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Major Article

Distribution of the bla_{OXA}, bla_{VEB-1}, and bla_{GES-1} genes and resistance patterns of ESBL-producing *Pseudomonas aeruginosa* isolated from hospitals in Tehran and Qazvin, Iran

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Abstract

Introduction: *Pseudomonas aeruginosa* is one of the most common nosocomial pathogens. The emergence of extended spectrum β-lactamases (ESBLs) has been increasingly reported as a major clinical concern worldwide. The main aim of the present study was to determine the distribution of bla_{OXA}, $bla_{PER-1,}$ $bla_{VEB-1,}$ and bla_{GES-1} genes among ESBL-producing *P. aeruginosa* isolated from two distinct provinces in Iran. **Methods:** In this study, a total of 75 (27.5%) ESBL-producing isolates were identified from 273 *P. aeruginosa* isolates collected from patients in Qazvin and Tehran. Phenotypic detection of ESBLs and antimicrobial susceptibility testing were performed according to the Clinical and Laboratory Standards Institute guidelines. PCR and sequencing were employed to detect bla_{OXA-1}, bla_{OXA}, $bla_{GES-1,}$ bla_{PER-1}, and bla_{VEB-1} genes. Isolate genetic relationships were evaluated by repetitive extragenic palindromic sequence-based PCR (REP-PCR). **Results:** In total, 59 (78.7%) of the ESBL-producing isolates showed multidrug resistance. The highest rates of susceptibility were observed against colistin (75 isolates, 100%) and polymyxin B (75, 100%) followed by amikacin (44, 58.7%), and piperacillin-tazobactam (40, 53.3%). The bla_{OXA-1} (37.3%) gene was the most common of the genes investigated, followed by bla_{OXA-4} (32%), bla_{GES-1} (16%), and bla_{VEB-1} (13.3%). REP-PCR identified three different genotypes: types A (89.3%), B (6.7%), and C (4%). **Conclusions:** We found a significant presence of bla_{OXA-1}, bla_{OXA-4}, $bla_{GES-1,}$ and bla_{VEB-1} genes among *P. aeruginosa* isolates, highlighting the need for suitable infection control strategies to effectively treat patients and prevent the further distribution of these resistant organisms.

Keywords: *Pseudomonas aeruginosa.* bla_{OXA}. bla_{VEB-1}. bla_{GES-1}. Repetitive extragenic palindromic sequence-based PCR.

INTRODUCTION

Pseudomonas aeruginosa is the most frequent bacterial species associated with infections such as urinary tract infections, respiratory infections, dermatitis, soft tissue infections, gastrointestinal infections, and a variety of systemic infections, particularly in patients with severe burns, cancer, and acquired immunodeficiency syndrome (AIDS)[1,2]. Infections caused by *P. aeruginosa* develop intrinsically and may exhibit acquired resistance against various commonly prescribed antimicrobial drugs. In addition, infections caused by this organism are often difficult to treat, as they eventually reveal the emergence of multi-drug resistant *Pseudomonas aeruginosa* (MDRPA) isolates[3]. Nosocomial infection with MDRPA is a serious growing concern worldwide and is associated with higher morbidity, mortality, and cost of therapy[4].

There are several mechanisms for the emergence of resistance to β-lactam antibiotics, with extended-spectrum β-lactamases (ESBLs) among the leading causes[5,6]. These enzymes are plasmid-encoded β-lactamases commonly found in *Klebsiella pneumoniae* and *Escherichia coli* and also observed in other clinical isolates of Enterobacteriaceae and *Pseudomonas*[7-9]. It is clear that the TEM (temoniera), SHV (sulfhydryl-variable), and CTX-M (cefotaximase) proteins are the principal types of ESBLs among clinically important Enterobacteriaceae species; however, the presence of less-studied types of ESBLs, including OXA (oxacillinase), VEB (Vietnamese extended spectrum β-lactamase), PER (Pseudomonas extended resistant), and GES (Guyana extended spectrum β-lactamase), has been reported in other bacterial species[10,11]. Recent studies have indicated that the dissemination of genes that encode ESBLs may play an important role in the spread of antibiotic resistance and may complicate the treatment of infections caused by *P. aeruginosa*

