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Research Article

MOLECULAR IDENTIFICATION OF ESBL GENES *BLA-$_{SHV}$*, *BLA-$_{VEB}$* AND *BLA-$_{PER}$*, IN *ACINETOBACTER BAUMANNII* STRAINS ISOLATED FROM PATIENTS ADMITTED TO A UNIVERSITY HOSPITAL IN ISFAHAN, IRAN

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ABSTRACT
Extended spectrum beta lactamase (ESBL) associated resistance among Acinetobacter species is now known. One hundred and twenty one (121) isolates of A. *baumannii* were collected from a university hospital in Isfahan, Iran. Over an eight-month period were included in our study. The samples were from different wards and of different specimens. Antimicrobial susceptibility was conducted by testing resistance against, eleven antibiotics using the disk diffusion method. The double disk synergy test (DDST) was used to determine the ESBL production. The detection of *bla*-$_{PER}$, *bla*-$_{VEB}$ and *bla*-$_{SHV}$ in ESBL production isolates of A. *baumannii* was carried out by PCR. DNA sequencing for 15 strains was performed in order to identify the detected bla genes. All of the isolates showed high resistance to different groups of antibiotics; the most effective antibiotic was ampicillin-sulbactam with a resistance rate of 33.9%. 62.8% of isolates were XDR and 100% were MDR. Among all samples, the *bla*-$_{SHV}$ was gene detected in 16%, *bla*-$_{VEB}$ in 26.6% and *bla*-$_{PER}$ in 36.8% of isolates. Followed by sequencing, three out of five isolates were identified as *bla*-$_{SHV-12}$ and two out of five as *bla*-$_{SHV-56}$. Furthermore, *bla*-$_{VEB-1}$ and *bla*-$_{PER-1}$ were identified in this study. This study shows relatively high levels (49.5%) of ESBL have been detected in Acinetobacter. large dissemination of the gene *bla*-$_{PER}$ among A. *baumannii* in Alzahra Hospital in Isfahan. ESBL production in Acinetobacter should be promptly detected and reported as it helps in treating individual cases and in controlling the spread of these resistant phenotypes to other individuals.

Keywords: *Acinetobacter Baumannii*, Extended Spectrum Beta-lactamase (ESBL), *bla*-$_{SHV}$, *bla*-$_{VEB}$ and *bla*-$_{PER}$

INTRODUCTION
A. *baumannii* is a gram-negative, pleomorphic, aerobic, nonmotile, catalase-positive and oxidase negative bacillus that exists in the natural environment (water and soil) and is typically isolated from hospital settings, respiratory secretions, wounds and the urine of patients (Doughari *et al.,* 2011). This organism is responsible for 10.2% of infections caused by gram-negative bacteria in America and Europe (Maragakis and Perl, 2008).
Acinetobacter baumannii (hereafter A. *baumannii*) is normally associated with serious nosocomial infections. The emergence of multidrug resistant A. *baumannii* strains, especially those resistant to carbapenems, has created strict therapeutic problems. Many kinds of β-lactamases demonstrating resistance to expanded-spectrum cephalosporins have been detected in Acinetobacter spp (Rodríguez-Martínez *et al.,* 2010).
ESBLs are a very important cause of multidrug resistance in gram-negative bacteria all over the world. These bacteria disperse rapidly and have become a serious threat for human well-being worldwide (Bali *et al.,* 2010). ESBLs are able to hydrolyse three and four generation cephalosporins and monobactams. The species that produce ESBL are inhibited by β_lactamase inhibitors (clavulanic acid, tazobactam and sulbactam) (Bradford, 2001).
In Acinetobacter associated nosocomial infections, the major problem encountered by ICU clinicians relates to the promptly transferable antimicrobial resistance expressed by these organisms. In addition to

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intrinsic resistance, A. *baumannii* has the ability to acquire resistance to many major classes of antibiotics, including recent β–lactams (AL-Thahab, 2013).

Typically, ESBLs are plasmid encoded, but also present on chromosomes, often in association with integrons. These enzymes are derivatives, predominantly of class A and D β-lactamases. Classical ESBLs evolved from class A, TEM (from TEM-1 or TEM-2) and SHV (from SHV-1) enzymes, and these remain the most prevalent types of ESBLs, though class D ESBLs (OXA family) have also been known for some time (AL-Thahab, 2013).

