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Antibiotic Resistance among *Klebsiella pneumoniae* Isolates by Detecting *blaVIM* and *blaNDM* Genes

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ABSTRACT

Introduction: The emergence of extended-spectrum β-lactamase (ESBL) and carbapenem-resistant *Enterobacteriaceae*, especially *Klebsiella pneumoniae* isolates, has become a severe concern worldwide. This study aimed to determine the prevalence of *blaVIM* and *blaNDM* genes among *K. pneumoniae* isolates. **Methods:** One hundred-eighty-one *K. pneumoniae* isolates were obtained from different clinical specimens of patients hospitalized at Firoozgar hospital, Tehran, Iran. The isolates were identified by standard biochemical tests, and their identity was confirmed by Vitek 2 (bioMérieux, France), a fully automated system for bacterial identification. The isolates were subjected to antimicrobial susceptibility testing and screened for ESBL by double-disc synergy test (DDST) and modified Hodge test (MHT) for the detection of carbapenemase. PCR was also used to detect the presence of *blaVIM* and *blaNDM* resistance genes in the isolates. **Results**: The Vitek 2 system confirmed the biochemical test results. The highest and lowest rates of resistance to antibiotics belonged to cefepime (83.9%) and imipenem (55.2%). Eighty-six and 100 isolates showed to produce ESB and KPC by DDST and MHT, respectively. About 71% and 97% of the 100 isolates were positive for *blaVIM* and *blaNDM* genes, respectively. **Conclusion:** The high rate of ESBL- and KPC-producing *K. pneumoniae* isolates in our hospital setting revealed resistance to conventional antibiotics, which limit our options in choosing appropriate antimicrobials. Although the management of infections associated with these organisms is challenging, it is essential to control such strains to prevent the outbreak.

INTRODUCTION

In recent decades, the World Health Organization (WHO) has reported antimicrobial resistance (AMR) as one of the leading public health problems worldwide [1]. The emergence of strains' resistant to nearly all conventional antimicrobials has become a global concern [2]. This issue includes various members of *Enterobacteriaceae* that express the resistance enzymes, such as carbapenemase- and extended-spectrum β-lactamases (ESBLs)-producing *Klebsiella pneumoniae* bacteria [3]. Among *Enterobacteriaceae*, the worldwide emerging ESBL-producing *K.pneumoniae* isolates pose a severe threat in infection control management [4]. Generally, carbapenemase enzymes, identified in *Enterobacteriaceae*, belong to one of the three classes of ß-lactamase, *i.e*., class A, B, or D [5]. To date, in class B carbapenemases, various types of metallo-β-lactamases (MBL) have been recognized, including *IMP*, *VIM*, *NDM*, *SPM*, and *GIM* [6]. New Delhi Metallo-β-lactamase (*NDM*) and Verona Integron-Mediated Metallo-β-lactamase (*VIM*) are the most common MBL in *Enterobacteriaceae* species [7]. *NDM* was first detected in Sweden in 2008 in a patient previously admitted for a urinary tract infection in India, and by 2010 was detected in many of the parts of the world [8]. The first type of *VIM* was identified in a *P. aeruginosa* clinical isolate in Verona, Italy [9]. In the past few years, many studies have reported the spread of *VIM*-type MBLs in *Enterobacteriaceae*. Recently, the prevalence of *VIM*-type MBLs producing *K. pneumoniae* strains was reported in Greece [10], France [11], Italy [12], and Algeria [13].

In the members of the *Enterobacteriaceae* family, these enzymes are on horizontally transferable plasmids and are transferred from one bacterium to another. Also, *blaNDM* and *blaVIM* are clinically active against many β- lactam antibiotics [4].

The prevalence of carbapenem-resistant bacteria is on the increase, and the data on the frequency of *blaVIM* and *blaNDM* genes in *K. pneumoniae* isolates in Iran is scarce. This study aimed to determine the prevalence of MBL-encoding genes, *blaVIM* and *blaNDM* in *K. pneumoniae*

isolates originated from clinical specimens of patients in Firoozgar hospital, Tehran, Iran.

MATERIAL AND METHODS

Bacterial Isolates. Between March and December 2018, 181 *K. pneumoniae* isolates were collected from different wards at Firoozgar Hospital, Tehran, Iran. The *K. pneumoniae* colonies were identified using standard biochemical tests, including Gram staining, indole, motility, citrate, urease, lactose fermentation, lysine decarboxylase, and MR-VP as described elsewhere [14]. For sample collection, written consent was obtained from each participant and/or their legal representative, as appropriate. The ethics committee of Qom University of Medical Sciences approved the study (No: 15430507951011).

