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Molecular Detection of bla_{VEB-1} Beta-Lactamase Encoding Gene Among Extended Spectrum B-Lactamase Positive Wound Isolates of *Pseudomonas aeruginosa*

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Abstract

Background: *Pseudomonas aeruginosa* is considered as a leading cause of nosocomial infections. Burn and wound infections are mainly caused by multidrug-resistant *P. aeruginosa* isolates. Drug resistance frequently occurs among nosocomial isolates and can usually resist a myriad of antibiotics such as novel β-lactam antibiotics. Detection of multidrug-resistant isolates could assist better drug administration.
Objectives: The aim of this study was to detect Extended Spectrum Beta-Lactamases (ESBL) positive wound isolates and the genes encoding bla_{VEB-1} ESBL among wound isolates of *P. aeruginosa*.
Materials and Methods: A total of 89 wound isolates of *P. aeruginosa* were collected from patients (47% (n = 42) were male and 53% (n = 47) were female) at six Iranian hospitals between years 2009 and 2011. Antibiotic susceptibility and phenotypic ESBL production tests were conducted. The combined disk was used to determine ESBLs production. The bla_{VEB-1} gene was detected with the polymerase chain reaction (PCR).
Results: The majority of the wound isolates were resistant to augmentin (90%, n = 80) and cefpodoxime (87.6%, n = 78). However, the majority was susceptible to imipenem and meropenem. Fifty-eight (42%) wound isolates were ESBL positive. The antibiotic resistance amongst ESBL positive isolates was relatively higher than ESBL negative isolates. Twenty-three (40%) ESBL-positive isolates amplified the bla_{VEB-1} gene.
Conclusions: More than behalf of the wound isolates were ESBL positive, and the presence of bla_{VEB-1} was determined in less than half of these isolates. Fortunately, resistance to imipenem and meropenem was low.

Keywords: Extended Spectrum Beta Lactamases, Wound Samples, bla_{VEB-1}, *Pseudomonase aeruginosa*

1. Background

Pseudomonas aeruginosa (*P. aeruginosa*) isolates are known as potential opportunistic organisms, frequently involved in infections of immune suppressed or hospitalized patients, and also cause outbreaks of hospital-acquired infections. These strains are inherently resistant to an extended spectrum of antibiotics, including novel β-lactam agents, and thereby can culminate in high morbidity and mortality rates (1). Useful antibiotics include extended spectrum beta-lactamases (ESBL) and carbapenems, though multidrug-resistant isolates have emerged in hospital settings (2). Of several primary antibiotic resistant mechanisms, the down-regulation of membrane porins (OprD), in addition to increase in the expression of multidrug efflux pumps (MexAB-OprM) help intrinsic drug resistance (3). Novel beta-lactamases, including AmpC, extended spectrum beta-lactamases (ESBLs) and likewise several metallo beta-lactamases (MBLs) have emerged around the world as genetic encoding reservoirs responsible for the antimicrobial resistance among different gram-negative isolates (4). The ESBL enzymes are encoded by plasmids and integrons and were first reported in isolates of *Klebsiella pneumonia*, in Germany (5). In *P. aeruginosa* several classes of enzymes including class A ESBLs, which are comprised of bla_{PER-1} and bla_{VEB-1}, and GES/IBC and BEL types have been identified; these were initially reported in Turkey, south Asia and France. These six types of ESBLs at the genetic level have a low similarity, but they are identical regarding hydrolysis profiles

(6). The ESBL enzymes have been demonstrated to be the derivatives of TEM- and SHV-lactamases, with minor genetic mutation in the active site (7). These enzymes have shown resistance to extended spectrum cephalosporins. Furthermore, there have reports of non-TEM and non-SHV ESBLs in several areas (8, 9). Among several acquired beta-lactamase enzymes, the bla_{VEB-1} has the greatest clinical importance as it causes resistance to oxyimino beta-lactams (10). On the other hand, resistance to carbapenems in *P. aeruginosa*, alongside *K. pneumoniae* and *Acinetobacter baumannii*, is of high concern (11). Implication for health policy/practice/research/medical education: *P. aeruginosa* is a nosocomial pathogen and likewise the increasing rate of antibiotic resistance has become a great concern. Among several mechanisms of drug resistance, there are ESBL enzymes that confer the resistance to a broad range of antibiotics in beta lactam family. Detection of ESBLs and their importance and prevalence can help for better follow up of this pathogen.

2. Objectives

The aim of this study was to detect the production of ESBLs and prevalence of bla_{VEB-1} gene among wound isolates of *P. aeruginosa*.

3. Materials and Methods

A total of 89 clinical isolates of *P. aeruginosa* were collected from wound samples in several hospitals between years 2009 and 2011. The isolates were identified by gram staining, catalase and oxidase tests, motility on Sulfide indole motility (SIM) medium, indole production, H_2S production, characteristics on the triple sugar iron (TSI) agar culture medium, methyl red (MR) test, voges proskauer (VP) medium, Simon citrate agar, oxidative/fermentative (OF) test, urea broth, growth on MacConkey agar and also on cetrimide agar media. The isolates were then subsequently stored at -70°C for future studies.

