

Molecular Characterization of Extended spectrum beta-lactamases (ESBLs) Carbapenem-Resistant Genes in *Klebsiella pneumoniae* Isolated from Iranian Hospital

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ABSTRACT

Background-Carbapenem-resistant *Klebsiella* isolates are described as one of the most significant problems worldwide. The aim of this study was to determine the prevalence of extended spectrum beta-lactamase (ESBL) subtypes and carbapenem-resistant genes in *Klebsiella pneumoniae* (*K. pneumoniae*) species isolated from patients at Shariatee Hospital in Tehran, Iran.

Methods: Fifty isolates of *K. pneumoniae* were isolated and identified from Shariatee Hospital in Tehran from January 2014 to February 2016. Disk diffusion and E-test minimum inhibitory concentration (MIC) testing methods were carried out to characterize the isolates. Modified Hodge Testing (MHT) and carbapenem inactivation method test (CIM), was used to confirm carbapenemase activity. Finally, Sequencing and polymerase chain reaction (PCR) was applied for further analysis.

Results: Our findings showed that the prevalence of blaCTX-M 33 (63.46%) genes was the most prevalent ones. blaCTX-M₁₅ 19 (36.53%) was found to be the most commonly detected subtype in CTX-M groups. blaKPC, blaOXA-48, and blaNDM1 were detected in 3 (5.76%), 6 (11.53%), and 12 (23.7%) of the carbapenem-resistant isolates, respectively.

Conclusions: Our study displayed that the CIM test has a great potential to detect carbapenem-resistant *Klebsiella* (CRK). Based on our results, there is a need for further considerations regarding the emergence and diagnosis of isolates harboring ESBL subtypes and carbapenem genes. Further attention should be paid for the treatment of patients with highly resistant isolates.

Keywords: *Klebsiella pneumoniae*, New Delhi metallo-beta-lactamase-1, Extended-spectrum β -lactamase

INTRODUCTION

In recent years, *Klebsiella pneumoniae* have become one of the important nosocomial pathogens causing main hospital-acquired infections¹. Outcome of antibiotic treatment for third-generation cephalosporin-resistant Gram-negative has led to the promotion of a selection of β -lactamase producer bacteria^{2,3,4}. Beta-lactam antibiotics are the most widely used class of antibiotics for the treatment of infections caused by *K. pneumoniae*; however, resistance related to these antibiotics has increased due to selection pressure. In general, unwarranted use of third-generation cephalosporins is an alarm for the incidence and distribution of extended spectrum beta-lactamase (ESBL)-producing bacterial isolates, becoming an emerging public health concern¹. The ESBL-producing isolates of *Klebsiella* spp., have been implicated in numerous outbreaks of nosocomial infections throughout the United States of America⁵, Europe⁶, the Far East, and Asia⁷. In recent years, ESBL-K strains have been widespread in Tehran hospitals, but there are relatively little data about β -lactamase genes. Additionally, carbapenem antibiotics are antibiotics of choice for the treatment of invasive infections caused by ESBL-producing isolates. Regarding the high prevalence of strains harboring carbapenem resistance genes, it is not surprising to see significant challenges in the treatment of patients in hospitals. Treatment of infection

caused by carbapenem-hydrolyzing beta-lactamase KPC strains has become a matter of concern in hospitals in Iran due to its multiple drug resistance. Hence, detection of carbapenem resistant-KPC is fundamental to infection control measures and to thwart the distribution of resistant *Klebsiella* strains in hospital settings. The aim of this study was to determine the prevalence of ESBL subtypes and carbapenem-resistant genes (KPC/OXA-48/NDM1) in *K. pneumoniae* spp. isolated from patients in Shariatee Hospital in Tehran, Iran.