VITEK® 2 SYSTEM. The identity of the isolates was confirmed by the automated system Vitek 2 (bioMérieux, France), an automated microbiology system utilizing growth-based technology. The VITEK 2 system accommodates the same colorimetric reagent cards that are incubated and interpreted automatically for 4 hours. Calculations are performed on raw data and compared to thresholds to determine reactions for each test [14].

Antibiotic Susceptibility Testing. Antibiogram analysis was performed for all 181 *K. pneumoniae* isolates using the disk diffusion method on Mueller-Hinton agar following the CLSI guideline, 2016 [15].

The 12 antibiotic disks used in this assay included ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), doripenem (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), piperacillin (100 µg), and aztreonam (30 µg). All the antibiotic disks were purchased from Mast Company, UK. The *K. pneumoniae* strain ATCC 13883 was used in assays as a positive control.

Double disc synergy test (DDST) for detection of ESBL. Four antibiotics were used in DDST including ceftazidime (30 µg), ceftazidime- clavulanic acid (30 µg/10 µg), cefotaxime (30 µg) and cefotaxime- clavulanic acid (30 µg/10 µg). The discs were placed on Muller Hinton agar plates at a distance of 1.5 cm. A 0.5 McFarland standards of suspected ESBL-producing isolates were inoculated and incubated at 37°C for 24 h. A difference of ≥5 mm between the zone diameter of ceftazidime/cefotaxime and clavulanic acid was defined as ESBL production. *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603 strains were used as the negative and positive controls in the assays, respectively [16].

Modified Hodge Test (MHT) for detection of *carbapenemase.* The MHT was done on all isolates, as recommended by the CLSI, 2016 [15]. Simply, an ertapenem disk was placed on an agar plate inoculated with *E. coli* ATCC 25922, and The isolates were inoculated in a straight line followed by incubation at 35ºC for 16–24 h. the appearance of a clover leaf-like shape exhibited the production of carbapenemase. *K. pneumoniae* ATCC1705 (carbapenemase positive) and *K. pneumoniae* ATCC1706 (carbapenemase negative) were used as controls [17].

Amplification of *blaVIM* and *blaNDM* genes. A PCR assay was used to detect *blaVIM* and *blaNDM* genes in the isolates that became positive with the modified MHT. The *blaVIM* and *blaNDM* genes were amplified with specific primers to prevent false-positive results [18]. The primers were designed by using IDT software and synthesized by a commercial company (Macrogen, Korea). The reaction contained 2.5 µl 10X PCR buffer, 0.5µl dNTPs 10 mM, 0.75µl $MgCl_2$ 10mM, 10 pmol of forward and reverse primers, 0.5 U *Taq* DNA polymerase, and H_2O to 25µl final volume. The amplifications included one cycle of 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 58C, and 30 s at 72°C and a final extension for 8 min at 72°C.

Table 1. The primers used for detection of *blaVIM* and *blaNDM* genes

Gene	Primer sequences 5' to 3'	Amplicon (bp)	Reference
blaVIM	F: CGGCAGCAGTTTGTTGATTG R: CAGACGACGGCATAGTCATTT	150	Present study
blaNDM	F: GAGGTCCGACTTTACCAGATTG R: AGAACTGCCGCTGTGTTT	161	Present study

Statistical analysis. The generated data were analyzed by SPSS version 23.0 (SPSS, Chicago, IL) using the Chi-square test. The value of $p< 0.05$ was considered a significant statistical difference.

RESULTS

***K. pneumoniae* isolates.** The *K. pneumoniae* isolates, identified by biochemical tests, belonged to 93 males and 88 females and included 35 isolates from urine samples, 26 from blood, 65 from aspirates, 40 from sputum, and 15 from other clinical samples. The automated Vitek 2 system with 99% probability identified *K. pneumoniae* isolates and confirmed the biochemical tests (Fig. 1).

Antibiotic susceptibility testing. The majority of isolates showed high resistance to most of the tested antibiotics with the highest rate to cefepime (83.9%), followed by gentamicin (82.7%) and cefotaxime (83.5%) (Fig. 2). Data analysis demonstrated that carbapenems, including imipenem, meropenem, ertapenem, and doripenem, were of higher antibacterial activity against *K. pneumonia* isolates. Based on the result of the combination disc method, 86 (47.51%) isolates were ESBLs positive, and 95 (52.48%) were ESBLs negative (Fig. 3). One hundred (55.24%) isolates exhibited KPC production by MHT assay, i.e., the appearance of a clover leaf-shape (Fig. 4).

PCR assay. The PCR showed the prevalence of *blaVIM* and *blaNDM* genes among carbapenems positive isolates to be 71 (71%) and 97 (97%), respectively. Moreover, both genes coexisted in 70 isolates (Table 2, Fig. 5A, Fig. 5B).