Production of ESBL genes is one of the important mechanisms of becoming resistant against β-lactam antibiotics in gram negative bacteria (Eraç *et al.,* 2013). PER-1, which is one of these enzymes, was first identified in a *Pseudomonas aeruginosa* strain isolated from a urine sample from a Turkish patient, and is known to be widespread in Turkey. PER-1 producing strains have been reported at various centres in Europe as well as in Asian countries. So far, PER-1 has been mainly identified in P. *aeruginosa* and A. *baumannii* complex isolates, but it has also been detected in various gram-negative species. ESBL-producing isolates coded for $bla\text{-}_{SHV}$ have been reported from various countries in Europe, such as Austria, France, Italy and Greece, as well as in the United States and Australia. Among the $bla\text{-}_{SHV}$ type of ß-lactamases, $bla\text{-}_{SHV5}$ was found to be responsible for outbreaks of nosocomial infection in several countries (Jemima and Susan, 2008).

Hence, the fundamental aim of this study is to identify the occurrence of ESBL genes in A. *baumannii* isolates recovered from a university hospital setting in Iran.

MATERIALS AND METHODS

The study material consisted of 121 non-duplicated clinical samples of A. *baumannii* collected during a period of eight months from patients being treated in a university hospital in Isfahan. Samples were cultured from the trachea (36%), urine (15%), blood (10%), wound (10%), cerebrospinal fluid (7%), abscess (5%), bronchial fluid (3%), pleural fluid (4%), sputum (2%) and other samples (8%). Isolates were collected from different wards, mostly from the ICU (50%), surgery ward (13%) and paediatric ward (12%).

Identification of the species was carried out using the biochemical and sugar utilization tests as described by Bouvet and Grimont (Bouvet and Grimont, 1987). Isolates were further identified by gram stain, acidity or alkalinity in triple sugar iron (TSI) agar slants, motility, catalase and oxidase tests, haemolytic patterns on blood agar, growth on citrate agar slants and ability to grow at 44°C (Yum *et al.,* 2002).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed for 11 different therapeutically relevant antibiotics by the Kirby–Bauer disc diffusion method according to Clinical Laboratory Standards Institute guidelines ("Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing, Twenty-second Informational Supplement. CLSI document M100-S22. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2012). Antibiotics tested included cefotaxime (30µg), ceftriaxone (30µg), ampicillin-sulbactam (10/10µg), cefepime (30µg), meropenem (10µg), tobramycin (10µg), amikacin (10µg), tetracycline (30µg), ciprofloxacin (5µg), trimethoprim- sulfamethoxazole (1.25/23.75 µg) and aztreonam (30µg). Escherichia coli ATCC 25922 was included as a negative control and Klebsiella pneumonia ATCC 700603 was included as a positive control (Tripathi and Gajbhiye, 2013). To analyse susceptibility rates in different wards and different age categories, we used the WHONET 5.6 software.

Detection of ESBL Production by Double Disc Synergy Test

The double disk synergy test (DDST) was used to determine ESBL production. Four different cephalosporin disks - ceftazidime, cefotaxime, ceftriaxone, cefepime (30 mg each) were placed around the amoxicillin-clavulanate disk (Mast, UK), at a centre-to-centre distance of 15 mm from the central disk. E. coli ATCC 25922 was used as the negative control and an in-house ESBL producing Acinetobacter strain was used as the positive control. When there was no enhancement between any of the cephalosporins and the clavulanate-containing disks, that isolate was considered as an ESBL negative.

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When there was any enhancement between any of the four cephalosporins and the clavulanate-containing disks, that isolate was considered as an ESBL producer (Sinha *et al.,* 2007).

DNA Extraction

The complete DNAs of the bacterial isolates were extracted by the DNA extraction kit (Bioneer Company, Korea, Cat. number K-3032-2).

