

Warning: spread of NDM-1 in two border towns of Iran

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Abstract: NDM-1 producing gram-negative bacteria can be resistant to every beta-lactam antibiotic, including carbapenem which is one of the last-lines of antibiotic therapy against multi-drug resistant bacteria. This study aimed to detect the metallo-beta-lactamase in the isolated gram-negative bacteria of the Iranian clinical specimens collected from two major cities in Iran. In this cross sectional study 171 *Acinetobacter baumannii*, 120 *Enterobacter* spp. and 145 *Klebsiella pneumoniae* isolated from clinical specimens of two training hospitals in Tabriz and Mashhad were evaluated. Carbapenem resistant screening was performed according to CLSI guide line. The antibiotic susceptibility testing was prepared for carbapenem resistant strains. Then, the metallo-beta-lactamase genes detection was also carried out by PCR assay and confirmed by sequencing. Sixty-eight, 12 and 22 carbapenem resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Enterobacter* spp. were respectively confirmed, respectively. *bla_{VIM}* in 9% and *bla_{NDM-1}* in 4% of isolated *A. baumannii* were observed. *bla_{NDM-1}* was also detected in 18% and 25% of *K. pneumoniae* and *Enterobacter* spp. isolates, respectively. This is the first report of NDM-1 producer *A. baumannii* and *Enterobacter* pp. in Iran. NDM-1 producing gram-negative bacteria can be resistant to all beta-lactam antibiotics and cause complicated challenges in health care systems.

Key words: NDM-1; *Acinetobacter baumannii*; *Enterobacter* spp.; Iran.

Introduction

Antibiotic resistance is one of the major global challenges as the world had already experienced (1-3). Failed antibiotic therapy related to presence of the multi-drug resistance (MDR), extensively-drug resistance (XDR) bacteria may lead to increasing mortality and morbidity in hospitalized patients, especially immune compromised individuals (1,4,5). Antimicrobial resistance is now growing to be an alerting health problem in the twenty-first century (2,3,6). Carbapenem is one of the broad-spectrum antibiotics that is used in treatment of antibiotic resistance microorganisms and in several instances it is the final choice belonging to the last line of antibiotic therapy (1-3,7-10). Unfortunately resistance to carbapenem has been widely reported related to production of carbapenemase enzymes such as metallo-beta lactamase (MBL) (7,8). One of the significant metallo-beta lactamases is the New-Delhi Metallo-beta lactamases-1 (NDM-1) isolated from *Klebsiella pneumoniae* and *Escherichia coli* for the first time from a patient admitted to a hospital in New Delhi (11,-13). NDM-1 producer gram-negative bacteria can be resistant to all beta-lactam antibiotics including carbapenems but excluding aztreonam (6). Different plasmids can harbor NDM-1 gene with large number of other antibiotic resistant genes simultaneously including other carbapenemase, plasmid born cephalosporinase genes,

Extended Spectrum Beta- Lactamase (ESBL) genes, aminoglycoside resistant genes, macrolide resistant genes, rifampin resistant genes and sulfamethoxazole resistant genes (14-18). Presence of MDR and XDR strains especially in nosocomial strains such as *Acinetobacter baumannii* and *K. pneumoniae* may lead to more mortality and morbidity (1, 2, 8, 14). The underlying problem in the NDM-1 producing strains is the plasmid location of the NDM-1 gene which is a mobile genetic element (6,12,13). The NDM-1 can horizontally transfer in bacteria strains as well as other plasmid born genes and may cause spread this antibiotic resistant gene in countless bacteria (6,12,13,19). Thus, the detection of NDM-1 in various bacteria like MDR *Acinetobacter baumannii*, carbapenem resistant *K. pneumoniae* and *Enterobacter* spp., which are big threat to human health, is important according to Centers for Disease Control and Prevention (CDC) reports (20). Enterobacteriaceae and *A. baumannii* carrying NDM-1 have been reported from clinical and environmental isolates in several countries (6,12,13,18) and indicating an alarming situation. As a result, rapid detection of NDM-1 producing microorganism could play a key role for inhibition of spreading of these microorganisms in the hospitals. Fortunately, gram-negative bacteria producing NDM type enzymes have been rarely reported in some countries such as Italy and Iran (11,21,22) although in some of these studies (11,16) the sequencing of the PCR pro-

Table 1. Sequence of primers.

