produced by various genes including bla_{BES}, bla_{VEB}, bla_{PER}, bla_{OXA}, $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{TEM} (12). The possibility of horizontal gene transfer via conjugation, transformation or transduction may increase spread of genes responsible for ESBL production in the bacterial community (12). The purpose of this study was to evaluate resistance patterns of *P. aeruginosa* isolates collected from various hospitals, classify the isolates into the PDR, XDR and MDR groups, and identify ESBL-positive isolates using the phenotypic and genotypic methods.

MATERIAL AND METHODS

In this cross-sectional study, 161 *P. aeruginosa* isolates referred to laboratory for identification of the bacterial species, were collected from different treatment centers in Isfahan from May 2017 to September 2017. Susceptibility of the isolates to different antimicrobial agents including tobramycin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), ceftazidime (30 μg), cefepime (30 μg), piperacillin (100 μg), imipenem (10 μg), meropenem (10 μg), azteronam (30 μg) and polymyxin B (10 μg) were assessed by the Kirby-Bauer method. Data were analyzed according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017) (13) and *P. aeruginosa* (ATCC 27853) was used as the control. The combined disk method was used to identify ESBL-producing bacteria. Briefly, suspensions of ceftazidime-resistant *P. aeruginosa* equaling 0.5 McFarland turbidity standard were cultured on Mueller-Hinton agar (CONDA, Spain). Then, ceftazidime and ceftazidime-clavulanic acid disks were placed 25 mm apart on the culture medium using sterilized forceps. If the inhibition zone diameter around the ceftazidime-clavulanic acid disks was ≥5 mm than that around the ceftazidime disk, the isolate was considered as ESBL-producing (14). After evaluating the antibiotic resistance patterns, the isolates were classified as PDR, XDR and MDR according to the standards introduced by the CDC (7). For this purpose, whole genomic DNA extraction was performed using a commercial DNA extraction kit (SinaClon, Iran) according to the manufacturer's protocol. Specific primers for the bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{OXA}, bla_{PER}, bla_{VEB} and bla_{GES} genes were used for molecular detection of ESBL-producing bacteria using polymerase chain reaction (PCR) (Table 1). DNA amplification was conducted in a 25 μl reaction mixture containing 1 μl of template DNA, 0.5 μl of each primer (10 pmol), 2.5 μl of 10X buffer, 1 μl of $MgCl_2$ (50 Mm), 0.5 μl of dNTPs (10 mM) and 1.5 units of Taq DNA polymerase (CinnaGen, Iran) (10). PCR products (5 μl)

were subjected to electrophoresis in 1% agarose gel (SinaClon, Iran). Presence and size of amplicons were analyzed by a Gel documentation system (Bio-Rad, USA).
Data were analyzed with GraphPad (GraphPad Software Inc., USA) using the Fisher's exact test. P-values less than 0.05 were considered statistically significant.

RESULTS

General characteristics and sources of the 161 *P. aeruginosa* isolates are presented in table 2. As shown in table 3, the highest level of resistance was recorded against ceftazidime (77.64%). In addition, none of the isolates was resistant to polymyxin B. In the phenotypic method, 64 isolates (39.75%) were identified as ESBL-positive, whereas 132 isolates (81.98%) had at least one of the resistant genes in the genotypic method. Moreover, 29 isolates (18.01%) did not contain any of the resistance genes. The resistance genes were not detected in eight isolates (12.5%) found as ESBL-positive in the phenotypic method. The number of ESBL-positive isolates identified in the genotypic method was significantly greater than that of the phenotypic method. The frequency of the bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{OXA} was 60.86%, 29.81%, 24.22% and14.28%, respectively. Bla_{PER}, bla_{VEB}, and bla_{GES} were not detected in any of the isolates. The frequency of the bla_{TEM} gene was significantly higher than the other genes (P<0.0001) (Table 4).

Table 1- Sequences of the specific primers and size of amplicons

Primer	Sequence (5' to 3')	Amplicon Size (bp)	Reference
bla_{TEM}	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	867	(25)
bla_{SHV}	GATGAACGCTTTCCCATGATG CGCTGTTATCGCTCATGGTAA	214	(26)
bla_{CTX-M}	TTTGCGATGTGCAGTACCAGTAA CGATATCGTTGGTGGTGCCATA	590	(27)
bla_{OXA}	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438	(28)
bla_{PER}	ATGAATGTCATTATAAAAGC AATTTGGGCTTAGGGCAGAA	925	(29)
bla_{VEB}	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	642	(30)
bla_{GES}	ATGCGCTTCATTCACGCAC CTATTTGTCCGTGCTCAGG	844	(28)

Table 2 -General characteristics of the 161 *P. aeruginosa* isolates used in the study

