ORIGINAL ARTICLE

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

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ABSTRACT

Background: Carbapenem-resistant *Klebsiella* isolates are describedas one of the most significant problemsworldwide. The aim of this study was to determinethe prevalence of extended spectrum beta-lactamase (ESBL) subtypes and carbapenem-resistant genes in *Klebsiella pneumoniae* (*K. pneumonia*) speciesisolatedfrom 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-M_{15} 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 are a need for further considerations regarding the emergence anddiagnosis of isolates harboring ESBL subtypes and carbapenem genes. Further attention should be paidfor the treatment of patients with highlyresistant-isolates.

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

INTRODUCTION

In recent years, Klebsiella pneumoniae have become one of the important nosocomial pathogens causing main hospital-acquired infections[1]. 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 analarm for the incidence and distribution of extended spectrum beta-lactamase (ESBL)-producing bacterial isolates, becoming an emerging public health concern[1]. The ESBL-producing isolates of Klebsiella. Spp., have been implicated in numerous outbreaks of nosocomial infections throughout the United States of America[5], Europe[6], the Far East, and Asia[7]. 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 surprised tosee 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 werecollected 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 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 described[8]. K. pneumonia, 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 minormodifications[10,11,12]. Briefly, the isolates were cultured on Mueller-Hinton Agar plates. Afterwards, 10 µl of each isolatewas taken by an inoculation loop, and dissolvedin 400 µl of sterile deionized water, followed by addition of an active susceptibility standard disc for meropenem (MEM). After two-hourincubationat 32°C, the disk was separated and situated on an M-HA plate, and the plate was inoculated at 35°C for 24 hourswith a suspension of OD595 1.25 with a sterile cotton swab. The resultsof 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 beCIM-positive[8, 11,12].

Polymerase chain reaction (PCR) and sequencing: The boiling method was used topreparebacterial genomic DNAfor PCR reaction. In brief, five colonies of each isolate was dissolved in 300 ml 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 conductedusing the specific primers for the blaKPC family.

PCR amplification was carried out forbla_{CTX-M}, bla_{TEM}, bla_{SHV}, blaOXA-48-like, blaNDM, blaVIM, and blaIMP genes, as previously described[13-18]. Alignment of sequences were carried outusing the online BLAST software. Sequences were registered in the GenBank nucleotide database under accession numbers MH359121, MH359122, MH359123, MH369836, MH369837, MH369839, and MH369840.

RESULTS

Colisitin 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).Table 1 indicates the results obtained fromdisc 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 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. 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 blaCTX-M_{15}19 (36.53%) gene was the most prevalent ESBL-encoding gene in the hospital. Resistant to cefotaxime and ceftazidime were detected inall 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 postive in all ESBL isolates. Prevalence of three resistant genes, including blaNDM1, blaOXA-48 and blaKPC, in carbapenem-resistant isolates was12 (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**

									/ 2016						
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	Entrobacter 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	360ات165	**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**

Positive	Positive	Positive	32	256	32	32	32	General Internal	April /2016	58	F	BAL	klebpne	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

