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ORIGINAL ARTICLE

Nosocomial emerging of (VIM1) carbapenemase-producing isolates of *Klebsiella pneumoniae* in North of Iran

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ABSTRACT

Background and Objectives: The rapid emergence and dissemination of carbapenemase-producing *Klebsiella pneumoniae* strains and other members of the *Enterobacteriaceae* poses a considerable threat to the care of hospitalized patients and to public health. The aim of this study was to determine the frequency of metallo-β-lactamases (MBL) and VIM-1 gene in multidrug-resistant strains of *K. pneumoniae*.
Methods: 50 isolates of non – duplicated *K. pneumoniae* cultured from patients at intensive care units were tested for their susceptibilities to 13 different antibiotics using microbroth dilution assay. Isolates showing resistance to at least one of the carbapenems were checked for production of metallo-β-lactamase (MBLs) using imipenem–EDTA synergy tests. PCR was used to detect the gene encoding VIM-1 metallo-β-lactamase (MBL).
Results: Of 50 clinical isolates, 26 (52%) were resistant to imipenem in disk diffusion method. Using imipenem–EDTA synergy tests, production of MBL was detected in 15 (30%) isolates. PCR assay showed that 15 isolates were positive for VIM and these included 10 and 5 isolates showing positive and negative results in phenotypic method of MBL detection test respectively. Amikacin was found as the most effective antibiotic against the MBL producers in this study.
Conclusion: The emergence of bla(VIM-1) producing *K. pneumoniae* in North of Iran is concerning. Microorganisms producing bla(VIM-1) constitute the prevalent multidrug-resistant population of *K. pneumoniae* in that region.

Keywords: *Klebsiella pneumoniae,* metallo-beta-lactamases, Imipenem-resistant, bla(VIM-1)gene.

INTRODUCTION

Klebsiella pneumoniae is an important cause of serious hospital-acquired and community-onset bacterial infections in humans (1). β-lactam antibiotics are a major drug class used to treat serious hospital-acquired infections. Resistance to these agents is a therapeutic challenge at the hospitals (2). The high prevalence of antibiotic resistance in *Enterobacteriaceae,* especially in *K. pneumoniae* has been reported in Iran (3-5). Over the last few years, the confidence on carbapenems has been challenged owing to the wide spread of acquired metallo-β-lactamases [MBLs]. Resistance to carbapenems in *K. pneumoniae* is related to two main mechanisms:i) production of extended-spectrum β-lactamase (cephalosporinase or ESBL) associated with porin loss, and ii) production of carbapenem-hydrolyzing β-lactamase such as Ambler's class A carbapenemases (KPC-type), class B metallo-β-

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lactamase (VIM-, NDM- or IMP-type) or class D carbapenemase OXA-48(6). Two dominant groups of acquired MBLs have been recognized: the IMP and VIM types (7-9). Among MBLs, the first member of the VIM- type of enzymes, VIM-1, was identified in *Pseudomonas aeruginosa* in Italy, but emerged in Enterobacteriaceae and become a major problem in some health care settings (10, 11). Recently, there have been several reports on the emergence of VIM-producing *K. pneumoniae* (VPKP) isolates. These strains are endemic in various hospitals and cause life-threatening infections (12, 13). Because MBL-producing *K. pneumoniae* are increasing globally, there is an urgent need to evaluate antibiotic regimens for the treatment of infections caused by these strains. Therefore, this study was designed to determine the phenotypic characteristics of multidrug-resistant *K. pneumoniae* producing VIM-1 gens.

MATERIALS AND METHODS

Bacterial isolates. The bacterial strains used in this study were selected from a collection of *K. pneumoniae* containing resistant to three or more antimicrobial classes used in the previous studies from hospitalized patients at ICU in Shahid - Beheshti hospital of Babol, Iran. The pure colonies from each sample collected in BHI broth (Heart Infusion Broth, Difco) and kept at -20 ºC for performing sensitivity test and DNA extraction.

