ORIGINAL ARTICLE

Detection of New Delhi Metallo-Beta-Lactamase-1 (NDM-1) in carbapenem-resistant *Klebsiella pneumoniae* isolated from a university hospital in Iran

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Abstract

Background: New Delhi metallo-beta-lactamase-1(NDM-1) is a novel type of metallo-beta-lactamase (MBL) which inactivates all β-lactam antibiotics except aztreonam. *Enterobacteriaceae* expressing NDM-1 have been identified worldwide. The aim of this study was to detect MBLs in carbapenem-resistant *K. pneumoniae* isolates obtained from patients hospitalized in one of the university hospitals in Isfahan, Iran.

Methods: Of the 112 isolates obtained from various clinical samples, 49 were selected for carbapenemase detection based on their reduced susceptibility to imipenem or meropenem according to the disc diffusion method. These isolates were screened for carbapenemase and MBL production using the Modified Hodge Test (MHT) and Epsilometer test (E-test) MBL strips. Polymerase chain reaction was performed on all 49 isolates using specific primers to detect genes encoding IMP (active on imipenem), VIM (Verona integron-encoded metallo-β-lactamase), SPM-1 (Sao Paulo metallo-β-lactamase) and NDM-1.

Results: Among 49 carbapenem-resistant isolates, 32 (65.3 %) were positive for MHT and 6 (12.2 %) were found positive for blaNDM-1. Other MBL genes were not detected.

Conclusion: This is the second report on the detection of blaNDM-1 in Iran since it was first reported by Shahcheraghi and colleagues in 2012. This study indicated that resistance to carbapenems and isolation of bacteria producing NDM-1 is increasing. Therefore, the rapid detection of isolates expressing NDM-1 is essential to control their spread.
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Keywords: New Delhi metallo-beta-lactamase-1, *Klebsiella pneumoniae*, carbapenem, Modified Hodge test, Epsilometer test

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Introduction

Klebsiella pneumoniae is an important nosocomial pathogen causing urinary tract, respiratory tract, and bloodstream infections[1]. It is the second most common cause of nosocomial Gram-negative bacteremia after *Escherichia coli* (*E. coli*). As an opportunistic pathogen, *Klebsiella* spp. often affects immunocompromised individuals receiving treatment for other severe underlying diseases, such as diabetes mellitus and chronic pulmonary obstruction[2]. Beta-lactam antibiotics are widely used to treat infections caused by Gram-negative pathogens. However, the efficacy of these drugs is reduced considerably due to the appearance of extended-spectrum beta-lactamases (ESBLs) and the consequent emergence of multi-drug resistant (MDRs) strains[3]. Carbapenems are the most important therapeutic agents used to treat infections caused by MDR Gram-negative bacteria. However, their efficacy is threatened by the emergence of resistant isolates[4,5]. Carbapenemases represent a heterogeneous group of beta-lactamases from various molecular classes (A, B and D).The emergence of acquired metallo-beta-lactamases (MBLs) among major Gram-negative pathogens has clinical and epidemiological implications and causes particular concern worldwide[6]. MBLs have been reported in many geographic areas. The most common acquired MBLs include IMP (active on imipenem), VIM (Verona integron-encoded metallo-β-lactamase), SPM (Sao Paulo metallo-β-lactamase), GIM (German imipenemase), SIM (Seoul imipenemase), and NDM-1 (New Delhi metallo-beta-lactamase-1) enzymes[7,8]. New Delhi Metallo-beta-lactamase was first reported in *K. pneumoniae* and *E .coli* isolated from a Swedish 59-year-old pa-

tient from India that was previously admitted to a hospital in New Delhi[7].

MBL genes are located within a variety of integron structures. Horizontal transfer of integrons carrying MBL genes that is associated with plasmids or transposons between bacteria, plays an important role in the occurrence of resistance to beta-lactam antibiotics in the community and hospitals[3,8]. Because of poor treatment and increased mortality rate related to the infections caused by these organisms, the early detection of MBL-producing organisms for infection control purposes and the prevention of nosocomial outbreaks is very important.