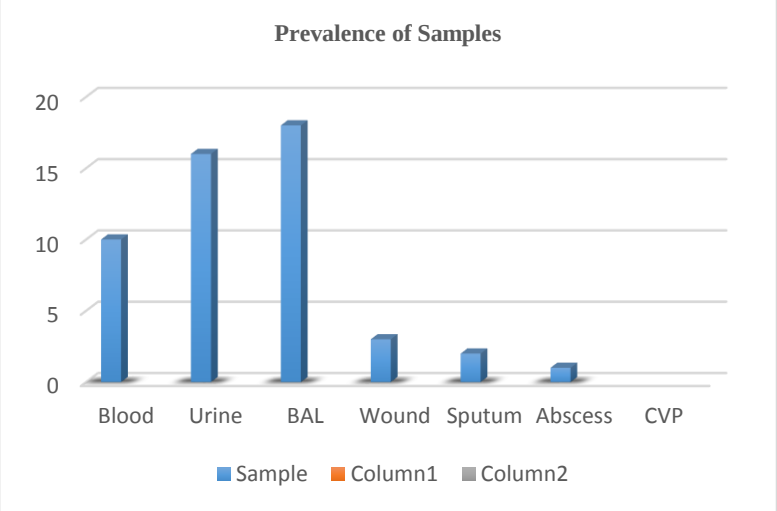


Fig. 2: Distribution of Klebsiella isolates based on Sex and Age

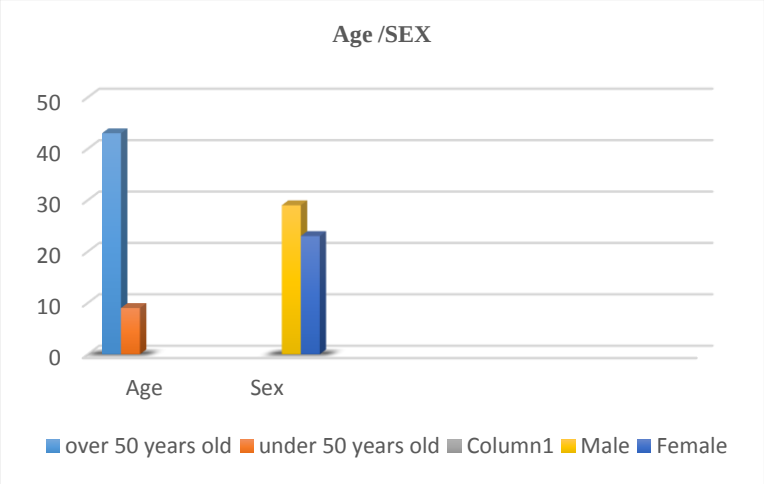
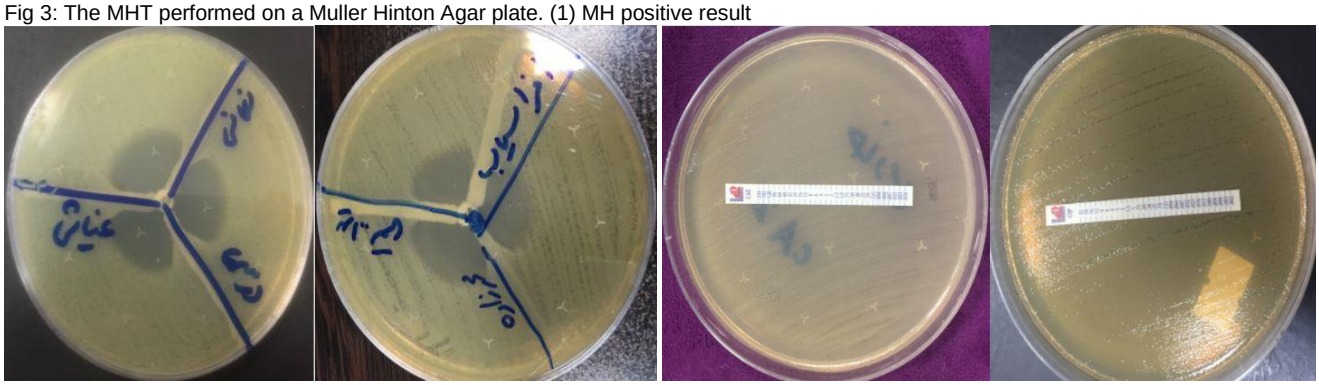


Fig 3: The MHT performed on a Muller Hinton Agar plate. (1) MH positive result



DISCUSSION

Nowadays, carbapenem is the only remaining option for the treatment of serious infections caused by Enterobacteria[19]. However, Due to extensive use of carbapenem, the emergence of carbapenem-resistant enterobacterial isolates has been increasing worldwide in the past decades[19]. Treatment of multidrug resistant bacteria is too difficult and may assist the development and worldwide spread of antibiotic resistance[19, 40]. 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 isolatesonce confined to hospitals but now widespread in communities[19]. ESBL carbapenem-resistant Klebsiella (CRK) strains have become distributed globally in an epidemic that is associated with extensiveantibioticuse[20]. 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 al[20]. 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 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 blaSHVgene is highly 18 (34.61%) prevalent in clinical ESBL producing strains.The mostcommon 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 thatin K. pneumoniae strains TEM-1, SHV-1, SHV-12were the most common subtypes in Hospitals of Tehran[30].

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-M15groups 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 thehigh prevalenceof CTX-M-15 in KPC isolates in Bosnia-Herzegovina[16]. 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 beCTX-M-15 in the United States whileother type of enzymes including CTX-M-2 and CTX-M-4 groups were infrequentlydistinguished[26]. Similar to our study, Yaghoubi et al. and van der Zwaluw et al. descried that CIM is a new test withhigh specificity and sensitivity for identification of carbapenemase producers[12,14]. Results fromboth tests (CIM and MHT) verified that all the isolates werepositive. Our results of the CIM test are completely consistentwith other investigationsstated in other regions of the world[11,12,15]. Therefore, the CIM test is an applicable, highlyefficient, and low-cost method for the detection of carbapenem-resistant isolates in our hospitals and clinical setting. PCR amplification showedthe presence of the carbapenemase-encoding gene in 21(40.38%) of the isolates. In our study, frequency of the blaNDM-1 gene in CR K. Pneumonia isolates was 12 (23.07).NDM and OXA-48 types were found to be the most common carbapenemasein the study conducted by Zowawi et al[33]. Prevalence of the blaOXA-48-like gene was reported in 6(11.53) isolates. Our findingsindicated 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 China[13]. The lack of known target genes in a number of carbapenem-resistant isolates may be due to the presence of other genes, AmpC betalactamases, and an ESBLs, and the reduced permeability of the outer membrane[13,34,35]. Frequency of the blaKPC 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-levelresistant KP isolates as well as the high coexistence of blaCTX-M and OXA-48 /NDM1in our clinical setting. We also introducedanapplicable method (the CIM test) forthe reliable detection of CRK strains in hospital settings in Tehran. Giving to our study, colisitin, regardless of its side effects, representsan appropriate empirical treatment forinfectionscausedby ESBL-producing pathogens.Becauseantibioticsare highlyexpensive 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 arefacing 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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