Detection of *VIM*- and *IMP*-type *Metallo-Beta-Lactamase* Genes in *Acinetobacter baumannii* Isolates from Patients in Two Hospitals in Tehran

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Background: *Acinetobacter baumannii*, is an opportunistic pathogen and is responsible for numerous nosocomial infections. In recent years, this microorganism has been resistant to a wide range of antibiotics. One of the most important mechanisms of resistance in this microorganism is production of *metallo-beta-lactamases* (MBLs).
Objectives: The aim of this study was to detect *VIM*- and *IMP*-type *metallo-beta-lactamase* genes in Acinetobacter baumanniiisolates from patients in two Hospitals in Tehran.
Materials and Methods: 104 isolates were tested using the PCR method for the identification of *VIM*- and *IMP*-type genes.
Results: *vim*1, *vim*2, *imp*1and *imp*2 genes were detected in 6.7%, 41.7%, 50% and 1.7% of the isolates from Tehran Heart Center, and in 29.5%, 38.6%, 4.5% and 4.5% of the isolates from Shahid Mutahhari Hospital respectively.
Discussion: Our analysis revealed that the majority of the isolates had at least one of these genes, indicating that MBLs production is an important resistance mechanism in *Acinetobacter baumannii*.

Keywords: *Acinetobacter baumannii*; *Imp*1; *Imp*2; *Metallo-beta-lactamase*; *Vim*1;*Vim*2

1. Background

Acinetobacter baumanni is a non-fermentative Gram-negative coccobacilli belonging to the Moraxellaceae family. The genus *Acinetobacter* has different species, most of which are environmental and usually do not cause disease in humans. In recent years, however, strains of *Acinetobacter baumannii* have exhibited multi-drug resistance (MDR) and caused various infections such as those of the respiratory tract, blood, and skin amongst others. This microorganism is deemed a major problem in hospitals, not least in the intensive care unit (ICU), where control of various infections is difficult (1). Different mechanisms of antibiotic resistance are identified in *Acinetobacter baumannii*, including the widespread production of hydrolyzing enzymes known as metallo-beta-lactamases (MBLs) such as IMP- and VIM-types, Class D carbapenemases. A new family of Class C cephalosporinases that has been recently detected in this genus, resist the porin loss, which changes the permeability of the bacterial outer membrane, and leads to the production of a broad spectrum of pumps named efflux pumps, which cause resistance to cefotaxime and a large number of other antibiotics (2).

The increased use of Beta-lactam antibiotics has prompted development in the production of Beta-lactamases in recent years (3). At least three major groups of MBLs have been identified: IMP, VIM and SPM types (4). The most effective mechanism of resistance relates to VIM and IMP MBLs. MBLs are the cause of *Acinetobacter baumannii* resistance to the newer generation of cephalosporins and carbapenems (2). Carbapenemases comprise a wide range of enzymes that are active against various antibiotics, including carbapenems such as imipenem, meropenem and ertapenem. IMP type carbapenemase was first detected in Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter* species in Japan in the 1990s, and then spread slowly in Asia and then was also detected in Europe, Canada and Brazil. VIM-types were first reported in Italy in 1999 and now have a geographical distribution in Europe, South America,

Asia and The United Sates (3). Different results have been reported about the frequency of VIM- and IMP-type MBLs in different studies. Shahcheraghi *et al.* evaluated the existence of these genes in 100 *Acinetobacter baumannii* isolates from seven hospitals in Tehran and reported that none of them carried VIM- or IMP-type MBL encoding genes (5). In this study, the VIM- and IMP-type MBL encoding genes were detected in *Acinetobacter baumannii* isolates from two hospitals of Tehran, IR Iran.

2. Materials and Methods

2.1. Separation and Collection of Strains

Totally, 104 clinical isolates of *Acinetobacter baumannii* were collected from patients in Tehran Heart Center (60 isolates) and Shahid Mutahhari Burns Hospital (44 isolates) in a 4-month period. Information regarding the specimens such as sex, age, site of specimen collection, etc. was recorded, and the isolates were identified via Gram staining and biochemical tests.

