Phenotypic and Genotypic Detection of Metallo-Beta-Lactamases among Imipenem Resistant Gram Negative Isolates

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ABSTRACT

***Background*:** Imipenem-resistant gram negative bacteria, resulting from metallo-beta-lactamase (MBLs)-producing strains have been reported to be among the important causes of nosocomial infections and of serious therapeutic problem worldwide. Because of their broad range, potent carbapenemase activity and resistance to inhibitors, these enzymes can confer resistance to almost all beta-lactams. The prevalence of metallo-beta-lactamase among imipenem-resistant *Acinetobacter* spp., *Pseudomonas* spp. and *Enerobacteriaceae* isolates is determined.

***Methods*:** In this descriptive study 864 clinical isolates of *Acinetobacter* spp., *Pseudomonas* spp. and *Enterobacteriaceae*, were initially tested for imipenem susceptibility. The metallo-beta-lactamase production was detected using combined disk diffusion, double disk synergy test, and Hodge test. Then all imipenem resistant isolates were tested by PCR for *imp*, *vim* and *ndm* genes.

***Results*:** Among 864 isolates, 62 (7.17 %) were imipenem-resistant. Positive phonetypic test for metallo-beta-lactamase was 40 (64.5%), of which 24 (17.1%) and 16 (9.2%) isolates were *Acinetobacter* spp. and *Pseudomonas* spp., respectively. By PCR method 30 (48.4%) of imipenem resistant *Acinetobacter*, and *Pseudomonas* isolates were positive for MBL-producing genes. None of the *Enterobacteriaceae* isolates were positive for metallo-beta-lactamase activity.

***Conclusion*:** The results of this study are indicative of the growing number of nosocomial infections associated with multidrug-resistant gram negative bacteria in this region leading to difficulties in antibiotic therapy. Thereby, using of phenotypic methods can be helpful for management of this problem.

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Introduction

The introduction of carbapenems into clinical practice represented a great advance in the treatment of serious bacterial infections caused bybeta-lactam resistant bacteria (1). Due to their broad spectrum of activity and stability to hydrolysis of most beta-lactamases, the carbapenems have been the drug of choice for treatment of infections caused by penicillin or cephalosporin-resistant Gram-negative bacilli especially, extended spectrum beta-lactamase (ESBL) producing Gram-negative infections (2,3).

Resistance to carbapenem is due to decreased outer membrane permeability, increased efflux systems, alteration of penicillin binding proteins and carbapenem hydrolyzing enzymes (carbapenemase) (4, 5).

Carbapenemases are carbapenem-hydrolyzing beta-lactamases that cause resistance to a broad spectrum of beta-lactams, including carbapenems. The carbapenemases based on amino acid homology in the Ambler molecular classification system are divided into four classes A to D. Class A, C, and D beta-lactamases have a serine residue in the active site. Class B or Metallo-beta-lactamase (MBL) belongs to a group beta-lactamase which requires divalent cations of zinc as cofactors for enzyme activity (6, 7, 8). These have potent hydrolyzing activity not only against carbapenem but also against other beta-lactam antibiotics except azetreonam (8). In the past decade, the emergence of acquired metallo-beta-lactamases (MBLs) in Gram-negative bacilli such *pseudomonas* spp., *Acinetobacter* spp. and *Enterobacteriaceae* family, is becoming a therapeutic challenge. Thus, MBL-producing Gram negative bacterial isolates have been reported to be important causes of nosocomial infections (9, 10, 11). The spread of metallo-beta-lactamases presents a major challenge both for treatment of individual patients and for policies of infection control, exposing the substantial unpreparedness of public health structures in facing up to this emergency. We undertook this study to examine the reliability of phenotypic tests to determine the incidence of MBL producing gram negative bacteria in patients admitted to the selected Tehran hospitals over a period of one year from 2014-2015.

Material and method

This was a descriptive study in which 864 clinical isolates of *Acinetobacter* spp., *Pseudomonas* spp. and *Enterobacteriaceae* family, which were collected from Emam Hossein and Loghman Hakim hospitals during 2014-2015.

