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ORIGINAL ARTICLE

Analysis of carbapenemases genes of carbapenem-resistant *Klebsiella pneumoniae* isolated from Tehran heart center

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ABSTRACT

Background and Objectives: Emerging of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is one of the major concerns among healthcare systems. This study aimed to investigate the antibiotic susceptibility pattern and carbapenemase genes of carbapenemase-producing *K. pneumoniae* isolates obtained from Iranian hospitalized patients.
Materials and Methods: This study was performed on 71 CRKP strains isolated from different clinical specimens collected in Tehran Heart Center (Tehran, Iran). A Modified Hodge test (MHT) was done for the detection of carbapenemase-producing *K. pneumoniae*. The presence of bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and bla_{OXA-48}-type carbapenemases was evaluated by the PCR method.
Results: We identified 8.82% (71/805) of *K. pneumoniae* isolates as CRKP by MHT test. The antibiotic susceptibility indicated that all isolates were resistant to imipenem, meropenem, cefotaxime, ceftazidime, cefepime, ceftriaxone, cephalothin, ciprofloxacin, and augmentin, and then mostly resistant to aztreonam, cefoxitin, gentamicin, and trimethoprim/sulfamethoxazole with 98.6%, 98.6%, 97.2%, and 94.4%, respectively. The lowest resistance was related to amikacin with 46.5% (33/71 isolates). The level of imipenem MIC for all carbapenem-resistant isolates was estimated ≥32 µg/mL. Among positive isolates for carbapenemase genes, the most frequent gene was bla_{OXA-48}. It was found in 48 (67.6%) isolates followed by bla_{VIM} in 28 (39.4%) isolates. $bla_{IMP,}$ bla_{NDM}, and bla_{KPC} genes were identified in 19 (26.8%), 13 (18.3%) and 5 (7.0%) isolates, respectively. These genes were not detected in nine isolates.
Conclusion: The relatively high frequency of some carbapenemase genes suggests major concern about the emergence of isolates containing carbapenem resistance genes as a potential health threat.

Keywords: Carbapenem-resistance; *Klebsiella pneumoniae*; bla_{VIM}; bla_{IMP}; bla_{NDM}; bla_{OXA-48}

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INTRODUCTION

In recent years, the worldwide prevalence of carbapenem-resistant *Enterobacteriaceae* became one of the most concerns among healthcare systems (1). The increasing prevalence of carbapenem-resistant strains poses a serious problem for prevention and control of infection, leading to increased morbidity and mortality of hospitalized patients (2). Carbapenems (imipenem, ertapenem, meropenem and doripenem) are broad-spectrum beta-lactam agents (3), which exhibit the highly effective activity against infections of multidrug-resistant (MDR) Gram-negative bacteria (4). Carbapenems have been frequently used as first-line agents in the treatment of severe infections caused by extended-spectrum beta-lactamase (ESBL)-producing pathogens (5). One of the major emerging carbapenem-resistant bacteria is *Klebsiella pneumoniae (K. pneumoniae)*, known as carbapenem-resistant *K. pneumoniae* (CRKP) (6). *K. pneumoniae* is an opportunistic Gram-negative bacterium that causes serious nosocomial infections such as septicemia, pneumonia, urinary tract infections (UTIs) (7). CRKP has a great role in hospital infections by increasing the length of hospital stay, cost, and mortality (8, 9).

Mechanisms of resistance to carbapenems may be related to the combination of the production of AmpC beta-lactamases, carbapenemases (specific β-lactamases with the ability to hydrolyze carbapenems), efflux pumps, and alteration of the expression and/or function of porins and penicillin-binding proteins (PBPs) (10, 11). The production of various carbapenemases is the most important mechanism of carbapenem-resistance by CRKP. According to the Ambler classification, three main classes of carbapenemases are mostly identified in *K. pneumoniae*, including class A or serine β-lactamases, class B, or metallo-beta-lactamase (MBLs), and class D or oxacillinase-hydrolyzing (OXA) (12). *K. pneumoniae* carbapenemase (KPC), encoded by the bla_{KPC} gene, is a particularly important enzyme-mediated the worldwide rise of CRKP (13, 14). KPC is an Ambler class A plasmid-encoded enzyme that can inactivate all beta-lactam antibiotics, containing monobactams, extended-spectrum cephalosporins, and carbapenems (15). The other carbapenemases include NDM (New Delhi metallo-β-lactamase-1), IMP (imipenem-resistant *Pseudomonas*), VIM (Verona integron-encoded metallo-β-lactamase), and OXA-48 (Oxacillinase-48) were also related to carbapenem resistance in *K. pneumoniae* (12, 16). VIM, IMP, and NDM belong to class B MBLs while OXA-48 is a class D carbapenemases (17). Moreover, some non-enzymatic mechanisms have been described in which outbreaks of CRKP. For example, a decrease in the expression of outer membrane proteins (OMPs), especially OmpK35 and OmpK36 porins, can be associated with increased carbapenem MICs (18). However, it serves only as a minor component of carbapenem resistance, i.e. only carbapenemase production without defective porin expression could strongly elevate carbapenem MICs (19).