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because of limitations in therapeutic choices[6,10]. OXA-type enzymes belong to a growing family of ESBLs that differ from the TEM and SHV enzymes as they belong to the molecular class D and functional group 2d[12,13]. These β-lactamase enzymes have been found mainly in *P. aeruginosa*[11]. VEB-1 was initially observed in a single isolate of *E. coli* obtained from a Vietnamese patient but was later observed in a *P. aeruginosa* isolate from a Thai patient[14]. GES-1 is reported to not be closely related to any other plasmid-mediated β-lactamase; nevertheless, it demonstrates 36% homology to a carbenicillinase produced by *Proteus mirabilis*[11]. The PER-1 β-lactamase was first discovered in strains of *P. aeruginosa* isolated from patients in Turkey[15]. There are limited reports describing the prevalence of the OXA, VEB, PER, and GES types of ESBLs in clinical isolates of *P. aeruginosa* in Iran. The main aim of the present study was to determine the distribution of the bla_{OXA}, bla_{PER-1}, bla_{VEB-1}, and bla_{GES-1}-genes and resistance patterns among *P. aeruginosa* isolated from two distinct provinces in Iran.

METHODS

Study design and bacterial isolates

In this cross-sectional study, a total of 75 (27.5%) ESBL-producing isolates were identified from among 273 *P. aeruginosa* collected from hospitalized patients (one isolate per patient) in the Tehran and Qazvin provinces from January 2014 to October 2015. The bacterial isolates were collected from different clinical specimens including urine, wound, sputum, bronchoalveolar lavage, trachea, and blood samples. These isolates were obtained from patients who were admitted to intensive care units (ICUs), as well as internal medicine, general surgery, neurology, neurosurgery, and infectious disease wards. Duplicate isolates from the same patient were excluded. The study was approved by the Ethics Committee of Qazvin University of Medical Sciences (code IR.QUMS.REC.1394.147). Written informed consent was obtained from all subjects enrolled in this study. All isolates were identified as *P. aeruginosa* through the application of standard microbiological tests such as Gram staining, oxidase test, growth at 42°C, growth on cetrimide agar medium (Liofilchem, Roseto, Italy), O/F (oxidation/fermentation) test, and pigment production[16]. Isolates were stored at –70°C in trypticase soy broth containing 20% glycerol and were subcultured twice prior to testing.

ESBL detection and antimicrobial susceptibility

ESBL production was tested by phenotypic combined disk (CD) assay, as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines[17]. All isolates were initially screened for ESBL production using the standard disk diffusion method, using ceftazidime (30μg), cefotaxime (30μg), ceftriaxone (30μg), cefpodoxim (30μg), and aztreonam disks (30μg) (Mast Diagnostics Group Ltd., Merseyside, UK). Isolates that were not susceptible to any of these antibiotics were further examined for ESBL production by the CD method, which compared the inhibition zones of disks containing cefotaxime or ceftazidime with and without clavulanic acid. ESBL production was confirmed by a ≥5mm increase in the diameter of the inhibition zone for ceftazidime/clavulanate (30/10μg) and cefotaxime/clavulanate (30/10μg) compared to the diameters of the inhibition zones in the absence of clavulanate. Antimicrobial susceptibility was further performed by the disk diffusion method using the following antibiotic disks (Mast Diagnostics Group Ltd.): cefepime (30μg), amikacin (30μg), aztreonam (30μg), ceftriaxone (30μg), imipenem (10μg), meropenem (10μg), gentamicin (10μg), piperacillin/tazobactam (100/10μg), piperacillin (100μg), carbenicillin (100μg), ceftazidim (30μg), cefotaxime (10μg), ciprofloxacin (5μg), levofloxacin (5μg), ofloxacin (10μg), ticarcillin (10μg), carbenicillin (10μg), tobramycin (10μg), colistin (30μg), and polymyxin B (300 units). *P. aeruginosa* American Type Culture Collection (ATCC) strain 27853 was used as the quality control strain in antimicrobial susceptibility testing.