All isolates were confirmed by oxidase test, motility and standard biochemical reactions. Then these isolates were initially tested for imipenem susceptibility and later for metallo-beta-lactamase production using combined disk diffusion, Double Disk Synergy Test (DDST), and Hodge test. Antibiotic susceptibility of positive metallo-beta-lactamase isolates were further evaluated by disk diffusion technique using CLSI methodology.

Screening of resistance to imipenem

The inoculum was adjusted to a 0.5 McFarland standard, and a 50-mm Mueller-Hinton agar plate was inoculated, then a 10 µg imipenem disk (Mast, England) was placed on the plate and was incubated in 37 °C for 18 hours.

The Modified Hodge Test (MHT)

The surface of a 150 mm MHA plate was inoculated overnight with a suspension of *Escherichia coli* ATCC 25922, adjusted to a 0.5 McFarland standard. After a brief drying period, a 10 µg imipenem disk (Mast, England) was placed at the center of the plate, and the test isolate was streaked heavily from the edge of the disk toward the edge of the plate. The MHT was considered positive, if *E.coli* growth was observed within the inhibition zone of the imipenem disk, giving a

distorted zone and interpreted as carbapenemase production. *P. aeuruginosa* ATCC 25853 was used as MBL positive control.

Combined disk diffusion Method

The test strain was inoculated on plates with Mueller Hinton agar as recommended by CLSI for antibiotic sensitivity testing by the disk diffusion method. The presence of MBL was determined by placing two 10 µg imipenem disks (Mast, England) on the inoculated plate, in which 10 µl of 0.5 M EDTA was added to one of the imipenem disks. After overnight incubation at 37 °C, the inhibition zones of imipenem and imipenem with EDTA were compared. A zone difference of over 7mm between the imipenem and the imipenem-EDTA inhibition zones confirmed the isolate to be MBL-positive.

Double Disk Synergy Test (DDST)

The inoculum was adjusted to a 0.5 McFarland standard, and a Mueller-Hinton Agar plate was inoculated. One blank filter paper disk was treated with 10 µl of 0.5 M EDTA and placed on the center of the MHA plate. Imipenem (10 µg) disk (Mast, England) was placed 15 mm away from the EDTA disk (measured center-to-center). After overnight incubation at 37 °C, any synergistic inhibition zone was interpreted to be positive by the MBL screening test.

Antibiotic sensitivity of imipenem resistant isolates

The Imipenem resistant isolates were inoculated on plates with Mueller Hinton agar as recommended by CLSI for antibiotic sensitivity testing by the disk diffusion (Kirby-Bauer) method. Antibiotic sensitivity to Ciprofloxacin (5µg), Gentamicin (10µg), Piperacillin (100 µg), Azetreonam (30 µg), Cefotaxime (30 µg), Cefepime (30µg) disks (Mast, England) were examined.

PCR assay

All imipenem resistant isolates were extracted by boiling method (12), Then PCR amplification was performed in a 25 µL reaction mixture containing 2 µL of DNA template, 12µL ready to use Mastermix (Fermentase. Germany), 9 µL of distilled water and 1µL of 20 pmols forward and reverses primers. For imp, vim and ndm genes, specific primers were used under conditions that listed in table 1. *A. baumannii* AC54/97, *P. aeruginosa* PO510 and *K. pneumoniae* ATCC BAA215 were used as controls for *imp*, *vim* and *ndm* genes respectively.

Table 1. Primer sequences, sizes of product fragments and PCR conditions.