2.2. Antibiotic Susceptibility Testing

Imipenem susceptibility patterns were determined via disk diffusion method (Kirby-Bauer technique) using imipenem disks (MAST Co., England) on Muller-Hinton agar (Hi-Media, India). The results were interpreted according to the CLSI standard.

2.3. DNA Extraction

The DNA content of the isolates was extracted using boiling method. Briefly, the isolates were cultured on Muller-Hinton agar for 24 h; a few colonies of each isolate were rinsed twice with sterile distilled water and boiled for 15 min, followed by 4 min of treatment on ice before final centrifugation (5000 rpm) for 3 min. The supernatant was preserved at -70ºC for further investigations.

2.4. Polymerase Chain Reaction (PCR)

In this study, four pairs of primers were used to detect *vim1*, *vim2*, *imp1* and *imp2* genes (Table 1). The reaction mixture consisted of 2.5 µL 10X buffer, 1.25 µL MgCl$_2$ (50 mM), 0.25 µL dNTPs (10 mM), 0.75 µL of each primer (10 µM), 50 µg template DNA, and 0.15 µL of *Taq* DNA polymerase (2500 U.µL^{-1}) (CinnaGen Co., Iran), in a final volume of 25 µL. PCR program for the amplification of *vim1* included the initial denaturation at 95ºC for 4 min and 35 cycles of 95ºC for 1 min, primer annealing at 55ºC for 30 seconds and polymerization at 72ºC for 45 seconds. The PCR program for amplification of *vim2*, *imp1* and *imp2* consisted of the initial denaturation at 95ºC for 4 min and 35 cycles of 95ºC denaturation for 1 min, primer annealing at 53ºC for 1 min and polymerization at 72ºC for 1.5 min. A final polymerization step was set at 72ºC for 7 min. A tube containing all components of the PCR reaction with human DNA was used as negative control. Strains of *Pseudomonas aeruginosa* VR-143/97 carrying *vim1*, *Pseudomonas putida* NTU-91/99 carrying *vim2* and two separate plasmids carrying *imp1* and *imp2* were used as positive controls.

2.5. Detection and Electrophoresis of PCR Products

Agarose gel (1%) in borate buffer was used for electrophoresis. The voltage was set at 90-100V. The ethidium bromide staining (0.5 µg.mL^{-1}) was used and the bands were observed using Gel-Doc instrument (Vilber Lourmat, France). A 50-1000 bp ladder (CinnaGen, Iran) was employed to determine the molecular weight of the DNA bands. All the data are presented as absolute frequencies and percentages. The data and result analyses were performed using statistical software SPSS version 13.

Table 1. Oligonucleotide primers and their related information

Primer Name	Oligonucleotide sequence	PCR product size (pb)	Annealing temperature (ºC)	Reference
blaVIM1	Forward: 5'-TTA-TGG-AGC-AGC-AAC-GAT-GT-3' Reverse: 5'-CAA-AAG-TCC-CGC-TCC-AAC-GA-3'	924	55	Shibata *et al.* (2003)
blaVIM2	Forward: 5'-ATG-TTC-AAA-CTT-TTG-AGT-AAG-3' Reverse: 5'-CTA-CTC-AAC-GAC-TGA- GGG-3'	801	53	Shibata *et al.* (2003)
blaIMP1	Forward: 5'-ACC-GCA-GCA-GAC-TCT-TTG-CC-3' Reverse: 5'-ACA-ACC-AGT-TTT-GCC-TTA-CC-3'	587	53	Shibata *et al.* (2003)
blaIMP2	Forward: 5'-GTT-TTA-TGT-GTA-TGC-TTC-C-3' Reverse: 5'AGC-CTG-TTC-CCA-TGT-A-C-3'	678	53	Shibata *et al.* (2003)

3. Results

Over a four-month period, 104 isolates of *Acinetobacter baumannii* were collected. Amongst a total of 60 isolates from Tehran Heart Center, 32 (53.3%) and 28 (46.7%) were isolated from male and female patients, respectively. At Shahid Mutahhari Burns Hospital, from a total of 44 isolates, 35 (79.5%) and 9 (20.5%) were isolated from male and female patients, respectively.