Sex		Specimen type							
Female	Male	Urine	Wound/Abscess	Respiratory	Dialysis-related fluids	Ascitic fluid	Blood	Cerebrospinal fluid	Plural fluid
90	71	31	8	85	4	3	22	5	3

Table 3- Antibiotic resistance pattern of *P. aeruginosa* isolates based on the phenotypic method

Antibiotic	Sensitive	Intermediate	Resistant
	Number (%)	Number (%)	Number (%)
Aminoglycosides			
Tobramycin	53 (32.91 %)	17 (10.56 %)	91 (56.52 %)
Amikacin	119 (73.91 %)	0 (0.0 %)	42 (26.09 %)
Quinolone and Fluoroquinolones			
Ciprofloxacin	77 (47.83 %)	32 (19.88 %)	52 (32.30 %)
Levofloxacin	83 (51.55 %)	0 (0.0 %)	78 (48.45 %)
Β-Lactams			
Ceftazidime	36 (22.36 %)	0 (0.0 %)	125 (77.64 %)
Cefepime	74 (45.96 %)	0 (0.0 %)	87 (54.04 %)
Piperacillin	42 (26.09 %)	4 (2.48 %)	115 (71.43 %)
Carbapenems			
Imipenem	37 (22.98 %)	0 (0.0 %)	124 (77.02 %)
Meropenem	73 (45.34 %)	3 (1.90 %)	85 (52.80 %)
Monobactams			
Azteronam	103 (63.97 %)	0 (0.0 %)	58 (36.03 %)
Polymyxin B	161 (100.0%)	0 (0.0 %)	0 (0.0 %)

Table 4- Resistance phenotypes and presence of resistance genes in ESBL-positive *P. aeruginosa*

Isolate	Resistance phenotypes [a]	Resistance genes	Sex [b]	Source [c]
Pae-1	TOB, PIP, CAZ, IMP	bla_{TEM}, bla_{SHV}	M	U
Pae-2	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}, bla_{CTXM}	M	PF
Pae-3	AN, PIP, CAZ, FEP, IMP	bla_{TEM}, bla_{OXA}	M	BL
Pae-5	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}	M	R
Pae-8	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT	bla_{TEM}, bla_{SHV}, bla_{CTXM}, bla_{OXA}	F	CSF
Pae-9	AN, PIP, CAZ, FEP, IMP	bla_{TEM}	M	U
Pae-15	TOB, AN, PIP, CAZ, FEP, IMP	bla_{TEM}	M	R
Pae-16	PIP, LOM, CAZ, IMP	bla_{TEM}	M	R
Pae-19	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT	bla_{TEM}, bla_{SHV} ,bla_{CTXM}, bla_{OXA}	F	W/A
Pae-20	TOB, AN, PIP, CAZ, FEP, IMP	bla_{TEM}	F	R
Pae-21	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}	F	R
Pae-25	CP, CAZ, FEP, MEN	bla_{TEM}, bla_{SHV}	F	U
Pae-28	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT	bla_{TEM}	F	R
Pae-30	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	---	M	BL
Pae-31	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}, bla_{SHV}, bla_{CTXM}	F	R
Pae-32	TOB, AN, PIP, CAZ, FEP, IMP, AZT	bla_{SHV}	F	R
Pae-33	TOB, AN, PIP, CAZ, FEP, IMP	bla_{TEM}	F	R
Pae-36	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}, bla_{SHV}, bla_{OXA}	M	R
Pae-40	TOB, AN, PIP, CAZ, FEP, IMP	bla_{SHV}	M	R
Pae-45	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}	F	U
Pae-47	TOB, PIP, LOM, CAZ, FEP, IMP, MEN, AZT	bla_{TEM}, bla_{SHV}, bla_{OXA}	M	R
Pae-48	TOB, AN, PIP, CAZ, IMP	---	F	R
Pae-53	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	bla_{SHV}	M	W/A
Pae-54	TOB, AN, PIP, CAZ, FEP, IMP	bla_{TEM}, bla_{SHV}, bla_{CTXM}	M	D/F
Pae-57	PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT	bla_{TEM}	F	CSF
Pae-58	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}	M	BL
Pae-62	TOB, AN, PIP, CAZ, IMP	bla_{TEM}	F	U
Pae-63	TOB, PIP, CAZ,IMP	bla_{TEM}	F	R
Pae-64	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}, bla_{SHV}	M	R
Pae-70	TOB, PIP, LOM, CAZ, FEP, IMP	bla_{CTXM}	F	R
Pae-72	TOB, PIP, LOM, CAZ, FEP, IMP,MEN	---	F	R
Pae-74	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT	bla_{OXA}	F	U
Pae-76	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}, bla_{SHV}, bla_{CTXM}, bla_{OXA}	M	BL
Pae-81	AN, PIP, CAZ, FEP, IMP, AZT	bla_{OXA}	M	U
Pae-82	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT	---	F	BL

a: TOB: tobramycin, AN: amikacin, CP: ciprofloxacin, LOM: levofloxacin, CAZ: ceftazidime, FEP: cefepime, PIP: piperacillin, IMP: imipenem, MEN: meropenem, AZT: azteronam
b: F: Female, M: Male
c: U: urine, W/A: wound/abscess, R: respiratory, D/F: dialysis-related fluids, AF: ascitic fluid, BL: blood, PF: plural fluid

