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Molecular Fingerprinting and Occurrence of *bla*$_{KPC}$ and *bla*$_{OXA}$-48 in *Klebsiella pneumoniae* Isolated From Clinical Specimens, Khorramabad, Iran

Gholamreza Goudarzi[1], Pegah Shakib[1], Khatereh Anbari[2], Shahram Shokri[3*]

Abstract

Objectives: Carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp) is created by various mechanisms mainly including the production of *Klebsiella pneumoniae* carbapenemase (KPC) and other class A, B, and D beta-lactamases (e.g., OXA-48). The present study was undertaken to assess the occurrence of *bla*$_{KPC}$ and *bla*$_{OXA-48}$ genes among *K. pneumoniae* isolates.

Materials and Methods: In this cross-sectional study, 110 *K. pneumoniae* strains were collected from clinical samples from 2 teaching hospitals in Khorramabad, Iran. Susceptibility testing and modified-Hodge test (MHT) for *K. pneumoniae* isolates were performed according to Clinical and Laboratory Standards Institute procedures, followed by carrying out the detection of *bla*$_{KPC}$ and *bla*$_{OXA-48}$ genes by polymerase chain reaction (PCR). Finally, genetic similarity among the tested strains tested was determined by the repetitive extragenic palindromic PCR (rep-PCR) method.

Results: Up to 110 *K. pneumoniae*, the highest resistance and susceptibility rates pertained to ampicillin and ertapenem, respectively. In addition, 10 (9%) isolates were considered as carbapenemase-producing *K. pneumoniae* by the MHT. Although 13 (11.8%) isolates were *bla*$_{OXA-48}$-positive, none of them were harboring *bla*$_{KPC}$. Eventually, 13 and 44 clusters were distinguished in hospitals A and B by rep-PCR, respectively.

Conclusions: The results of this study indicated that resistance to carbapenems among *K. pneumoniae* isolates were relatively common in our region. Moreover, the disseminated clones in our hospitals had mainly diverse origins.

Keywords: Carbapenemase, *Klebsiella pneumoniae*, *bla*$_{OXA-48}$, *bla*$_{KPC}$

Introduction

Klebsiella pneumoniae, as an opportunistic gram-negative pathogen, causes nosocomial infections such as septicemia, pneumoniae, and urinary tract infection (1-3). The growing occurrence of extended-spectrum β-lactamases-producing *K. pneumoniae* led to the administration of carbapenem antibiotics potentially used to combat the multidrug-resistant strains of this bacterium (4). The first carbapenem-resistant *K. pneumoniae* (CR-Kp) was detected in 1996 in the United States (5).

Unfortunately, in the recent decade, CR-Kp strains have been reported from several regions across the world (6-8). According to some studies (2,9,10), CR-Kp is created by the acquisition of one or more of the beta-lactamase classes of Ambler class A (i.e., KPC, NMC, IMI, SME, and GES), class B (i.e., IMP, VIM, GIM, SPM, and NDM), and class D (OXA-48).

bla$_{KPC}$, as a major carbapenem-resistant gen, has been reported in some studies worldwide (11,12). Today, 21 KPC *(Klebsiella pneumoniae* carbapenemase) variants have been identified (13) which are found worldwide (9). This gene could be carried by mobile genetic elements such as transposons and plasmids (14,15). As a plasmid-borne carbapenemase, *bla*$_{OXA-48}$ is barely related to other oxacillinases. On the other hand, its carbapenem-hydrolysing potential is as strong as that of the KPC-1 enzyme (16). OXA-48 producers are mostly identified in *K. pneumoniae* and *Escherichia coli*. Nevertheless, they are reported to exist in the species of *Enterobacteriaceae* (17-19).

Common molecular typing methods such as repetitive extragenic palindromic polymerase chain reaction (rep-PCR), multi-locus sequence typing, and pulsed-field gel electrophoresis to find the origin and relatedness of resistant clones could be pivotally useful for adopting subsequent controlling and preventing strategies (8,14).

Hence, this study aimed to assess the occurrence of *bla*$_{KPC}$ and *bla*$_{OXA-48}$ genes and find the similarity of the tested strains by rep-PCR among *K. pneumoniae* isolated from clinical specimens obtained from certain hospitals in Khorramabad, Iran.

