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RESEARCH ARTICLE

Investigation of NDM-1 and OXA-48 producing carbapenem resistant *Klebsiella pneumoniae* ST15 in Iran

HEDAYAT BOZORGI MOHAJER[1], HIMEN SALIMIZAND[2,3]*, DAHIEH GHARANIZADEH[1], AFRA HOSSAINPANAHI[3] and RASHID RAMAZANZADEH[4]

[1] Student Research Committee, Kurdistan University of Medical Sciences, Sanandaj, Iran

[2] Cellular and Molecular Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran

[3] Department of Microbiology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran

[4] Department of Microbiology, Faculty of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran

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ABSTRACT

The aim of this study was to determine the frequency of carbapenem resistant *Klebsiella pneumoniae* (CRKP) sequence types (STs) in Iran. Samples were collected from three university hospitals in Sanandaj, Iran, from December 2016 to March 2018. Antibiotic susceptibility testing, phenotypic and genotypic detection of carbapenemases were performed. Common *K. pneumoniae* capsular types were sought for all isolates. The genetic relatedness of isolates was investigated by multilocus sequence typing (MLST). Plasmids were detected by PCR-based Replicon Typing (PBRT). During the study, 67 *K. pneumoniae* isolates were identified. Of which, 18 (26.9%) isolates were detected as carbapenem-resistant. The most effective antibacterial agent was tigecycline (97%, 65 isolates) followed by imipenem and ertapenem (73.13%, 49 isolates). PCR showed that 13 isolates (19.4%) had $bla_{\text{NDM-1}}$ gene and 5 (7.5%) harbored $bla_{\text{OXA-48}}$. Examination of common capsular types showed that 2 isolates had K2 and 2 others had K54. REP-PCR revealed 10 clones and 11 singleton strains. MLST analysis of CRKP found ST15 as the most common type (13 isolates, 72.2%), but other STs were also detected namely, ST19, ST117, ST1390, and ST1594. ColE1 and IncL/M plasmids were the carriers of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$, respectively. The results showed that CRKP spread in our health centers. Our results, therefore, indicate a worrying trend of resistance to carbapenems in *K. pneumoniae*.

KEYWORDS

Klebsiella pneumoniae, PCR-based replicon typing, carbapenemase, MLST, high-risk clone

1. INTRODUCTION

Klebsiella species are opportunistic pathogens that primarily cause infections in people with disruption in immune barriers as well as those suffering from underlying diseases such as diabetes or chronic pulmonary obstruction. In the United States, *Klebsiella* spp. account for 3–7% of the reported nosocomial bacterial infections and these are among the eight major infectious pathogens in hospitals [1].

Antibiotic-resistant bacteria are a great threat to human health, which has expanded dramatically in many countries. Carbapenemase-producing *Enterobacteriaceae* (CPE) are important causes of nosocomial infections that have been reported worldwide [2]. Studies have shown that mortality due to infection with metallo-β-lactamase (MBL) producing gram

*Corresponding author. Kurdistan University of Medical Sciences, Pasdaran Ave., Sanandaj, Kurdistan Province, Iran. P.O. Box: 6617713446; Tel.: +988733664546.
E-mail: hsalimizand@muk.ac.ir, hsalimizand@gmail.com

negative rods are much higher than MBL-negative strains. Frequently reported MBLs include VIM and IMP, and recently NDM-1 variants [3].

Significant mortality and economic aspect of antibiotic resistance are the great concern for health systems [4, 5]. On the other hand, tracing drug-resistant bacteria and related resistance genes is essential to obtain basic and fundamental information to find the origin of such bacteria. The multilocus sequence typing (MLST) technique provides globally valuable data in this regard. This work sought carbapenemase-producing *Klebsiella pneumoniae* sequence types in the west of Iran, a resource-constrained setting country with limited information about high-risk clones.

2. MATERIALS AND METHODS

2.1. Bacterial isolates and hospital setting

From December 2016 to March 2018, samples were collected from three educational-medical centers at the same time in Sanandaj, located in the west of Iran.

2.2. Identification of *K. pneumoniae*

Bacterial isolates were characterized by biochemical tests. To confirm the collected isolates as *K. pneumoniae*, a PCR was performed using the 16–23S Internal transcribed spacer (ITS) gene-specific to *K. pneumoniae* [6]. DNA was extracted by simple boiling method and isolates that amplified 130 bps band was confirmed as *K. pneumoniae*.