Detection of bla_{OXA}, bla_{PER}, bla_{VEB}, and bla_{GES} genes

ESBL-producing isolates were subjected to polymerase chain reaction (PCR) for detection of bla_{OXA}, bla_{PER-1} bla_{VEB-1}, and bla_{GES-1} genes using the specific primers listed in **Table 1**[18-24]. Total deoxyribonucleic acid (DNA) was extracted from bacterial isolates using an extraction kit (Bioneer, Daejeon, Korea). PCR amplification was performed in a thermocycler (Applied Biosystems, Carlsbad, CA, USA) as follows: 96°C for 5 min; 35 cycles of 1 min at 96°C, 1 min at a specific temperature for each primer, and 1 min at 72°C; and a final extension step of 10 min at 72°C. Amplification reactions were prepared in a total volume of 25μl including 12.5μl Taq DNA polymerase 2× Master Mix with 1.5mM $MgCl_2$ (Ampliqon, Odense, Denmark), 0.5μM forward primer, 0.5μM reverse primer, 9μl nuclease free water, and 2.5μl DNA template (50pg concentration). PCR products were electrophoresed on a 1% agarose gel at 100V, stained with ethidium bromide solution, and finally visualized with a gel documentation system (UviTec, Cambridge, UK). Purified PCR products were sequenced by the Macrogen Company (Seoul, Korea), and sequence alignment and analysis were performed online using the basic local alignment search tool (BLAST) program of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Clonal analysis

Repetitive extragenic palindromic sequence-based PCR (REP-PCR) reactions were carried out in a final volume of 25μl, containing 2.5μl 10× PCR buffer, 0.5μl deoxynucleotide triphosphate (dNTP) Mix (10 mol), 5μl $MgCl_2$, 25pM REP-primer F, 25pM REP-primer R[24], 2U Taq DNA polymerase, 3μl extracted template DNA, and 16.1 μl distilled water. Amplification conditions were as follows: initial denaturation for 5 min at 95°C; 30 cycles of 1 min at 94°C, 1 min at 45°C, and 2 min at 72°C; and a final extension of 16 min at 72°C. PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. Analysis of REP-PCR profiles was performed by visual comparison of band patterns. Isolates with similar patterns (up to two bands different) were considered to belong to the same DNA group.

Statistical analysis

Statistical data analysis was performed using descriptive statistics including frequencies and cross-tabulation of

TABLE 1
Primers used for detecting ESBL-encoding genes in this study.

Primer	Target	Sequence (5'-3')	Annealing temperature (°C)	Reference
VEB-F	VEB-1	CGACTTCCATTTCCCGATGC	55	18
VEB-B		GGACTCTGCAACAAATACGC		
OXA-1-A	OXA-1-group III	AGCCGTTAAAATTAAGCCC	53	19
OXA-1-B		CTTGATTGAAGGATTGGGCG		
OXA-2-F	OXA-2-group II	GCCAAAGGCACGATAGTTGT	62	20
OXA-2-B		GCGTCCGAGTTGACTGCCGG		
OXA-10-F	OXA-10-group I	TCTTTCGAGTACGGCATTAGC	56	18
OXA-10-B		CCA ATGATGCCCTCACTTTCC		
OXA-4-F	OXA-4	TCAACAGATATCTCTACTGTT	50	21
OXA-4-B		TTTATCCCATTTGAATATGGT		
PER-F	PER-1	AATTTGGGCTTAGGGCAGAA	53	22
PER-B		ATGAATGTCATTATAAAAGC		
GES-1-A	GES-1	ATGCGCTTCATTCACGCAC	53	23
GES-1-B		CTATTTGTCCGTGCTCAGG		
REP-1	REP-PCR	IIIGCGCCGICATCAGGC	45	24
REP-2		ACGTCTTATCAGGCCTAC		

ESBL: extended spectrum β-lactamase; **VEB:** Vietnames*e* extended spectrum β-lactamase; **OXA:** oxacillinase; **PER:** *Pseudomonas* extended resistant; **GES:** Guyana extended spectrum β-lactamase; **REP:** repetitive extragenic palindromic sequence-based.

microbiological and clinical features and demographic characteristics using the computer software program Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) version 19.