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[1]Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran. [2]Department of Community Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran. [3]Department of Infectious Diseases, Lorestan University of Medical Sciences, Khorramabad, Iran.
***Corresponding Author:** Shahram Shokri, Tel: +989166609743, Email: shahram35040@yahoo.com

Materials and Methods

In this cross-sectional study conducted from June 2016 to April 2018, 110 *K. pneumoniae* isolates were collected from various clinical samples of two referral teaching hospitals of Khorramabad, Iran. *K. pneumoniae* isolates were collected from urine, blood, wound, sputum, or lung discharge, tissue, body fluids, and catheter. One isolate per patient was evaluated and all specimens were streaked onto a MacConkey agar medium (Merck, German). After the incubation of inoculated plates at 37˚C for 18 to 24 hours, presumptive colonies were further identified as *K. pneumoniae* species using conventional biochemical approaches (20).

Then, susceptibility testing was performed on Mueller-Hinton agar (Merck, Germany) against ciprofloxacin (5 µg), ceftazidime (30 µg), imipenem (10 µg), cefotaxime (30 µg), cefepime (30 µg), amikacin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), and gentamicin (10 µg, Roscoe, Denmark) antibiotics by the Kirby-Bauer disc diffusion technique according to the Clinical and Laboratory Standards Institute (CLSI) recommended procedures (21). The isolates which were resistant to at least three classes of antimicrobial agents were designated as multidrug-resistant (22). Next, the modified-Hodge test (MHT) was carried out as a confirmatory test to identify carbapenemase-producing *K. pneumoniae* using the ertapenem disk (10 µg) and *E. coli* ATCC 25922 according to the CLSI guidelines (20). In addition, *K. pneumoniae* ATCC 13883 was used as a carbapenemase-positive strain (21).

The total DNA was extracted from all bacterial suspensions of the tested isolates using the Sinapure DNA Extraction Kit (SinaClon, Tehran, Iran) according to the manufactures' instructions. The presence of bla_{KPC} gene was authenticated via previously designed and, the following PCR conditions:

Initial denaturation at 94˚C for 10 minutes, 30 cycles of 94, 55, and 72˚C for 40 seconds, 40 seconds, and 1 minute, respectively, and the final extension of 72˚C for 7 minutes (22,23). Further, bla_{OXA-48} gene was separately PCR amplified using OXA-48 primers and PCR conditions as the initial denaturation at 95˚C for 2 minutes, 35 cycles of 95, 55, and 70˚C for 30 seconds, 45 seconds, and 1 minute, respectively, along with the final extension of 70˚C for 5 minutes (24). Moreover, 5 µL of PCR products were subsequently loaded on the agarose gel (1.5%, 1X TAE) and electrophoresed. Positive controls harboring the tested genes (bla_{KPC} and bla_{OXA-48}) were prepared from the Pasteur Institute of Iran, Tehran.

The genetic relatedness among the tested strains was determined using strain clustering by the rep-PCR typing method. The total extracted DNA from 109 tested strains were subjected to amplify by primers REP1R-I (5'-IIIICGICGICATCIGGC-3') and REP2-I (5'-ICGICTTATCIGGCCTAC-3'. Then, rep-PCR was performed according to the original method described by Versalovic et al with some modifications (25). Next, the PCR reactions were prepared in a final volume of 25 µL of ready-to-use 2X PCR Master Mix (Ampliqon, Denmark) plus 40 pmol of each primer. Briefly, the PCR process was carried out in a Bio-Rad thermal cycler system with an initial denaturation at 95˚C for 7 minutes, followed by 30 cycles amplification (90, 40, and 65 ˚C for 30 seconds, 1 minute, and 8 minutes, respectively) and a final extension at 65˚C for 16 minutes (25).

The gels were scanned using UV gel Doc (Cambridge, UK) and saved as an inverted TIFF format. The images were then normalized with the reference marker. Additionally, the rep-PCR profiles were analyzed and compared with BioNumerics, version 7.5 (Applied Maths, Saint-Martens-Latem, Belgium) using the Dice coefficient to calculate similarities. Moreover, the unweighted paired group method based on average linkages (was used to create dendrograms and cluster analysis. A cluster of closely related isolates was defined as the same type when the pattern shared 80% or more similar bands (25).