Continue of table 4- Resistance phenotypes and presence of resistance genes in ESBL-positive *P. aeruginosa*

Isolate	Resistance phenotypes	Resistance genes	Sex	Source
Pae-84	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT	---	M	U
Pae-86	LOM, CAZ, FEP, IMP, MEN	bla_{TEM}	M	R
Pae-87	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	bla_{TEM}	F	R
Pae-90	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT	---	F	BL
Pae-92	TOB, PIP, CP, LOM, CAZ, FEP, IMP, MEN, AZT	bla_{TEM}, bla_{CTXM}	M	R
Pae-94	TOB, PIP, LOM, CAZ, FEP, IMP, MEN	bla_{SHV}	F	U
Pae-95	PIP, LOM, CAZ, IMP, MEN	---	M	U
Pae-96	TOB, AN, PIP, sCAZ, FEP, IMP, MEN, AZT	bla_{TEM}	M	BL
Pae-97	TOB, PIP, CAZ,IMP, MEN, AZT	bla_{TEM}, bla_{SHV}, bla_{OXA}	F	PF
Pae-98	PIP, CP, LOM, CAZ, IMP, MEN	bla_{TEM}, bla_{CTXM}, bla_{OXA}	M	R
Pae-108	CP, LOM,CAZ, IMP, MEN	bla_{TEM}	M	BL
Pae-110	TOB, PIP, CP, LOM, CAZ, IMP, MEN, AZT	bla_{SHV}	M	U
Pae-115	TOB, PIP, CP, LOM, CAZ, IMP, MEN, AZT	bla_{TEM}, bla_{SHV}, bla_{CTXM}, bla_{OXA}	M	U
Pae-118	TOB, AN, PIP, CAZ, IMP, AZT	bla_{TEM}, bla_{SHV}, bla_{CTXM}	M	W/A
Pae-121	CP, LOM, CAZ, MEN	bla_{TEM}, bla_{SHV}	F	R
Pae-123	TOB, AN, PIP, CAZ, IMP, MEN, AZT	bla_{TEM}	F	R
Pae-125	FEP, MEN	bla_{TEM}, bla_{CTXM}	M	R
Pae-127	TOB, PIP, CP, LOM, CAZ, IMP, MEN	bla_{TEM}, bla_{SHV}, bla_{CTXM}	F	U
Pae-128	TOB, AN, PIP, CAZ, IMP, AZT	bla_{SHV}, bla_{OXA}	F	U
Pae-133	TOB, AN, PIP, CAZ, IMP, AZT	bla_{TEM}, bla_{CTXM}, bla_{OXA}	F	A/F
Pae-137	TOB, PIP, CP, LOM, CAZ, IMP, MEN, AZT	---	M	R
Pae-138	TOB, AN, PIP, CAZ, IMP, AZT	bla_{OXA}	F	U
Pae-140	CAZ,FEP, MEN	bla_{SHV}	M	BL
Pae-143	PIP, CP, LOM, CAZ, IMP, MEN	bla_{TEM}, bla_{SHV}	M	R
Pae-150	CAZ,FEP	bla_{TEM}, bla_{SHV}, bla_{CTXM}	F	BL
Pae-155	CAZ,FEP	bla_{TEM}, bla_{SHV}	F	R
Pae-156	CP, LOM,CAZ, IMP, MEN	bla_{TEM}	M	U
Pae-160	TOB, PIP, CP, LOM, CAZ, IMP, MEN, AZT	bla_{TEM}, bla_{SHV}	F	R
Pae-161	CAZ,FEP, MEN	bla_{TEM}, bla_{CTXM}, bla_{OXA}	F	CSF

DISCUSSION

Production of broad-spectrum β-lactamases is one of the ways through which *P. aeruginosa* becomes resistant to antibiotics of the β-lactam family. Since *P. aeruginosa* is an important cause of hospital-acquired infections, spread of ESBL-producing genes among *P. aeruginosa* strains can make treatment of infections more challenging (12).