The aim of the present study was to determine the presence of MBL genes among carbapenem-resistant *K. pneumoniae* isolates obtained from hospitalized patients in a university hospital in Isfahan, Iran.

Methods

Clinical isolates

One hundred twelve *K. pneumoniae* isolates were collected from various clinical specimens including: urine (n =46), tracheal aspirate (n =21), bronchoalveolar-lavage (BAL) fluid (n =10), wound (n =10), abscess (n =7), cerebrospinal fluid (n =3), sputum (n =2), catheter (n =2), and eye (n =1) between March 2012 and March 2013 from hospitalized patients at the main university hospital of Isfahan, Iran. The specimens were identified by standard laboratory methods and API20E system (BioMerieux, Marcy l'Etoile, France). Isolates were then preserved at -80˚C in Brain-heart infusion (BHI) broth containing 20% glycerol.

Antimicrobial susceptibility testing

The antibiotic susceptibility of isolates was determined using the disc diffusion technique (Kirby-Bauer) on Muller-Hinton agar plates. Results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), January 2012[9]. The antibiotic discs used in this study were amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), ceftazidime (30 µg), cefepime (30 µg), piperacillin (100 µg), piperacillin/tazobactam (110 µg) and aztreonam (30 µg) (Mast Group Limited, Merseyside, UK). *E. coli* ATCC® 25922™ was used as the quality control for antimicrobial susceptibility test. The minimum inhibitory concentrations (MICs) of imipenem were determined using E-test strips (Liofilchem, Roseto degli Abruzzi, Italy) for imipenem and meropenem resistant isolates.

Phenotypic detection of MBLs

All imipenem and meropenem-resistant isolates were examined for MBL production using the Modified Hodge test (MHT) and Epsilometer test (E-test) MBL strips. The MHT was carried out according to CLSI guidelines using a 10µg disc of ertapenem on Muller–Hinton agar plates. Following overnight incubation, the presence of a "cloverleaf shaped" inhibition zone was interpreted as a positive result[10]. *E. coli* ATCC® 25922™ was used as the carbapenem-susceptible strain. The detection of MBLs was carried out using E-tests according to the manufacturer's instructions (Liofilchem, Roseto degli Abruzzi, Italy). The E-test MBL strips contained imipenem (4 to 256 µg/ml) and imipenem (1 to 64 µg/ml)-EDTA combinations. A three-fold or greater reduction in imipenem MIC in the presence of EDTA was interpreted as the positive result. Furthermore, the presence of a "phantom" zone between the two gradient sections or a deformation of the imipenem ellipses was considered as a marker of MBL production[11]. *Pseudomonas aeruginosa* ATCC® 27853™ strain was used as a negative control.

Polymerase chain reaction (PCR) amplification and DNA sequencing

Genomic DNA was extracted using the DNA Extraction Kit (Sinaclon, Iran) according to the manufacturer's directions. All carbapenem-resistant isolates were screened by standard PCR using specific primers for MBL genes (Table 1). Standard strains for blaVIM, blaIMP, and blaSPM genes were provided by the Pasteur Institute of Iran. PCR fragments were sequenced at Macrogen Inc. (Seoul, Korea). The sequences were then confirmed using the NCBI web site (http://www.ncbi.nlm.nih.gov/).

Ethics Statement

The Ethics Committee of the Faculty of Medical School, Isfahan University of Medical Sciences approved the study (no 290284), and the author group collected written informed consent from the patients.

Results

Antimicrobial susceptibility test

The antimicrobial susceptibility results for all 112 isolates are summarized in Table 2. In total, 80.3 % (n =90) of the isolates were resistant to 3 or more antibiotics of different classes and were determined as multidrug resistant (MDR). High-level resistance to piperacillin, ceftazidime and ciprofloxacin was observed in 88.4 % (n =99), 83 % (n =93) and 63.4 % (n =71) of the isolates respectively.