Statistical Analysis

Statistical analysis was carried out via SPSS software, version 16, and the chi-square test was used to compare characteristics between MHT-positive and -negative isolates. A P value of ≤ 0.05 was considered statistically significant.

Results

Out of the 110 tested *K. pneumoniae,* 69, 10, 9, 16, 3, and 2 isolates were collected from urine, blood, wound, sputum or lung discharge, tissue, body fluids, respectively, and 1 isolate belonged to the catheter. The isolates were collected from the outpatient or emergency (n = 63), intensive care unit (n = 10), coronary care unit (n = 3), infectious (n = 4), surgical (7n =), internal (n = 4), women (n = 5), urology (n = 8), orthopedic (n = 4), and neurology (n = 2), wards of hospitals A (n = 89) and B (n = 21). It should be noted that 56 (50.9%) and 54 (49.1%) isolates were collected from women and men, respectively.

The highest and the lowest resistance rates were observed in ampicillin (96.4%, 106/110) and ertapenem (10%, 11/110), respectively. In addition, 32 (29%) isolates were resistant to ampicillin, along with all tested broad-spectrum cephalosporins. Further, 42 (38.1%) isolates had non-susceptible phenotypes that were either resistant or intermediate to at least one tested carbapenem. Table 1 presents the antimicrobial susceptibility profile of the isolates. Among the 110 *K. pneumoniae*, 62 (56.4%) cases were considered as multidrug-resistant (MDR). According to the MHT results, 10 (9.0%) isolates were CPKp from which 9 isolates were resistant to imipenem or ertapenem. The comparison of the tested variables among MHT-positive (carbapenemase-producing) and -negative isolates is provided in Table 2. No significant differences were statistically (chi-square test) found between the

Table 1. Antimicrobial Susceptibility Pattern of *Klebsiella pneumoniae* Using Disc Diffusion Method

Antibiotic Class	Antibiotic	Susceptible No. (%)	Resistant No. (%)	Intermediate No. (%)
Beta-lactams	Cefepime	54 (49.1)	50 (45.4)	6 (5.5)
	Aztreonam	54 (49.1)	56 (50.9)	0 (0)
	Ampicillin	3 (2.7)	106 (96.4)	1 (0.9)
	Cefotaxime	34 (30.9)	67 (60.9)	9 (8.2)
	Ceftazidime	46 (41.8)	52 (47.3)	12 (10.9)
	Imipenem	75 (68.2)	30 (27.3)	5 (4.5)
	Ertapenem	99 (90)	11 (10)	0 (0)
Aminoglycosides	Gentamicin	58 (52.7)	52 (47.3)	0 (0)
	Amikacin	88 (80)	20 (18.2)	2 (1.8)
Quinolones	Ciprofloxacin	49 (44.5)	56 (51)	5 (4.5)
	Nalidixic Acid	40 (36.4)	58 (52.7)	12 (10.9)
Folate synthesis inhibitor	Trimethoprim/sulfamethoxazole	46 (41.8)	62 (56.4)	2 (1.8)

subgroups except for the sample type (Table 2).

As illustrated in Figure 1, the polymerase chain reaction (PCR) results for the detection of bla_{KPC} and bla_{OXA-48} genes showed that none of the tested isolates were the carriers of bla_{KPC} while 13 (11.8%) of them carried the bla_{OXA-48} gene. Unfortunately, the majority of bla_{OXA-48}-carrying isolates were MDR (84.6%). In other words, 10 (76.9%) isolates were carbapenemase-producing by MHT, 11 (84.6%) isolates were phenotypically resistant to imipenem or ertapenem, and interestingly, 8 (61.5%) isolates were obtained from outpatient/emergency subjects. Table 3 summarizes the characteristics of 13 bla_{OXA-48}-positive *K. pneumonia* isolates.