Organism Quantity: cfu/mL
Selected Organism : Klebsiella pneumoniae ssp pneumoniae

Source: urine Collected:

Comments:	

Identification Information	Analysis Time:	4.00 hours	Status:	Final
Selected Organism	99% Probability Bionumber:	Klebsiella pneumoniae ssp pneumoniae 2207734653164010		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	+	4	PyrA	-	5	IARL	-	7	dCEL	+	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	-	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Fig. 1. Identification of *K. pneumoniae* isolates by automated system Vitek 2

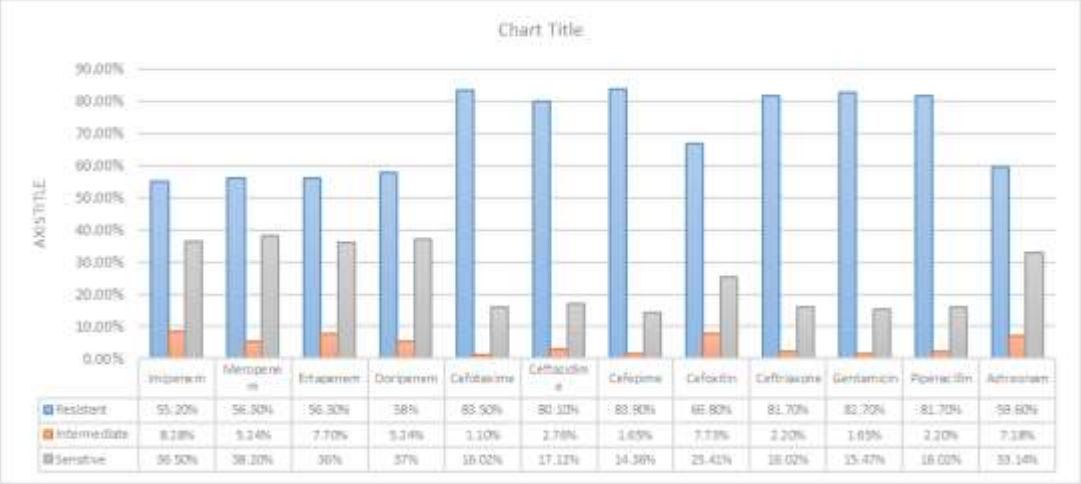

Fig. 2. Antibiotic susceptibility patterns among *K. pneumoniae* isolates.

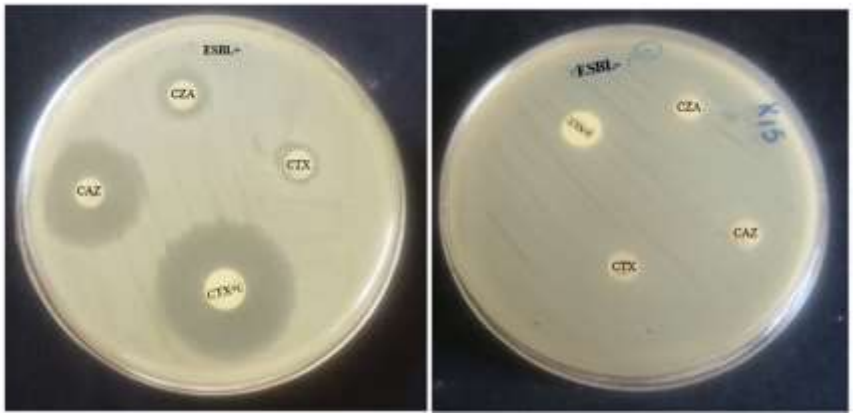

Fig. 3. Detection of ESBL production tested by the Double disc synergy test (DDST). CZA (ceftazidime + avibactam), CAZ (ceftazidime), CTX (cefotaxime) and CTX+ C (cefotaxime + clavulanic acid)

Fig. 4. Modified Hodge test (MHT) showing positive and negative results. 1, Negative control *K. pneumoniae* BAA1706; 2, MHT positive isolate; 3, MHT negative isolate; 4, positive control, *K. pneumoniae* BAA1705.

Table 2. The frequency of *blaVIM* and *blaNDM* genes in different clinical specimens.

