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Antibiotic Resistance Patterns and a Survey of Metallo-β-Lactamase Genes Including *bla-$_{IMP}$* and *bla-$_{VIM}$* Types in *Acinetobacter baumannii* Isolated from Hospital Patients in Tehran

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Key Words

Acinetobacter baumannii · Metallo-β-lactamase · *bla-$_{IMP}$* · *bla-$_{VIM}$* · Antibiotic resistance · PCR

Abstract

Background: Metallo-β-lactamases (MBLs) producing strains of *Acinetobacter baumannii* are serious etiological agents of hospital infections worldwide. Among the β-lactams, carbapenems are the most effective antibiotics used against *A. baumannii*. However, resistance to these drugs among clinical strains of *A. baumannii* has been increasing in recent years. In this study, the antimicrobial sensitivity patterns of *A. baumannii* strains isolated from eleven different hospitals in Tehran, Iran, and the prevalence of MBL genes (*bla-$_{VIM}$* and *bla-$_{IMP}$*) were determined. ***Method:*** During a period of 5 months, 176 isolates of *A. baumannii* were collected from different clinical specimens from hospitalized patients in Tehran. All isolates were confirmed by biochemical methods. The isolates were tested for antibiotic sensitivity by the Kirby-Bauer disk diffusion method. Following minimum inhibitory concentration determination, imipenem-resistant isolates were further tested for MBL production by the double disk synergy test (DDST) method. PCR assays were performed for the detection of the MBL genes *bla-$_{IMP}$* and *bla-$_{VIM}$*. ***Results:*** The DDST phenotypic method indicated that among the 169 imipenem-resistant isolates, 165 strains were MBL positive. The PCR assays revealed that 63 of the overall isolates (36%) carried the *bla-$_{VIM}$* gene and 70 strains (40%) harbored *bla-$_{IMP}$*. ***Conclusions:*** It is obvious that nosocomial infections associated with multidrug-resistant *Acinetobacter* spp. are on the rise. Therefore, the determination of antibiotic sensitivity patterns and screening for MBL production among *A. baumannii* isolates is important for controlling clinical *Acinetobacter* infections.

Introduction

Acinetobacter baumannii is a nosocomial pathogen that is responsible for a diverse set of serious infections that include bacteremia, ventilator-associated pneumonia, postsurgical meningitis and skin infections [1]. Some factors associated with these infections include antibiotic exposure, the length of stay in intensive care units (ICUs)

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and use of mechanical ventilation [2]. This bacterium causes infections associated with medical devices such as vascular catheters and Foley catheters, etc. [3, 4]. *Acinetobacter* species are Gram-negative, aerobic and nonmotile bacilli that can colonize skin surfaces and are considered important agents for hospital-acquired infections [5]. They are often resistant to many antimicrobial agents. The cause of resistance could be due to the extensive use of broad-spectrum antibiotics [6]. Many species are resistant to more than two different classes of antimicrobial agents, including sulbactam [7]. Infections due to *A. baumannii* are often difficult to treat due to their high levels of resistance to many antibiotics as a result of both intrinsic and acquired mechanisms [8, 9]. Carbapenems are often effective against *A. baumannii*, but metallo-β-lactamases (MBLs) can potently hydrolyze all β-lactam antibiotics except aztreonam [10]. MBLs are classified in group B of the Ambler classification and group III of the Bush classification [11, 12]. Group B of the Ambler classification is divided into four categories according to molecular structure: the IMP, VIM, GIM and SPM types [12]. The IMP and VIM types are the most prevalent acquired MBLs [7]. These enzymes require zinc ion as a cofactor [10]. Their activity is inhibited by chelators like ethylenediaminetetraacetic acid (EDTA), sodium mercaptoacetic acid, 2-mercaptopropionic acid and dipicolinic acid. Sulbactam, tazobactam and clavulanic acid, which are often used to inhibit β-lactamase enzymes, are not effective against MBLs [13, 14]. There are several phenotypic methods for the detection of MBLs producing bacteria. All these methods are based on the ability of metal chelators such as EDTA to inhibit the activity of MBLs. The double disk synergy test (DDST) method was employed in this investigation [10]. The goal of this study was to determine the antibiotic resistance pattern in *A. baumannii* species isolated from eleven hospitals in Tehran, Iran, and evaluate the prevalence of the most important MBL genes, *bla-*$_{VIM}$ and *bla-*$_{IMP}$, in imipenem-resistant strains.

