ISSN: 2588-4115; Infection Epidemiology and Microbiology. 2022;8(4):285-295 doi 10.52547/iem.8.4.285

High Incidence of MDR and XDR *Pseudomonas aeruginosa* Isolates Harboring bla_{GES}, bla_{VEB}, and bla_{PER} Extended-Spectrum Beta-Lactamase Genes in Iran

ARTICLE INFO

Article Type
Original Article

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How to cite this article
Beiki F., Tavakoli Kareshk A., Askari P, Hashemi A., Yousefi M. High Incidence of MDR and XDR *Pseudomonas aeruginosa* Isolates Harboring bla_{GES}, bla_{VEB}, and bla_{PER} Extended-Spectrum Beta-Lactamase Genes in Iran. Infection Epidemiology and Microbiology. 2022;8(4): 285-295.

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Article History
Received: June 14, 2022
Accepted: November 04, 2022
Published: December 19, 2022

ABSTRACT

Backgrounds: The ever-increasing incidence of multidrug resistance in ESBL-producing *Pseudomonas aeruginosa* is one of the most serious public health threats. This study aimed to investigate the antibiotic resistance profile and molecular characteristics of ESBL-producing *P. aeruginosa* isolates.

Materials & Methods: Antimicrobial susceptibility testing was performed for 120 *P. aeruginosa* clinical isolates using the Kirby-Bauer disk diffusion and broth microdilution assays. Combined disk test (CDT) was applied to screen for ESBL production among *P. aeruginosa* isolates. PCR assays determined the presence of bla_{GES}, bla_{PER}, and bla_{VEB} genes in all isolates.

Findings: The clinical isolates of *P. aeruginosa* showed the highest resistance to cefotaxime (86.7%) and gentamicin (65.8%). Of 120 *P. aeruginosa* isolates, 60.8% were MDR, and 53.3% were XDR. The prevalence of these strains was significantly higher in hospitalized patients than in out-patients (p<.001). Also, 58 *P. aeruginosa* strains (48.3%) were considered as phenotypic ESBL producers. Furthermore, 15, 35, and 24.2% of *P. aeruginosa* isolates harbored bla_{GES}, bla_{VEB}, and bla_{PER}, respectively. The incidence of MDR (71.4% vs. 41.9%, p= .001) and XDR (63.6% vs. 34.9%, p= .002) was significantly higher in ESBL-producing *P. aeruginosa* isolates compared to non-ESBL producers. The highest incidence rate of MDR was reported in bla_{VEB} gene-positive *P. aeruginosa* isolates (95.2%), followed by isolates harboring bla_{PER} (79.3%) and bla_{GES} (55.6%) genes.

Conclusion: This study findings show a high prevalence of MDR ESBL-producing *P. aeruginosa* isolates, indicating the importance of correct identification of these superbugs and judicious use of various antibiotics to prevent their spread.

Keywords: *Pseudomonas aeruginosa*, Drug resistance, Extended spectrum beta-lactamase, Beta-lactamase VEB, Beta-lactamase GES, Beta-lactamase PER.

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[DOI: 10.52547/iem.8.4.285]

Introduction

Pseudomonas aeruginosa is one of the most important infectious agents, especially in hospital wards. This opportunistic pathogen could survive on inanimate surfaces (including medical equipment), which causes the spread of this bacterium in hospital settings. Recently, the prevalence of *P. aeruginosa* infections has increased among hospitalized patients, particularly among those receiving extensive antibiotic treatment with long-term hospital stays [1-3].

In recent years, the widespread use of antibiotics has made bacteria resistant to broad-spectrum antibiotics of various antibiotic groups; thus, there are increasing reports of multiple drug resistant (MDR) strains. The ever-increasing incidence of antibiotic resistance among *P. aeruginosa* strains has become a worldwide health concern and resulted in severe adverse outcomes such as increased in-hospital mortality, cost, and hospital stay [4, 5]. Eradication of *P. aeruginosa* has become increasingly difficult due to its significant antibiotic resistance. For early detection and prevention of antibiotic resistance in *P. aeruginosa* and other pathogenic bacteria, continuous microbiological surveillance should be performed [6, 7].