The antibiotic susceptibility test of the isolates was performed on the basis of Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibiotic disks used in the present study have been depicted in Table 1.

Production of ESBLs by *P. aeruginosa* isolates was determined using the usual combined disk test. In the combined disk test, both ceftazidime and cefotaxime disks in the presence or absence of clavulanic acid were used on the muller hinton agar (MHA) culture Plates. A positive test was indicated when the difference in the diameter in the absence of clavulanic acid was equal or more than 5 mm when compared to the diameter in the presence of clavulanic acid.

Following the suspension of a colony of each bacterial isolate in 10 mL of Luria Bertani (LB) broth medium, and then incubation overnight at 37°C, the tubes were centrifuged for 10 minutes at 4000 rpm and the obtained precipitate was re-suspended in sterile H_2O for DNA extraction. Furthermore, for DNA isolation, the boiling and DNA Extraction kit (DIAtom DNA Prep 100) methods were used.

The PCR was performed for the detection of the ESBL encoding gene of bla_{VEB-1} by the employment of specific primers, as shown in Table 2.

The reaction mixture for these genes included: 10X PCR buffer = 2.5 µL, dNTP (10 Mm) = 0.75 µL, $MgCl_2$ (50 mM) = 1.5 µL, forward primer (100 µM) = 2.5 µL, reverse primer (100 µM) = 2.5 µL, template (DNA) = 1 µL, Taq DNA polymerase (5 U/µL) = 0.2 µL, and nuclease-free H_2O = 14.05 µL.

3.1. Statistical Analysis

The analysis of data was performed with application of the Student's t-test.

Table 1. Three Families of Antibiotics Used in This Study

Antibiotic Family	Disks and Concentrations
Beta-lactams, µg	Aztreonam (30), piperacillin (100), carbenicillin (100), meropenem (10), netilmicin (30), ticarcillin (75), piperacillin-tazobactam (110), cefoperazone (75), augmentin (30), cefotaxime (30), imipenem (10), cefpodoxime (10), ceftriaxone (30), ceftazidime (30) and cefepime (30)
Fluoroquinolones, µg	Ofloxacin (5), ciprofloxacin (5), levofloxacin (5)
Aminoglycosides, µg	Amikacin (30), tobramycin (10), gentamicin (120)

Table 2. The Primers Used in This Study

Primer	Sequence 5 to 3	Product Size	Reference
bla_{VEB-1}		699	(12)
	F: AATGGCAATCAGCGCTTC		
	R: GCGCGACTGTGATGTATA		

4. Results

The wound isolates of *P. aeruginosa* were collected from six hospitals of Tehran (Shariati, burn center), Shiraz (Namazi), Ilam (Imam Khomeini), Kerman (Bahonar), Kermanshah (Imam Khomeini), and Ahvaz (Imam) city. These isolates were identified with conventional biochemical tests.

The majority of the wound isolates were susceptible to imipenem and meropenem antibiotics. However, most were resistant to augmentin disk and cefpodoxime. The difference between ESBL- and non-ESBL-producing isolates regarding resistance has been classified in Table 3.

Fifty-eight (56.1%) of the wound *P. aeruginosa* isolates were ESBL positive, among which 40% (n = 26) were isolated from males and 60% (n = 32) from females. The antibiotic resistance pattern was higher in these isolates relative to ESBL-negative isolates, although no significant difference was observed ($P \leq 0.05$), as shown in Table 3. The prevalence of ESBLs in each hospital were as follows; Tehran (n = 11), Shiraz (n = 6), Ilam (n = 8), Kerman (n = 11), Kermanshah (n = 8) and Ahvaz (n = 6).

Table 3. The Antibiotic Susceptibility Test Pattern for the Extended Spectrum B-Lactamases Positive and Negative Wound *Pseudomonas aeruginosa* Isolates

Disks/Isolates	ESBL-Positive (Resistance %), n = 35	ESBL-Negative (Resistance %), n = 54
Augmentin	100	92
Cefepime	96	68
Ceftazidime	89	66
Cefpodoxime	86	54
Carbenicillin	87	63
Ceftriaxone	99	63
Piperacillin	88	46
Aztreonam	86	62
Cefoperazone	89	46
Cefotaxime	98	67
Ticarcillin	68	56
Imipenem	22	17
Meropenem	23	18
Ciprofloxacin	89	65
Levofloxacin	78	44
Ofloxacin	76	55
Netilmicin	63	45
Amikacin	82	42
Gentamicin	68	43
Tobramycin	68	46
Piperacillin	56	37

Table 4. Association Between Third-Generation Cephalosporins Resistance and Extended Spectrum Beta-Lactamase Genotypes[a]

Third Generation Cephalosporins Resistant Isolates	bla_{VEB-1}, %
CTX	80
CAZ	89.3
CPM	78
CRO	78.4
CTX, CAZ, CRO, CPM	97.6

[a]Abbreviations: CTX: cefotaxime, CAZ: ceftazidime, CPM: cefpodoxime, CRO: ceftriaxone.

