Journal of Medical Microbiology and Infectious Diseases

JoMMID
ISSN: 2345-5349
eISSN: 2345-5330

The Prevalence of *VIM*, *IMP*, and *NDM-1* Metallo-beta-Lactamase Genes in Clinical Isolates of *Klebsiella pneumoniae* in Qom Province, Iran

Marzieh Saremi[1], Leila Saremi[2], Razieh Nazari[1], Fatemeh Feizy[3], Somayeh Vafaei[4], Ali Lashkari[5], Zohreh Saltanatpour[6]*

[1]Department of Microbiology, Qom Branch, Islamic Azad University, Qom, Iran; [2]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran; [3]Department of Nursing, Hamadan University of Medical Sciences, Hamadan, Iran; [4]Department of Molecular Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences (IUMS), Tehran, Iran; [5]Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran; [6]Stem Call and Regenerative Medicine center of Excellence, Tehran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Original Article

Keywords: *Klebsiella pneumonia*, Metallo-beta-lactamase genes, Antibiotic resistance, PCR

Received: Aug. 12, 2019
Received in revised form: Jun. 27, 2020
Accepted: Jun. 27, 2020
DOI: 10.29252/JoMMID.8.1.34

***Correspondence**
Email: zohre_saltanatpour@yahoo.com
Tel: +98 2166927411
Fax: +98 2166927411

ABSTRACT

Introduction: An increase in the consumption of antibiotics has raised significant concerns over the treatment of *Klebsiella pneumonia*-infected patients. In this study, the resistance pattern of *K. pneumoniae* to antibiotics such as imipenem, meropenem, and ertapenem, as well as the frequency of Metallo-beta-lactamase (MBL) genes, namely *VIM*, *IMP*, and *NDM-1* were investigated. **Methods:** Following the isolation of 200 *K. pneumoniae* isolates from 650 clinical samples, the antibiotic resistance pattern of these isolates against different antibiotics was evaluated. The isolates resistant to imipenem, meropenem, and ertapenem were identified, and the presence of *VIM*, *IMP*, and *NDM-1* genes was examined by using PCR methods. **Results:** The *K. pneumoniae* isolates exhibited different resistance patterns in response to various antibiotics. The frequency of *VIM*, *IMP*, and *NDM-1* genes showed that 48 strains are resistant to imipenem, meropenem, and ertapenem in which 15.6% was positive for *IMP*, 2.42% for *VIM*, and 1.92% positive for *NDM-1* gene. The isolates showed the highest antibiotic resistance to ampicillin (97.5%) and the lowest to meropenem (5.5%). **Conclusion:** Considering carbapenem antibiotics such as imipenem, meropenem, and ertapenem which are known to be among the most frequently used antibiotics for the treatment of *K. pneumoniae* infections and the involvement of MBL genes in this scenario, we aimed to screen and identify MBL genes responsible for the resistance of *K. pneumoniae* to imipenem, meropenem, and ertapenem.

INTRODUCTION

Klebsiella pneumoniae causes a wide range of infections, including urinary tract infections, pneumonia, liver abscesses, and bacteremia [1]. The high prevalence of broad-spectrum MBL genes in different bacteria such as *Escherichia coli* and *K. pneumoniae* isolated from clinical specimens has resulted in the emergence and speared of antibiotic resistance and mortality of patients. Beta-lactam antibiotics are among the most frequently used antibiotics with a higher percentage of resistance comparing to the other antibiotics [2]. Several reports about the internal and external transmission of *K. pneumoniae* showed that there are multiple ways of transmission, such as from one patient to another, from one hospital to another, or even from patients to home [3].

Carbapenems are categorized as beta-lactam antibiotics including imipenem, meropenem, and ertapenem, which are characterized by the presence of beta-lactam ring and synthesized from Tunicamycin. These compounds are resistant to many β-lactamases and chromosomal class A beta-lactamase which neutralize the efficacy of third-generation cephalosporin [4, 5].