In recent decades, beta-lactam antibiotics have been attractive in the treatment and prevention of infections caused by Gram-negative bacteria. Therefore, resistance to beta-lactam antibiotics is a serious challenge in the treatment of *P. aeruginosa* infections [8, 9]. The most important mechanism of resistance to beta-lactam antibiotics in Gram-negative bacteria is the production of beta-lactamase enzymes, especially extended-spectrum beta-lactamases (ESBLs) that confer resistance to most beta-lactam antibiotics, including penicillins, cephalosporins, and monobactam. The transmission of beta-lactamase genes between different bacterial strains could be accomplished by highly diverse mobile genetic elements, which play a significant role in their rapid dissemination in hospital settings. ESBL-producing *P. aeruginosa* is one of the critical members of antibiotic-resistant bacteria whose spread in hospital environments could lead to the outbreak of infection and treatment failure [10, 11].

In the last decade, several classes of beta-lactamases (A, B, and D) were identified in *P. aeruginosa* strains. All ESBL-type enzymes belong to Ambler classes A and D. The most commonly encountered ESBLs in *P. aeruginosa* strains belong to the PER, GES, and VEB (class A) as well as OXA (class D) families. Moreover, the presence of ESBLs typical of the Enterobacteriaceae family, such as TEM, SHV, and CTX-M-type, has been described in a few *P. aeruginosa* isolates. GES-1 (Guiana extended-spectrum beta-lactamase) is the first member of the GES family, identified in *Klebsiella pneumoniae*. It exhibits strong activity against most beta-lactams and results in an ESBL phenotype. VEB (Vietnamese extended-spectrum beta-lactamase) and PER (*Pseudomonas* extended resistant) enzymes are frequently found in non-fermenting bacteria such as *P. aeruginosa*, and their abundance is increasing worldwide [12-14].

Objectives: Given the treatment failures of ESBL-producing *P. aeruginosa* infections worldwide and the importance of identifying and treating infections caused by these pathogens with high morbidity and mortality rates, this study aimed to evaluate the antibiotic resistance pattern and molecular characteristics of ESBL-producing *P. aeruginosa* isolates.

Materials and Methods

Study design: This cross-sectional study was conducted on 120 non-duplicate *P. aeruginosa* clinical isolates collected from patients referring to Imam Reza hospital (60 isolates) in Birjand and the burn center

[DOI: 10.52547/iem.8.4.285]

of Shahid Motahari hospital (60 isolates) in Tehran, Iran, from Jan 2018 to May 2019. Clinical samples collected from Imam Reza hospital in Birjand included urine, wound swab, blood, and lung secretion specimens, while those collected from the burn center of Shahid Motahari hospital in Tehran included only wound swab specimens. The Research Ethics Committee of Birjand University of Medical Sciences approved the study (IR.BUMS.REC.1397.198).

Isolation and identification of *P. aeruginosa*: All clinical samples were cultured on MacConkey agar (Merck, Germany) and 5% sheep blood agar (Merck, Germany) culture media, and then standard microbiological methods were employed to identify *P. aeruginosa* isolates (colony appearance, Gram stain, the alkaline reaction on Triple Sugar Iron [TSI] agar, oxidation/fermentation of glucose using OF media, growth at 42°C, motility, and oxidase test). Notably, the phenotypic identification of the isolates was confirmed by PCR and 16S rRNA gene sequencing using specifically targeted primers (Table 1).

Antibiotic susceptibility testing (AST): The antibiotic resistance pattern of the isolates was determined using the Kirby-Bauer disk-diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. The antibiotic disks (MAST, UK) used for susceptibility testing in this study were Amikacin (30 µg), Aztreonam (30 µg), Piperacillin/tazobactam (100/10 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Cefepime (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), and Imipenem (10 µg). In addition, the minimum inhibitory concentration (MIC) for Colistin (Sigma-Aldrich, USA) was determined using the microdilution broth method. *P. aeruginosa* ATCC 27853 was used for quality control of antibiotic susceptibility testing. It should be noted that multidrug-resistant (MDR) and extensively drug-resistant (XDR) *P. aeruginosa* isolates were defined as those that were non-susceptible to at least one agent in three or more antimicrobial classes as well as those that were non-susceptible to at least one agent in all but two or fewer antimicrobial categories, respectively [16].

