

ORIGINAL ARTICLE

Distribution of *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} genes among ESBL-producing *P. aeruginosa* isolated from Qazvin and Tehran hospitals, Iran

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Keywords

Pseudomonas aeruginosa • ESBL • *bla*_{TEM} • *bla*_{SHV} • *bla*_{CTX-M}

Summary

Introduction. *Pseudomonas aeruginosa* is as an important opportunistic human pathogen, which is associated with several clinical infections that are usually difficult to treat because of resistance to multiple antimicrobials. The production of extended-spectrum β -lactamases (ESBLs) is an important mechanism of β -lactam resistance. The aims of this study were to determine the prevalence of ESBLs, antimicrobial susceptibility, and to detect the *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} genes.

Methods. In this study, carried out from March 2013 to December 2014, 266 *P. aeruginosa* isolates were collected from patients admitted to teaching hospitals of Qazvin and Tehran, Iran. All isolates were initially screened for ESBL production by disk diffusion method and were further confirmed using a combined disk method. Antimicrobial susceptibility of ESBL-producing isolates was determined by standard disk diffusion method. Polymerase

Chain Reaction (PCR) and sequencing techniques were employed for detection of *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} genes.

Results. In total, 262 (98.5%) *P. aeruginosa* isolates were non-susceptible to the used extended spectrum cephalosporins, and, among these, 75 (28.6%) isolates were ESBL producers. Fifty-nine (78.7%) of ESBL-producing isolates showed multidrug-resistance pattern. Of 75 ESBL-positive isolates, the *bla*_{TEM-1} (26.7%) was the most common gene, followed by *bla*_{CTX-M-15} (17.3%), *bla*_{SHV-1} (6.7%), and *bla*_{SHV-12} (4%), either alone or in combination.

Conclusions. The results of this study showed the notable prevalence of ESBLs among the clinical isolates of *P. aeruginosa* in Iran, indicating the urgency for the implementation of appropriate follow-up measures for infection control and proper administration of antimicrobial agents in our medical settings.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an important opportunistic clinical pathogen, causing a variety of healthcare-associated infections, such as pneumonia, sepsis, wounds, and urinary tract infections [1, 2]. This organism is an important cause of septic mortality in burn patients [3]. *P. aeruginosa* is a major cause of chronic lung infections in children and young adults with cystic fibrosis and can be especially severe in neutropenic or cancer patients [4]. Infections caused by *P. aeruginosa* are often difficult to treat because of its intrinsic and acquired resistance to many commonly prescribed antimicrobial agents, eventually leading to the emergence of multidrug-resistant *P. aeruginosa* (MDR-PA) strain [5]. *P. aeruginosa* is physiologically resistant to many antibacterial agents, especially to the extended-spectrum cephalosporins, due to the production of different classes of extended spectrum β -lactamases (ESBLs), overproduction of chromosomal AmpC cephalosporinase, and non-enzymatic mechanisms, such as efflux pumps and outer membrane impermeability [6], among others. Nosocomial infections due to MDRPA strains are increasingly recognized throughout the world

and are associated with increased morbidity, mortality, and cost of therapy [7, 8].

ESBLs are one of the main leading causes of resistance to β -lactam antibiotics among Gram-negative bacteria [6]. These enzymes are plasmid-encoded β -lactamases that mediate resistance to penicillins, first-, second- and third- generation cephalosporins, such as cefotaxime, ceftriaxone, and ceftazidime [6, 9]. TEM, SHV, and CTX-M are the major genetic groups of ESBLs amongst clinically important Gram-negative bacteria [9, 10].