Various groups of MBLs, such as Verona integron encoded Metallo-beta-lactamase (*VIM*), Imipenemase (*IMP*), New Dehli Metallo β-lactamase (*NDM-1*), Sao Paulo Metallo β-lactamase (SPM), and Seol Imipenemase Metallo β-Lactamase (*SIM*) have been identified in *K. pneumonia* [6, 7]. The genes responsible for these enzymes are usually transported by integrons. The ability of MBLs to transfer and hydrolyze carbapenems has raised many concerns about the development of effective antibiotics [8, 9].

NDM-1, a new enzyme that enables bacteria to resist many antibiotics, has raised a serious challenge in hospitals [10]. *NDM-1* is a wide-spectrum MBL that can halt the

[DOI: 10.29252/JoMMID.8.1.34]

activation of all β-lactam antibiotics [11] such as penicillin, cephalosporin [12], and carbapenems [13]. In 2011, NDM-1 and KPC enzymes were introduced as global problems, and their impact was assessed in infectious diseases like AIDS, tuberculosis, and malaria [14]. The resistance to carbapenems induced by various types of IMP has become a serious problem in Korea and other Pacific countries [15]. Studies demonstrated that VIM and IMP enzymes share less than 40% identity across the amino acid sequence of the full-length protein [16]. The genes encoding these two enzymes are part of a gene cassette located on class 1 integrals. In Italy, *Pseudomonas putida* isolates originated from nosocomial infections contained the *VIM* gene and exhibited resistant to imipenem, meropenem, and ertapenem [17].

The increased rate of gram-negative MBL-producing organisms, especially isolated from the ICUs, has been reflected in numerous annual reports, [18]. The Center for Disease Control and Prevention (CDC) has proposed PCR and Real-Time PCR techniques for the identification of the genes responsible for the development of resistance [19]. Many scholars in different countries have deployed these methods to identify these genes. In the present study, we aimed to determine the antibiotic resistance pattern among *K. pneumonia* isolates, we also examined the frequency of resistance among the strains to imipenem, meropenem, and ertapenem, as well as the prevalence of genes encoding *VIM*, *IMP*, and *NDM-1* Metallo-beta-lactamase in the isolates obtained from patients living in Qom province.

MATERIAL AND METHODS

Clinical specimens. We collected the clinical specimens from patients hospitalized in different hospitals of Qom, a city 140 km to the south of Tehran, from October 2014 to September 2016. The specimens comprised urine, blood, burn and surgical wounds, respiratory secretions, and secretions from ears, nose, and eyes. The demographic features of the patients, including name, gender, age, duration of hospitalization, as well as the type of clinical specimen were documented in a questionnaire and immediately transferred to a research laboratory of Islamic Azad University, Qom, Iran. The specimens were cultured in the MacConkey Agar as a differential-selective medium; the bile salts and violet crystals in this medium inhibit the growth of gram-positive bacteria. The *K. pneumoniae* colonies were identified based on morphological features and used for further investigations.

Isolation and identification of *K. pneumoniae.* From the solid medium, a few colonies of bacteria were removed by a wire loop and a mixture with a drop of the physiology serum was placed on clean slides and fixed by air drying. The slides were stained with crystal violet dye and then rinsed with water for one min. In the next step, the extension was done with adjacent to lugol iodin staing for 1 min. The slides were washed with water, destained with alcohol, and rinsed with water. The slides were stained with fuchsine for 30 s, and then thoroughly washed and dried. All of the slides were examined for the presence of gram-negative bacilli by optical microscopy. For gram-negative colonies of bacteria, the streak plate method was used several times, to make sure that only one *K. pneumoniae* strain was present in the culture. After gram staining of single-cell colonies and ensuring their purity, differential tests were performed to detect *K. pneumonia.*

Triple Sugar Iron test. At the first stage, the Triple Sugar Iron (TSI) test was used to prove that the isolated gram-negative bacteria were *K. pneumoniae*. The bacteria were cultured deep and superficial on TSI medium cultures and incubated at 37°C for 24 h.

Catalase test. To detect catalase activity of the isolated bacteria, a drop of hydrogen peroxide (30%) was poured on the slide, then a colony of the bacterium was mixed. The bubbles, presumably oxygen gas, indicated the presence of catalase enzyme.