Phenotypic detection of ESBL production: ESBL production by *P. aeruginosa* isolates was detected using the combined disk test (CDT) method based on CLSI recommendations [15]. Briefly, the CDT phenotypic test was performed by comparing the inhibition zone diameter of disks containing cefotaxime (30 µg) and ceftazidime (30 µg) alone and in combination with clavulanic acid (30 µg/10 µg). An increase of 5 mm or more in the inhibition zone diameter was considered as an ESBL-positive isolate. *Escherichia coli*

Table 1) Target genes and their primers used in this study

Primer	Sequence (5'-3')	Products Sizes (bp)	Annealing (ºC)	Ref.
bla_{GES}	Fw- ATGCGCTTCATTCACGCAC Rv- CTATTTGTCCGTGCTCAGG	864	50	[17]
bla_{VEB}	Fw- CGACTTCCATTTCCCGATGC Rv- GGACTCTGCAACAAATACGC	643	60	[17]
bla_{PER}	Fw- AATTTGGGCTTAGGGCAGAA Rv- ATGAATGTCATTATAAAAGC	925	53	[17]
16srRNA (*P. aeruginosa*)	Fw- GGGGGATCTTCGGACCTCA Rv- TCCTTAGAGTGCCCACCCG	956	58	[18]

[DOI: 10.52547/iem.8.4.285]

ATCC 25922 was used as a negative control strain, and *K. pneumoniae* ATCC 700603 was used as a positive control strain.

Genotypic detection of ESBL genes: In the current study, the presence of bla_{GES}, bla_{VEB}, and bla_{PER} genes responsible for ESBL production was investigated among all *P. aeruginosa* isolates using PCR assays based on specific primers shown in Table 1. DNA extraction was performed from *P. aeruginosa* clinical isolates using the High Pure PCR Template Preparation Kit (Roche, Germany) according to the manufacturer's instructions. PCR amplification was conducted in 25 µl volumes including 12.5 µl 2X Hot Star Taq Master Mix (Amplicon, Denmark), 1 µl forward and reverse primers (10 pmol/µl), 2 µl DNA, and 8.5 µl ddH2O using a thermocycler (PEQLAB, Erlangen, Germany). The reaction conditions were an initial denaturation at 94°C for 5 minutes; followed by 35 amplification cycles including denaturation at 94°C for 45 seconds, annealing at various temperatures for different genes for 45 seconds (Table 1), and extension at 72°C for 45 seconds; and finally an additional extension step of 10 minutes at 72°C. PCR products were electrophoresed on 1% agarose gel containing DNA green viewer (Parstous, Iran).

Statistical analysis: The results related to antibiotic resistance pattern and ESBL production in the isolates were analyzed in SPSS statistical software (Version 21) using Pearson Chi-square and Fisher's exact tests with *p*-values of less than 0.05, which were regarded as statistically significant.

Findings

Patients and bacterial isolates: In the present study, 120 non-repetitive *P. aeruginosa* clinical isolates were collected from two centers of Imam Reza hospital in Birjand and Shahid Motahari hospital in Tehran (60 isolates each). The majority of the isolates were isolated from wounds (69.2%, 83 isolates), followed by lung secretions (19.2%,

Table 2) Resistance pattern of *P. aeruginosa* isolates to different antimicrobial agents

Antimicrobial Agents	Resistance (%)	Intermediate (%)	Susceptible (%)
Piperacillin/tazobactam	67 (55.8)	4 (3.4)	49 (40.8)
Amikacin	68 (56.7)	1 (0.8)	51 (42.5)
Aztreonam	70 (58.3)	8 (6.7)	42 (35)
Ciprofloxacin	64 (53.3)	2 (1.7)	54 (45)
Gentamicin	79 (65.8)	2 (1.7)	39 (32.5)
Ceftazidime	71 (59.2)	1 (0.8)	48 (40)
Cefotaxime	104 (86.7)	2 (1.7)	14 (11.6)
Cefepime	69 (57.5)	2 (1.7)	49 (40.8)
Imipenem	63 (52.5)	1 (0.8)	56 (46.7)
Colistin	0 (0)	0 (0)	120 (100)
MDR Status			
Yes		73 (60.8)	
No		47 (39.2)	
XDR Status			
Yes		64 (53.3)	
No		56 (46.7)	