These enzymes are most commonly found in *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*) and are also observed in other clinical isolates of Enterobacteriaceae and *Pseudomonas* [11, 12]. The first TEM-type β -lactamase, produced by a clinical *E. coli* strain, was reported in 1965. The TEM-type ESBLs are derivatives of TEM-1 and TEM-2 [9]. The SHV-type ESBLs may be found in clinical isolates more frequently than any other types of ESBLs and have been reported from several countries in Europe, such as Austria, France, Italy, and Greece, as well as in the United States and Australia [6, 9, 10]. The CTX-M-type ESBLs developed from TEM and SHV and can be divided into five subgroups according to their amino acid sequence simi-

larities, including CTX-M-I, CTX-M-II, CTX-M-III, CTX-M-IV, and CTX-M-V [13, 14]. Detection of ESBLs is important for the surveillance and epidemiological studies of their transmission in medical settings [15]. One major concern regarding the spread of ESBL-producing *P. aeruginosa* within hospital settings is the treatment failure to infections caused by this organism due to the limitations in therapeutic choices [9, 16]. There are few reports describing the prevalence of TEM- or SHV-type ESBLs in *P. aeruginosa* in Iran. The aims of this study were to determine the prevalence of ESBLs and to detect the *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}-types ESBL genes among clinical isolates of *P. aeruginosa* collected from hospitals of Tehran and Qazvin, two central provinces of Iran.

Methods

BACTERIAL ISOLATES

The clinical isolates of *P. aeruginosa* (one isolate per patient) were collected from hospitalized patients of Tehran and Qazvin provinces from March 2013 to December 2014. The bacterial isolates were collected from different clinical specimens, including urine, wounds, sputum, bronchoalveolar lavage (BAL), trachea, and blood. These isolates were obtained from patients admitted to intensive care units (ICUs), internal medicine, general surgery, neurology, neurosurgery, and infectious disease wards. Specimens of these patients were sent to the microbiology laboratory of the hospitals under study. 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* using standard microbiological and biochemical tests [17]. The isolates were stored

at -70°C in trypticase soy broth (TSB) containing 20% glycerol and subcultured twice prior to testing.

ESBL SCREENING

All isolates were initially screened for ESBLs production using the standard disc diffusion method, using ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), cefpodoxim (30µg), and aztreonam (30µg). Isolates which were non-susceptible to any of third generation cephalosporins were selected for ESBLs detection phenotypically. The antibiotic disks were purchased from Mast (Mast Diagnostics Group Ltd). *P. aeruginosa* ATCC 27853 was used as a control strain in antimicrobial susceptibility testings.

CONFIRMATION OF ESBL PRODUCTION

Phenotypic confirmatory tests [18], which were designed for detecting ESBLs in *K. pneumoniae* and *E. coli*, were also performed by comparing the inhibition zone of disks containing cefotaxime or ceftazidime with and without clavulanic acid. *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603 were used as the quality control strains in antimicrobial susceptibility testing.

MOLECULAR DETECTION OF ESBL-ENCODING GENES

ESBL-producing isolates were subjected to Polymerase Chain Reaction (PCR) targeting *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M-1}, *bla*_{CTX-M-2}, *bla*_{CTX-M-8}, *bla*_{CTX-M-9}, and *bla*_{CTX-M-25} genes using the specific primers listed in Table I. Plasmid DNA was extracted using plasmid mini extraction kit (Bioneer Company, Korea). The PCR amplifications were performed in a thermocycler (Applied Biosystems, USA) as follows: 96° C for 5 min and 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 25 µl (24 µl of PCR master mix plus 1 µl of

Tab. I. Primers used in this study for detecting ESBL-encoding genes.