Detection of *bla-$_{SHV}$*, *bla-$_{VEB}$* and *bla-$_{PER}$*:

The detection of *bla-$_{SHV}$*, *bla-$_{VEB}$* and *bla-$_{PER}$* in clinical isolates of A. *baumannii* was carried out by PCR with primers illustrated in table 1. For the confirmation of the identity of the isolates, the *bla$_{-OXA-51}$* gene was also detected by PCR test. The PCR conditions were as follows: initial denaturation at 95°C for five minutes followed by 35 cycles of 94°C for 30 seconds, (53°C for *bla$_{-OXA-51}$*, 54 °C for *bla-$_{SHV}$*, 58°C for *bla-$_{VEB}$* and 56°C for *bla-$_{PER}$*) and 72°C for 45 seconds and then 72°C for 10 minutes. Reactions were performed with 2µl DNA template and PCR amplification was performed in a total volume of 25 µl. PCR products were analysed by electrophoresis in 1.2% agarose gel in a TAE buffer at 90 volts along with a DNA ladder. The gel was finally visualized in a gel documentation system (Farajnia *et al.,* 2013a).

Table 1: Primers used for amplification of ESBLs genes

Primer	Sequence	Product Size (bp)	Annealing TM	Reference
oxa$_{-51-like}$F	TAATGCTTTGATCGGCCTTG	353	53	(Jane *et al.,* 2006)
oxa$_{-51-like}$R	TGGATTGCACTTCATCTTGG			
bla$_{SHV}$F	ATGCGTTATATTCGCCTGTG	753	54	(Kristine *et al.,* 2006)
bla$_{SHV}$-R	TGCTTTGTTATTCGGGCCAA			
bla$_{VEB}$ -F	CGACTTCCATTTCCCGATGC	643	58	(Koo, 2010)
bla$_{VEB}$ -R	GGACTCTGCAACAAATACGC			
bla$_{PER}$ -F	AAAGAGCAAATTGAATCCATAGTC	835	56	This study
bla$_{PER}$ -R	GTTAATTTGGGCTTAGGGCAG			

Sequencing of the PCR Products

DNA sequencing for 15 strains was performed in order to identify the detected *bla* genes. The PCR products of the above genes were further purified with PCR purification kits (Fermentas) and subjected to direct sequencing performed by the Macrogen Company (Seoul, Korea), as described previously (Shacheraghi, Shakibaie, & Noveiri, 2010). The nucleotide sequences were analysed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST), CROMASPRO-2 and MEGA-4 software.

RESULTS AND DISCUSSION

Results

One hundred and twenty one clinical isolates of A. *baumannii* were identified with biochemical methods. These isolates were confirmed by PCR for the *bla$_{-OXA-51-like}$* gene. Analysis for the presence of the *bla$_{-OXA-51-like}$* gene showed that all isolates were positive for the *bla$_{-OXA-51-like}$* gene, which confirmed they are A. *baumannii*. Among these isolates, 44% was female and 56% was male. Acinetobacter infections were more common in patients aged over 40 years. Most of these patients had respiratory problems, such as bronchial asthma and respiratory failure. 44 Acinetobacter (36%) isolates were obtained from tracheal samples and 18 of these bacteria (15%) were obtained from blood (table 2). 61 out of 121 isolates (50%) were from patients admitted to the intensive care units (ICU) of the hospital. 13% and 12% of specimens were from the surgery and paediatric wards, respectively. Acinetobacter have a great capacity to develop antibiotic resistance extremely rapidly in response to being challenged with new antibiotics. The resistance rate between antibiotics was high: ceftriaxone (100%), cefotaxime (100%), ciprofloxacin (100%), aztreonam (100%), cefepime (99.2%), meropenem (100%), tobramycin (86.8%), amikacin (87.6%), tetracycline (92.6%), trimethoprim-sulfamethoxazole (99.2%) and ampicillin-sulbactam (33.9%). All isolates (100%) were resistant in at least one agent in ≥3 antimicrobial categories, which

means they were MDR, and 62.8% were XDR, which means them are resistant in all classes of antibiotics except one or two groups. The maximum sensitivity of Acinetobacter was observed to ampicillin-sulbactam (31.4%). This was 12.5% resistance for paediatrics and 37.1% for adults. In addition, there was no resistance to ampicillin-sulbactam in paediatric blood specimens. However, in 33.3% of blood samples of adults, resistance to ampicillin-sulbactam was observed.