RESULTS

ESBL-producing isolates were recovered from different clinical specimens including blood (24 isolates, 32%), urine (17, 22.7%), trachea (13, 17.3%), wound (9, 12%), sputum (6, 8%), and bronchoalveolar lavage (6, 8%) samples. Isolates were obtained from patients admitted to intensive care units (33 isolates, 44%) and internal medicine (19, 25.3%), infectious disease (12, 16%), general surgery (8, 10.7%), neurosurgery (2, 2.7%), and neurology (1, 1.3%) wards. There were 39 (52%) male and 36 (48%) female patients. A total of 59 (78.7%) ESBL-producing isolates were found to be multi-drug resistant *P. aeruginosa* (MDRPA), showing intermediate or complete resistance to at least three different classes of antimicrobial agents including β-lactams, aminoglycosides, and fluoroquinolones. The highest rates of susceptibility to the antimicrobials tested in this study were observed for colistin (75 isolates, 100%) and polymyxin B (75, 100%), followed by amikacin (44, 58.7%) and piperacillin-tazobactam (40, 53.3%). In addition, 41 (54.7%) and 40 (53.5%) isolates demonstrated complete or intermediate resistance to meropenem and imipenem, respectively **(Table 2)**.

Among the 75 isolates, $bla_{\text{OXA-1}}$ (28/37.3%) was the most common ESBL-encoding gene among those investigated, followed by $bla_{\text{OXA-4}}$ (32%), $bla_{\text{GES-1}}$ (16%), and $bla_{\text{VEB-1}}$ (13.3%). The study isolates were negative for $bla_{\text{OXA-10,}}$ $bla_{\text{PER-1}}$, and $bla_{\text{OXA-2}}$. The $bla_{\text{OXA-1}}$ gene was found to coexist with $bla_{\text{OXA-4}}$ in 14 (18.7%) isolates; two (2.7%) isolates also carried both $bla_{\text{OXA-1}}$ and $bla_{\text{GES-1}}$, and one (1.3%) isolate carried the $bla_{\text{OXA-1}}$, $bla_{\text{OXA-4,}}$ $bla_{\text{GES-1}}$, and $bla_{\text{VEB-1}}$ genes **(Table 3)**. Overall, $bla_{\text{OXA-1}}$-positive (24%) and $bla_{\text{OXA-4}}$-positive (20%) isolates were mostly collected from Tehran hospitals, whereas $bla_{\text{GES-1}}$-positive (16%) and $bla_{\text{VEB-1}}$-positive (13.3%) isolates all derived from Qazvin hospitals.

Molecular genotyping by REP-PCR showed that the 75 ESBL-producing isolates belonged to three distinct genotypes, named the A, B, and C genotypes. Among ESBL producers, 67 (89.3%), five (6.7%), and three (4%) isolates belonged to types A, B, and C, respectively. As shown in **Table 3**, the most frequent ESBL gene detected among the isolates of the current study, $bla_{\text{OXA-1}}$, was found in isolates belonging to the A (24, 31.9%) and B (3, 4%) genotypes, either alone or in combination.

DISCUSSION

ESBL-producing *P. aeruginosa* has recently emerged as a major cause of health-care associated infections[25,26]. The treatment of infections caused by these resistant organisms is increasingly complicated owing to the high levels of resistance to the most commonly prescribed antibiotics in hospital settings[27]. Several studies carried out by Mirsalehian et al.[28], Farshadzadeh et al.[29], and Shakibaie et al.[30] covering three

TABLE 2

Antimicrobial susceptibility of ESBL-producing *Pseudomonas aeruginosa* isolated from hospitals in Qazvin and Tehran (n = 75).