Oxidase test. A filter paper was moistened with a drop of oxidase reagent, and some bacterial colonies were rubbed onto it. The appearance of a purple color, after a few seconds, indicated oxidation activity.

Citrate test. A colony was removed from the media and diagonally cultured in a tube containing citrate and the agar medium. The gradual color changes of the cultures to dark blue after 24-48 h at 37°C, indicated citrate consumption of bacteria and citrate positivity. Moreover, the MR-VP and SIM tests were performed as described by others [20].

Antibiotic sensitivity test. Antibiotic resistance of the isolates was evaluated by the Kirby-Bauer disc method according to the CLSI guidelines.

Identification of Metallo-beta-lactamase isolates. Double Disk Synergy Test (DDST) method was used to identify the isolates bearing MBL. In this experiment, 5ml of 0.5 M EDTA solution was added to imipenem discs, allowed to dry, and then incubated for 12 weeks at 4°C. Imipenem-resistant strains were cultured on Muller Hinton medium. Then, one imipenem disc (10 μg) containing 930 μg EDTA was placed at an appropriate distance on a plate surface and incubated for 16-18 h at 37°C. An increase in the diameter of the inhibition zone around the imipenem disc containing EDTA indicated the production of Metallo-β-lactamase.

PCR detection of Metallo-β-lactamase genes. PCR assay was used to detect the MBL genes in *K. pneumoniae* isolates obtained from clinical specimens. Three genes, namely *VIM*, *IMP* and *NDM-1* were amplified using the primers designed by using the primers which were evaluated using the Gene Runner software (Table 1).

Bacterial DNA was extracted by using the boiling method as described elsewhere [21]. The amplification conditions for *IMP* reactions were as follows: one cycle at 94°C for 5 min, 36 cycles at 94°C for 30 s, 52°C for 40 s and 72°C for 50 s, followed by a final extension at 72°C for 5 min. The amplification conditions for *NDM-1* and *VIM* were similar to *IMP*, except the annealing temperatures, which were 48°C and 50°C, respectively. PCR products were electrophoresed on 0.8% agarose gels, stained with the ethidium bromide, and photographed.

[DOI: 10.29252/JoMMID.8.1.34]

Table 1. The list of designed primers

Primer type	Sequence
IMP F	5' GGA ATA GAG TGG CTT AAC TCT C 3
IMP R	5' CCA AAC CAC TAG GTT ATC T 3'
VIM F	5'GGT CTC ATT GTC CGT GAT GGT GAT 3'
VIM R	5'CTC GAT AGT CCT TCT AGAG 3'
NDM-1 F	5'GGTTTGGCGATCTGGTTTTC3'
NDM-1 R	5'CGG AAT GGC TCA TCA CGATC3'

RESULTS

Isolation of *K. pneumonia.* For identification of *K. pneumonia* isolates, the gram-negative colonies on McCanky Agar medium were selected based on morphological features, and then positive catalase and negative oxidase bacteria were screened. The colonies that possessed the *K. pneumoniae* features were chosen. The biochemical and differential tests are shown in Table 2.

Out of the 200 biochemically identified *K. pneumoniae* isolates, 10 (5%) were from respiratory secretions, 173 (86.5%) from urine, 4 (2%) from ears, nose, eyes secretions, 10 (5%) from burn wounds, and one (0.5%) from cerebrospinal fluid.

Antibiotic resistance pattern. Among the 200 *K. pneumonia* isolates, resistance to ticarcillin/clavulanic acid and tobramycin showed the highest and lowest prevalence, respectively (Table 3).

Among the 200 examined isolates, 48 (24%) were resistant to imipenem, ertapenem, and meropenem, and were used for phenotypic experiments to detect MBL genes responsible for the resistance of *K. pneumoniae*.

Table 2. Biochemical characteristics used to identify *K. pneumoniae* isolates obtained from patients in hospitals, Qom, Iran.