Therefore, periodic studies on the extent of resistance shown by this bacterium are of great importance. Based on the results, the highest rate of resistance was observed against ceftazidime, while none of the *P. aeruginosa* isolates was resistant to polymyxin B. Moreover, 39.75% of the isolates were ESBL-producing. A study by Ullah et al. showed that the lowest and highest level of antibiotic resistance was against meropenem (5.66%) and ampicillin (94.34%), respectively. Furthermore, 25.47% of the isolates were resistant to amikacin (15).

Tavajjohi and Moniri reported that 9.2% of the *P. aeruginosa* isolates were ESBL-positive. Furthermore, more than 30% of the isolates were MDR, 13% of which were resistant to all studied antibiotics. The highest and lowest

resistance ratewas observed against piperacillin (36.8%) and ciprofloxacin (11.9%), respectively (16). In another study, the highest and lowest level of antibiotic resistance was recorded against cefepime (4.7%) and ciprofloxacin (97.2%), respectively. In addition, 88.7% of the isolates were identified as MDR, 58.3% of which were ESBL-positive (17). In a study by Zafer et al., rate of resistance to cefuroxime, polymyxin B, ceftazidime and aztreonam was 87.7%, 2.4%, 60.6% and 45.1%, respectively. Additionally, 7.4% of isolates were identified as ESBL-positive (18). Tawfik et al. reported that none of 156 *P. aeruginosa* isolates was resistant to polymyxin B, and Shahcheraghi et al. stated that 95.5% of *P. aeruginosa* isolates were susceptible to polymyxin B (19, 20). Inconsistency between the results of our study and other studies could be due to differences in the geographical location, sources of the bacterial isolates and number of isolates. In our study, a relatively high percentage of the isolates were ESBL-producing and MDR, which can increase the risk of treatment failure. Since very few strains were resistant to polymyxin B, it can be concluded that administration of this drug can be effective for treatment of infections caused by MDR and/or ESBL-positive *P. aeruginosa*. Frequency of the bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ and bla_{OXA} genes was 60.86%, 29.81%, 24.22% and 14.28%, respectively. However, bla_{PER}, bla_{VEB} and bla_{GES} were not detected in any of the isolates. In a study by Shakibaie et al., the frequency of bla_{TEM}, bla_{PER} and bla_{SHV} in ESBL-positive isolates was 2.5%, 4.1% and 6.6%, respectively. In a study by Shahcheraghi et al., the frequency of bla_{VEB}, bla_{SHV}, bla_{PER} and bla_{TEM} among ESBL-positive strains was 24%, 22%, 17% and 9%, respectively (20). In 2014, Zafer et al. stated that $bla_{OXA\text{-}10}$ and $bla_{VEB\text{-}1}$ were found in 41.7 and 10.4% of isolates, respectively (18). According to statistics, the frequency of broad-spectrum β-lactamase genes in *P. aeruginosa* strains is increasing. Moreover, the results showed that the genotypic method of identifying ESBL-positive strains was significantly more accurate than the phenotypic method. This could be due to presence of gene(s) required for production of broad-spectrum β-lactamases that are not expressed because they are not under environmental pressure (presence of the β- lactam antibiotics). Employment of the phenotypic method could produce false negative results and disrupt the treatment process since antibiotics of the β-lactam family may be utilized to treat infections caused by ESBL-positive bacteria. Therefore, it is recommended to use the genotypic method for the detection of ESBL-positive isolates. In the present study, 12.5% of the ESBL-producing isolates had none of the studied resistance genes, probably because the enzyme was produced by genes (such as $bla_{NDM\text{-}1}$, bla_{BES}, *etc*.) other that those investigated in the present study. Bacteriophages can be considered as alternatives to antibiotics considering the emergence and rising incidence of multi-drug resistance (21). The natural potential of virulent phages to infect and destroy specific bacterial host cell makes them safe antibacterial agents for treatment of various infections caused by *Shigella* spp. (21, 22), *Salmonella* spp. (23), *Proteus mirabilis* (24), *Klebsiella pneumoniae* (14), etc. Since most *P. aeruginosa* strains isolated in the present study were MDR, they can be utilized as host for phage isolation and phage cocktail preparation against *P. aeruginosa*.

CONCLUSION

The resistance patterns in bacteria should be periodically and systematically studied. Reporting the results of such studies to physicians could help specify resistance frequencies and bacterial susceptibility to various antibiotics, increasing the chance of successful treatment of infections. Furthermore, it is highly recommended to use the genotypic method along with the phenotypic method for the detection of ESBL-positive *P. aeruginosa* strains because of the better accuracy. This can greatly influence the effectiveness of the treatment process. Moreover, we suggest using the CDC protocol for identification of MDR, PDR and XDR isolates.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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