Materials and Methods

Collection of Strains

A total of 176 strains of *A. baumannii* were collected during a 5-month period from September 2011 to January 2012 from the Imam Khomeini, Imam Hossein, Labbafi Nejad, Khatam Al Anbia, Loghman, Mostafa Khomeini, Milad, Motahari, Shariati, Hashemi Nejad and Mofid hospitals in Tehran, Iran. These strains were isolated from wound, trachea, blood, sputum, catheter, pleural fluid, urine and cerebrospinal fluid (CSF) samples. Ninety-five isolates were obtained from male patients and eighty-one isolates were from female patients. These isolates were subcultured on LB agar and their identification was performed by biochemical methods such as oxidase, lysine decarboxylase test, growth at 45 and 37°C, hydrolyses of gelatin, the ability to use citrate, the production of acid following growth and hydrolysis of blood in agar medium containing 5% sheep blood.

Antibiotic Susceptibility Tests

Antimicrobial sensitivity tests were performed on Mueller-Hinton agar (Biolab, Budapest, Hungary) by a disk diffusion method [15], which were interpreted according to CLSI (Clinical and Laboratory Standards Institute) standard tables [16]. *Pseudomonas aeruginosa* $ATCC_{27853}$ was used as a control for the susceptibility tests. The antibiotic disks used were levofloxacin (5 μg), ceftazidime + clavulanate (30 + 10 μg), ticarcillin + clavulanate (75 + 10 μg), piperacillin + tazobactam (100 + 10 μg), cefotaxime + clavulanate (30 + 10 μg), ciprofloxacin (5 μg), cefotaxime (30 μg), ceftazidime (30 μg), trimethoprim + sulfametoxazole (1.25 + 23.7 μg) and imipenem (10 μg; Rosco, Budapest, Hungary; Padtan Teb, Tehran, Iran). At first the bacteria were cultured in tryptic soy broth and incubated at 35°C for 24 h. The microbial suspension was prepared equivalent to the turbidity of 0.5 McFarland standard. The antibiotic disks were placed on the inoculated Mueller-Hinton agar plate and incubated at 35°C for 24 h. After incubation, the diameters of the zone of inhibition were measured.

Minimum Inhibitory Concentration

The determination of minimum inhibitory concentration (MIC) was performed for imipenem-resistant strains by an agar dilution method according to CLSI standards [16]. Isolates with an MIC value ≥16 μg/ml were considered as preliminary evidence for MBL-producing strains. *P. aeruginosa* $ATCC_{27853}$ was used as a control strain for the susceptibility testing.

Detection of MBLs Producing Isolates by the DDST Method

Imipenem-resistant isolates were investigated for MBL production by the DDST method. The bacterial suspension with a turbidity equivalent to 0.5 McFarland standard was prepared and cultured on Mueller-Hinton agar. For preparation of the IMP-EDTA disk, 750 μg of EDTA solution was added to 10 μg of imipenem disk and dried in an incubator [17]. Then, two 10-μg imipenem and imipenem-EDTA disks were placed on a bacteria-inoculated agar surface. After 24 h of incubation at 35°C, the inhibition zone of the imipenem disk and IMP-EDTA were measured. An increase of 7 mm or more in the zone diameter of the IMP-EDTA disk in comparison with the imipenem disk alone was considered to indicate an MBL-producing isolate [12].

DNA Extraction

Genomic DNA from *A. baumannii* isolates were extracted by a boiling method. In this method, about 10 colonies of *A. baumannii* were inoculated in a 1.5-ml microtube containing 500 μl of sterile water. The microtubes containing the bacterial cells were placed in boiling water for 15 min. Cell debris were removed by centrifugation at 12,000 *g* at room temperature for 10 min. Supernatants were transferred into new tubes and used as the template DNA for PCR reactions. For purity assurance, the template DNA was electrophoresed on agarose gel.

Table 1. Characteristics of the MBL-producing and non-MBL-producing *A. baumannii* isolates based on the source of specimens collected from 11 hospitals in Tehran

Source	*bla*-IMP-positive isolates (n = 70)	*bla*-VIM-positive isolates (n = 63)	Non-*bla*-VIM and *bla*-IMP MBL-positive isolates (n = 32)	Total MBL-positive isolates (n = 165)	Total MBL-negative isolates (n = 11)
Wound	29	25	5	59	–
Trachea	17	5	11	33	1
Blood	10	8	–	18	–
Sputum	–	3	–	3	8
Catheter	4	5	1	10	–
Pleural fluid	4	7	2	13	–
Urine	2	9	11	22	2
CSF	4	1	2	7	–