2.3. Antimicrobial susceptibility testing and phenotypic determination of carbapenemases

The minimum inhibitory concentration (MIC) of 15 antibiotics was measured by VITEK2 (Biomeriux, France) using AST-GN76 cards and interpreted according to CLSI guidelines [7]. The phenotypic detection of carbapenemases was performed by three methods. Modified Hodge test (MHT), Carbapenem Inactivation Method (CIM), modified Carbapenemase Inhibitory Method (mCIM). Those that were positive in CIM and mCIM were subjected to EDTA/meropenem combination disk (EMCD) [8, 9]. These methods are based on meropenem inactivation and observation of the inhibition zone. Positive and dubious results were considered for the molecular detection of carbapenemases.

2.4. Molecular detection of carbapenemases

Multiplex PCR was used to identify bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{GES}, bla_{SPM}, bla_{DIM}, bla_{BIC}, bla_{SIM}, bla_{GIM}, bla_{AIM}, and bla_{OXA-48} as described previously [10].

2.5. Molecular typing

Two methods were used to find the genetic relationship of isolates. Repetitive-palindromic extragenic element polymerase chain reaction (REP-PCR) was done using the REP1 and REP2 primers [11]. Taq2x MasterMix (Parstous, Iran) and primers with the concentration of 50 pM (final concentration of 2 µM) were used. PCR products were run in 1.8% agarose gel and 0.5X TAE buffer. Documented pictures analyzed by GelJ software (V. 1.3) with tolerance of 2, dice coefficient, and 95% similarity to draw dendrogram and find their genetic relatedness.

MLST was performed on carbapenem-resistant *K. pneumoniae* (CRKP) isolates. In this method, PCR was performed for seven house-keeping genes, as previously described by Diancourt [12]. PCR products were then sequenced, and the results were submitted to https://bigsdb.pasteur.fr/klebsiella/klebsiella.html, where the target ST was identified.

2.6. Capsular detection

Based on the study of Turton et al., all isolates were sought for common *K. pneumoniae* capsular types including k1, k2, k5, k54, k57, k20 [13].

2.7. Sequencing and accession numbers

To identify variants of bla_{NDM} and bla_{OXA-48} genes, these genes were sequenced from both sides [14, 15]. The obtained nucleotide sequences were submitted in the NCBI database at https://www.ncbi.nlm.nih.gov/genbank/update/ and the corresponding accession number was recorded.

2.8. Plasmid-based replicon typing

Carbapenemase producing *K. pneumoniae* isolates were subjected to find their plasmid profile, in multiplex format [16, 17]. This technique targets replicase gene in plasmids.

2.9. Conjugation

Several selected isolates and Escherichia coli J53 sodium azide resistant was cultured in the LB broth medium. After 24 h, 100 µL of the inoculated bacteria were transferred to a fresh LB medium, placed in a shaker incubator at 120 Rpm for 3 h, to reach 0.6 OD at 600 nm. 500 µL of donor bacteria and 500 µL of the recipient were mixed and placed in a shaker incubator for 2–3 h at 60 Rpm. Then, the tubes were rapidly shaken to stop the conjugation process. A loopful bacteria was transferred to the EMB medium containing 2 µgr/mL of meropenem and 100 µgr/mL of sodium azide incubated for 18 h [18].

2.10. Plasmid extraction and electroporation

Plasmids of carbapenemase producing isolates were extracted by the modified Kieser method [19]. Briefly, isolates were cultured in 5 mL TSB (BD, Sparks, MD, USA) containing 2 µgr/mL meropenem (Sigma, Germany) with overnight 150 RPM shaking. The protocol was followed as described previously. Extracted plasmids were run on 0.7% agarose gel for six hours on ice. All bands were cut and extracted from agarose gel by Gene JET Gel Extraction kit (ThermoFisher Scientific). Plasmid replicon typing and PCR for bla_{NDM} were

repeated on extracted bands. Plasmid that harbored bla_{NDM} and bla_{OXA-48} was considered for electroporation.

E. coli K12 competent strain was cultured in LB broth as described by Tu et al. [20]. Electrocompetent cells were streaked on LB agar containing 2 µgr/mL meropenem.