Molecular typing via the repetitive extragenic palindromic PCR (rep-PCR) method was carried out to determine the clonal patterns of 109 *K. pneumoniae* isolates (one isolate was not determined). Our isolates displayed different genetic backgrounds so that 109 of them were placed into 51 distinct clusters. The results of the rep-PCR analysis also revealed that 88 and 21 isolates from hospitals A and B were placed in 44 distinct and 13 diverse clusters, respectively (Figure 2). Two isolates in clusters 12 and 40 from hospital A, as well as, 2 isolates in cluster 3

Table 2. Characteristics of the MHT-negative and MHT-positive CPKp Isolates

Characteristics of Origin for Isolates	MHT-Positive (n=10)	MHT-Negative (n=100)	*P* Value[b]
Age group (n)			
≤ 20 (10)	1 (10)	9 (90)	
21-40 (30)	1 (3.3)	29 (96.7)	
41-60 (39)	3 (7.7)	36 (92.3)	0.284
61-80 (19)	2 (10.5)	17 (89.5)	
Over 80 (12)	3 (25)	9 (75)	
Gender (n)			
Male (54)	6 (60)	48 (48)	0.469
Female (56)	4 (40)	52 (52)	
Hospital ward (n)			
ICU (10)	2 (20)	8 (80)	
CCU (3)	0 (0)	3 (100)	
Infectious (4)	0 (0)	4 (100)	
Surgical (7)	0 (0)	7 (100)	
Internal (4)	0 (0)	4 (100)	
Outpatient and emergency (63)	5 (7.9)	58 (92.1)	0.563
Women (5)	0 (0)	5 (100)	
Urology (8)	2 (25)	6 (75)	
Orthopedic (4)	1 (25)	3 (75)	
Neurology (2)	0 (0)	2 (100)	
Sample type (n)			
Urine (69)	4 (5.8)	65 (94.2)	
Blood (10)	2 (20)	8 (80)	
Wound (9)	1 (11.1)	8 (88.9)	
Sputum and lung discharge (16)	1 (6.3)	15 (93.7)	0.001
Tissue (3)	0 (0)	3 (100)	
Body fluids (2)	2 (100)	0 (0)	
Catheter (1)	0 (0)	1 (100)	

Note. MHT: Modified Hodge test, a confirmatory test for carbapenemase production; CPKp: Carbapenemase-producing *K. pneumonia*.
[a] Chi-square test.

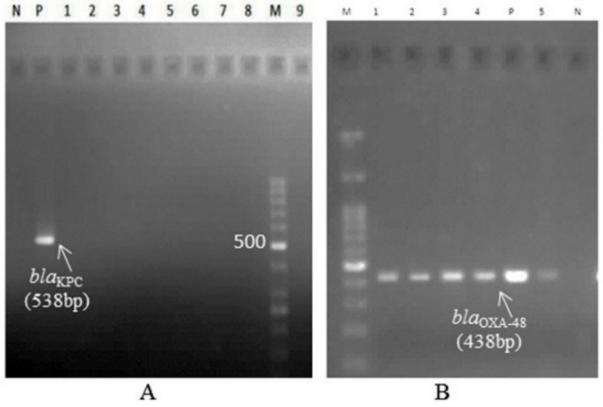

Figure 1. Agarose Gel Electrophoresis of DNA Fragments Generated by PCR
Note. DNA: Deoxyribonucleic Acid; PCR: Polymerase chain reaction; **A.** PCR products to detect the bla_{KPC} gene; Lanes 1-9: bla_{KPC}-negative *K. pneumoniae* strains; Lane M: DNA size marker (50-bp ladder; Fermentas, Lithonia); **B.** PCR products for the detection of the bla_{OXA-48} gene; Lane M: DNA size marker (100-bp ladder; Fermentas, Lithonia); Lane 1-5: bla_{OXA-48}-positive *K. pneumoniae* strains; Lanes P: Positive controls (prepared from Pasteur Institute of Iran); Lanes N: Negative control. The electrophoresis was run on 1.5% agarose gel, followed by staining using GelRed.