MATERIALS AND METHODS

Bacterial strains: This cross-sectional study was conducted from January 2014 to February 2016. Fifty strains of *Klebsiella* were isolated from Shariatee Hospital in Tehran. The *K. pneumoniae* 7881 strain containing the blaSHV and blaTEM genes was applied as a control. Samples were collected from different districts, including Neurology, ICU General, Post HSCT, Internal ICU, BAL (Bronchoalveolar lavage), Post-hematopoietic Stem Cell Transplantation, Blood, Oncology, Hematology, Gland wards, and Emergency. The antimicrobial susceptibility testing was carried out based on the clinical Clinical and Laboratory Standards Institute (CLSI) procedure [11]. The antibiotics used in this study were as follows: Gentamicin

(GM: 10 µg), Meropenem, Ampicillin-sulbactam, (ZOX: 30 µg), Amikacin (AN: 30 µg), Ciprofloxacin (CIP: 5 µg) (BBL), Ceftriaxone (CRO: 30 µg), Cefotaxime (CTX: 30 µg), Imipenem (IMP: 10µg), and Ceftazidime (CAZ: 30 µg). The minimum inhibitory concentration (MIC) was used to determine of Imipenem (IMP: 10 µg), Cp, IMP, MP, CAZ, and CRO (UK, MAST, Merseyside,) against isolates with reduced susceptibility using the E-test MIC assay according to CLSI guidelines.

Modified Hodge Testing (MHT): Carbapenemase-producing bacteria are identified using MHT as previously described⁸. *K. pneumoniae*, MHT Negative *Klebsiella pneumoniae* ATCC1706 positive control and a positive control MHT *Klebsiella pneumoniae* ATCC1705 were used as a control. The presence of a distorted zone (Clover-leaf shaped zone of inhibition) in plates was considered as positive for carbapenemase producing isolates^{8,9}.

Carbapenem Inactivation Method Test (CIM): CIM was performed as previously described with minor modifications^{10,11,12}. Briefly, the isolates were cultured on Mueller-Hinton Agar plates. Afterwards, 10 µl of each isolate was taken by an inoculation loop, and dissolved in 400 µl of sterile deionized water, followed by addition of an active susceptibility standard disc for meropenem (MEM). After two-hour incubation at 32°C, the disk was separated and situated on an M-HA plate, and the plate was inoculated at 35°C for 24 hours with a suspension of OD595 1.25 with a sterile cotton swab. The results of the test were analyzed after 24-hour incubation. Inhibition zone around each disk in plates was measured. Plates with inhibition zones less than 10 mm in diameter were confirmed to be CIM-positive^{8,11,12}.

Polymerase chain reaction (PCR) and sequencing: The boiling method was used to prepare bacterial genomic DNA for PCR reaction. In brief, five colonies of each isolate was dissolved in 300 µl of distilled water for 10 min, followed by centrifugation at 12,000 rpm for 10 min. The resultant supernatant was used for PCR amplification. PCR test was conducted using the specific primers for the blaKPC family.

PCR amplification was carried out for bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{OXA-48}-like, bla_{NDM}, bla_{VIM}, and bla_{IMP} genes, as previously described¹³⁻¹⁸. Alignment of

sequences were carried out using the online BLAST software. Sequences were registered in the GenBank nucleotide database under accession numbers MH359121, MH359122, MH359123, MH369836, MH369837, MH369839, and MH369840.

RESULTS

Colistin was found to be the most active antibiotic as compared with other antibiotics used, while that all of tested isolates showed sensitivity to others antibiotics. The prevalence of obtained samples included Urine (16; 30.76%), BAL (18; 34.61%), Wound (3; 5.76%), Abscess (1; 1.92%), Blood (10; 19.23%), and Sputum (2; 3.8%) (Fig.1). Of all the isolates studied, twenty-nine (55.7%) males and 23 (44.2%) females were infected with *Klebsiella* (Fig.2.3.4). Table 1 indicates the results obtained from disc susceptibility testing with antibiotics. Nine (17.3%) and 43 (83.7%) isolates of *Klebsiella* spp. were detected in the groups containing patients less and more than 50 years old, respectively, showing to be statistically significant ($P < 0.05$) (Fig.2). Isolates with positive MHT were defined as carbapenem-resistant (Fig.3). The result of MIC testing for a particular antibiotic including IMP, Cp, MP, CAZ, and CRO presented a high level of resistance with MIC > 2 (Fig.4). Phenotypic testing revealed that 52 (100%) were ESBL producers. PCR amplification using gene-specific primers displayed that 33 (63.46%), 18 (34.61%), and 20 (38.46%) of the isolates were positive for bla_{CTX-M}, bla_{SHV}, and bla_{TEM} genes, respectively. Moreover, the most prevalent the SHV types were considered as SHV-27 (Fig. 1). Our findings revealed that the bla_{CTX-M}₁₅19 (36.53%) gene was the most prevalent ESBL-encoding gene in the hospital. Resistant to cefotaxime and ceftazidime were detected in all CTX-M-harboring *Klebsiella* isolates. Among 52 *Klebsiella* isolates, 21 (40.38%) were found to carry a carbapenemase-encoding gene, while all PCR-positive isolates showed CIM-positive results (Table 2). The MHT test was positive in all ESBL isolates. Prevalence of three resistant genes, including bla_{NDM}1, bla_{OXA-48} and bla_{KPC}, in carbapenem-resistant isolates was 12 (23.07), 6 (11.53), and 3 (5.76), respectively.