From 112 *K. pneumoniae* isolates, a total number of 49 isolates were screened for carbapenemase production based on their reduced sensitivity to imipenem and meropenem.

MBL detection

From 49 carbapenem-resistant isolates, MHT indicated that 65.3% (32/49) of the isolates were carbapenemase-producers. Moreover, E-test MBL strips demonstrated that 10.2% (5/49) of the isolates were MBL-producers.

PCR screening for MBL-encoding genes and sequencing

PCR using specific primers for VIM, IMP, SPM and NDM-1 genes was performed on all the IMP-resistant isolates. A 291 bp fragment representing blaNDM-1 gene was amplified in 12.2% (n =6) of the isolates, includ-

Table 1: Forward (F) and Reverse (R) primer pairs used for the detection of metallo-beta-lactamases (MBLs) in *K. pneumoniae* isolates.

Primers	Sequence (5′–3′)	Gene	Product size (bp)	Reference
IMP-F	GAAGCTTGGCCAAAGTCCG	*bla* IMP	108	Current study
IMP-R	TGTAAGTTTCAAGAGTGATGCGTC			
VIM-F	GATTGATACAGCGTGGGGTG	*bla* VIM	165	Current study
VIM-R	GGTGATGCGTACGTTGCC			
SPM-F	GCCGTTTGAAAATCTGGGTAC	*bla* SPM	244	Current study
SPM-R	CCTTCGCTTCAGATCCTCG			
NDM-F	CAACTGGATCAAGCAGGAGA	*bla* NDM	291	Teo et al[12]
NDM-R	TCGATCCCAACGGTGATATT			

bp: basepairs, F: Forward primer pairs, R: Reverse primer pairs.

Table 2: Antimicrobial susceptibility results. In this study, high-level resistance to piperacillin, ceftazidime and ciprofloxacin was observed. Also, from 112 *K. pneumoniae* isolates, 49 isolates were resistant to imipenem and meropenem.

Antimicrobial agent	No of isolates	Sensitive %	Intermediate %	Resistant %
Piperacillin/Tazobactam	112	23.2	21.4	55.4
Piperacillin	112	6.2	5.4	88.4
Meropenem	112	52.7	6.2	41.1
Imipenem	112	50	8	42
Levofloxacin	112	47.3	2.7	50
Ciprofloxacin	112	34.8	1.8	63.4
Amikacin	112	35.7	12.5	51.8
Gentamicin	112	33.9	1.8	64.3
Ceftazidime	112	13.4	3.6	83
Cefepime	112	21.4	12.5	66.1
Aztreonam	112	14.3	0.9	84.8

Susceptibility of selected antimicrobials was obtained using the disc diffusion technique on Muller-Hinton agar plates and were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), January 2012. No: number.

ing all those that were positive by the E-test MBL strips. Therefore, there was no false positive result for E-test MBL strips. However, there was one MBL-positive isolate that was negative for the E-test MBL strips. Alignment of the blaNDM-1 PCR fragment sequence with other blaNDM-1 sequences displayed on the GenBank database revealed a 97-100% similarity. We submitted two nucleotide sequences of blaNDM-1 gene, determined in this study, to the GenBank nucleotide sequence database under the accession numbers of KF734093 and KF734094. The characteristics of the MBL positive isolates are summarized in Table 3.

Discussion

The current emergence of carbapenemase-producing bacteria represents a major threat to the clinical application of all beta-lactam antibiotics[6]. The clinical significance of bacteria possessing an MBL gene is their

Table 3: Characteristics of metallo-beta-lactamase-producing isolates. From 49 carbapenem-resistant isolates, 6 isolates were positive for New Delhi metallo-beta-lactamase-1 (blaNDM-1).