Table 2: Number and percent of Acinetobacter Species isolates from different clinical specimens and different wards

Clinical samples	No.	%	Wards	No.	%
Tracheal	44	36%	ICU	61	50%
Urine	18	15%	Surgery	16	13%
Blood	12	10%	Paediatric	14	12%
Wound	12	10%	Internal brain and vessel	7	6%
Cerebrospinal fluid	8	7%	Infectious disease	5	4%
Pleural fluid	5	4%	General	4	3%
Other samples	22	18%	Other	14	12%
Total	121	100%	Total	121	100%

By applying the phenotypic method for the detection of ESBL producers, 60 of 121 isolates (49.5%) were identified by the DDST method as ESBL positive. The PCR method was used to detect the $bla_{\text{-}VEB}$ gene, which was observed in 26.6% of samples, $bla_{\text{-}PER}$ in 36.8% and $bla_{\text{-}SHV}$ in 16% of isolates (figures 1, 2 and 3).

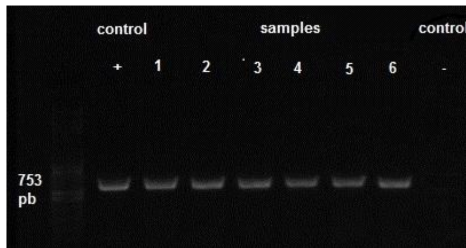

Figure 1: Gel electrophoresis of bla_{SHV} gene

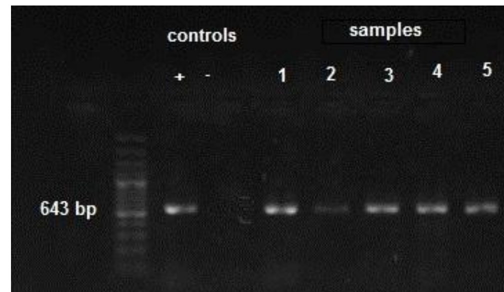

Figure 2: Gel electrophoresis of bla_{VEB} gene

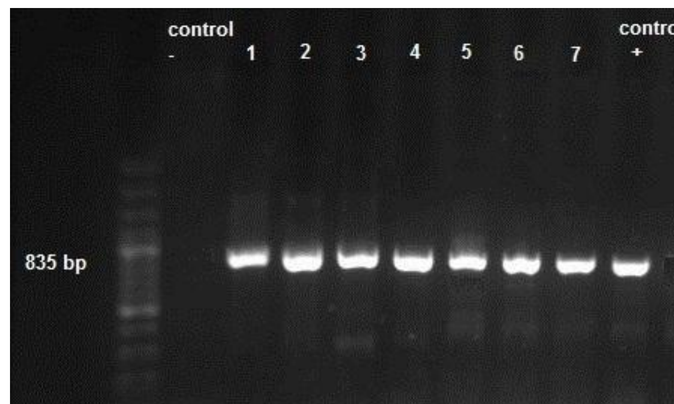

Figure 3: Gel electrophoresis of bla_{PER} gene

The distribution of the $bla\text{-}_{SHV}$, $bla\text{-}_{VEB}$ and $bla\text{-}_{PER}$ among the samples of various clinical specimens is given in table 3.

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Table 3: Distribution of ESBL genes among different samples

Source	bla_{SHV}	bla_{VEB}	bla_{PER}
Trachea	3	5	3
Urine	3	1	2
Wound	2	4	2
Csf	0	2	0
Pleural Fluid	1	2	2
Bronchal	0	3	1
Blood	1	0	3

Followed by sequencing results, three out of five isolates were identified as $bla_{-SHV-12}$ and two out of five as $bla_{-SHV-56}$ and bla_{-SHV-5}. Furthermore, bla_{-VEB-1} and bla_{-PER-1} were identified in this study (figure 4).