PCR Assays for the Detection of MBL Genes

For the design of universal primers, the nucleotide sequences of *bla*-$_{VIM}$ and *bla*-$_{IMP}$ genes in *A. baumannii* were obtained from the NCBI gene bank and aligned with Clastalw2 software (alignment program). After the identification of the commonality region, the Gene Runner program was used for primer design. Finally, for confirmation of primer specificity, they were subjected to the BLAST program. PCR reactions for *bla*-$_{VIM}$ and *bla*-$_{IMP}$ genes were performed in a final volume of 25 μl, containing the following:

bla-$_{IMP}$. PCR buffer (×10) 2.5 μl, $MgCl_2$ (50 mM) 1 μl, dNTPs (10 mM) 1 μl, forward (5′-GTTTGAAGAAGTTAACGGGTGG-3′) and reverse (5′-ATAATTTGGCGGACTTTGGG-3′) primers (designed) 459 bp (10 pmol/μl) 1 + 1 μl, Taq DNA polymerase (5 U/μl) 1 μl, template DNA 3 μl and distilled water 14.5 μl. The thermocycler program for the *bla*-$_{IMP}$ gene consisted of 4 min of initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min.

bla-$_{VIM}$. PCR buffer (×10) 2.5 μl, $MgCl_2$ (50 mM) 1 μl, dNTPs (10 mM) 1 μl, forward (5′-TGGTGTTTGGTCGCATATCG-3′) and reverse (5′-GAGCAAGTCTAGACCGCCCG-3′) primers (designed) 595 bp (10 pmol/μl) 1 + 1 μl, Taq DNA polymerase (50 U/μl) 1 μl, template DNA 2 μl and distilled water 15.5 μl. The scheduled program for the *bla*-$_{VIM}$ gene by thermocycler was 4 min of initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 5 min.

Water was used as a negative control and the *P. aeruginosa* strains producing the MBL genes (*bla*-$_{VIM}$ and *bla*-$_{IMP}$; provided by the Tehran Pasteur Institute) were used as positive controls for MBL detection. The PCR products were confirmed by gel electrophoresis in 1% (w/v) agarose gel (HT Bioscience, Cambridge, UK) in TBE buffer and visualized with ethidium bromide staining and photographed with UV waves through Gel Documentation (Technogen, Tehran, Iran).

Results

A total of 176 strains of *A. baumannii* were collected for this study and confirmed by bacteriological tests. They were obtained from clinical specimens such as wound (n = 59), trachea (n = 34), urine (n = 24), blood (n = 18), pleural fluid (n = 13), sputum (n = 11), catheter (n = 10) and CSF (n = 7). The majority were from patients in ICUs (n = 91) and the least were from patients in surgery units (n = 1; table 1).

Antibiotic Susceptibility

The antibiotic susceptibilities of the 176 isolates from the disk diffusion method against the 10 antibiotics are presented in table 2. The isolates showed high resistance to trimethoprim + sulfametoxazole and cefotaxime (98%), and the least resistance was toward ticarcillin + clavulanate (85%).

Minimum Inhibitory Concentration

The determination of the MIC for imipenem by the agar dilution method indicated that 96% (n = 169) of the strains were resistant to imipenem (MIC ≥16 μg/ml).

Detection of MBLs Producing Isolates by the Double Disk Method

In total, 165 strains were shown to be positive by this phenotypic method performed on the 169 imipenem-resistant isolates.

Molecular Analysis

The PCR assays indicated that 63 (36%) of the total strains (n = 176) contained the *VIM* gene, whereas 70

Table 2. Antimicrobial susceptibility patterns of the 176 *A. baumannii* isolates from 11 hospitals in Tehran using the disk diffusion method

Antibiotics	Susceptible, n (%)	Intermediate, n (%)	Resistant, n (%)
Trimethoprim + sulfamethoxazole	4 (2.27)	–	172 (97.72)
Cefotaxime	2 (1.13)	2 (1.13)	172 (97.72)
Imipenem	3 (1.70)	2 (1.13)	171 (97.15)
Ceftazidime	5 (2.84)	–	171 (97.15)
Piperacillin + tazobactam	5 (2.84)	–	171 (97.15)
Cefotaxime + clavulanate	2 (1.13)	3 (1.70)	171 (97.15)
Ciprofloxacin	2 (1.13)	5 (2.84)	169 (96.02)
Levofloxacin	7 (3.97)	4 (2.27)	165 (93.75)
Ceftazidime + clavulanate	8 (4.54)	13 (7.38)	155 (88.06)
Ticarcillin-clavulanic acid	16 (9.09)	10 (5.68)	150 (85.22)

(40%) of them harbored the *IMP* gene. Among the 165 isolates that were positive in the DDST method, 32 were negative by PCR for the *VIM* and *IMP* genes. All of these 32 isolates were resistant against all the studied antibiotics. Seven (4%) of the total isolates contained both the *VIM* and *IMP* genes.