Table 1:

Phenotypic Tests			MIC value (µg/ml)					Depart	Isolation date Date	Age	Sex	Specimen	Strain	Reception code	
DDST	MHT	CIM	CRO	CAZ	MP	IMP	Cp								
DDST	Positive	Positive	16	256	32	32	32	POST HSCT	April /2016	50	F	BAL	Kleb spp.	4203	51
Positive	Positive	Positive	32	256	24	32	32	POST HSCT	March /2016	33	F	Urine	klebpne	45395	52
Positive	Positive	Positive	32	192	32	24	24	ICU (Heart)	April /2016	30	M	Urine	klebpne	92765	53
Positive	Positive	Positive	32	256	32	32	32	ICU (Nerves)	April /2016	72	F	Blood	klebpne	50578	54
Positive	Positive	Positive	32	192	32	24	32	ICU (General)	April /2015	64	M	BAL	Kleb spp.	28929	55
Positive	Positive	Positive	32	192	32	24	12	Internal General	March /2015	86	M	Sputum	klebpne	34168	56
Positive	Positive	Positive	12	192	24	32	24	ICU General	December /2016	59	M	BAL	Kleb spp.	34166	57
Positive	Positive	Positive	24	256	32	32	12	OP	December /2016	54	M	Urine	klebpne	150236	58
Positive	Positive	Positive	24	256	32	32	32	ICU	December /2016	53	M	Blood	klebpne	69518	59
Positive	Positive	Positive	32	256	32	32	32	ICU	November	53	M	Blood	Kleboza	67239	60