Carbapenemases are generally expressed from transferable genetic elements such as plasmids or transposons, which contain a wide range of resistance genes and can be freely transferred between bacteria, from within and between hospitals/region/country and then spread all over the world (5, 20). Therefore, carbapenemase-encoding genes play a vital role in the antimicrobial resistance in *K. pneumoniae*, and their detection and the genome arrangement of particular functions are key areas of current research into CRKP. Notably, the detection of CRKP strains may be difficult relying on routine antibiotic susceptibility testing (3). The molecular techniques have been considered a simple and accurate methods for the quick and precise identification of carbapenemase genes (21). Therefore, studying the frequency of resistant genes is essential for the control of such infections.

The present study aimed to investigate the prevalence and the antibiotic susceptibility pattern of CRKP at a university hospital in Iran with an emphasis on the characterization of bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and bla_{OXA-48}-type carbapenemases in CRKP.

MATERIALS AND METHODS

Bacterial isolates. The study was accomplished as a single-center, cross-sectional study on 71 CRKPs (imipenem and meropenem resistant and Modified Hodge test (MHT)-positive strains) from 805 *K. pneumoniae* isolates collected at Tehran Heart Center (Tehran, Iran) from 2014 to 2018. Strains were isolated from different clinical specimens with *K. pneumoniae* infection [urine culture, blood culture, wound culture, tracheal secretion culture, other body fluids, and central venous catheter (CVP)] and then re-iden-

tified through confirmatory and genotypic tests. Doubtful *K. pneumoniae* colonies were identified according to the Gram staining, growth on MacConkey agar medium, and standard biochemical tests such as indole test, motility, glucose and lactose fermentation, citrate and urease test, lysine decarboxylase, and MR-VP (22).

Antimicrobial susceptibility testing. Susceptibility testing was performed by disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (23). Briefly, strains in liquid medium at a 0.5 McFarland standard concentration were grown on Muller–Hinton agar medium and antibiotic discs (Mast Group Ltd., Bootle, UK) was placed on the medium and was incubated for 18-24 h at 37°C and then the zone of inhibition diameter around the disks was measured. Antibiotic tested comprise: [carbapenems: imipenem (IMP: 10 µg), meropenem (MEM: 10 µg)], cefotaxime (CTX: 30 µg), ceftazidime (CAZ: 30 µg), cefepime (CPM: 30 µg), cefoxitin (FOX: 30 µg), ceftriaxone (30 µg), gentamicin (GEN: 10 µg), amikacin (AMK: 30µg), aztreonam (ATM: 30 µg), cephalothin (CET: 30 µg), ciprofloxacin (CIP: 5 µg), augmentin (amoxicillin 20 µg + clavulanic acid 10 µg), trimethoprim/sulfamethoxazole (CMX: 30 µg).

Modified hodge test. The MHT was performed based on the CLSI guidelines for carbapenem-resistant strains. A 0.5 McFarland suspension of *E. coli* ATCC 25922 was diluted 1:10 in sterile saline and was streaked as a lawn onto a Mueller Hinton agar plate. The plate was left for 5 min for drying and a 10 µg ertapenem disc was placed in the center of the agar plate. The test isolates were inoculated in a straight line from the edge of the disc to the edge of the plate. After 24 h incubation at 37°C, the clover leaf-like indentation between the test streaks near the disk was taken as positive for carbapenemase production (24). An MHT-positive *K. pneumoniae* ATCC BAA-1705 was used as a positive control.