Antibiotic	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Colistin	75 (100.0)	-	-
Polymyxin B	75 (100.0)	-	-
Amikacin	44 (58.7)	10 (13.3)	21 (28.0)
Piperacillin-tazobactam	40 (53.3)	9 (12.0)	26 (34.7)
Imipenem	35 (46.7)	9 (12.0)	31 (41.3)
Meropenem	34 (45.3)	7 (9.3)	34 (45.3)
Cefepime	30 (40.0)	1 (1.3)	44 (58.7)
Piperacillin	26 (34.7)	8 (10.7)	41 (54.7)
Ciprofloxacin	24 (32.0)	1 (1.3)	50 (66.7)
Gentamicin	22 (29.3)	-	53 (70.7)
Ceftazidime	22 (29.3)	4 (5.3)	49 (65.3)
Tobramycin	22 (29.3)	3 (4.0)	50 (66.7)
Ofloxacin	21 (28.0)	2 (2.7)	52 (69.3)
Levofloxacin	18 (24.0)	2 (2.7)	55 (73.3)
Ticarcillin	17 (22.7)	1 (1.3)	57 (76.0)
Aztreonam	14 (18.7)	10 (13.3)	51 (68.0)
Carbenicillin	14 (18.7)	3 (4.0)	58 (77.3)
Ceftriaxone	10 (13.3)	7 (9.3)	58 (77.3)
Cefotaxime	5 (6.7)	8 (10.7)	62 (82.7)

ESBL: extended spectrum β-lactamase.

TABLE 3

REP-PCR genotyping of ESBL-positive *Pseudomonas aeruginosa* isolates.

	Isolates			
Gene	Type A n (%)	Type B n (%)	Type C n (%)	Total n (%)
bla_{OXA-1}	10 (13.3)	1 (1.3)	-	11 (14.7)
bla_{OXA-4}	6 (8.0)	-	1 (1.3)	7 (9.3)
bla_{GES-1}	6 (8.0)	-	-	6 (8.0)
bla_{VEB-1}	5 (6.7)	2 (2.7)	-	7 (9.3)
bla_{OXA-1} and bla_{OXA-4}	12 (16.0)	2 (2.7)	-	14 (18.7)
bla_{OXA-1} and bla_{GES-1}	1 (1.3)	-	1 (1.3)	2 (2.7)
bla_{OXA-4} and bla_{GES-1}	2 (2.7)	-	-	2 (2.7)
bla_{VEB-1} and bla_{GES-1}	1 (1.3)	-	-	1 (1.3)
bla_{OXA-1}, bla_{OXA-4}, $bla_{GES-1,}$ and bla_{VEB-1}	1 (1.3)	-	-	1 (1.3)
No bla_{OXA-1}, $bla_{OXA-4,}$ $bla_{GES-1,}$ or bla_{VEB-1}	23 (30.7)	-	1 (1.3)	24 (32.0)
Total	**67 (89.3)**	**5 (6.7)**	**3 (4.)**	**75 (100.)**

REP-PCR: repetitive extragenic palindromic sequence-based-PCR; **ESBL:** extended spectrum β-lactamase.

distinct burn centers throughout the country, as well as a study by Alikhani et al.[31] in two training hospitals affiliated with the Hamadan University of Medical Sciences have described the existence of ESBLs among clinical isolates of *P. aeruginosa*. We previously revealed the appearance of metallo-β-lactamases (MBLs) among clinical isolates of *P. aeruginosa* in Iran[32]. ESBL producers are reported to be present in other Asian countries, such as India and Bangladesh, from where several reports by Senthamaria et al.[33] Umadevi et al.[34], and Begum et al.[35] are available. In the current study, 78.7% of ESBL-producing isolates were found to be MDRPA, exhibiting relatively high resistance rates to most antibiotics tested with the exception of polymyxin B and colistin, indicating that available choices for the treatment of these infections are currently limited.