MDR: Multidrug-resistant, XDR: Extensively drug-resistant

[DOI: 10.52547/iem.8.4.285]

Table 3) Distribution of MDR/XDR *P. aeruginosa* isolates by patient characteristics

Item/Status	*P. aeruginosa* Isolates		*P*-Value	*P. aeruginosa* Isolates		*P*-Value
	MDR (%)	Non-MDR (%)		XDR (%)	Non-XDR (%)	
Hospitalization Status						
In-patients	73 (67)	36 (33)	< .001	64 (58.7)	45 (41.3)	< .001
Out-patients	0 (0)	11 (100)		0 (0)	11 (100)	
Hospital Wards						
Surgery	2 (16.7)	10 (83.3)	< .001	0 (0)	12 (100)	< .001
Burn	67 (83.8)	13 (16.2)		61 (76.2)	19 (23.8)	
ICU	4 (23.5)	13 (76.5)		3 (17.7)	14 (82.3)	

MDR: Multidrug-resistant, XDR: Extensively drug-resistant

23 isolates), urine (10.8%, 13 isolates), and blood (0.8%, one isolate). Out of 120 *P. aeruginosa* isolates, 80 (66.7%) strains were isolated from males, and 40 (33.3%) strains were isolated from females. This study results showed that the mean age of patients was 39.04±16.71 years (ranging from 1 to 74 years), of which 109 (90.3%) cases were hospitalized patients (hospital stay >48 hours at the time of specimen collection), and 11 (9.7%) cases were out-patients.

Antibiotic resistance pattern of *P. aeruginosa*: The antibiotic resistance pattern of *P. aeruginosa* clinical isolates is shown in Table 2. The highest resistance of *P. aeruginosa* isolates was reported to be against cefotaxime (86.7%) and gentamicin (65.8%), while colistin and imipenem were the most effective antimicrobials with 100 and 46.7% susceptibility rates, respectively. The findings revealed that out of 120 *P. aeruginosa* isolates, 60.8% were multidrug-resistant (MDR), and 53.3% were extensively drug-resistant (XDR) (Table 2). Furthermore, the statistical analysis results indicated that MDR (67% vs. 0%, $p< .001$) and XDR (58.7% vs. 0%, $p< .001$) *P. aeruginosa* isolates were more prevalent in hospitalized patients than in out-patients. The prevalence of these MDR (83.8% vs. 23.5% in ICU and 16.7% in the surgical ward, $p< .001$) and XDR (76.2% vs. 17.7% in ICU and 0% in the surgical ward, $p< .001$) isolates was significantly higher among patients admitted to the burn ward compared to other wards (Table 3).

Distribution of phenotypic ESBL-producing *P. aeruginosa* and ESBL genes: Out of a total of 120 *P. aeruginosa* isolates, 48.3% were considered as phenotypic ESBL producers. The results indicated that 15, 35, and 24.2% of *P. aeruginosa* isolates harbored bla_{GES}, bla_{VEB}, and bla_{PER} genes, respectively (Figure 1). PCR amplification products of ESBL genes among *P. aeruginosa* isolates are shown in Figure 2.

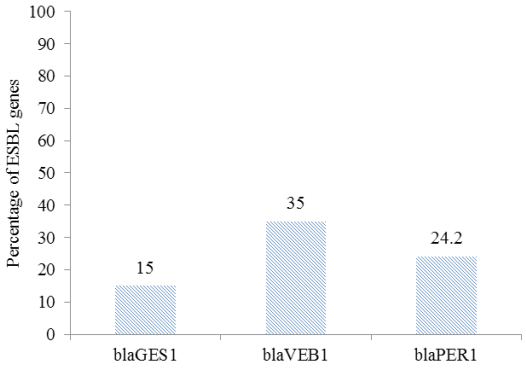

Figure 1) Distribution of ESBL genes among *P. aeruginosa* isolates

Distribution and antibiotic resistance pattern of ESBL-producing *P. aeruginosa*: In this study, out of 77 *P. aeruginosa* iso-