3. RESULTS

3.1. Bacterial isolates and hospital setting

During the 16-month isolate collection, 67 *K. pneumonia* isolates were identified. The highest frequency of *K. pneumoniae* was isolated from ICU wards (29/43.3%). Isolates were mainly collected from urine (36/53.7%). Demographic data from each hospital were collected during sampling as shown in Table 1.

3.2. Antimicrobial susceptibility testing and phenotypic determination of carbapenemases

The antimicrobial susceptibility pattern of isolates is shown in Table 2. The highest susceptibility was related to tigecycline (65/97.97%) followed by imipenem and ertapenem (49/73.13%). Nitrofurantoin was the least effective antimicrobial (50/74.6%). Overall, 17 (25.37%) and 25 isolates (37.31%) were identified as MDR and XDR, respectively. The antibiotic susceptibility results for 15 antibiotics measured by VITEK2 with MIC_{50} and MIC_{90} are presented in Table 2.

Table 1. Demographic information of the studied samples

Demographics	Number (%)
Age range (years)	24–94
*Sex**	
Male	35 (55.55)
Female	28 (44.44)
Internal medicine	12 (17.91)
ICU	29 (43.28)
Infectious Diseases	3 (4.47)
postpartum	6 (8.95)
Emergency	11 (16.41)
Dialysis clinic	1 (1.49)
Neurology	2 (2.98)
Pulmonary	1 (1.49)
Gastroenterology	1 (1.49)
Cardiology	1 (1.49)
Specimens	
Urine	36 (53.73)
Lung	10 (14.92)
Wound	3 (4.47)
Tracheal tube	4 (5.97)
Blood	8 (11.94)
Cerebral Spinal Fluid	1 (1.49)
Bag valve mask	1 (1.49)
Flat Guard	2 (2.98)
Trolley	1 (1.49)
Barometer	1 (1.49)

*Gender was calculated from 63 samples because 4 isolates were peripheral and non-gendered.

Regarding 18 CRKP isolates, MHT had no positive result. CIM protocol revealed 14 (20.9%) carbapenemase producers. In mCIM method, 17 isolates (25.4%) had an inhibition zone less than 15 mm around the meropenem disc that was considered as carbapenemase producers. Moreover, 13 isolates were positive in the EMCD test (19.4%).

3.3. Molecular detection of carbapenemases

Multiplex PCR was performed for 18 carbapenem-resistant isolates. Of which, 13 (72.2%) isolates had bla_{NDM} and 5 (27.8%) isolates had bla_{OXA-48}. Other carbapenemases were not found. Detailed information regarding CRKP isolates is listed in Table 3.

3.4. Molecular typing

Genetic analysis of all isolates by REP-PCR showed 70%–100% similarity between *K. pneumoniae* isolates (Fig. 1). The size of the REP fragments varied between 100 bps and 1000 bps. Based on the 95% similarity, 21 different patterns were identified, including 10 A-J clones and 11 unique singleton strains. The clone with the most members was clone G with 14 isolates. Three OXA-48 producing isolates with different STs, ST15, ST117, and ST1390 were in the B clone. These three isolates were urine isolated from different wards. The other two strains that carried OXA-48 were not allocated in any colon. Isolates with NDM-1 were embedded in D, E, F, G, I, and J clones, but most of them were in clones I and J, indicating the clonal distribution of these strains (Fig. 1).

A total of five STs were identified in 18 CRKP isolates. The most common ST in carbapenem-resistant strains was ST15 that was detected in 13 isolates (72.22%). Of which, 12 had bla_{NDM-1} and 1 isolate harbored bla_{OXA-48}. The other ST, ST117, with two members, carried bla_{OXA-48}. Two other STs were also identified as ST19 and ST1390 carried the $bla_{OXA-48.}$ A single isolate, ST1594, was bla_{NDM-1} producer (Table 3).

3.5. Capsule detection

Multiplex PCR results for K1, K2, K5, K54, K57, and K20 capsular types showed that two isolates had K2 and two isolates with K54 capsular type. In the other isolates, none of the studied capsular types were found. The merely CRKP that produce K2 was ST19 that was not frequently isolated (Table 3).

3.6. Sequencing and accession number

The accession number submitted in NCBI GenBank is MK263176 for bla_{NDM-1} and MK680005 for bla_{OXA-48}.