Primer	Sequence (5'→3')	PCR product size (bp)	References
VIM F	TTGACACTCCATTTACDG	390	
VIM R	GATYGAGAATTAAGCCACYCT		
Imp F	GATGGTGTGGTTCGCATA	139	24
Imp R	CGAATGCGCAGCACCAG		
NDM-1 F	CCCGGCCACACCAGTGACA	129	
NDM-1 R	GTAGTGCTCAGTGTGGCAT		25
SPM-1 F	GGGTGGCTAAGACTATGAAGCC	447	
SPM-1 R	GCCGCCGAGCTGAATCGG		

duct have been not prepared for presence confirmation of the NDM-1. The aim of this study was to detect metallo-producing *A. baumannii*, *Enterobacter* spp. and *K. pneumoniae* in variety clinical samples obtained from different specimens.

Materials and Methods

Bacterial strains

In this study, 171 *A. baumannii*, 120 *Enterobacter* spp. and 145 *K. pneumoniae* were collected from 436 specimens from different clinical samples (wound, blood, urine etc.) from July 2016 to October 2016 in two training hospitals in Tabriz and Mashhad, Iran. Identification of the isolated strains was conducted by the conventional biochemistry and microbiological methods.

Antibiotic susceptibility testing

In the first step, Carbapenem resistance was confirmed by imipenem and meropenem discs (Mast Company, England) according to the Clinical & Laboratory Standards Institute (CLSI) standard data-bases (23). Antibiotic susceptibility testing was prepared according to CLSI guide line for carbapenem resistant strains. The antibiotic susceptibility testing was performed by disc diffusion method on Mueller–Hinton agar for carbapenem resistant strains by using cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg) amikacin (30 µg), colistin (10 µg), and trimethoprim-sulfamethoxazole (1.25/23.75).

Polymerase Chain Reaction (PCR) amplification for carbapenemase producing genes

Extraction of bacterial DNA was performed with a plasmid Mini kit (Thermo, USA) based upon the manufacturer's instructions.

Two different sets of multiplex PCR for *bla*_{VIM}, *bla*_{IMP} and *bla*_{NDM-1}, *bla*_{SPM-1} genes were designed. The lists of utilized primers were summarized in table 1.

The first set of multiplex PCR (*bla*_{VIM} and *bla*_{IMP} genes) were performed and expressed as follows:

DNA thermal-cycler was programmed: The first denaturation at 94°C for 10 minutes and 30 cycles of 94°C for 40 seconds, annealing at 60°C for 40 seconds, extension at 72°C for 60 seconds and followed by the final extension at 72°C for 7 minutes.

The second set of multiplex PCR (*bla*_{NDM-1} and *bla*_{SPM-1} genes) were performed in following condition:

DNA thermal-cycle was programmed as follows: The first denaturation at 94°C for 60 second and 30 cycles of 94°C for 30 seconds, annealing at 60°C for 40 seconds, extension at 72°C for 60 seconds for and the ultimate extension at 72°C for 7 minutes.

Direct sequencing of amplified PCR products was also carried out using ABI 3730X capillary sequencer (Pishgam, Macrogen, Seoul, Korea).

Results

In this cross sectional study 68, 12 and 22 carbapenem resistant isolates of *A. baumannii*, *K. pneumoniae* and *Enterobacter* spp, have been confirmed according to conventional biochemical, microbiological, and antibiotic susceptibility tests, respectively.

In addition to carbapenem, all of these strains were resistant to the cefotaxime, ceftazidime and cefepime; antibiotic resistance pattern is indicated in table 2. As can be seen in Table 2, the Colistin is the most effective antibiotic and all of the strains are sensitive to colistin. The NDM-1 producing strains showed resistance to all tested antibiotics except colistin. According to the antibiotic susceptibility testing the 68 (40%) of *A. baumannii* isolates were resistant to three family of tested antibiotics and considered as MDR strains. Twenty-two

Table 2. Percentage of antibiotic resistant in carbapenem resistant isolates.

	imipenem	meropenem	cefepime	cefotaxime	ceftazidime	ciprofloxacin	gentamicin	amikacin	trimethoprim-sulfamethoxazole	colistin
<i>A. baumannii</i>	100%	100%	100%	100%	100%	99%	91%	93%	97%	—
<i>K. pneumoniae</i>	100%	100%	100%	100%	100%	100%	49%	59%	100%	—
<i>Enterobacter Spp.</i>	100%	100%	100%	100%	100%	80%	80%	20%	40%	—

Table 3. Characterization of collected bacteria.