Discussion

Acinetobacter species, and *A. baumannii* in particular, are responsible for increasing rates of hospital-acquired infections. This bacterium causes a significant number of nosocomial outbreaks worldwide, which commonly occur in settings with high antibiotic resistance, such as ICUs [18]. Carbapenems are potent β-lactam antibiotics commonly used against MBL-producing and multidrug-resistant bacteria [10]. Therefore, the detection of MBL-producing strains is essential for the treatment of infected patients and to control the spread of resistance among other bacteria. Reports of carbapenem resistance are growing steadily, which raises concern. Carbapenem-resistant *Acinetobacter* isolates are being reported worldwide [19, 20]. In recent years, MBLs have been identified from clinical isolates and the strains that produced these enzymes were responsible for prolonged treatment and acute infections [21]. Resistance to imipenem has been increasing in Iran in recent years. In a study conducted between 2005 and 2006, Rahbar et al. [22] reported that the rate of imipenem-resistant *A. baumannii* was 1.1%. In another study conducted between 2006 and 2007, Soroush et al. [23] reported that 50% of *A. baumannii* isolates were resistant to imipenem. From 2008 to 2009, Peymani et al. [7] observed an increase in the rate of resistance of *A. baumannii* to imipenem (54%). In our study, which was conducted from September 2011 to January 2012, we noted that the resistance rate to imipenem has substantially increased (97%) compared to those reported previously. Since MBL genes are carried on plasmids, this may explain the higher prevalence of resistance to many antibiotics in MBL-positive isolates [24]. In strains which were resistant to imipenem but were MBL negative, resistance to imipenem may be due to other mechanisms, such as the production of oxacillinase, deficiency in porin or reduced expression of outer membrane proteins [7]. For the confirmation of MBL-producing strains, PCR can be an important and accurate method [14]. In this study all isolates were screened for *VIM* and *IMP* genes by PCR, which indicated that 63 isolates had the *VIM* gene and 70 isolates harbored the *IMP* gene. The 32 isolates that were positive in the DDST method were negative by PCR. This shows that these two genes are not the major source of resistance against imipenem. Other MBL genes, such as *SPM*, *GIM* and *SIM* may contribute to resistance against MBLs. Peymani et al. [7] isolated 100 *A. baumannii* strains from patients admitted to hospitals in Tabriz, Iran, from 2008 to 2009. The production of MBL in these isolates was determined by E-test strips which showed that 19 strains carried *bla-$_{IMP}$* and 9 strains carried *bla-$_{VIM}$*. In another study conducted in Egypt from 2013 to 2014, Fattouh et al. [25] collected 24 *A. baumannii* strains from patients in ICUs, 13 of which turned out to be MBL positive. In another study conducted in Iran in 2013 [26], PCR was carried out on 93 *A. baumannii* isolates, 85% of which were resistant to imipenem. The *VIM-1* and *IMP-1* genes were not detected in any of the isolates. In 2009, Eser et al. [27] collected 124 *A. baumannii* strains in Turkey. Among these isolates, 64 (51.6%) yielded a positive result by the IMP-EDTA combined disk test, and all the isolates were negative in terms of the tested MBL genes by PCR. In another study in India [28], 55 *A. baumannii* strains were collected from a south Indian tertiary care hospital. Thirty-nine (70.9%) strains were positive for MBLs by the DDST method, and *bla-$_{IMP-1}$* was only seen

in 23 isolates (42%). Interestingly, none of the isolates showed amplification of the *bla*-$_{VIM-2}$ gene. In this study, the percent of strains that carried *bla*-$_{VIM}$ and *bla*-$_{IMP}$ genes was higher than those reported in previous investigations. More MBL genes are found to be located on the class I integron and can spread rapidly to other Gram-negative bacilli, making them resistant to other antibiotics [29]. As a limitation of this study, genes other than *VIM* and *IMP* that can be responsible for bacterial resistance were not investigated.

Conclusion

This study illustrated that MBL-producing *A. baumannii* strains have become resistant to various antibiotics in recent years and represent a serious concern. The rapid identification of MBL-producing strains, use of appropriate infection control measures and efforts to reduce the rate of transfer between different strains are necessary for appropriate treatment of *A. baumannii* infections.

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