Targets	Primer Sequences (5'-3')	Annealing Temperature (°C)	References
TEM	ATGAGTATTCACATTTCCG GACAGTTACCAATGCTTAATCA	50	19
TEM (Sequencing)	TAACCATGAGTGATAACACT CCGATCGTTGTCAGAAGTAA	50	19
SHV	CTTTACTCGCCTTTATCG TCCGCGAGATAAATCACCA	50	19
SHV (Sequencing)	ACTGCCTTTTTGCGCCAGAT CAGTTCGGTTTCCAGCGGT	56	19
CTX-M-1 group	ATGGTTAAAAAATCACTGCGTC TTGGTGACGATTTTAGCCGC	55	20
CTX-M-2 group	ATGATGACTCAGAGCATTCG TGGGTTACGATTTTCGCCGC	55	20
CTX-M-8 group	ACTTCAGCCACACGGATTCA CGAGTACGTACGACGACTT	55	20
CTX-M-9 group	ATGGTGACAAAGAGACTGCA CCCTTCGGCGATGATTCTC	55	20
CTX-M-25	CACACGAATTGAATGTTTCA TCACTCCACATGGTGAGT	50	21

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

Antibiotics	S (%)	I (%)	R (%)
Amikacin	44 (58.7)	10 (13.3)	21 (28)
Piperacillin-tazobactam	40 (53.3)	9 (12)	26 (34.7)
Imipenem	35 (46.7)	9 (12)	31 (41.3)
Meropenem	34 (45.3)	7 (9.3)	34 (45.3)
Cefepime	30 (40)	1 (1.3)	44 (58.7)
Piperacillin	26 (34.7)	8 (10.7)	41 (54.7)
Ciprofloxacin	24 (32)	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)	50 (66.7)
Ofloxacin	21 (28)	2 (2.7)	52 (69.3)
Levofloxacin	18 (24)	2 (2.7)	55 (73.3)
Ticarcillin	17 (22.7)	1 (1.3)	57 (76)
Aztreonam	14 (18.7)	10 (13.3)	51 (68)
Carbenicillin	14 (18.7)	3 (4)	58 (77.3)
Ceftriaxone	10 (13.3)	7 (9.3)	58 (77.3)
Cefotaxime	5 (6.7)	8 (10.7)	62 (82.7)

S: Sensitive, I: Intermediate, R: Resistant.

template DNA) including 5 ng of genomic DNA, 2.0 U of Taq DNA polymerase, 10 mM dNTP mix at a final concentration of 0.2 mM, 50 mM MgCl₂ at a final concentration of 1.5 mM, 1 μM of each primer, and 1X PCR buffer (final concentration). PCR products were electrophoresed on a 1% agarose gel at 100 volts and then were stained with ethidium bromide solution and finally visualized in gel documentation system (UVtec, UK). The purified PCR products were sequenced by the Macrogen Company (Seoul, Korea). The sequence alignment and bioinformatics analyses were performed using the Basic Local Alignment Search Tool (BLAST) online program of the National Center for Biotechnology Information (freely available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical analysis was performed for descriptive statistics, including frequencies, cross-tabulation of microbiological, clinical, and demographic characteristics, using the commercial software Statistical analysis software package (version 16, IBM SPSS Corporation, Armonk, NY, USA).

Results

During the study period from March 2013 to December 2014, a total of 266 isolates of *P. aeruginosa* were collected from different clinical specimens including blood (94 isolates; 35.3%), urine (80 isolates; 30.1%), wounds (29 isolates; 10.9%), trachea (26 isolates; 9.8%), sputum (19 isolates; 7.1%), and BAL (18 isolates; 6.8%). Isolates were obtained from patients admitted to intensive care units (99-37.2%), internal medicine (64-24.1%), infectious diseases (61-22.9%), general surgery (26-9.8%), neurosurgery (12-4.5%), and neurology (4-1.5%) wards.