Gene	Primers	Product size	PCR conditions	Refrence
imp	F-GAAGGCGTTTATGTTCATAC R-GTATGTTTCAAGAGTGATGC	587 bp	(95°(5 min), 95°(30sec) , 56°(30sec), 72°(30sec), 72°(5min))×30	13
vim	F-GTTTGGTCGCATATCGCA AC R-AATGCGCAGCACCAGGATAG	382 bp	(95°(5 min), 95°(30sec) , 60°(30sec), 72°(30sec), 72°(5min))×30	13
ndm	F-GGGCAGTCGCTTCCAACGGT R-GTAGTGCTCAGTGTCGGCAT	475 bp	(95°(5 min), 95°(30sec) , 58°(30sec), 72°(30sec), 72°(5min))×30	14

Results

Among 864 gram negative isolates, including 140 *Acinetobacter* spp., 173 *Pseudomonas* spp. and 548 *Enterobacteriaceae*, 62 isolates (7.17 %) were imipenem-resistant. Of the imipenem resistant isolates 35 (56.45%), 24 (38.70 %) and 3 (4.85 %) isolates were *Acinetobacter* spp., *Pseudomonas* spp. and *Enterobacteriaceae*, respectively. The Modified Hodge test showed that all (100%) imipenem resistant *Acinetobacter* isolates, 19 (79.2%) *Pseudomonas* isolates and 3

(100%) *Enterobacteriaceae* were produced carbapenemase. The metallo-beta-lactamase was produced by 24 (68.57 %) and 16 (66.66 %) isolates of imipenem resistant *Acinetobacter*, and *Pseudomonas*, respectively, according to both Combine Disk Diffusion and Double Disk Synergy test methods. On the other hand 24 (17.1%) and 16 (9.2%) of all *Acinetobacter*, and *Pseudomonas* isolates were produced metallo-beta-lactamase. All the carbapenemase producing/imipenem resistant *Enterobacteriaceaee* isolates were negative for MBL. Among 35 imipenem resistant *Acinetobacter* isolates 14 (40%), 8 (22.8%) were positive for *vim* and *imp*, respectively. In 24 imipenem resistant *Pseudomonas* isolates, the *imp* and *vim* genes were detected in 18 (75%) and 6 (25%) isolates, respectively. No *ndm* gene was detected in imipenem non-susceptible *Acinetobacter* and *Pseudomonas* isolates. On the other hand, *imp* and *vim* genes were detected among 100% of *Pseudomonas*, 91.7% of *Acinetobacter* isolates which were MBL-producer by phenotypic methods. No MBL-producer *Enterobacteriaceae* was detected by phenotypic and genotypic methods. The antibiotic sensitivity to other antibiotics among MBL positive *Acinetobacter*, and *Pseudomonas* isolates was listed in Table 2.

Table 2. Antibiotic sensitivity in MBL producer *Acinetobacter*, and *Pseudomonas* isolates.

Antibiotic disks / Imipenem Resistant isolates	Ciprofloxacin (5µg)	Gentamicin (10µg)	Piperacillin (100 µg)	Aztreonam (30 µg)	Cefotaxime (30 µg)	Cefepime (30 µg)
Acinetobacter spp	92%	75%	96%	79%	100%	100%
Pseudomonas spp	57.8%	36.8%	84.2%	47.3%	94.7%	94.7%

The multi-drug resistance was seen in 10 (58.8%) and 5 (31.2%) of MBL positive *Acinetobacter* and *Pseudomonas* isolates. Other detailed results were shown in the tables 3 and 4.

Table 3. Distribution of imipenem resistant isolates from clinical samples.

		Sputum	Blood	Wound	Others
Pseudomonas spp	*aeruginosa* 33 (94.3%)	23 (69.8%)	5 (15.2%)	3 (9%)	2 (6%)
	Others 2 (5.7%)	2 (100%)	0	0	0
Acinetobacter spp	*baumanii* 21 (87.5%)	19 (79.2%)	3 (12.5%)	0	2 (8.3%)
	Others 3 (12.5%)	3 (100%)	0	0	0
Enterobacteriaceae	*Klebsiella pneumoniae*	0	1 (33.3%)	0	0
	Enterobacter aerogenes	0	1 (33.3%)	0	0
	Citrobacter freundii	0	0	0	1 (33.3%)

Table 4. Distribution of carbapenemase and MBL producer isolates among hospital units**.**