Biochemical tests	Catalase	Oxidase	VP	MR	*Simon citrate*	TSI	SIM
K. pneumoniae	+	-	+	-	+	A/A	-

Table 3. Frquency of antibiotic-resistant isolates

Antibiotics	Resistant isolates (%)	Frequency of resistant isolates (No.)
Amikacin	31.5	63
Gentamacin	27.5	55
Ceftazidime	55	110
Ciprofloxacin`	28.5	57
Ceftriexon	42.5	85
Cefotaxim	51.5	103
Meropenem	5.5	11
Ertapenem	11.5	22
Imipenem	7.5	15
Amoxicillin	93	186
Amoxicillin-clavulanic acid	93.5	187
Ticarcillin	72.5	145
Ticarcillin-clavulanic acid	85	170
Ampicillin	97.5	195
Cephtazoxim	32.5	65
Aztreonam	71	142
Terometoprim-solfometaxazoll	63.5	127
Cefepime	36	72
Piperacillin	45	95
Piperacillin-tazobactam	37	74
Leofloxacin	48	96

Phenotypic test for Metallo-β-lactamase. The phenotypic test was carried out to evaluate the characteristics of MBL-producing strains. The results showed that among 48 strains resistant to imipenem, meropenem, and ertapenem, 15 (31.25%) showed the phenotype belonging to MBL. The minimum inhibitory concentration (MIC) of imipenem among imipenem-resistant *K. pneumoniae* isolates revealed 4 isolates (1.92%) with a MIC of 4 µg/L, 5 (2.4%) with a MIC of 8 µg/L, 9 (2.06%) with a MIC of 16 µg/L, 2 (0.95%) with a MIC of 32 µg/L, 3 (1.44%) with a of 64 µg/L, 2 (0.95%) with a MIC of 128 µg/L, 3 (1.44%) with a MIC of 256 µg/L, and 5 (2.44%) with a MIC of 512 µg/L.

Amplification of *IMP*, *VIM*, and *NDM-1* genes. The PCR assay showed that among 48 imipenem-resistant *K. pneumonia* isolates, 13 were positive for the *IMP* gene confirmed by the presence of a 188 bp amplicon (Fig. 1A). Also, five isolates yielded a 290 bp amplicon, indicative of the *VIM* gene (Fig. 1B). Finally, four isolates were positive for the *NDM-1* gene as confirmed by the presence of 623 bp amplicon (Fig. 1C).

[DOI: 10.29252/JoMMID.8.1.34]

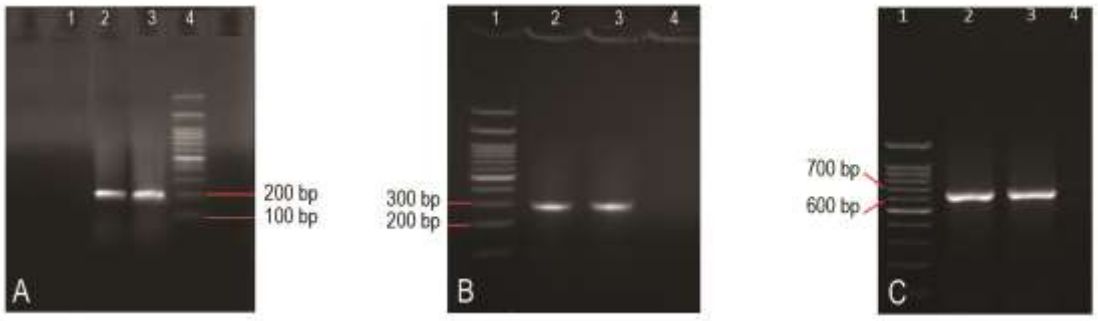

Fig. 1. A) PCR amplification of the *IMP* gene. Lane 1, negative control; lanes 2 and 3, IM- positive *K. pneumoniae* isolates; lane 4, a 100-1500 bp molecular marker. B) PCR amplification of the *VIM* gene. Lane 1, a 100-150 bp molecular marker; lanes 2 and 3, *VIM* positive *K. pneumoniae* isolate; lane 4, negative control. C) The PCR amplification of the *NDM-1* gene. Lane 1, 100-1500 bp DNA marker; lanes 2 and 3, *K. pneumonia* isolated positive for the *NDM-1* gene; lane 4, negative control.