Genes	Source of Sample					
	Blood	**Sputum**	**Aspirate**	**Urine**	**Other**	Total
blaVIM	10	17	26	13	5	71
blaNDM	17	19	34	19	8	97
blaVIM and *blaNDM*	10	17	25	13	5	70

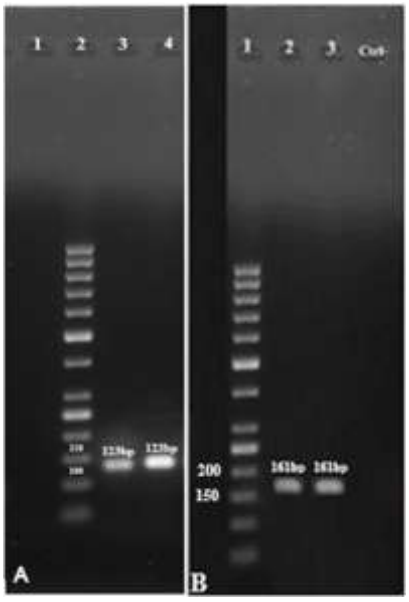

Fig. 5. Gel electrophoresis of amplified *blaNDM* and *blaVIM* genes. **A.** *blaNDM,* lane 1, negative control; lane 2, 50 bp DNA ladder; lane 3, clinical specimen; lane 4, positive control (*K. pneumoniae* ATCC 13883 strain). **B.** *blaVIM,* lane 1, 50 bp DNA ladder; lane 2, clinical specimen; lane 3, positive control, *K. pneumoniae* ATCC 13883 strain.

DISCUSSION

Antibiotic resistance is one of the most significant concerns in public health. Infections by extended-spectrum β-lactamase-producing *K. pneumoniae* (ESBL-KP), and carbapenem-resistant *K. pneumoniae* (CRKP) can cause a severe nosocomial infection [19-20].

In the present study, we investigated the prevalence of ESBL and carbapenemase-associated genes by phenotypic and genotypic methods in *K. pneumoniae* clinical isolates. Phenotypic assays showed a remarkable prevalence in ESBL- and KPC-producing *K. pneumoniae* isolates, and PCR confirmed the results. In this study, of 181 *K. pneumoniae* clinical isolates, 86 (47.5%) were ESBL producers. In a similar study, the prevalence of ESBL producing *K. pneumoniae* isolates in a teaching hospital was 35%, which is almost in agreement with our results [19]. Also, a recent study reported that 55.4% of *K. pneumoniae* clinical isolates from Imam Khomeini hospital in Tehran were ESBL producers [21]. Other similar studies have reported the prevalence rates of 53.8% [4], and 57.14% [22]. In contrast, in the Middle East and some countries, lower rates of ESBL producing isolates were reported, which might reflect different policies in using antibiotics in each region [23].

The Vitek 2 system can identify gram-negative rods within 4 h. This system has shown to be an accurate, easy, and rapid tool for the identification of *K. pneumoniae* isolates [24]. In our study, the Vitek 2 system demonstrated a similar rate of accuracy in the identification of *Klebsiella* and identified the isolates to the species level. Similar studies have employed the Vitek 2 system for the identification of carbapenem-resistant *Enterobacteriaceae* and the investigation of antibiotic susceptibility of Gram-negative rods and Gram-positive cocci [6].

Currently, PCR is the gold standard for the detection of ESBL-producing and KPC-producing bacteria [25-26]. In our study, among 100 KPC-producing isolates, PCR detected *blaVIM* and *blaNDM* genes in 71 (71%) and 97 (97%) isolates, respectively. However, we did not perform the assay to detect other KPC-producing genes, including *blaKPC*, *blaGES*, *blaOXA-48*, and *blaIMP*. Some studies have reported lower prevalence rates compared to the current study. For example, in Brazil, only 2.06% isolates contained *blaNDM*, and 0.72% *blaVIM* [27]. In another study, of 170 *K. pneumoniae* clinical isolates four became positive for *blaNDM* [28].

K. pneumoniae clinical isolates showed the highest resistance rates to cefepime (83.9%), gentamicin (82.7%), and cefotaxime (83.5%). According to our findings, the isolates had a higher resistance rate to cephalosporins. The current study revealed that the *K. pneumoniae* KPC-producing were more susceptible to the carbapenems (meropenem 38.2%, imipenem 36.5%, ertapenem 36%, and doripenem 37%) compared to the cephalosporins (cefotaxime 16.02%, cefepime 14.36%, and ceftazidime 17.12%). These findings could be of significance for treating Gram-negative infections with carbapenems, which is consistent with the result of a similar study [29].

In the present study, 55.2% (100 of 181) strains were positive for KPC by the MHT method, which is similar to the results obtained by Hagihashemi et al. (2012), and Cury et al. (2012) [30, 31]. Therefore, MHT suggests as a useful method for detecting carbapenemase production. The most prevalent *NDM* and *VIM* types of carbapenems have become a warning topic due to their unexpected epidemiological changes in antibiotics resistance, idiotypic diversity, variable region gene, and rapid and global outbreak in *Enterobacteriaceae*, particularly *K. pneumoniae* [32]. Strict supervision on the usage of antibacterial medications and the application of suitable and fast molecular methods for the detection of the contributing genes can assist us in eliminating or reducing the spread of type ESBLs and carbapenemases.

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CONFLICT OF INTEREST

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

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