The specimens collected from patients at these two hospitals were from different sources (Figure 1). 13 (21.7%), 3 (5%) and 44 (73.3%) of the isolates from Tehran Heart Center and 10 (22.7%), 8 (18.2%) and 26 (59.1%) of the isolates from Shahid Mutahhari Hospital had sensitive, intermediate and resistant pattern to imipenem, respectively.

PCR results demonstrated that 4 (6.7%), 25 (41.7%), 30 (50%) and 1 (1.7%) of the isolates from Tehran Heart Center and 13 (29.57%), 17 (38.6%), 2 (4.5%) and 2 (4.5%) of the isolates from Shahid Mutahhari Hospital carried *vim1*, *vim2*, *imp1* and *imp2* genes respectively (Figure 2).

From the 60 isolates at Tehran Heart Center, 2 (3.3%) carried both *vim1* and *vim2* genes, 13 (21.7%) isolates carried both *imp1* and *vim2* genes, and 15 (25%) isolates carried none of them. Of the 44 isolates examined at Mutahhari Hospital, 4 (9.1%) isolates carried both *vim1* and *vim2* genes, 2 (4.5%) isolates carried both *imp1* and *vim2* genes, 1 (3.3%) isolate carried both *imp2* and *vim1* genes and 17 (38.6%) isolates carried none of them. 75% of the isolates at Tehran Heart Center and 61% of the isolates at Shahid Mutahhari Burns Hospital carried at least one of these four genes.
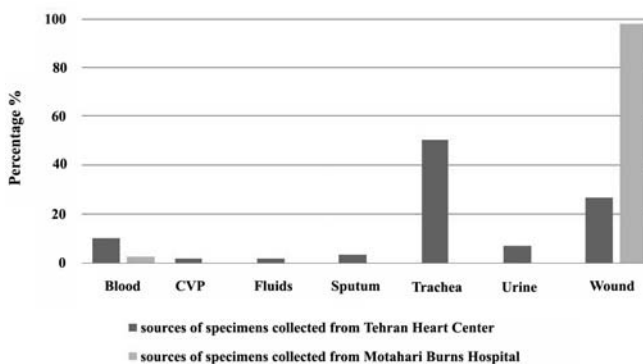

Figure 2. A: Gel electrophoresis of *vim1* PCR products. Lines 1, 2 and 7 show the negative control, positive control and marker respectively; lines 3 and 5 show the positive isolates; and lines 4 and 6 show the negative isolates. B: Gel electrophoresis of *vim2* PCR products. Lines 1, 2 and 3 are related to the negative control, positive control and marker, respectively; and lines 4 and 5 are related to the positive isolates. C: Gel electrophoresis of *imp1* PCR products. Lines 2, 3 and 6 show the negative control, positive control, and marker respectively; and lines 1, 4, and 5 show the positive isolates. D: Gel electrophoresis of *imp2* PCR products. Lines 2, 3, and 6 are related to the negative control, positive control and marker respectively; line 4 is related to the positive isolate; and lines 1 and 5 are related to the negative isolates