	Total isolates	Carbapenem resistant	NDM-1 producing
<i>A. baumannii</i>	171	68	3
<i>Enterobacter spp.</i>	120	14	3
<i>K. pneumoniae</i>	145	22	4

of 145 (15.2%) *K. pneumoniae* and 14 of 120 (11.6%) of *Enterobacter spp.* were carbapenem resistant strains. According to the PCR results, the *bla*_{NDM-1} was present in 3 (4%) samples of the MDR *A. baumannii*, 4 (18%) samples of *K. pneumoniae* and 3 (25%) samples of *Enterobacter spp.* (Table 3) (Fig.1). The city and unit list of collected *bla*_{NDM-1} positive strains are summarized in table 4.

Only 6 (9%) of carbapenem resistant *A. baumannii* strains were positive for *bla*_{VIM} (Fig. 2) and the *bla*_{VIM}, *bla*_{IMP} and *bla*_{SPM-1} were found in none of the other bacteria. In this study, PCR results were confirmed by sequencing.

Discussion

Antibiotic resistance is dramatically increasing worldwide despite of many carried research, reports, and cautions in health care centers (1-5). Limited therapeutic options in many cases could lead to increasing mortality and morbidity by appearance of MDR bacteria in hospitals, especially in immune compromise patients such as hospitalized ones (1-5). Carbapenem is one of the best choices for antibiotic therapy by its broad spectrum efficacy, especially on ESBL-producing bacteria (1-3). Thus, presence of resistance to this group of antibiotics may cause to very serious problems for physicians (1-3). NDM-1 is one of the global rare MBL

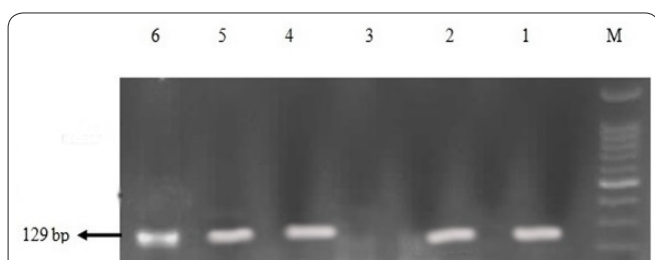


Figure 1. NDM-1 gene. M; 100bp marker. 1, 2, 4, 5; positive NDM-1 strains. 3; Negative control. 6; Positive control.

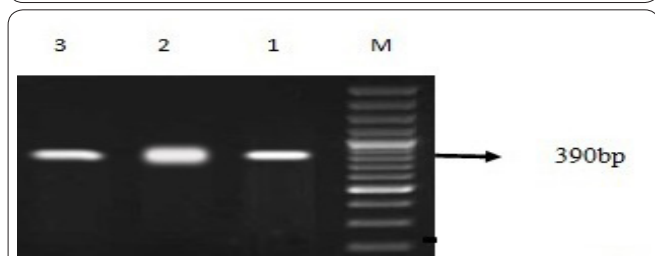


Figure 2. VIM gene. M; 100bp marker. 1, 3; positive VIM strains. 2; Positive control.

Table 4. Details of NDM-1 positive strains.

	City	Hospital units	Number of NDM-1 producing strains
<i>A. baumannii</i>	Tabriz	ICU and neurosurgery unit	3
<i>Enterobacter spp.</i>	Tebriz	ICU, lung and emergency unit	3
<i>K. pneumoniae</i>	Tabriz and Mashhad	ICU and lung unit	4

that can cause of resistant not only to carbapenem but also to all of the beta-lactam antibiotics, except aztreonam (6). Therefore, emergence of these bacteria can be a threat for health. Carbapenems are the last-line antibiotics used against hospital infections. In this study, NDM-1 producing strains illustrated a high resistant to the aminoglycoside and fluoroquinolone. Other studies have reported a high rate of aminoglycoside and fluoroquinolone resistance in NDM-1 producing strains (14,15,26, 27). The observed high rate of antibiotic resistant in NDM-1 producing strains can be attributed to the plasmid that can carry the NDM-1 gene because of harboring other antibiotic resistance genes by NDM-1 simultaneously (14-16).