		ICU	Surgery	Ortopedi	Others
Pseudomonas spp	*Carbapenemase* + (19 isolates)	16 (84.2%)	1 (5.3%)	0	2 (10.5%)
	MBL + (16 isolates)	16 (100%)	0	0	0
Acinetobacter spp	*Carbapenemase* + (24 isolates)	19 (79.2%)	1 (4.2%)	2 (8.3%)	2 (8.3%)
	MBL + (24 isolates)	19 (79.2%)	1 (4.2%)	2 (8.3%)	2 (8.3%)
Enterobacteriaceae	*Carbapenemase* + (3 isolates)	2 (66.7%)	0	0	1 (33.3%)
	MBL +	0	0	0	0

Discussion

Carbapenems have been the beta-lactam antibiotics used most successfully to evade bacterial resistance, but acquired carbapenem resistance due to the production of metallo-beta-lactamases (MBLs) has been increasingly reported, particularly for *Pseudomonas aeruginosa* and *Acinetobacter* spp. (15, 16). Also an increasing rate of carbapenem resistance among *Enterobacteraceae* isolates, were observed in the last years (17).

This study showed that 17.1% (phenotype) and 15.7% (genotypes) and 9.2% (phenotypic and genotypic) among *Acinetobacter*, and *Pseudomonas* which were isolated from clinical samples from Tehran hospitals were produced the MBLs that suggests expanding the resistance to imipenem and other beta-lactam antibiotics in this region. Also, we showed that VIM and IMP are dominant MBLs in imipenem resistant *Acinetobacter*, and *Pseudomonas* isolates in our region.

In Brazil in 2008 Carvalho et al by Combine Disk showed that 38.3% of *P. aeruginosa* isolates and 5.6% of *A. baumannii* isolates were positive for MBLs (18).

John et al in 2011 in India demonstrated that 99% of *Acinetobacter* isolates are resistant to the imipenem which 14.8% of the resistant isolates were produced MBLs, While 27.7% of imipenem-resistant *Pseudomonas* isolates were MBL positive (19).

The MBL producing *Acinetobacter baumannii* isolates phenotypically determined by Double Disk Synergy Test (DDST) and E-test method by Gholamine et al in 2008 in the west of Iran. They reported that 82% of isolates were produced MBLs (20).

The MBLs in imipenem resistant *P. aeruginosa* isolates were tested by Khosravi et al in 2012 in Malaysia using double-disk synergy test (DDST), combined disk test (CDT), and imipenem/imipenem-inhibitor (IP/IPI) E-test and PCR and were concluded that phenotypic methods have specificities as similar to that of PCR (21).

Wadekar et al in 2013 determined MBL producers *Enterobacteriaceae* in 18% of *E. coli*, *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp. isolates from clinical samples in India by combined disc diffusion test (22).

The results of current and reviewed studies showed a significant increasing in MBL related resistance in various regions, especially in our region that indicate an increasing number of nosocomial infections associated with multidrug-resistant gram negative bacteria leading to difficulties in antibiotic therapy.

A few studies compared two phenotypic and genotypic methods (21, 23). The results of these studies suggested that phenotypic tests have enough sensitivity to detect MBL producing isolates. In the current study, we used CDT and DDST after primary screening Hodge test for differentiation non-MBL carbapenemase producing isolates from MBL producers. Our results showed similar sensitivity of CDT and DDST while other studies (21, 23) indicated that DDST is a more sensitive method for this aim. All phenotypic MBL-positive isolates in our study showed positive PCR results which confirm the reliability of phenotypic methods for detection of MBL-producer gram negative bacteria. The phenotypic tests have an advantage in comparison with molecular methods which these tests distinguish all types of MBLs while PCR needs to detect all MBL-encoding genes using specific primers which are not cost and time consuming for routine laboratories.

Conclusion

According to simplicity and sensitivity of phenotypic methods for distinguishing MBLs and carbapenemases, using these methods can be very helpful for hospital laboratories to control of serious nosocomial infections and management of antibiotic therapy specially, for MDR isolates.

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Conflict of interests

The authors report no conflicts of interest.

Financial disclosure

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