DNA extraction. DNA extraction was carried out by the boiling method as described by Sepehriseresht et al. (25). Briefly, a single *Klebsiella* colony was harvested and washed twice with 200 µl TE buffer [10 mM Tris, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8.0] and was centrifuged at 5000 × g for 2 min. The final pellet was re-suspended in 200 µl sterile distilled water. The cell suspension was heated for 10 min at 100°C and then centrifuged at 8000 × g for 3 min to precipitate the bacterial debris. The supernatant was transferred to a new DNase-free, RNase-free sterile tube. Then, 3 µl of the supernatant was used as a source of template DNA for PCR.

Detection of specific carbapenemase genes by PCR. The existence of carbapenemase genes includes the bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, and bla_{OXA-48} were evaluated using PCR assays. PCR experiments were performed using specific oligonucleotide primers are listed in Table 1 (16, 26). The mixture for the amplification of this gene consisting of 12.5 µl of PCR Master mix 2× (PCR buffer, 1.25 mM $MgCl_2$, 0.2 mM of each dNTPs and 1.25 U of *Taq* DNA polymerase), 1 µl of each primer, and 3 µl of DNA template, in a final volume of 25 µl. Amplification was performed using a BioRad MJ MiniTM PCR system. Amplification was carried out with the following thermal cycling conditions: an initial denaturation step at 94°C for 5 min, and 35 cycles of amplification consisting of 30 s at 94°C, 40 s at specific annealing temperature (bla_{KPC} 55°C, bla_{VIM} 57°C, bla_{IMP} 57°C, bla_{NDM} 52°C, and bla_{OXA-48} 55°C), and 45 s at 72°C. The final extension was at 72°C for 5 min (16, 27). A tube containing all PCR reaction mixtures and *E. coli* DNA as a template was used as a negative control. A carbapenemase-resistant *K. pneumoniae* isolate, which was previously characterized in our laboratory, was used as a positive control. PCR products were separated 1% agarose gel electrophoresis and the band's pattern was analyzed in a gel doc. Positive control and negative control were included in each PCR run.

Statistical analysis. The results are expressed as absolute frequencies and percentages. For the statistical analyses, the statistical software SPSS version 21.0 for Windows (SPSS Inc., Chicago, IL) was used. The curves were plotted using Excel software version 2010 (Microsoft Corporation, USA).

RESULTS

A total, 71 of 805 (8.82%) *K. pneumoniae* isolates were identified as CRKP based on MHT-test. It is worthy to note that 79 isolates exhibited resistance to imipenem and meropenem, but 71 of 79 (89.8%) isolates were characterized as CRKPs by developing

Table 1. The list of primers, annealing temperatures, and expected amplicon sizes for molecular detection of carbapenemases-producing *K. pneumoniae* isolates.

Gene	Primer	Sequence (5'→3')	Tannealing	Product size (bp)	References
bla_{KPC}	KPC-F	CGTCTAGTTCTGCTGTCTTG	55°C	798	
	KPC-R	CTTGTCATCCTTGTTAGGCG			
bla_{VIM}	VIM-F	GATGGTGTTTGGTCGCATA	57°C	390	
	VIM-R	CGAATGCGCAGCACCAG			
bla_{IMP}	IMP-F	GGAATAGAGTGGCTTAAYTC	57°C	232	(16, 26)
	IMP-R	TCGGTTTAAYAAAACAACCACC			
bla_{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTC	52°C	621	
	NDM-R	CGGAATGGCTCATCACGATC			
bla_{OXA-48}	OXA-48-F	CCAAGCATTTTTACCCGCATCKACC	55°C	438	
	OXA-48-R	GYTTGACCATACGCTGRCTGCG			

Abbreviations: bp: base pair; F: forward; R: reverse.

cloverleaf shapes in MHT-test. Totally, of 71 confirmed CRKP isolates, 31 (43.7%) were obtained from females and 40 (56.3%) from male patients. The isolates originated from hospitalized patients with an age range of 18 to 93 years old. Regarding the source of specimens, the highest proportion belonged to urine and tracheal secretion [each 25 (35.2%)] and wound samples [12 (16.9%)]. Meanwhile, 35 (49.3%) isolates were obtained from hospitalized patients in the intensive care units (ICUs). The sources of clinical isolates according to specimens, wards, and sources are shown in Table 2. Moreover, there was no statistical association between the wards and gender with a rate of carbapenem resistance.