Two NDM-1 producing *Enterobacter cloacae* have been reported in China in 2016 (23), but in this study 25% of carbapenem resistant *Enterobacter spp.* were positive for the NDM-1. This difference can be related to spread a specific clone of the NDM-1 producing *Enterobacter spp.* in two selected training hospitals in that period.

Twelve percent of carbapenem resistant *K. pneumoniae* were positive for NDM-1 in the study conducted by Fazeli et al. in Iran, in 2015 (11). In the current study, 18% of the carbapenem resistant *K. pneumoniae* found to be a carrier of *NDM-1* gene. Due to the same country for performing these studies, the results of these two studies are presumably similar, because of both of these studies have been performed in Iran. carbapenem resistant *A. baumannii* that harbored NDM-1 has been reported 22% in Turkey (17), in 2016. In this study 12% of carbapenem resistant *A. baumannii* were positive for NDM-1. These differences can be related to the differences in the prevalence of NDM-1 in both countries. It should be stressed that all NDM-1 producing *A. baumannii* in this study showed resistance to all tested antibiotics, except colistin and are categorized as a XDR gram-negative bacteria and can be a super bug.

NDM-1 gene is located on plasmid and can transfer among bacteria. Our results showed that NDM producing *Enterobacter spp.*, *A. baumannii* and *K. pneumoniae* were found in intensive care unit (ICU) in a certain hospital in Tabriz. The spread of NDM can be related to movement and transfer of NDM gene among bacteria and spread of NDM producing gram-negative bacteria in ICU of certain hospital. These results represent the first report of *A. baumannii* and *Enterobacter spp.* producing NDM-1 according to our knowledge. *A. baumannii* is one of the important causes of nosocomial infection in hospitals and appearance of NDM-1 producing strains can be considered as alarming factor for health care system in Iran. Another important point is that NDM-1 is located on mobile genetic element (6) and can transfer to other bacteria and spread rapidly. NDM-1 is a transmissible agent according to CDC (14). Thus, one of the highlight of the results in this study can be related to emergence of NDM-1 pro-

ducing *A. baumannii* in Mashhad. Mashhad is one of the pilgrimage towns in Iran with many annual visitors from many countries, especially neighboring countries such as Pakistan. Pakistan is one of the reservoirs of the NDM-1 that could have been transferred to Mashhad. On the other hand, other NDM-1 producing strains have been isolated from Tabriz that is neighbor with Turkey. NDM-1 producing *A. baumannii* has been reported in 2015 from Turkey (28) and NDM-1 producing *A. baumannii* has been detected in Tabriz as well. Many Iranians travel to Turkey annually. As a result, this superbug can be transferred by passengers traveling between these two countries. Due to the rare report of NDM-1 producing gram-negative bacteria in Iran and neighboring countries, identification and isolation of patients infected by NDM-1 producing bacteria can be counted as useful health measures in order to help to design an inhibition plan that could prevent spread of them from the hospitals. Based on a review article published about global spread of NDM-1 in Iran (14) no NDM-1 has been reported from Iran. Another study includes other countries with spread of NDM-1 in 2011 (29). The *K pneumoniae* NDM-1 producing strains have been recorded in 2013 and 2015 from Iran and this study is the first report of *A. baumannii* and *Enterobacter* spp. producing NDM-1 from Iran

Emergence of NDM-1 producing strains can be very important and considerable problem because of NDM-1 genes can carry on plasmids with the pool of antibiotic resistant genes and appear as a superbug and health care system must pay more attention to this issue. Some studies consider NDM-1 as important as HIV, tuberculosis and malaria (14,30). Control and screen of passengers that travel to Iran especially from the reservoir countries of NDM-1 or any person who come back to Iran from these countries could be one of the reasons that helps transfer and spread of NDM-1 producing bacteria in Iran.

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Conflict of interest

The authors declare no conflicts of interest.

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