[DOI: 10.52547/iem.8.4.285]

Table 4) Distribution and antibiotic resistance of ESBL-producing *P. aeruginosa* isolates

Item/Status	*P. aeruginosa* Isolates		*P*-Value
	ESBL (%) (n=77)	Non-ESBL (%) (n=43)	
Hospitalization Status			
In-patients	71 (65.1)	38 (34.9)	.349
Out-patients	6 (54.5)	5 (45.5)	
Hospital Wards			
Surgery	8 (66.7)	4 (33.3)	.076
Burn	56 (70)	24 (30)	
ICU	7 (59.5)	10 (40.5)	
Multidrug-Resistant			
Yes	55 (71.4)	18 (41.9)	.001
No	22 (28.6)	25 (58.1)	
Extensively Drug-Resistant			
Yes	49 (63.6)	15 (34.9)	.002
No	28 (36.4)	28 (65.1)	

ESBL: Extended-spectrum beta-lactamase

Table 5) Antibiotic resistance pattern in *P. aeruginosa* isolates harboring different ESBL genes

	P. aeruginosa Harboring ESBL Genes		
	bla_{GES} Positive (%) (n=18)	bla_{VEB} Positive (%) (n=42)	bla_{PER} Positive (%) (n=29)
Multidrug-Resistant			
Yes	10 (55.6)	40 (95.2)	23 (79.3)
No	8 (44.4)	2 (4.8)	6 (20.7)
Extensively Drug-Resistant			
Yes	9 (50)	36 (85.7)	20 (69)
No	9 (50)	6 (14.3)	29 (31)

lates that were phenotypically and/or genotypically ESBL-producers, 71.4 and 63.6% were MDR and XDR, respectively. The incidence of MDR (71.4% vs. 41.9%, *p*= .001) and XDR (63.6% vs. 34.9%, *p*= .002) was significantly higher among ESBL-producing *P. aeruginosa* isolates compared to non-ESBL producers. Furthermore, although the prevalence of ESBL-producing *P. aeruginosa* isolates was higher in hospitalized patients than in out-patients (65.1% vs. 54.5%, *p*= .349), especially among patients admitted to the burn ward compared to other wards (70% vs. 59.5% in ICU and 66.7% in the surgical ward, *p*= .076), this difference was not significant (Table 4).

The highest incidence rate of MDR was reported in bla_{VEB} gene-positive *P. aeruginosa*

[DOI: 10.52547/iem.8.4.285]

isolates (95.2%), followed by isolates harboring bla_{PER} (79.3%) and bla_{GES} (55.6%) genes. Furthermore, the results indicated that 50, 85.7, and 69% of *P. aeruginosa* isolates carrying bla_{GES}, bla_{VEB}, and bla_{PER} genes were XDR, respectively (Table 5).

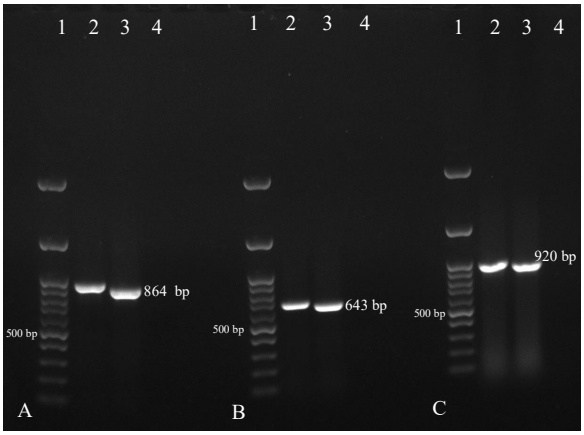

Figure 2) PCR amplification products of ESBL genes in *P. aeruginosa* isolates. **(A)** PCR amplification of bla_{GES} gene: Lane 1: DNA marker (100 bp), Lane 2 and 3: clinical isolates harboring bla_{GES} gene (864 bp), and Lane 4: negative control. **(B)** PCR amplification of bla_{VEB} gene: Lane 1: DNA marker (100 bp), Lane 2 and 3: clinical isolates harboring bla_{VEB} gene (643 bp), and Lane 4: negative control. **(C)** PCR amplification of bla_{PER} gene: Lane 1: DNA marker (100 bp), Lane 2 and 3: clinical isolates harboring bla_{PER} gene (920 bp), and Lane 4: negative control