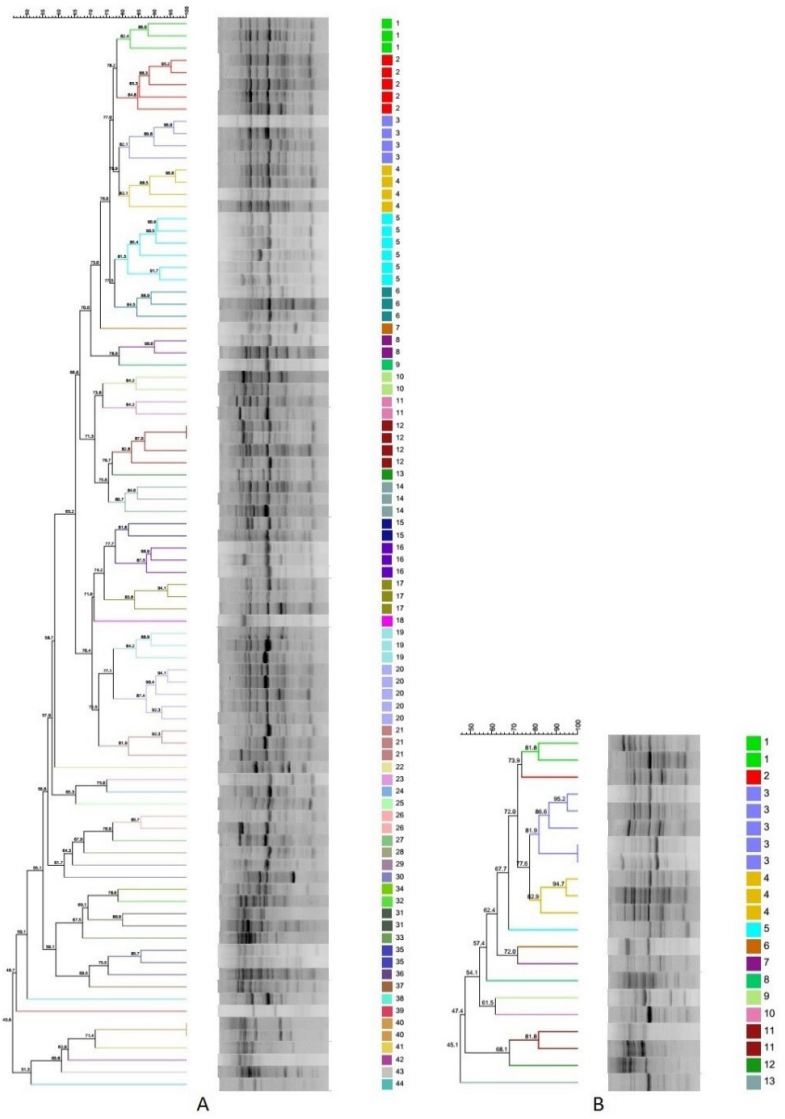

Figure 2. Rep-PCR Analysis of 109 *K. pneumoniae* Isolates Collected From the Clinical Samples of Two Hospitals of Khorramabad, Iran
Note. Rep-PCR: Repetitive extragenic palindromic polymerase chain reaction. **A.** Dendrogram resulting from electrophoretic pattern of 88 isolates from hospital A, showing 44 distinct clusters; **B.** Dendrogram of 21 isolates from hospital B revealed 13 diverse clusters. BioNumerics software was used to draw the linkage dendrograms of fragment patterns.

from hospital B exhibited 100% similarity, and each of them formed one real clone. Clusters 5 (hospital A) and 3 (hospital B) were the major clusters (Figure 2). Finally, the rep-PCR analysis revealed that bla_{OXA-48}^{+} isolates could be distributed to 10 different clusters (Table 3).

Discussion

Klebsiella pneumoniae and, in a lower extent, *K. oxytoca* are as 2 significant opportunistic pathogens worldwide, which are responsible for many hospital-acquired infections. *K. pneumoniae* carries genes for resistance to a majority of antimicrobial drugs, including carbapenems (26,27). The disk susceptibility testing results revealed that although the highest susceptibility rate was related to ertapenem (10%), the greatest resistance rate was observed to ampicillin (96.4%) and then trimethoprim/sulfamethoxazole (59.1%). The susceptibility profile of our study was consistent with the results of the study conducted by Bina et al (28). In another study, Shibl et al reported a high degree of resistance to amikacin, gentamicin, ciprofloxacin, and trimethoprim/sulfamethoxazole among 60 examined *K. pneumoniae* isolates (29). Based on previous evidence, the rates of antibiotic resistance vary in different regions. This phenomenon is partly due to differences in antibiotic consumption and administration patterns. Unfortunately, approximately 81% of the tested isolates had non-susceptible phenotypes to the third-generation of cephalosporins. This issue increases the therapeutic challenge in the removal of resistant isolates in our hospital settings, highlighting the role of colistin as the last drug of choice. However, the availability and the use of this antibiotic are actually restricted and it is

Table 3. Distribution and Characteristics of 13 bla_{OXA-48} Positive *K. pneumonia* Isolates