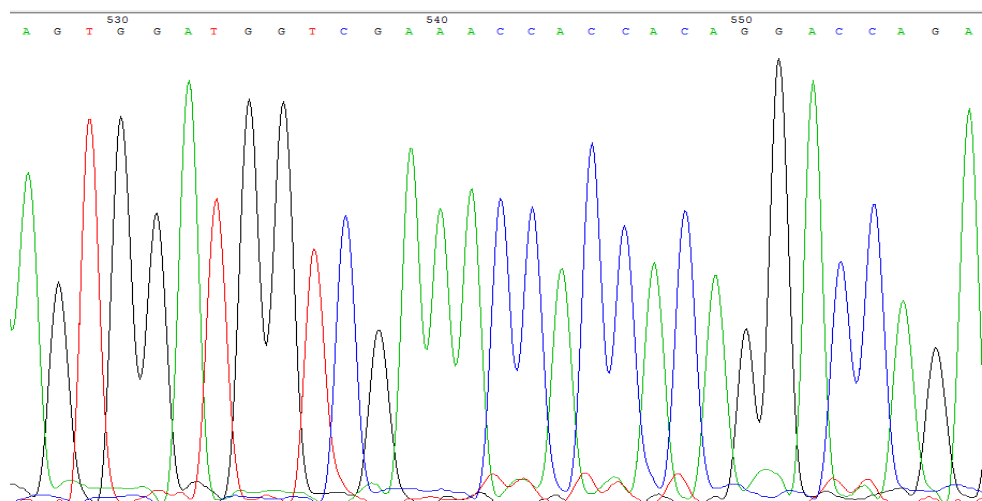

Figure 4: Sequences and Chromatogram of bla_{-PER-1} of *A. baumannii*. The nucleotide sequences was analyzed with CROMASPRO-2 and MEGA 4 softwares and confirmed by blast system in internet

Discussion

A. *baumannii* is one of the most important nosocomial pathogens that cause nosocomial outbreaks in hospitals all around the world. Most of the strains are highly resistant to different groups of antibiotics, so therapeutic options are becoming increasingly limited (Koo *et al.,* 2010). Production of ESBLs is one of the main mechanisms for antibiotic resistance in gram-negative bacteria including A. *baumannii* and the detection of ESBL production and related genotypes is critical for the surveillance of drug resistance in different hospitals.

In this study, the rate of ESBL production, the prevalence of ESBL genotypes bla_{-SHV}, bla_{-VEB} and bla_{-PER} genes were investigated in A. *baumannii* isolates recovered from patients admitted to Alzahra Hospital in Isfahan, which is the largest hospital in this city.

The results of the present study showed that 49.5% of the studied Acinetobacter isolates were positive for ESBL following phenotypic tests; Owlia *et al.,* study identified 21% of isolates were positive for ESBL, while another study conducted in Poland reported this at 20% (Owlia *et al.,* 2012; Sacha *et al.,* 2012). In Iran, Farajnia *et al.,* (2009) reported 70% ESBL phenotypically in the northwest of the country and Sharif *et al.,* (2013) reported 51% in Tehran (Farajnia *et al.,* 2013b; Sharif *et al.,* 2014). The high rate of ESBL prevalence in Iran and its widespread dissemination is a cause for concern. In this study, the prevalence of bla_{-PER} genes among ESBL producing A. *baumannii* isolates was 36.8%. According to the present study, PER-1 is the most common ESBL genotype among A. *baumannii* strains. The prevalence of this genotype in Iran was reported at 51% in Farajnia et al.'s study and 78.03% in Fallah *et al.,* study, 46% in Turkey and 54.6% in South Korea. Screening for VEB genotype revealed that (26.6%) of A. *baumannii* isolates

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contained VEB-1 gene. The prevalence of this gene was reported to be 10% in Farajnia et al.'s study and 39.5% in Fallah *et al.,* study in Iran and 47.61% in the USA (Farajnia *et al.,* 2013b; Pasterán *et al.,* 2006). The prevalence of β-lactamase-producing isolates and their isolation from life-threatening infections is dramatically increasing worldwide. Intense pressure for the usage of PER-1 antimicrobial drugs called for by patients has resulted in the eradication of normal flora and given rise to a situation of MDR isolates substitution. To conclude, phenotypic methods are only screening methods for the detection of ESBLs in a routine laboratory. The genotypic methods help us to confirm the genes responsible for ESBL production This study showed that β-lactamase producing A. *baumannii* strains are an emerging threat in ICUs and should be supervised by the implementation of timely identification and strict isolation methods that will help to reduce their severe outcomes and the mortality rate of patients.

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