Antimicrobial Susceptibility Testing. Susceptibilities to antibiotics were determined by Broth dilution and disc diffusion tests, according to CLSI guidelines (14). All experiments were performed in triplicate .

The antibiotics; cefepime, ceftriaxone, cefazolin, ceftizoxime, imipenem, cefotaxime, amikacin, ofloxacin, ciprofloxacin and gentamicin were purchased from Sigma Chemical Company.

Phenotypic detection of MBL activity. All isolates with reduced susceptibility to imipenem were also subjected to a phenotypic analysis by EDTA combination disk test. For the imipenem-EDTA double-disk synergy test (DDST), an overnight culture of the test strain was suspended to the turbidity of a McFarland standard of 0.5 and used to inoculate a Mueller-Hinton agar plate. A disc of either imipenem alone (10 µg) or imipenem (10 µg) in combination with EDTA (750 µg /disc) was placed at the distance of 20 mm (centre to centre). After incubation overnight at 35°C, the difference of ≥7mm between the inhibition zone diameter of the IPM-EDTA disk and that of IPM only disk was considered to be a positive for the presence of MBLs(15) . The procedure was repeated twice to ensure the reproducibility of results .

PCR assay. DNA was extracted using a Kit supplied by Roche, (Roche Diagnostics, Germany). PCR amplification for detection of blaVIM-1 genes was carried out on thermal cycler (Eppendorf, Hamburg, Germany).

The primer sequences were F: 5'-AGTGGTGAGTATCCGACAG-3' and R: 5'-TGAAAGTGCGTGGAGAC-3' for the amplification of blaVIM-1 gene, which could produce 261bp (12). The PCR reaction was performed in 50 µL volumes which contained 10 µL extractions of DNA (equal to 1 µg), 5 pmol/L from each primer, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTPs and 1.5 unit of Taq DNA polymerase enzyme. The PCR program consisted primary denaturation at 94 ºC for 5 minutes and then, was followed by 30 cycles of denaturation at 94 ºC for 25 seconds, annealing at 52 ºC for 40 seconds and extending at 72 ºC for 50 seconds. Moreover one cycle for the final extension at 72 ºC for six minutes was performed.

After performing PCR reaction, electrophoresis of PCR products was conducted in 1.5% agarose gel for 60 minutes. Then, the results were illuminated under UV light on the UV gel document. Positive and negatives controls were *K. pneumoniae* ATCC 1029 and *K. pneumoniae* ATCC 1053 respectively. The data was analyzed using the SPSS statistical software version 18.

RESULTS

Among these 50 clinical isolates used in this study, 26 (%52) were found to be resistant to imipenem in disk diffusion method. Phenotypic testing revealed synergy between imipenem and EDTA for 20(%40) between samples were resistant to imipenem. PCR experiments revealed the presence of bla_{VIM-1} gene in 15 (%30) isolates, of these 10 were positive in phenotypic test (Fig. 1). Five isolates were positive

in PCR but these were negative in phenotypic test (Table 1).

MIC was performed for all isolates (Table 2). The carbapenem MICs for the 15 VPKP (VIM-1 metallo-beta lactamase producing *Klebsiella pneumonaie*) strains ranged from 0. 5 to 32 µg/ml; 4 isolates were resistant to imipenem (MIC > 4 µg/ml), and 11 were susceptible or intermediate to imipenem (MIC ≤ 4 µg/ml). Most the VPKP isolates were multidrug-resistance, 5 isolates of MBL- producing were also found to be resistant to at least 5 antibiotics. The highest resistances observe for VPKP included cephazolin 12 (80%). None of VPKP isolates was resistant to amikacin (Table 2). Significant relationship was found in VPKP isolates, in 35 negative sample and 15 positive sample, in compared to antibiotics cefotaxim and ceftizoxim ($p<0.05$).