3.7. Plasmid-based replicon typing and carbapenemase-carrier plasmids

In all the NDM-producing isolates ColE1 and IncHIB were detected (Table 3). PCR for carbapenemases on extracted plasmids revealed ColE1 as bla_{NDM-1} carrier. Various Inc

Table 2. Antibiotic susceptibility of *K. pneumoniae* isolates (%)

Antibiotic	Susceptible	Intermediate	Resistance	MIC_{50}	MIC_{90}
Amikacin	38 (56.71)	5 (7.46)	24 (35.82)	2	64
Gentamicin	40 (59.70)	1 (1.49)	26 (38.80)	1	16
Cefazolin	19 (28.35)	0	48 (71.64)	64	64
Cefepime	25 (37.31)	16 (23.88)	26 (38.80)	8	64
Cefoxitin	35 (52.23)	10 (14.92)	22 (32.83)	4	64
Ceftazidime	22 (32.83)	9 (13.43)	36 (53.73)	16	34
Ceftriaxone	21 (31.34)	3 (4.47)	43 (64.17)	64	64
Ciprofloxacin	32 (47.76)	0	35 (52.23)	4	4
Levofloxacin	32 (47.76)	0	35 (52.23)	8	8
Ertapenem	49 (73.13)	0	18 (26.86)	0.5	8
Imipenem	49 (73.13)	2 (2.98)	16 (23.88)	0.25	16
Nitrofurantoin	7 (10.44)	10 (14.92)	50 (74.62)	128	512
Pip/Taz	35 (52.23)	8 (11.94)	24 (35.82)	16	128
Tigecycline	65 (97.01)	0	2 (2.98)	1	2
Tri/Sul	28 (41.79)	0	39 (58.20)	320	320

Abbreviations: Pip/Taz, piperacillin/tazobactam; Tri/Sul, trimethoprim/sulfamethoxazole.

groups were detected in subjected isolates. On the other hand, IncL/M plasmid was found to contain bla_{OXA-48}. IncHIB was also found in OXA-48 producing isolates.

3.8. Conjugation and electroporation

The mating experiment, as well as electroporation, were not successful even by three-time repeating experiment and with various isolates.

4. DISCUSSION

In the present study, 67 isolates of *K. pneumoniae* were collected from three university hospitals in Sanandaj, west of Iran. Based on the results of susceptibility testing by the Vitek2 device, 18 isolates (26.86%) were identified as carbapenem-resistant. CRKP has been frequently reported in the countries of Asia [21–25]. Furthermore, other studies were in line with this study, in which NDM producers were more resistant than OXA-48 producers. Isolates that harbor NDM usually contain other resistance factors, simultaneously [26–28].

NDM-1 was first reported in India, has also been reported in other Asian countries such as China, Pakistan, and Saudi Arabia. However, the NDM-1 first report in Iran was in 2013, and a dramatically increasing trend of spreading carbapenemases has been issued [21, 27, 29–31]. OXA-48 producing *Enterobacteriaceae* was first identified in Turkey in 2001 and subsequently reported from several countries in the Middle East, North Africa, and Europe [32, 33]. The OXA-48 gene was first reported by Azimi et al. in 2014 in 27 *K. pneumoniae* isolates in Iran [34]. In the Study of Moghadampour et al., in 2018, 46 OXA-48 carriers of *K. pneumoniae* were identified [35]. Overall, given that Pakistan and Turkey as the frequently reported NDM and OXA-48 genes, respectively, the prevalence of these genes in Iran, which shares the common borders, was not unexpected.

A wide range of plasmids has a predominant role in distributing resistance determinants. In regard to CRKP, IncL/M has been revealed as the bla_{OXA-48} transfer [36]. This plasmid was shown to be the carrier of bla_{OXA-48} in our centers. On the other hand, in our isolates, bla_{NDM-1} was lied in a ColE1 plasmid that has not been reported, previously. Aslani et al. determined this gen on the other Inc groups [37]. The transferability of these plasmids in our lab was not successful which might be due to the large size of plasmids. With the techniques in our hands, the approximate size of plasmids was not available. More studies are required to evaluate the precise characteristics of these plasmids.