Isolate	Sample Site	Hospital Ward	Hospital	MDR	Rep Cluster No.
1	Urine	OP	A	Yes	6
51	Sputum	ICU	A	Yes	6
3	Body fluid	Urology	A	Yes	16
6	Urine	OP	A	Yes	5
14	Body fluid	Urology	A	Yes	5
71	Blood	Orthopedic	A	No	2
88	Urine	OP	A	Yes	2
94	Urine	OP	A	Yes	37
100	Wound	OP	A	Yes	19
25	Urine	ICU	B	Yes	6
48	Blood	OP	B	Yes	1
63	Urine	OP	B	Yes	4
98	Sputum	OP	B	Yes	3

Note. Rep: Repetitive extragenic palindromic; OP: Outpatient and emergency; MDR: Multidrug-resistant (resistance to at least three classes of antibiotics); ICU: Intensive care unit.

extremely expensive. The PCR results demonstrated that the presence of bla_{KPC} among *K. pneumoniae* was negative. Likewise, it seems that the KPC enzyme is not the main source of resistance to carbapenems in our region. Although bla_{KPC} has frequently been reported from various countries (8,13,30,31), the published data from the Persian Gulf region as well as Iran indicate that the occurrence of this gene is generally low (29,32,33). Conversely, OXA-48 was relatively prevalent (11.8%) among our strains. Various studies conducted in Iran and the Middle East have also identified that *K. pneumoniae* strains carry OXA-48 (29,34-36).

Turkey, which is in close geographical proximity to Iran, is one of the well-known endemic reservoirs of *K. pneumoniae* harboring OXA-48. Hence, the transmission of this gene via enteric bacteria originated from healthy tourism or travelers is not unlike. All bla_{OXA-48} harboring strains were also MHT-positive, indicating that MHT could be a simple and sensitive laboratory test for detecting OXA-48 producing isolates. The emergence of carbapenem resistance is often associated with co-resistance to other antimicrobial agents (37). Interestingly, this survey showed that the majority of bla_{OXA-48} positive strains were MDR (92.3%) and mostly derived from outpatients or emergency wards (61.5%). The tragedy is most likely due to the co-carriage and transferring of multiple genes of resistance to mobile genetic elements. The results of rep-PCR genotyping demonstrated that bacterial isolates involved in our study were highly diverse (i.e., polyclonal). This polyclonality nature could partly be because 57.2% of the tested samples were collected from outpatients or emergency wards. On the other hand, the selected hospitals were of referral type, and many patients had been admitted from other health care centers and neighboring cities in order to receive specialized services. Hence, the tested isolates had less genetic similarity and higher diversity. Unfortunately, clone diversity increases the potential for generating new evolutionary variants so that it causes the expansion of antibiotic resistance in the future. Even though our research was the first study that reported the occurrence of the two significant genes of resistance to carbapenems in Western Iran, the limited sample size, the lack of other typing methods such as multi-locus sequence typing, pulsed-field gel electrophoresis (PFGE), and the non-including of all health care centers in the project were the weaknesses of our study. Although OXA-48 is only one of the mechanisms that cause phenotypic resistance to carbapenems, Metallo-beta-lactamase enzymes are considered as the major sources of this phenomenon (38). Therefore, future studies should aim at detecting class B Metallo-beta-lactamases genes (i.e., IMPs, VIMs, NDM-1, and the like), involving in resistance to these valuable bactericidal drugs and typing the method of PFGE. In conclusion, the results of the present research revealed that resistance to carbapenems among *K. pneumoniae* isolates was relatively common in Khorramabad hospitals. Moreover, although bla_{KPC} was not detected, the presence of bla_{OXA-48} was proven to be partly responsible for carbapenem resistance. Considering that bla_{OXA-48} and many other genes of resistance are readily transported via mobile genetic elements, there is the potential to further spread resistant clone intra- and inter-hospital in our region. Hence, adherence to the essential standards to prevent the development of hospital infections through health care personnel and inpatients colonized with carbapenem-resistant isolates and the surveillance of resistance to carbapenems in any region could be an essential measurement and critical to the reduction of the development of resistance to these valuable drugs.

Conflict of Interests

The authors declare that they have no competing interests.

Ethical Issues

The study was conducted after obtaining the approval of

the Ethics Committee of Lorestan University of Medical Sciences (ID: IR.LUMS.REC.1397126).

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