Positive	Positive	Positive	32	192	32	24	32	General Internal	November / 2016	45	F	Urine	klebpne	72496	61
Positive	Positive	Positive	32	256	24	32	32	Blood	March /2015	59	M	Blood	klebpne	150695	62
Positive	Positive	Positive	24	256	24	32	32	ICU General	December /2016	66	M	BAL	Kleb spp.	430833	63
Positive	Positive	Positive	32	256	12	32	24	D	December /2016	80	M	BAL	Kleb spp.	470989	64
Positive	Positive	Positive	32	256	32	32	32	ICU General	March /2015	82	F	BAL	klebpne	409526	65
Positive	Positive	Positive	32	256	32	24	32	OP	December /2015	49	M	Wound	klebpne	410911	66
Positive	Positive	Positive	32	256	32	32	32	ICU	December /2016	69	F	Urine	klebpne	401189	67
Positive	Positive	Positive	32	256	32	32	32	ICU	December /2016	55	F	BAL	klebpne	392170	68
Positive	Positive	Positive	32	256	32	32	32	Lung	December /2016	83	M	Sputum	Kleb spp.	391728	69
Positive	Positive	Positive	32	256	32	32	32	ICU Internal	August /2015	81	F	BAL	Kleb spp.	405853	70
Positive	Positive	Positive	24	256	32	32	32	urology	October/ 2015	70	F	Urine	Kleb spp.	391002	71
Positive	Positive	Positive	32	256	32	32	32	Emergency (blood)	September / 2015	17	F	Urine	E.coli	399711	72
Positive	Positive	Positive	32	256	32	24	24	ICU General	September / 2015	64	M	BAL	Kleb spp.	392702	73
Positive	Positive	Positive	32	256	32	32	32	ICU General	October/ 2016	70	M	BAL	Kleb spp.	407274	74
Positive	Positive	Positive	32	256	32	32	32	ICU General	October/ 2016	43	F	Urine	Kleb spp.	237945	75
Positive	Positive	Positive	32	256	32	32	32	ICU General	September / 2015	95	M	Urine	Kleb spp.	276638	76
Positive	Positive	Positive	16	256	32	32	12	ICU General	September / 2015	90	F	BAL	Kleb spp.	315867	77
Positive	Positive	Positive	32	256	32	24	32	ICU General	October/ 2016	71	F	BAL	Kleb spp.	325680	78
Positive	Positive	Positive	24	256	32	32	32	OP	September / 2015	59	M	Urine	E.coli	327509	79
Positive	Positive	Positive	32	256	32	32	32	ICU General	December / 2015	71	F	Blood,Wound	Kleb spp	314331	80
Positive	Positive	Positive	32	256	24	32	32	General Internal	August /2015	63	M	BAL	Kleb spp.	308230	81
Positive	Positive	Positive	32	256	32	32	32	ICU General	December /2015	73	M	BAL	klebpne	333962	82
Positive	Positive	Positive	32	256	32	24	32	General Internal	September / 2015	86	M	CVP	Kleb spp.	407331	83
Positive	Positive	Positive	32	256	32	32	32	ICU General	December /2015	62	M	BAL	Kleb spp.	334746	84
Positive	Positive	Positive	32	192	32	32	32	General Internal	August /2015	81	F	Urine	klebpne	277377	85
Positive	Positive	Positive	32	256	32	32	32	Orthopedic	October/ 2015		M	Wound	klebpne	265726	86
Positive	Positive	Positive	32	256	32	32	32	General Internal	September / 2015	90	F	Urine	Kleb spp.	316178	87
Positive	Positive	Positive	32	256	24	32	32	Urology	September / 2015	85	M	Wound	Kleb spp.	3177642	88
Positive	Positive	Positive	32	256	24	24	24	General Internal	December / 2015	85	M	Urine	Enterobacter spp.	322305	89
Positive	Positive	Positive	32	256	32	32	32	Blood	August /2015	66	M	Blood	E.coli	340581	90
Positive	Positive	Positive	32	192	32	32	32	Lung	December /2015	54	F	Urine	Kleb spp.	308603	91
Positive	Positive	Positive	32	192	32	32	32	ICU General	September / 2015	62	F	Blood	Kleboza	362760	92
Positive	Positive	Positive	32	256	32	32	32	ICU General	April /2016	34	M	Blood	klebpne	360165	93
Positive	Positive	Positive	32	256	32	32	32	surgical	March /2016	16	F	Abscess	klebpne	348912	94
Positive	Positive	Positive	32	256	32	32	32	General Internal	April /2016	62	M	Urine	Kleb spp.	305054	95
Positive	Positive	Positive	32	256	32	32	32	ICU General	April /2016	71	F	CVP	Kleb spp.	320471	96
Positive	Positive	Positive	24	192	24	32	32	ICU General	April /2016	64	M	BAL	Kleb spp.	339712	97
Positive	Positive	Positive	32	256	24	32	32	ICU General	April /2016	67	F	BAL	klebpne	32347	98

Prevalence of carbapenem-resistant *Klebsiella*

Positive	Positive	Positive	32	256	32	32	32	General Internal	April /2016	58	F	BAL	kleb pne	34574	99
Positive	Positive	Positive	32	256	32	32	32	General Internal	March /2016	44	F	Blood	Kleb spp.	33254	100
Positive	Positive	Positive	24	256	32	32	32	General Internal	April /2016	64	M	Urine	Kleb spp.	31245	101
Positive	Positive	Positive	32	256	32	32	32	General Internal	April /2016	84	M	Blood	Kleb spp.	33546	102

Abbreviations: POST HSCT; MHT = Modified Hodge test; DDST = Double disk synergy test; ICU = Intensive care unit; Cp= Ciprofloxacin; IMP=Imipenem; MP=Meropenem; CAZ=Ceftazidime; CRO=Ceftriaxone; CIM=Carbapenem Inactivation method; N= Isolates number; F=Female;M=Male; Cp Genes=Carbapenem Genes; Post-hematopoietic stem cell transplantation, BAL; Broncho alveolar lavage, MIC = Minimum inhibitory concentration; IMP = Imipenem.