Discussion

P. aeruginosa is one of the most important causes of nosocomial infections, and severe conditions caused by this pathogen are often life-threatening. The incidence of antibiotic resistance in *P. aeruginosa* strains is increasing, and identifying effective therapeutic options for managing MDR *P. aeruginosa* infections is still a challenge for physicians [4, 5, 13]. In this study, the highest resistance of 120 *P. aeruginosa* isolates was reported to be against cefotaxime (86.7%) and gentamicin (65.8%), while colistin and imipenem were the most effective antimicrobials with 100 and 46.7% susceptibility rates, respectively. Similar antimicrobial resistance patterns have been reported for *P. aeruginosa* in previous studies [19-21]. The ever-increasing incidence of antibiotic resistance in *P. aeruginosa* isolates has become a global health concern, especially in developing countries, which could be due to the relatively high level of over-the-counter antibiotic sales and frequent self-medication with antibiotics [19, 22].

The findings indicated that the prevalence rate of MDR and XDR *P. aeruginosa* isolates was 60.8 and 53.3%, respectively. Several studies have reported similar prevalence rates for MDR and XDR *P. aeruginosa* isolates [23-25]; however, a few studies have reported lower frequencies for MDR and XDR *P. aeruginosa* isolates [26-28]. This could be explained by differences in study populations, clinical sample type, geographic location, hospitalization status, hospital wards, and history of antibiotic use. Furthermore, the statistical analysis results indicated that MDR (67% vs. 0%, $p< .001$) and XDR (58.7% vs. 0%, $p< .001$) *P. aeruginosa* isolates were significantly more prevalent in hospitalized patients than in out-patients. The prevalence of these MDR/XDR isolates was considerably higher among patients admitted to the burn ward compared to other wards. In line with the present study findings, Mahto et al. (2021) also indicated that *P. aeruginosa* isolates obtained from inpatients were significantly more likely to be MDR/XDR than those obtained from out-patients [29]. In another study, the statistical analysis results showed that the incidence rate of MDR/XDR in uropathogenic bacteria (including *P. aeruginos*), obtained from inpatients was significantly higher than in those obtained from out-patients [30]. In a study by Hashemi et al. (2017), resistance to most antibiotics was higher in *P. aeruginosa* isolates isolated from hospitalized patients compared to those isolated from out-patients [31]. In addition, Farajzadeh Sheikh et al. (2020) found that the prev-

[DOI: 10.52547/iem.8.4.285]

alence of MDR *P. aeruginosa* isolates was higher among patients admitted to the burn ward compared to other wards [32]. It is noteworthy that the high prevalence of MDR and XDR *P. aeruginosa* isolates in hospitalized patients, especially in burn patients, is not unexpected due to long-term hospital stays and the use of various antibiotics to treat these patients.