The prevalence of bla_{VEB-1} in each hospital was as follows; Tehran (n = 10), Shiraz (n = 2), Ilam (n = 8), Kerman (n = 3), Kermanshah (n = 5) and Ahvaz (n = 4). Isolates that contained bla_{VEB-1} gene also showed higher resistance to third generation antibiotics. As described previously, several ESBL positive isolates in some hospitals did not amplify the bla_{VEB-1} gene. Table 4 shows the association between third-generation cephalosporins resistance and extended spectrum beta-lactamase genotypes.

5. Discussion

Drug resistance is increasingly developing amongst nosocomial pathogens (13, 14). Approximately 0.3% of *P. aeruginosa* genes encode agents for antibiotic resistance (15). The ESBL-positive *P. aeruginosa* strains are resistant to the extended-spectrum cephalosporins with several estimated mechanisms (16). In this study, more than 45% of the ESBL positive wound isolates contained the bla_{VEB-1} gene, suggesting that several other mechanisms can also interfere in decreased resistance to third generation cepgalosporins; such as reducing the levels of antibiotics accumulated in bacteria or increasing the expression of efflux pumps that are important in gram negative strains. In the present study, the majority of the wound *P. aeruginosa* isolates were resistant to disks of cefpodoxime and augmentin/co-amoxi clav. We observed that most of our wound isolates were sensitive to imipenem and meropenem. Moreover, about 40% of the isolates were resistant to cefpodoxime, aztreonam, ceftriaxone and cefotaxime. The combined disk is routinely used for detection of phenotypic positive ESBLs with use a third generation cephalosporin with or without inhibitory clavulanate (17). However, resistance to the inhibitor indicates the possible presence of AmpC or other consistent enzymes (18). Several previous studies that aimed to detect ESBLs have demonstrated a high level of resistance among *P. aeruginosa* isolates to antibiotics (19). In this study, more than half of the wound ESBL positive isolates could amplify bla_{VEB-1} (47%). As mentioned above, the antibiotic resistance pattern was considerably at a higher level in ESBL positive isolates (not significant). Interestingly, the bla_{VEB-1} was detected in isolates that were resistant to all

the used third generation cephalosporins. There are limited results regarding the prevalence of the bla_{VEB-1} gene in Middle Eastern countries. Amongst the results from our country, 24% of ESBL positive isolates in the study of Shacheraghi et al. contained this gene (20). Furthermore, in Tehran, Mirsalehian demonstrated that 49.25% and 31.34% of ESBL positive isolates collected from burn patients amplified bla_{PER-1} and bla_{VEB-1} genes, respectively (21). However, in Korea, none of the *P. aeruginosa* isolates could amplify the bla_{VEB-1} gene (10). Although we detected this gene at a low prevalence, because of its plasmid borne nature, there is a possibility of rapid transmission amongst gram-negative bacteria. Fortunately, carbapenem resistance was not high, as found by the study of Mirsalehian (21). However, another study exhibited that 95% of ESBL positive isolates of *P. aeruginosa* were resistant to imipenem and meropenem; such findings may be warning of a crisis, as these drugs are the best choices for ESBL positive isolates. In an Iranian study, conducted in the Semnan province during 2010, 88% of gram negative isolates harbored ESBLs (22). However, Aminzadeh in a study conducted in Tehran during 2011, determined that 13.7% of enteric pathogens (a total of 292 species) were ESBL positive (23). Khosravi in 2012 investigated several isolates of *Klebsiella pneumonia* and found that 47.27% were ESBL positive containing TEM-1 (34.61%), SHV-1 (46.15%) and CTX-M-1 (26.92%) genes (24). In the research of Fazeli, 71% of *K. pneumonia* isolates were ESBL positive (25). In the study of Kapur from India, 61% of urinary tract pathogens were ESBL positive (26). For resistant isolates combination therapy (usually including a class of β-lactam and an aminoglycoside) is recommended that would contribute to the curing of pseudomonal infections (27). Less than half of our wound isolates of *P. aeruginosa* produced ESBLs among which an approximate half could amplify the bla_{VEB-1} gene. These isolates showed a higher drug resistance compared to ESBL negative strains. On the other hand, the resistance to carbapenems was low.

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Footnote

Authors' Contributions: Elham Davodian designed the experiments, Nourkhoda Sadeghifard advised the study, Abdolmajid Ghasemian helped with the data analysis and writing of the manuscript, and Samileh Noorbakhsh helped with the study in the laboratory process.

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