Fig. 1: Distribution of *Klebsiella* positive samples

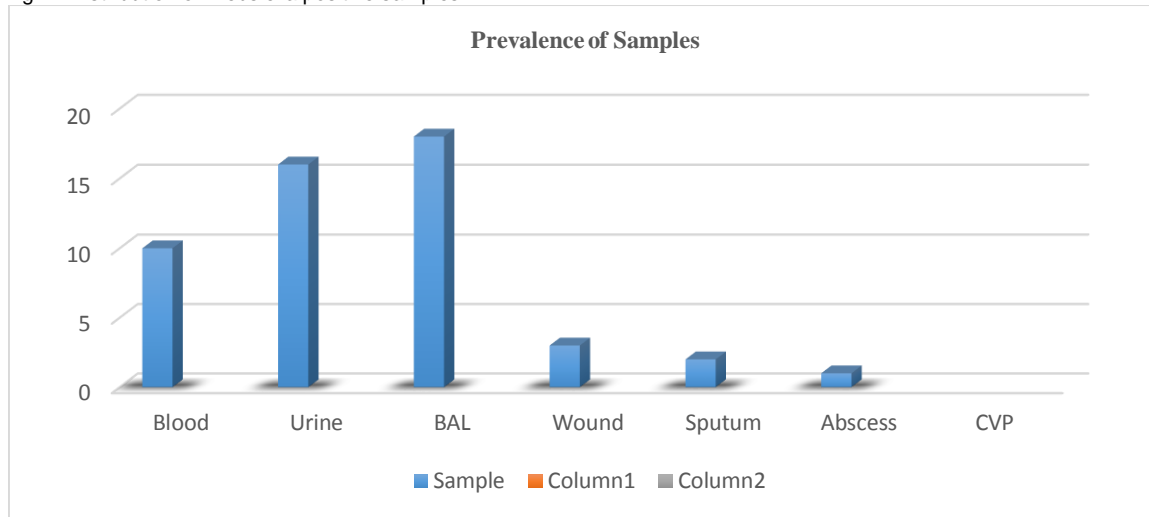


Fig. 2: Distribution of *Klebsiella* isolates based on Sex and Age

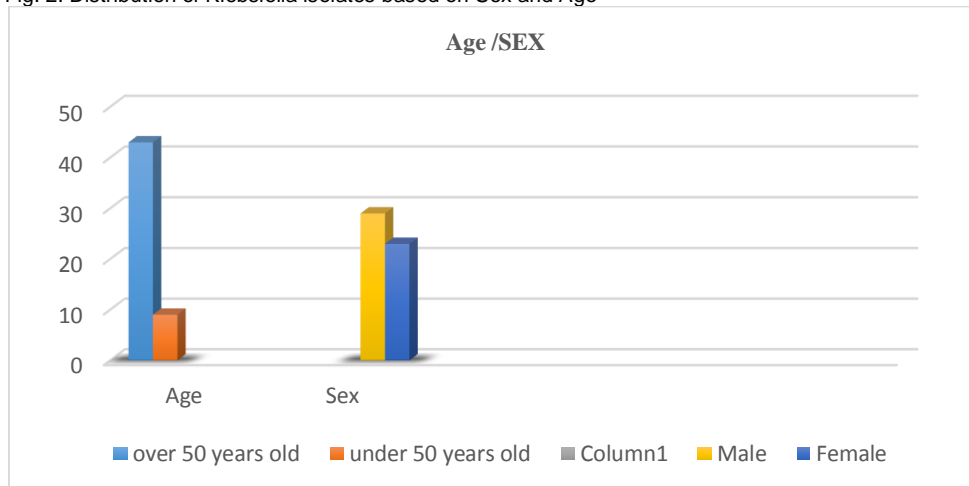


Fig 3: The MHT performed on a Muller Hinton Agar plate. (1) MH positive result

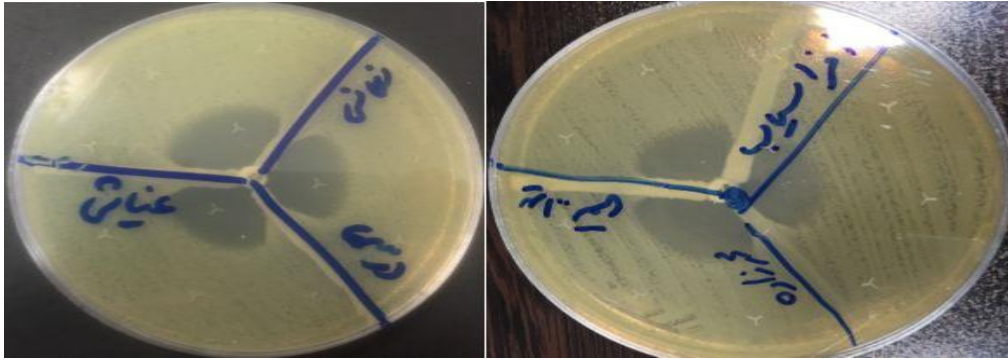
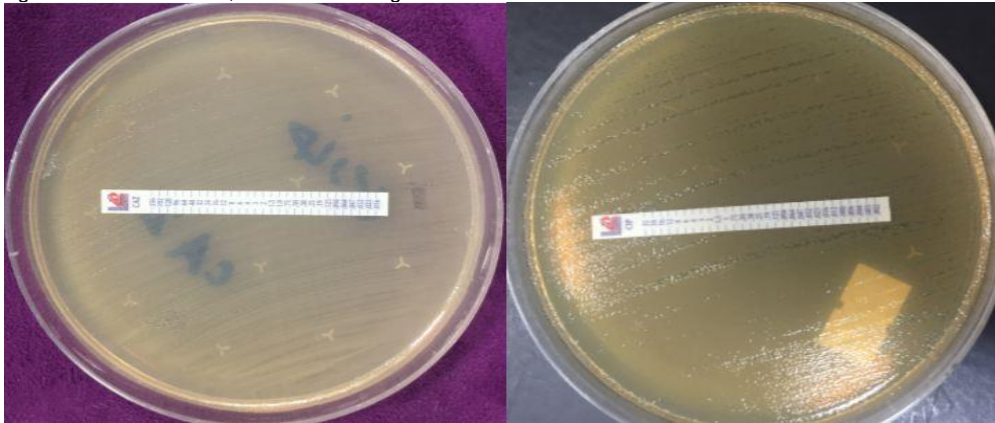


Fig 4: Result of MIC test, indicated that high level of resistance in isolates



DISCUSSION

Nowadays, carbapenem is the only remaining option for the treatment of serious infections caused by Enterobacteria¹⁹. However, Due to extensive use of carbapenem, the emergence of carbapenem-resistant enterobacterial isolates has been increasing worldwide in the past decades¹⁹. Treatment of multidrug resistant bacteria is too difficult and may assist the development and worldwide spread of antibiotic resistance¹⁹. The prevalence rate of infections can be caused by organisms resistant to betalactam antibiotics, making it an increasing challenge worldwide. Therefore, there is a need for detection of ESBL-producing isolates once confined to hospitals but now widespread in communities¹⁹. ESBL carbapenem-resistant *Klebsiella* (CRK) strains have become distributed globally in an epidemic that is associated with extensive antibiotic use²⁰. In this situation, it is important to detect high-level resistant CPK strains as early as possible. In this study, the rates of resistance in *K. pneumoniae* isolates to cefotaxime, ceftazidime, ceftriaxone antibiotics were higher than those described by Ghafourian et al²⁰. Their data demonstrated a trend toward growing resistance to these antibiotics in Iran. We identified a high frequency of ESBL-producing isolates, which is approximately similar to those reported in Turkey (69%) (10), Taiwan (97%) (11), India (97%) (19), Saudi Arabia (55%) (20), and Nepal (62.7%) (21), but higher than those reported in Lebanon (20%), the United States (7.6%), and Canada (4.9%)²¹⁻²⁹. The present study further presented that the blaSHV gene is highly 18 (34.61%) prevalent in clinical ESBL producing strains. The