Table 2. Clinical characteristics of patients with carbapenem-resistant *K. pneumoniae*

Characteristics	No. (%) of Strains
Specimens	
Urine	25 (35.2)
Blood	6 (8.5)
Wound	12 (16.9)
Tracheal secretion	25 (35.2)
Other body fluid	2 (2.8)
Central venous catheter (CVP)	1 (1.4)
Ward	
ICU	35 (49.3)
Non_ICU	36 (50.7)
Gender	
Male	40 (56.3)
Female	31 (43.7)

Antimicrobial susceptibility testing. The results of antibiotic susceptibility showed that all isolates were resistant to imipenem, meropenem, cefotaxime, ceftazidime, cefepime, ceftriaxone, cephalothin, ciprofloxacin, and augmentin, and then mostly resistant to aztreonam, cefoxitin, gentamicin, and trimethoprim/sulfamethoxazole with 98.6%, 98.6%, 97.2%, and 94.4%, respectively. The lowest resistance was related to amikacin [33 (46.5%) of the isolates] (Table 3). The level of imipenem MIC for all of the carbapenem-resistant isolates was ≥32 µg/mL. In our work, the number of carbapenemase resistance strains showed an increasing trend over the five years at Tehran Heart Center (Fig. 1).

Carbapenemase genes analysis. Fig. 2. shows the results of molecular tests for the detection of genes encoding the carbapenemase. The PCR products of the bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM} and bla_{OXA-48} genes

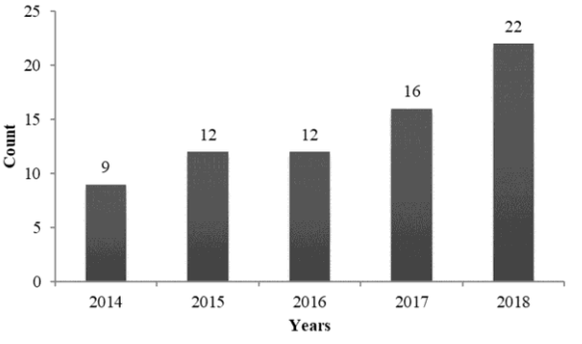

Fig. 1. Distribution of CRKP isolates by years

Table 3. Antimicrobial resistance profile of CRKP isolates

Antibiotic		No. (%) of Antibiotic resistance		
		Resistance	Intermediate	Sensitive
1.	imipenem	71 (100.0)	0 (0)	0 (0)
2.	meropenem	71(100.0)	0 (0)	0 (0)
3.	cefotaxime	71 (100.0)	0 (0)	0 (0)
4.	ceftazidime	71 (100.0)	0 (0)	0 (0)
5.	cefepime	71 (100.0)	0 (0)	0 (0)
6.	cefoxitin	70 (98.6)	0 (0)	1 (1.4)
7.	ceftriaxone	71 (100.0)	0 (0)	0 (0)
8.	gentamicin	69 (97.2)	0 (0)	2 (2.8)
9.	amikacin	33 (46.5)	8 (11.3)	30 (42.2)
10.	aztreonam	70 (98.6)	0 (0)	1 (1.4)
11.	cephalothin	71 (100.0)	0 (0)	0 (0)
12.	ciprofloxacin	71 (100.0)	0 (0)	0 (0)
13.	augmentin	71(100.0)	0 (0)	0 (0)
14.	trimethoprim/sulfamethoxazole	67 (94.4)	1 (1.4)	3 (4.2)

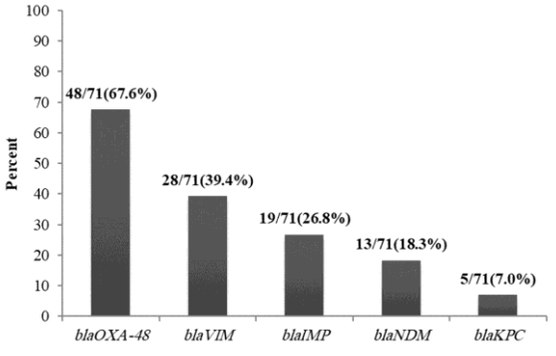

Fig. 2. Presence and distribution of carbapenemase genes in carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates