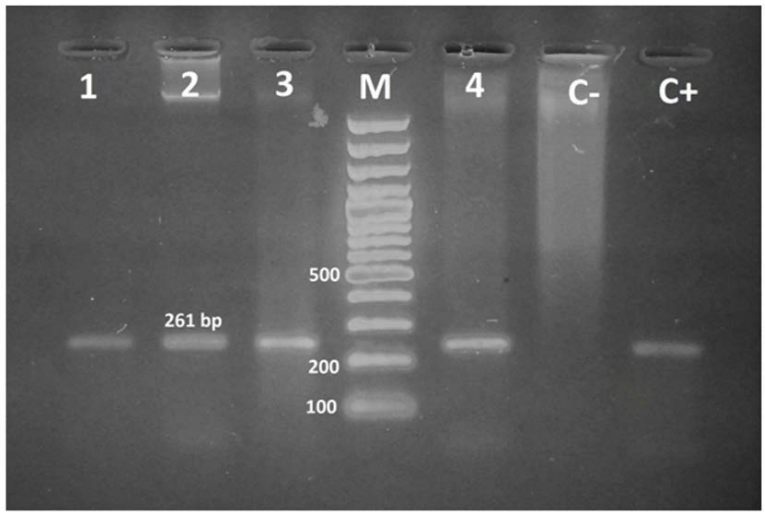

Fig.1. Gel-Electrophoresis of the PCR products of VIM-type MBLs of *Klebsiella pneumoniae*: Lane 1 -3,4 :positive isolate, LaneM: DNA size marker, Lane C+: positive control, Lane C-: negative control.

Table1. Performance of phenotypic methods for detecting VIM enzymes and antimicrobial susceptibility testing results of K. pneumoniae

Carbapenem phenotype	MBLenzyme	MIC(µg/ml)								
		*CTX	AN	GM	FEP	CZ	OFX	Cefti	CP	IPM
Positive										
N3	VIM- I	4	32	2	8	128	4	16	4	16
D13[1]	VIM- I	32	4	64	64	128	4	2	8	2
B17	VIM- I	32	16	8	8	256	16	16	4	32
P5	VIM- I	8	2	8	2	64	8	2	32	2
A5	VIM- I	256	32	8	64	256	4	32	16	16
D8	VIM- I	32	2	8	64	≥256	1	1	1	1
N2[2+]	VIM- I	64	1	4	2	≥ 256	1	0.5	1	0.5
23	VIM- I	0.5	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5
D6	VIM- I	1	1	0.5	0.5	2	0.5	1	0.5	0.5
N2[1]	VIM- I	0.5	0.5	0.5	0.5	4	0.5	1	0.5	0.5
Negative										
D8[5]	VIM- I	16	32	8	2	128	8	8	8	1
O14	VIM- I	0.5	0.5	1	4	8	0.5	0.5	0.5	0.5
T17	VIM- I	4	2	1	1	16	1	8	0.5	2
S13	VIM- I	8	32	1	1	8	2	16	1	1
W2	VIM- I	8	16	2	16	8	2	32	1	4

*Cefotaxim (CTX), Amikacin(AN), Gentamicin (GM), Cefepim(FEP), Cephazolin(CZ), Ofloxacin(OFX), Ceftizoxim, Ciprofloxacin(CP), Ticarcillin , Ceftriaxon(CRO), Imipene(IPM)