most common subtypes were considered as SHV-27. In a study, Al-Agamy et al. exhibited that 214 (97.3%) of 220 strains isolated in Saudi Arabia carry the blaSHV gene, which is higher than those shown in other studies. Inconsistent with our study, Feizabadi et al. showed that in *K. pneumoniae* strains TEM-1, SHV-1, SHV-12 were the most common subtypes in Hospitals of Tehran³⁰.

In the clinical setting, distribution of ESBL-producing *K. pneumoniae* has been considered an important therapeutic concern. Our findings indicated that CTX-M1 and CTX-M15 groups had a high prevalence in clinical ESBL-producing isolates and the most common subtype, respectively. According to reports in different European countries and Iran, CTX-M.15, CTX-M-14, and CTX-M-2 have been the most prevalent CTX-M enzyme^{16,31}. In agreement with our study, Dedeic-Ljubovic et al showed the high prevalence of CTX-M-15 in KPC isolates in Bosnia-Herzegovina¹⁶. However, results in other regions, such as South America, are actually different from our findings. Different studies showed that CTX-M-8 and CTX-M-2 enzymes are as the common ESBL types^{32,33}. In agreement with our study, the most common genotype was found to be CTX-M-15 in the United States while other type of enzymes including CTX-M-2 and CTX-M-4 groups were infrequently distinguished²⁶. Similar to our study, Yaghoubi et al. and van der Zwaluw et al. described that CIM is a new test with high specificity and sensitivity for identification of carbapenemase producers^{12,14}. Results from both tests (CIM and MHT) verified that all the isolates were positive. Our results of the CIM test are completely consistent with other investigations stated in other regions of the world^{11,12,15}. Therefore, the CIM test is an applicable,

highly efficient, and low-cost method for the detection of carbapenem-resistant isolates in our hospitals and clinical setting. PCR amplification showed the presence of the carbapenemase-encoding gene in 21 (40.38%) of the isolates. In our study, frequency of the bla_{NDM-1} gene in CR K. Pneumonia isolates was 12 (23.07). NDM and OXA-48 types were found to be the most common carbapenemase in the study conducted by Zowawi et al³³. Prevalence of the bla_{OXA-48}-like gene was reported in 6 (11.53) isolates. Our findings indicated that *Klebsiella* isolates harboring OXA-48 are increasing dramatically when compared with other regions of the world including Russia, Turkey, France, Saudi Arabia, Taiwan, and China¹³. The lack of known target genes in a number of carbapenem-resistant isolates may be due to the presence of other genes, AmpC beta-lactamases, and an ESBLs, and the reduced permeability of the outer membrane^{13,34,35}. Frequency of the bla_{KPC} gene was detected in three (5.76) CR isolates with high level of resistance. Several reports from other regions suggested that KPC-harboring isolates had a resistance to the majority of antimicrobial agents. In general, we determined high-level resistant KP isolates as well as the high coexistence of bla_{CTX-M} and OXA-48 /NDM1 in our clinical setting. We also introduced an applicable method (the CIM test) for the reliable detection of CRK strains in hospital settings in Tehran. Giving to our study, colistin, regardless of its side effects, represents an appropriate empirical treatment for infections caused by ESBL-producing pathogens. Because antibiotics are highly expensive and can cause more complications, therefore, reasonable prescription of cephalosporins and the precise control may have better outcomes. Our findings led to the idea that the CIM test is an achievable and fast method for detection of CRK in our clinical setting. According with increased emergence of OXA-48, KPC, CTX-M, and NDM1 (ESBL-resistant/carbapenem-resistant) harboring CRK strains, we are facing a big challenge in the future. All of the results clarify the necessity of further control in our health facility locations and initiation of appropriate antimicrobial therapy.

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