amplification on 71 CRKP isolates extracted DNAs revealed that nine of the CRKP isolates did not carry any of the genes. Among CRKP isolates, the most frequent gene was $bla_{\text{OXA-48}}$, which was found in 48 (67.6%) isolates followed by bla_{VIM} in 28 (39.4%) isolates. The bla_{IMP}, bla_{NDM} and bla_{KPC} genes was identified in 19 (26.8%), 13 (18.3%) and 5 (7.0%) isolates, respectively. The data showed among all CRKP, $bla_{\text{OXA-48}}$ was the most frequent gene. In addition, the genes code VIM-type enzyme (bla_{VIM}) was the most frequent MBLs. Interestingly, among gene-positive CRKPs (n=62) isolated from different sources, tracheal aspirates were obtained through intubated patients 92.0% (23/25), urine 84.0% (21/24), and wound 75.0% (9/12) of the specimens with at least one carbapenemase-related gene (Table 4).

DISCUSSION

The possession of the different combinations of genes encoding carbapenemases in CRKP isolates is the basis for the high prevalence of its resistance. Here, we aimed to investigate the presence and the distribution of *K. pneumoniae* carbapenemase genes in the CRKP isolates with phenotypic and genotypic methods in patients with different types of infections. Among phenotypic tests that have been established for the recognition of CRKP isolates, the MHT is a specific and sensitive method for the detection of carbapenemase approved by the CLSI (24). In the MHT, 89.8% (71/79) of strains were positive which is relatively similar to the previous findings by Bina et al. (33/41; 80.5%) (28), Haji Hashemi et al. (32/38; 84%) (29) and Hosseinzadeh et al. (27/29; 93.1%) (30). However, the MHT represents a lower sensitivity to MBLs detection and is not specific to serine carbapenemases. The variable results and the false positivity in the MHT test are the other concerns (31). In addition, the MHT is not helpful for the detection of carbapenemase production in non-fermenting Gram-negative bacilli. Recently, other methods such as the Carba NP and the modified carbapenem inactivation method (mCIM) are more reliable phenotypic methods for carbapenemase detection (32).

In this study, most of the isolates were resistant to different antibiotics, suggesting that these isolates may have complex mechanisms of antibiot-

Table 4. Distribution of carbapenemase genes in CRKP isolates by sources

Isolates	Urine (n=25)	Blood (n=6)	Wound (n=12)	Tracheal secretion (n=25)	Other body fluids (n=2)	Central venous catheter (CVP) (n=1)
Non-gene carriers	4 (16.0%)	0	3 (25.0%)	2 (8.0%)	0	0
At least one-gene carriers	21 (84.0%)	6 (100%)	9 (75.0%)	23 (92.0%)	2 (100%)	1 (100%)

ic resistance at the same time. For example, the ESBL activity and outer membrane permeability defects enhance the hydrolysis of carbapenems in OXA-48-producers strains (33). It is demonstrated that the co-existence of beta-lactamase genes enhances antimicrobial resistance, particularly in $bla_{\text{OXA-48}}$ isolates (34). The high incidence of resistance pattern was also probably due to the widespread use of numerous antimicrobial agents and the presence of integrons and plasmids in ESBL-producing bacteria (35). The source of infections and geographical distribution can also affected the rate of antibiotic resistance. The carbapenem-resistance in the isolates without any of the targeted genes (9 isolates) may be due to other mechanisms (or a combination of them) such as decreased susceptibility to ESBLs, AmpC-type betalactamases, reduced permeability of outer membrane or efflux pumps activity, or probably the existence of other genes that were not studied in this research (19).

The most prevalent carbapenemase gene was $bla_{\text{OXA-48}}$, which was detected in 67.6% (48/71) of the isolates (Fig. 2). It was relatively lower than the first cases of OXA-48-producing *K. pneumoniae* in Iran recovering from burn patients (27/28; 96.4%) (26). However, Hosseinzadeh et al. detected $bla_{\text{OXA-48}}$-like genes only in 2 (0.9%) isolates in southwestern Iran (30). Numerous studies in other country had confirmed the presence of the $bla_{\text{OXA-48}}$gene among CRKP isolates in the wide ranges from 1.9% (4/210) in Taiwan (36) to 90.5% in Spanish hospitals (37). To date, OXA-48 is considered the most common carbapenemase in the Middle-East countries (38). The high prevalence of OXA-48-positive CRKP strains has been reported in these countries including 90.3% (84/93) in Turkey (39), 78% (47/60), and 81.5% (44/54) in Saudi Arabia (34, 38), 77.7% (35/45) recovered from countries around the Persian Gulf (40), 96.4% (27/28) (26) and 67.6% (this study) in Iran confirm this. In our study, the bla_{KPC} gene was observed in only 5 (7%) of the isolates which is lower than the results of Farajzadeh et al. (41) and Sedighi et al. (42) who showed the presence of bla_{KPC} gene in 51.8%, and 23% of isolates, respectively. Whereas, bla_{KPC} were not detected by Bina et al. (28), Azimi et al. (26), and Khorvash et al. (43). This indicates that a limited number of our CRKP strains in the present study produce KPC-type carbapenemase.