DISCUSSION

Klebsiella pneumoniae strains are significant causes of nosocomial infections and carbapenems are considered the drug of choice in these cases (3, 16). To our knowledge, this is the first study that compares the (VIM-1) positive and (VIM-1) negative *K. pneumoniae* nosocomial infections in Iran. The prevalence of VIM-1 producing carbapenemases among our isolates was found to be 30% (in 15 samples) during 2-year period. The prevalence of (VIM-1)evaluated in this study is much higher than described in several other parts of Iran (17). The prevalence of carbapenemases reported by Japoni-Nejad et al. was 12%. The sensitivity and specificity of disk diffusion for their identification using imipenem disk were 66.6% and 100%, respectively, whereas meropenem disk demonstrated 100% sensitivity and specificity. Only10% of the isolates were carried a carbapenemase gene, the most common being bla_{VIM}(18) . Recently, there have been several reports on the emergence of VPKP, mainly from several countries (19, 20) .The rate of VIM-1 producers in our study is lower than the result reported by Psichogiou *et al*. and Giani *et al.* (12, 21). This difference in the prevalence of MBL-producing *K. pneumoniae* strains seems to be the result of the variation among the different patients studied and the different rates of antibiotic uses in different hospitals.

Carbapenem MICs are typically low and variable for MBL-producing *K. pneumoniae* . In our study the MICs of imipenem against 15 VPKP isolates ranged from 0.5 to 32 mg/L. Most bla(VIM-1) producing *K. pneumoniae* isolates included in this study did not show a definitive carbapenem resistant phenotype and their carbapenem MICs were still in the susceptible range. For five strains of fifteen, the MIC of imipenem was >1 mg/l .The wide diversity in the MICs of carbapenems from the susceptible to fully resistance range has been attributed to loss of porins and duplications of the blaVIM-1 gene in some of VPKP isolates . Susceptibility to carbapenems

was also documented for most strains isolated of VIM-1 positive *K.pneumoniae* (9, 12). As reported previously, the VPKP isolates were distributed into a relatively large number of chromosomal types, most of which included both carbapenem-resistant and susceptible organisms. The low carbapenem MICs of a significant proportion of the VPKP isolates makes their direct phenotypic identification difficult.

In our study, the most isolates were resistant to more than three antibiotics (Table 2). The highest resistance observed for VPKP included cephazolin 12 (80%). Thirty-hree percent of VPKP isolate (5 strains) were resistant to at least 5 antibiotics including cefotaxim, cephazolin, cefepim, ceftizoxim, and ciprofloxacin. This investigation can be compared with the study of Rastegar Lari and his colleagues in Iran where 19 isolates that were resistant to imipenem, 9 isolates were also resistant to all other antibiotics (17).

None of VPKP isolates was resistant to amikacin. Therefore, carbapenems in combination with another active agent (an aminoglycoside) may provide some therapeutic benefit against VIM-positive carbapenem-susceptible organisms. Our data confirm the need for a systematic screening to detect MBL-producing strains among isolates circulating in high-risk wards such as ICUs can be an effective step in reducing the prevalence of such genes.

Table 2. Results of antibiotic susceptibility testing (MIC) for isolates of *K. pneumoniae* from Babol

Antibiotics						
	R	I	S	R	I	S
CARBAPENEMS						
Imipene	19(38%)	5(10%)	26(52%)	4(26.7%)	3(20%)	8(53.3%)
CEPHEMS						
Ceftizoxim	8(16%)	12(24%)	30(60%)	7(46.7%)	2(13.3%)	6(40%)
Cefotaxim	18(36%)	5(10%)	27(54%)	11(73.3%)	0(0%)	4(26.7%)
Cefepim	12(24%)	6(12%)	32(64%)	3(20%)	3(20%)	9(60%)
Cephazolin	35(70%)	4(8%)	11(22%)	12(80%)	0(0%)	3(20%)
AMINOGLYCOSIDES						
Amikacin	7(14%)	0(0%)	43(86%)	0(0%)	4(26.7%)	11(73.3%)
Gentamicin	17(34%)	4(8%)	29(58%)	1(6.7%)	6(40%)	8(53.3%)
FLUOROQUINOLONES						
Ciprofloxacin	21(42%)	6(12%)	23(56%)	6(40%)	0(0%)	9(60%)
Ofloxacin	13(26%)	12(24%)	25(50%)	3(20%)	3(20%)	9(60%)

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