Figure 1. Different specimen sources for *Acinetobacter baumannii* isolates of Tehran Heart Center and Shahid Mutahhari Burns Hospital patients. The majority of the specimens from Shahid Mutahhari Burns Hospital patients were collected from wound, whereas trachea was the most frequent source of *A. baumannii* isolated from Tehran Heart Center patients
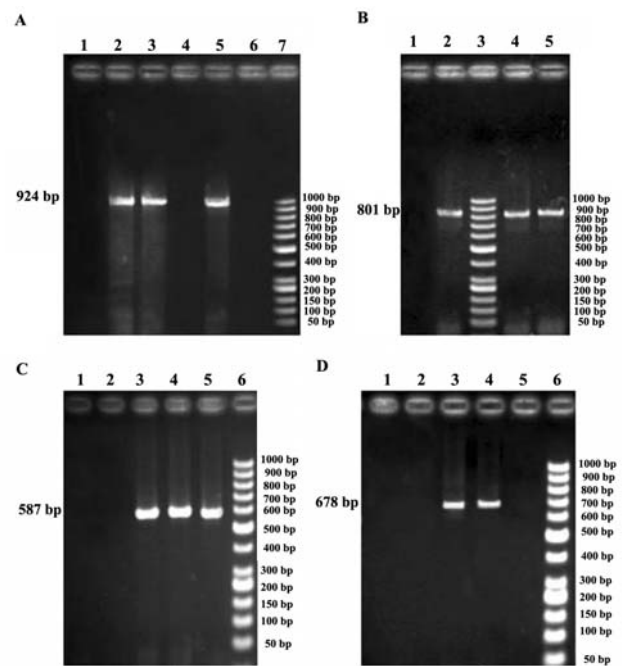
4. Discussion

Acinetobacter baumannii is the sixth most common Gram-negative bacterium isolated from ICU patients. The bacterium's high rate of mortality, increase in the MDR phenotype, easy spread and potential for opportunism constitute a serious threat to the clinical centers. The clinical importance of *Acinetobacter baumannii* has increased, especially in recent years, due to its remarkable ability to acquire resistance. Indeed, it is due to this ability that *Acinetobacter* has become a problematic organism in recent years. The most important mechanism of resistance to beta-lactam antibiotics in this bacterium is the production of beta-lactamases (2). Imipenem is an effective antibiotic against *Acinetobacter baumannii* and is resistant to most beta-lactamases; be that as it may, it is inhibited by a group of these enzymes, metallo-beta-lactamases, which fortunately has been found only in a few strains of bacte-

ria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii. Acinetobacter* resistance to imipenem is growing and one of the ways to acquire resistance to imipenem in this organism is the acquisition of MBL-encoding genes (Gordon and Wareham, 2010) such as *vim1*, *vim2*, *imp1* and *imp2*.

In this study, *Acinetobacter baumannii* resistance frequencies to imipenem at Tehran Heart Center and Shahid Mutahhari Burns Hospital were 73.3% and 59.1%, respectively. Halstead *et al.* Studied 851 *Acinetobacter* isolates and showed that 39.8% of them, collected from 76 centers in the United States between 2004 and 2005, were resistant to imipenem (Halstead *et al.*, 2007). In a study on 75 *Acinetobacter* isolated from civilian and military patients from Iraq and Afghanistan in 2006, Hujer *et al.* showed that 20% of the isolates were resistant to imipenem (Hujer *et al.*, 2006). In contrast, in a study on the American servicemen injured in Iraq in 2007, Scott *et al.* reported that only 10% of the *Acinetobacter baumannii* strains were resistant to carbapenems (Scott *et al.*, 2007). Hawly *et al.,* in their study on the American servicemen wounded in 2007, showed that amongst 142 strains of *Acinetobacter baumannii*, 37% were resistant to imipenem (Hawley *et al.*, 2007). In Latin America in a broad study between 2002 and 2004, Unal *et al.* demonstrated that 29% of the *Acinetobacter* isolates were resistant to meropenem or imipenem (Unal and Garcia-Rodriguez, 2005). In this study at Tehran Heart Center and Shahid Mutahhari Burns Hospital, imipenem resistance to *Acinetobacter baumannii* was higher than that reported by previous studies. The differences are probably due to the difference between geographical regions, medical centers and different regimens of antibiotic therapy.