Isolate no.	Source of isolation	Hospital unit	MIC (µg/ml) of imipenem	MHT	E-test MBL	Detection of MBL
123	Urine	ICU	>256	Positive	Positive	NDM-1
293	Sputum	ICU	>256	Positive	Positive	NDM-1
583	Urine	ICU	>256	Positive	Positive	NDM-1
590	Urine	ICU	>256	Positive	Positive	NDM-1
592	Urine	ICU	>256	Positive	Positive	NDM-1
317	Urine	ICU	>256	Positive	ND	NDM-1

ND: non determinable, MBL: Metallo-Beta-Lactamase, MHT: modified Hodge test, E-test: Epsilometer test, MIC: minimal inhibitory concentrations; ICU, intensive care unit.

ability to hydrolyze β-lactams.

MBL genes are also often associated with aminoglycoside resistant genes, and thus, these bacteria are often co-resistant to aminoglycosides, further compromising therapeutic regimes[13,14].

In recent years, detection of MBLs and other antibiotic resistance genes among Gram-negative bacilli has been reported worldwide. The presence of MBL genes on mobile genetic elements, such as integrons and plasmids helps their widespread dissemination and is a serious concern in nosocomial infection control[15,16].

Nowadays, *K. pneumoniae* is recognized as an important reservoir for a variety of resistance determinants including β-lactamases, such as KPC (*K. pneumoniae* carbapenemase) and metallo-β-lactamases VIM, IMP, and NDM[17]. In this study, PCR results demonstrated that 6 out of 49 (12%) carbapenem-resistant isolates expressed the New Delhi metallo-beta-lactamase, five of which were also positive by the MHT and E-test MBL strips. This is the second report on the detection of NDM-1 in Iran. It was first reported by Shahcheraghi et al in 2012 during a study on *Enterobacteriaceae* family isolates collected from five hospitals in Tehran. Shahcheraghi et al detected a *K. pneumoniae* isolate carrying blaNDM-1[18]. The data obtained in this study indicate that the results of MBL detection using phenotypic tests and PCR correspond to the findings of other studies, such as Ikonomidis, Kassis-Chikhani, Psichogiou, Roy and Nordmann[19-23]. Previous studies showed that the sensitivity of MBL-producing isolates to carbapenems varied from intermediate to resistant. However, all the isolates in this study that expressed blaNDM-1 were completely resistant to imipenem and meropenem with MICs >256 for imipenem.

NDM-1 is an Ambler class B beta-lactamase that confers resistance to all β-lactams except aztreonam. In this study, isolates harboring NDM-1 were resistant to all tested antibiotics, including aztreonam. This may probably indicate the simultaneous presence of other antibiotic resistance mechanisms in these isolates. The NDM-1-encoding gene can spread from one strain of bacteria to another by horizontal gene transfer and moreover, plasmids possessing the bla NDM gene often carry other resistance genes including: ESBLs and 16SrRNA methylase genes (armA, rmtA, rmtB, rmtC, rmtD, rmtE, rmtFand npmA), conferring resistance to all β-lactams and aminoglycosides. Therefore, the early detection of NDM-1 producing bacteria causing clinical infections and/or colonization is essential to prevent their spread[24-26]. Some studies have suggested the use of tigecycline and colistin as the only available options for the treatment of infections caused by MBL- or KPC-producing Gram-negative bacilli. However, reports of the emergence of colistin and tigecycline-resistant isolates and also unfavorable pharmacokinetics and toxic side-effects of these antibiotics, may limit their use[18]. Recently, FIM-1 (Florence Imipenemase), a new type of acquired MBL was detected in a multidrug-resistant *P. aeruginosa* clinical isolate in Florence, Italy. This novel MBL enzyme has 40% amino acid similarity to the NDM enzyme[27].

The isolation of NDM-1 expressing strains in Iran is still very rare. However, it is expected that the number of such isolations will increase in the near future. Therefore, the early detection of NDM-1 expressing bacteria using molecular methods could be a useful control strategy. Moreover, it is necessary that antibiotics should be applied appropriately to prevent the emergence and spread of MDR bacterial strains in hospitals and the community.

Conflict of interest

All authors report no conflicts of interest relevant to this article.

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