One hundred and twenty-five (47%) were male and 141 (53%) were female. Out of the 266 *P. aeruginosa* isolates, 262 (98.5%) isolates were non-susceptible to at least one of the antibiotics used in the screening test and, among these, 75 (28.6%) isolates were identified as potential ESBL producers using combined disk method. Fifty-nine (78.7%) of ESBL-producing isolates were found to be multidrug-resistant (MDR), i.e. showed intermediate or fully resistance to at least three different classes of antimicrobial agents including β-lactams, aminoglycosides, and fluoroquinolones. Amikacin (58.7%) and piperacillin-tazobactam (53.3%) showed the highest rates of susceptibility among antimicrobials tested in this study, respectively (Table II). Further, 41 (54.7%) and 40 (53.5%) ESBL-producing isolates were fully or intermediate resistant to meropenem and imipenem, respectively. ESBL-producing isolates were mainly recovered from urine (30.2%), followed by wound (28.3%) samples. The patients affected were mainly those admitted in ICU (54.7%) and the internal wards (45.1%), respectively (Table III). Of the 75 *P. aeruginosa* isolates with ESBL phenotype, *bla*_{TEM-1} (20-26.7%) was the most common gene, followed by *bla*_{CTX-M-15} (13-17.3%), *bla*_{SHV-1} (5-6.7%), and *bla*_{SHV-12} (3-4%), either alone or in combination (Table IV). In this study, isolates were, instead, negative for *bla*_{CTX-M-2}, *bla*_{CTX-M-8}, and *bla*_{CTX-M-9} group genes, as well as for *bla*_{CTX-M-25}.

Discussion

P. aeruginosa has recently emerged as a major cause of healthcare-associated infections, especially in immunocompromised people and burn patients [22, 23]. The treatment of *P. aeruginosa* infections is increasingly

Tab. III. Frequency of ESBL-producing isolates broken down by hospitals ward and source of clinical specimen.

Hospital wards	n (%)
ICU	33 (44)
Internal medicine	19 (25.3)
Infectious disease	12 (16)
Surgery	8 (10.7)
Neurosurgery	2 (2.7)
Neurology	1 (1.3)
Clinical specimens	(n/%)
Blood	24 (32)
Urine	17 (22.7)
Trachea	13 (17.3)
Wound	9 (12)
BAL	6 (8)
Sputum	6 (8)

ICU: intensive care unit, BAL: bronchoalveolar lavage

Tab. IV. Distribution of *bla*_{SHV}, *bla*_{TEM}, and *bla*_{CTX-M} genes in ESBL-producing *P. aeruginosa* isolates.

Resistance genes	n (%)
<i>bla</i> _{SHV} -1	4 (5.3)
<i>bla</i> _{SHV} -12	3 (4)
<i>bla</i> _{TEM} -1	12 (16)
<i>bla</i> _{CTX-M} -15	4 (5.3)
<i>bla</i> _{SHV} -1 and <i>bla</i> _{CTX-M} -15	1 (1.3)
<i>bla</i> _{TEM} -1 and <i>bla</i> _{CTX-M} -15	8 (10.7)
No <i>bla</i> _{TEM} -1, <i>bla</i> _{SHV} -1 and <i>bla</i> _{CTX-M} -15	43 (57.3)

complicated due to both intrinsic and acquired resistance to the most commonly prescribed antibiotics in hospital settings [2, 24]. The emergence of ESBL has become a matter of serious concern for the treatment of patients in Iran. ESBL detection is not routinely tested in most laboratories in Iran. There are only few reports on prevalence of ESBL among *P. aeruginosa* isolates in our country. In the present study, 262 (98.5%) *P. aeruginosa* isolates were non-susceptible to at least one of the antibiotics used in the ESBL screening test and, among these, 75 (28.6%) isolates were ESBL positive. The prevalence rate of ESBL in our study is lower than the rate reported by Mirsalehian et al. (39.4%) [25] and Shakibaie et al. (34%) [26] from two burn centers in Iran, Begum et al. from Bangladesh (37.8%) [27], and Senthamaria et al. from India (42.3%) [28], but higher than the rate found by Zafar et al. from Egypt (7.4%) [29], Umadevi et al. from India (19.4%) [30] and Woodford et al. from United Kingdom (3.7%) [31]. In our study, 78.7% of ESBL-producing isolates were found to be MDR and showed relatively higher resistance rates to most antibiotics test-