The frequencies of *vim1*, *vim2*, *imp1* and *imp2* genes were 6.7%, 41.7%, 50% and 1.7% at Tehran Heart Center and 29.57%, 38.6%, 4.5% and 4.5% at Shahid Mutahhari Burns Hospital, respectively. In a study conducted between 2000 and 2001 in 28 hospitals in Korea, Lee *et al.* studied 38 MBL-producing isolates of *Acinetobacter*, of which 27 (10.1%) and 11 (4.1%) carried *vim2* and *imp1*, respectively (Lee *et al.*, 2003). In 2003 in Korea, Oh *et al.* examined 31 isolates of *Acinetobacter baumannii* in terms of the presence of *vim1*, *vim2* and *imp1* genes and amongst them only 4 (12.9%) isolates carried *vim2* while the others lacked each of the four genes (Oh *et al.*, 2003). In 2004, Sader *et al.* studied 33 isolates of *Acinetobacter* in Latin America, amongst which 7 isolates (21%) had *imp1* gene (Sader *et al.*, 2004). In 2007 in Spain, Ruiz *et al.* examined 83 isolates of *Acinetobacter bauman-*

nii with respect to the presence of *vim* and *imp* genes and demonstrated that all of them lacked these two genes (Ruiz *et al.*, 2007). In 2007 in Greece, Iknomidies *et al.* studied 87 isolates of *Acinetobacter baumannii* for the presence of *vim1* gene and found that only 2 (2.3%) isolates carried *vim1* (Iknomidies *et al.*, 2007). In 2010, in India, Azim *et al.* examined 38 isolates of *Acinetobacter baumannii* and found that 20 (52.6%) and 14 (36.8%) isolates carried *imp* and *vim* genes, respectively (Azim *et al.*, 2010). In Iran, Shahcheraghi *et al.* detected no VIM- or IMP-type MBL-encoding genes in 100 *Acinetobacter baumannii* isolates from seven hospitals of Tehran (Shahcheraghi *et al.*, 2011).

As can be seen, there has been a considerable differences between the frequencies of VIM- and IMP-type MBL-encoding genes in various regions of the world in different years. These differences could also result from differences in geographical regions, antibiotic therapy regimens, and the number of isolates examined. To resolve this controversy and to achieve reliable statistics on the frequency of MBL-encoding genes, a comprehensive study comprising a collection of specimens obtained from several countries at a given time interval with complete data on specimens and patients such as the site of specimen collection, ward of hospitalization and antibiotic treatment regimen through a well-defined and constant technique is recommended.

Overall 75% of the isolates in Tehran Heart Center and 61.4% of the isolates in Shahid Mutahhari Burns Hospital had at least one of the above-mentioned four genes. This indicates that the production of MBLs is one of the most important mechanisms of resistance to imipenem by *Acinetobacter baumannii* in these two hospitals. In addition, in this study 25% of the isolates in Tehran Heart Center and 38.6% of the isolates in Shahid Mutahhari Burns Hospital lacked these four genes. The resistance to imipenem in these isolates may have been the consequence of other mechanisms of resistance in this organism. Resistance among the isolates that lacked these four genes may have been due to other MBL enzymes, whose genes were not examined in this study.

Given the high frequency of MBL-coding genes, high levels of resistance to imipenem amongst the *Acinetobacter baumannii* isolates from hospitalized patients in Tehran Heart Center and Shahid Mutahhari Burns Hospital, the possibility of resistance transfer amongst different bacteria and amongst different strains of a bacterium, and the likelihood of the indiscriminate use of antibiotics inducing resistance in bac-

teria, it is vitally important that arrangements be made to reduce usage of key antibiotics such as imipenem in hospitals, perform accurate health protocols to prevent the spread of imipenem-resistant bacteria in the hospitals through personnel or hospital equipment, and finally carry out regular and periodic examination of imipenem resistance and frequency of its encoding genes.

Acknowledgments

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