DISCUSSION

K. pneumoniae infections are widely reported in the hospital. These bacteria are considered opportunistic pathogens and cause various types of infectious diseases in hospitalized patients, especially in surgical, burn, and ICU wards. Numerous studies have shown that most of *K. pneumoniae* strains are resistant to the majority of available antibiotics, and these multi-drug-resistant strains are rapidly expanding among hospitalized patients, making difficulties in the treatment procedures. There are several mechanisms underlying the antibiotic resistance of *K. pneumonia* bacteria. The production of MBL enzymes is one of the main underlying mechanisms of *K. pneumoniae* resistance. In the present study, the antibiotic resistance patterns and the frequency of MBL genes (*IMP*, *VIM*, and *NDM-1*) in strains isolated from hospitals of Qom were investigated. Our results showed that 48 strains are resistant to imipenem, meropenem, and ertapenem in which 15.6% was positive for *IMP*, 2.42% for *VIM*, and 1.92% positive for *NDM-1* gene. Some studies showed that, with respect to the relative increase in resistance, ciprofloxacin and its derivatives such as quinolone, Amikacin such as aminoglycoside, and Imipenem such as beta-lactam have been the most effective antibiotics for the treatment of *Pseudomonas* infections [22-24]. In our study, the most effective aminoglycoside, quinolone, and beta-lactam were tobramycin, ciprofloxacin, and imipenem, respectively. Consistent with our results, various research has implicated that imipenem is a highly effective antibiotic for the treatment of *K. pneumonia* [25]. In Japan, the resistance to carbapenem is annually reported, and only two MBL genes, namely *IMP* and *VIM* are responsible for this resistance [26]. The prevalence of the *VIM* gene in Iran is lower than that of other countries such as South Korea, Japan, and France. Other resistance mechanisms such as efflux pumps, defects, and the lack of external membrane proteins, including OprD and Carbapenema class D might be involved in the emergence of resistance to Carbapenems [27].

A study in India showed that most bacteria resistant to Carbapenems were positive for the *NDM-1* gene. In June 2011, the CDC reported the presence of the *NDM-1* gene in *Providencia stuartii* isolated from the blood of a soldier burned in Bagram, Afghanistan. Given the importance of this type of resistance [28], and the neighborhood of Iran with countries in which the presence of NDM-1 has reported, it would be necessary to screen and examine such resistance in infected individuals [29]. The long-term use of a specific antibiotic not only results in the development of bacteria resistance to that combination, but also plays a crucial role in the emergence of resistance to other antibiotics.

In addition the lack of attention to antibiotic susceptibility testing, whether in the stage of testing and reporting the results, or in terms of considering the reported results at the time of prescribing and treating the disease, is also effective in causing resistance [30, 31]. Several preventive strategies must be addressed the prevalence of resistant organisms, including the avoidance the over self-prescriptions and choosing the proper antibiotic in the treatment phase.

ACKNOWLEDGMENT

The authors are grateful to the Islamic Azad University of Qom for financial support.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

ETHICAL APPROVAL

All assays in the present study that included human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

All the participants consented to be enrolled in the study under the direction of the Iranian Foundation of Martyrs and Veterans Affairs review board. Before admission to the study, informed consent was obtained from all participants.

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[DOI: 10.29252/JoMMID.8.1.34]

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Cite this article:

Saremi M, Saremi L, Nazari R, Feizy F, Vafaei S, Lashkari A, Saltanatpour Z. The Prevalence of *VIM*, *IMP*, and *NDM-1* Metallo-beta-Lactamase Genes in Clinical Isolates of *Klebsiella pneumoniae* in Qom Province, Iran. J Med Microbiol Infect Dis, 2020; 8 (1): 34-39. DOI: 10.29252/JoMMID.8.1.34.

[DOI: 10.29252/JoMMID.8.1.34]