The emergence and distribution of ESBL-producing *P. aeruginosa* strains represent a significant public health threat worldwide. ESBL-producing *P. aeruginosa* infections could lead to severe adverse clinical outcomes. Efforts to control the outbreak of these infections should emphasize prevention and management strategies and the judicious use of antimicrobial agents to reduce the spread of these resistant pathogens [10, 33]. In the current study, 48.3% of *P. aeruginosa* isolates were identified as phenotypic ESBL producers. In line with the present study, significant outbreaks of ESBL-producing *P. aeruginosa* infections have been reported in Saudi Arabia [13], Iraq [25], India [26, 34], Africa [33], Sudan [35], and Nigeria [36]. Furthermore, 15, 35, and 24.2% of *P. aeruginosa* isolates harbored bla_{GES}, bla_{VEB}, and bla_{PER}, respectively. Several studies have reported similar results regarding the distribution of ESBL genes in *P. aeruginosa* isolates with the predominance of the bla_{VEB} gene [13, 25, 37, 38]. In this study, the prevalence of MDR (71.4% vs. 41.9%, p= .001) and XDR (63.6% vs. 34.9%, p= .002) was significantly higher in ESBL-producing *P. aeruginosa* isolates compared to non-ESBL producers. Furthermore, the prevalence of ESBL-producing *P. aeruginosa* isolates was higher among hospitalized patients compared to out-patients as well as among patients admitted to the burn ward compared to other wards. Consistent with the present study results, Alikhani et al. (2014) also indicated that resistance to many antibiotics was higher in ESBL-positive *P. aeruginosa* isolates than in ESBL-negative isolates [39]. Another study found a relatively high prevalence of MDR and ESBL production among *P. aeruginosa* isolates [40]. In a study by Shaikh et al. (2015), ESBL-producing *P. aeruginosa* isolates showed concomitant resistance to an array of antimicrobial agents [41]. In a previous study, the prevalence of ESBL-producing Gram-negative isolates, including *P. aeruginosa,* was significantly higher in hospitalized patients (21.2%) than in out-patients (15.3%) [42]. In another study, the highest prevalence of ESBL genes was observed in *P. aeruginosa* isolates collected from the burn ward, but no statistically significant relationship was observed in this regard [38]. The high frequency of MDR/XDR in ESBL-producing *P. aeruginosa* isolates indicates that these pathogens exhibit co-resistance to many antibiotic classes, which could lead to limited treatment options. Therefore, ESBL-related infections are increasing worldwide, and it is necessary to identify and control them in hospital settings. Finally, in this study, the highest incidence rate of MDR/XDR was reported in bla_{VEB} gene-positive *P. aeruginosa* isolates, followed by isolates harboring bla_{PER} and bla_{GES} genes. In a similar study, Shahcheraghi and colleagues (2009) showed that *P. aeruginosa* isolates containing bla_{VEB} were resistant to almost all antibiotics (except imipenem) compared to isolates positive for other ESBL genes [37]. In another study, resistance to most beta-lactam antibiotics was higher in bla_{VEB} gene-positive *P. aeruginosa* isolates than in isolates harboring bla_{PER} and bla_{GES} genes [38]. Moreover, Nasser et al. (2020) showed a high frequency of bla_{VEB} and bla_{GES} genes in MDR *P. aeruginosa* isolates compared to other ESBL genes [43]. The higher incidence of MDR in *P. aeruginosa* isolates harboring the bla_{VEB} gene may be explained by the fact that this gene is carried on plasmids containing other resistant genes. Overall, accurate tracking and reporting of ESBL-producing *P. aeruginosa* iso-

[DOI: 10.52547/iem.8.4.285]

lates would help select appropriate treatment options and prevent further spread of these superbug pathogens.

Conclusion

The present study findings show a high prevalence of MDR ESBL-producing *P. aeruginosa* isolates, indicating the importance of correct identification of these superbugs and judicious use of various antibiotics to prevent their spread. The results also show that colistin and imipenem are still effective antibiotics for the treatment of *P. aeruginosa* infection. The high prevalence of these resistant bugs reflects the need for more infection control surveillance and the establishment of effective antimicrobial stewardship programs in hospital settings.

Acknowledgments

The authors would like to express their gratitude and thanks to the staff of microbiology laboratories of Imam Reza hospital in Birjand and the Shahid Motahari hospital in Tehran.

Ethical permissions: The Research Ethics Committee at Birjand University of Medical Sciences approved the study (IR.BUMS.REC.1397.198).

Conflicts of interests: The authors declare that they have no competing interests.

Author's contributions: FB and PK collected the data. ATK and AH collected the data, drafted the manuscript, and edited the paper. MY designed the study, collected the data, and revised the manuscript. All authors read and approved the final manuscript.

Fundings: This research was supported by Birjand University of Medical Sciences, Birjand, Iran (Grant Number 455695).

Consent to participate: Written informed consents were obtained from all patients.

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[DOI: 10.52547/iem.8.4.285]