Pseudomonas aeruginosa isolates exhibited the highest rates of susceptibility to the antimicrobials tested in this study. The inappropriate management of infections in terms of excessive use of antibiotics is likely to be a major predisposing factor in the emergence of resistant bacteria in hospital settings. These data highlight the necessity for establishing both a local and nationwide antimicrobial resistance surveillance system to monitor the possible appearance of resistance within our healthcare system. Moreover, the results of the present study show that ESBL-producing isolates mostly derive from patients admitted to ICUs. This may reflect a greater clinical impact, as the patients admitted to these wards likely face more chronic underlying conditions and are exposed to broad-spectrum antibiotics and medical manipulations through the use of invasive devices. However, it should be noted that the distribution of nosocomial microorganisms varies widely on different continents and in different regions, countries, hospitals, and even in different areas of the same hospital. Patient factors such as age, infection severity, immune response, and length of hospital stay also affect the presence of these microorganisms. Furthermore, hospital-related factors, such as the availability and use of broad-spectrum antibiotics and the diagnostic and therapeutic procedures employed, should also be taken into account, though data on these factors are limited.

In the present study, 37.3%, 32%, 16%, and 13.3% of ESBL-producing *P. aeruginosa* isolates carried the $bla_{\text{OXA-1}}$, $bla_{\text{OXA-4}}$, $bla_{\text{GES-1}}$, and $bla_{\text{VEB-1}}$ genes, respectively, alone or in combination. In a study from Iran, Shahcheraghi et al. reported that 24%, 17%, and 0% of MDRPA isolates harbored the bla_{VEB}, bla_{PER}, and bla_{GES} genes, respectively[23]. Mirsalehian et al. reported that 74.6%, 49.2%, and 31.3% of ESBL-producing isolates of *P. aeruginosa* collected from patients at Motahhari Burn Hospital in Tehran were positive for $bla_{\text{OXA-10}}$, $bla_{\text{PER-1}}$, and $bla_{\text{VEB-1}}$, respectively[28]. In another study conducted by Rafiee et al. at the same burn center, it was shown that 21.6% of *P. aeruginosa* isolates carried the $bla_{\text{PER-1}}$ gene[13]. Farshadzadeh et al., in a study carried out at the Taleghani Burn Hospital in Ahvaz (Iran), reported that 54.2% and 68.7% of ESBL-producing isolates of *P. aeruginosa* harbored $bla_{\text{PER-1}}$ and $bla_{\text{OXA-10}}$, respectively[29]. Among the studies reported from Asian countries, Lee and colleagues from South Korea showed that 6.3%, 13.1%, 4.3%, 2.0%, 2.3%, and 0.4% of *P. aeruginosa* isolates carried the $bla_{\text{PSE-1}}$, $bla_{\text{OXA-10}}$, $bla_{\text{OXA-4}}$, $bla_{\text{OXA-30}}$, $bla_{\text{OXA-2}}$, and $bla_{\text{OXA-17}}$ genes, respectively[36]. In another study performed in Saudi Arabia, the presence of genes encoding VEB-1, OXA-10, and GES was confirmed among extended-spectrum cephalosporin-non-susceptible *P. aeruginosa* clinical isolates from burn patients[10]. Together, these data indicate the successful spread of ESBL-encoding genes around the world.

In the present study, the application of REP-PCR for molecular genotyping showed the presence of three separate clones of ESBL-producing *P. aeruginosa*. Genotype A was the most common (89.3%) within the hospitals under evaluation, which is strongly associated with the clonal spread of these resistant organism and patient-to-patient transmission. The distribution of ESBL genes among different clones in the current study suggests not only the clonal spread of ESBL-producing isolates among different wards but also the spread of resistance genes among these bacterial strains.

In conclusion, the presence of ESBL-producing *P. aeruginosa* in clinical settings has become a serious concern owing to the fact that these strains exhibit a wide range of resistances not only to β-lactam but frequently to other classes of antibiotics. Moreover, the genes encoding these enzymes are often carried on mobile elements that can quickly spread between different strains via horizontal transfer. The identification of ESBLs, introduction of appropriate infection control measures, and justified antibiotic use are necessary to prevent the further spread of infections by these organisms.

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Conflict of interest

The authors declare that have no conflicts of interest.

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