In the current study, bla_{VIM} was the highest frequency MBLs gene followed by bla_{IMP} and bla_{NDM}. The high prevalence of bla_{VIM} is similar to the prevalence of bla_{VIM} in Zanjan, Iran (41.6%; 5/12) (35). Other studies reported 33% (42), 11.9% (5/42) (44), 10.3% (3/29) (43), and 3.5% (1/28) (26) of isolates harbored bla_{VIM} gene. Given that Zeighami et al. founded bla_{IMP} in 100% (12/12) of MBLs-producing *K. pneumoniae* strains (35), other previous studies did not detect IMP enzyme in clinical CRKP isolates (26, 30, 42), and bla_{IMP} was detected in only one (3.4%) isolate in another study (43). However, other studies speculated that these enzymes producing isolates are not a main problem in the Middle-East countries (34, 38, 39); based on our results, it is worth following their incidence in this region. It has been also suggested that the Middle-East region might be a reservoir for the spread of bla_{NDM} isolates (34, 45). For the first time, $bla_{\text{NDM-1}}$-containing *K. pneumoniae* has been identified in Iran by Shahcheraghi et al. (46). Other studies identified $bla_{\text{NDM-1}}$ gene in 7.1% (3/42) (44), 12.2% (6/49) (47), 11.1% (20/181) (48), and 2.3% (4/170) of *K. pneumoniae* isolates (49). One general concept from previous studies and our results (18.3%) is the low prevalence of $bla_{\text{NDM-1}}$-harboring *K. pneumoniae* strains in Iranian hospitals. However, the high prevalence of bla_{NDM} was reported in 79.3% (23/29) of *K. pneumoniae* isolates in southwestern Iran (30). Therefore, the bla_{NDM}-associated resistance may be increased soon as a potential health warning in Iran and raising the possibility of dissemination of these isolates into our neighboring countries. Therefore, it is necessary to apply effective infection control to prevent the overall spread of bla_{NDM}-carrying

strains in Iran.

In this study, for the first time in Iran, we investigated a possible relationship between genes encoding carbapenemases in CRKP strains and the types of clinical samples. It was found that 92.0% of tracheal aspirates patients have exhibited the presence of at least one carbapenemase-related gene whereas this rate was 84.0% in urine samples. This indicates that patients with *K. pneumoniae*-related respiratory infection should be receiving an appropriate antimicrobial treatment to avoid the distribution of CRKP in Iran. It is also recommended that the clinical microbiology laboratories routinely check OXA- and MBL-producing genes in *K. pneumoniae* isolates with the PCR method. Imipenem and meropenem are considered common drugs for the treatment of nosocomial infections but increasing resistance to these antibiotics has restricted their effectiveness. It can be noticed that the use of another anti-CRKP drug such as amikacin may be probably suitable for treat-ment of *K. pneumoniae* infections.

In conclusion, we have shown that increasing trends of CRKP isolates from 2014 to 2018 at Tehran Heart Center in Tehran wherever the most CRKP isolates were from urine and tracheal secretion specimens. The antibiotic susceptibility indicated that all CRKP isolates were resistant to imipenem, meropenem, cefotaxime, ceftazidime, cefepime, ceftriaxone, cephalothin, ciprofloxacin, and augmentin. Moreover, this study illustrated that $bla_{\text{OXA-48}}$ was the most frequent carbapenemase genes that found in 48 (67.6%) isolates followed by bla_{VIM} in 28 (39.4%) isolates. bla_{IMP}, bla_{NDM}, and bla_{KPC} genes was identified in 19 (26.8%), 13 (18.3%) and 5 (7.0%) isolates, respectively. Therefore, the VIM- and OXA-48- producing *K. pneumoniae* strains is an emerging threat in this medical center and should be focused on timely identification and strict control of infections caused by *K. pneumoniae*.

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