ed. These results are in accordance with those of other studies, such as those conducted by Fallah et al. and Hakemi Vala et al. in Iran [32, 33]. Our findings demonstrated that amikacin (58.7%) and piperacillin-tazobactam (53.3%) showed the highest rates of susceptibility among the antimicrobials tested, whereas cefotaxime and ceftriaxone revealed low susceptibility rates of 6.7% and 13.3%, respectively. The inappropriate management of infections through unnecessary and widespread administration of antibacterial agents is likely to be the main predisposing factor leading to the emergence of resistant bacteria in our medical centers. Moreover, the high resistance rate of ESBLs found among the isolates in this study emphasizes the need for a local and national antimicrobial resistance surveillance system for monitoring the administration of antimicrobials in our hospital settings.

It should be noted that, in this study, 71.5% of extended spectrum cephalosporin non-susceptible isolates were ESBL negative, which can be associated with other resistance mechanisms such as overproduction of chromosomal cephalosporinase (AmpC), up-regulation of efflux systems or decreased outer-membrane permeability. Additionally, our data showed that 54.7% and 53.5% of ESBL-producing isolates were non-susceptible to meropenem and imipenem, respectively. Since in hospital setting the appropriate treatment of infections caused by MDR Gram-negative bacteria is generally achieved by the administration of carbapenems, this finding indicated that the available therapeutic choices are currently limited. This has a relevant clinical impact, especially in the future in case these strains should become more prevalent. This study, in line with previous studies [34, 35], showed that ESBL-producing isolates were mostly collected from the patients admitted to ICUs. It seems that prolonged period of ICU stay, exposure to broad spectrum antibiotics, chronic underlying conditions, and the use of invasive techniques and devices predispose patients to infection caused by these resistant isolates.

In the present study, 26.7%, 17.3%, 6.7%, and 4% of ESBL-producing *P. aeruginosa* isolates carried *bla*_{TEM}-1, *bla*_{CTX-M}-15, *bla*_{SHV}-1, and *bla*_{SHV}-12 genes alone or in combination, respectively. In the literature, there have been only rare reports of *bla*_{SHV}-12 and *bla*_{CTX-M}-15-types ESBLs among *P. aeruginosa* worldwide. We believe that this is the first report of *bla*_{CTX-M}-15 and *bla*_{SHV}-12-related ESBL genes among *P. aeruginosa* isolates collected from Qazvin and Tehran hospitals. Shakibaie et al. reported that 6.6%, 4.1%, and 2.5% of ESBL-producing *P. aeruginosa* isolates carried *bla*_{SHV}, *bla*_{PER}, and *bla*_{TEM} family genes, respectively [26]. In another study from Iran, Shahcheraghi et al. reported that 24%, 22%, 17%, and 9% of MDR isolates of *P. aeruginosa* harbored *bla*_{VEB}, *bla*_{SHV}, *bla*_{PER}, and *bla*_{TEM} genes, respectively [36]. In Japan, Uemura et al. showed the presence of *bla*_{SHV}-12 among *P. aeruginosa* isolates collected from burn patients [37]. Polotto et al. reported that the *bla*_{CTX-M}-2 (19.6%) gene was the most prevalent ESBL gene in Brazil [38]. Together, these data indicate successful spread of the ESBL-encoding genes around the world.

Conclusions

Findings of the our study show a high prevalence of ESBL-producing *P. aeruginosa* isolates carrying *bla*_{CTX-M-15} and *bla*_{SHV-12} genes in Iran. The presence of ESBL-producing bacteria within the healthcare setting in Iran should be considered a public health concern both therapeutically and epidemiologically. As such, the identification, treatment, and infection control and management of patients infected with these organisms is of prime necessity.

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The authors declare no conflict of interest

Authors' contributions

AP initiated the study design, project and protocol development, and data analysis; TNF performed the data quality control and manuscript writing; EZ collected data and performed microbiological and molecular experiments; KHA was involved in the editing of the manuscript. All authors read and approved the final draft of the manuscript.

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