In this study, carbapenem-resistant and susceptible strains were in the same clones, indicating the inappropriateness of REP-PCR technique to distinguish these strains. Resistant isolates from different hospitals have no genetic similarity indicating the presence of multiple clones of CRKP in the studied health centers. Carbapenem-resistant isolates have been also analyzed by MLST technique for global surveillance. A total of 5 STs were identified in 18 carbapenem-resistant isolates. The most common ST was ST15 in 13 isolates out of 18 CRKP (72.22%). *K. pneumoniae* ST15 is the dominant ST in the ST258 clonal group that was associated with a wide range of beta-lactamases including NDM, OXA-48, KPC, and CTX-M-15 [38]. *K. pneumoniae* ST15 carrying the NDM has been issued in many countries [39]. In Bulgaria, this clone was responsible for the outbreak of KPC-2 producing *K. pneumoniae* [38]. In addition, *K. pneumoniae* ST15 carries OXA-48 in various continents [39]. In studies in Turkey and Pakistan, NDM-1 producing ST15 *K. pneumoniae* were identified [40–42], alongside ST15 *K. pneumoniae* ESBL producer isolates in Iran [43]. Numerous reports of CRKP ST15 from various locations indicate the worldwide prevalence of this high-risk clone. Therefore, these strains can be easily transferred to Iran by various factors, including international travelers, especially from neighboring countries such as Turkey and Pakistan.

Table 3. Comprehensive information of carbapenem resistant isolates

Strain	Ward, Specimen, Hospital	MIC (mg L^{-1})		Carbapenemase	CIM/ mCIM	EMCD	Resistotype	Antimicrobial Susceptibility	PBRT	Plasmid harbor carbapenemase	Capsular type	ST
		IMP	ETP									
KP1	Internal, Urine, A	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	GEN, TIG	ColE1, IncHIB, IncF1B-M	ColE1	ND	15
KP2	ICU, Urine,A	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	GEN, TIG	ColE1, IncHIB, IncL, IncY, IncA/C,	ColE1	ND	15
KP4	ICU, Bag valve mask, A	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1, IncHIB, IncF1B-M	ColE1	ND	15
KP13	ICU, blood, A	8	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1, IncHIB, IncF1B-M	ColE1	ND	15
KP14	ICU, Flat Guard,A	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1, IncHIB, IncF1B-M	ColE1	ND	15
KP15	ICU, Trolley,A	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1, IncHIB, IncF1B-M	ColE1	ND	15
KP19	ICU, Lung, A	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1, IncHIB	ColE1	ND	15
KP20	ICU, Urine, A	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1, IncHIB, IncK, IncF1B-M	ColE1	ND	15
KP21	ICU, Barometer, A	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1, IncHIB, IncL, IncA/C	ColE1	ND	15
KP27	Dialysis clinic, Urine, B	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1	ColE1	ND	15
KP53	ICU, Tracheal aspirate, B	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	-	ColE1, IncHIB, IncF1B-M, IncX3, IncA/C	ColE1	ND	15
KP62	Emergency, Urine, B	2	≥8	bla_{OXA-48}	−/+	−	XDR	TIG	IncL/M, IncHIB	IncL/M	ND	15
KP63	ICU, Urine, B	≥16	≥8	bla_{OXA-48}	−/+	−	XDR	TIG	IncL/M, IncHIB	IncL/M	ND	117
KP67	Infection, Urine, B	≥16	4	bla_{OXA-48}	−/+	−	MDR	AMK, CIP, LEV, NIT, GEN, TIG	IncL/M	IncL/M	ND	1,390
KP73	ICU, Lung, B	8	≥8	bla_{OXA-48}	−/+	−	XDR	-	IncL/M, IncHIB	IncL/M	ND	117
KP76	ICU, Lung, C	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1, IncHIB, IncF1B-M	ColE1	ND	1,594
KP79	Emergency, Wound, C	2	4	bla_{OXA-48}	−/−	−	MDR	AMK, CIP, GEN, LEV, TIG, Tri/Sul	IncL/M	IncL/M	K2	19
KP82	ICU, Urine, C	≥16	≥8	bla_{NDM-1}	+/+	+	XDR	TIG	ColE1, IncHIB	ColE1	ND	15

Abbreviations: Besat hospital, A; Tohid hospital, B; Kosar hospital, C; PBRT, PCR-Based Replicon Typing; MIC, minimum inhibitory concentration; mCIM, modified carbapenem inactivation method; EMCD, EDTA/meropenem combined disk; MDR, multi-drug resistant; XDR, extensive drug resistant; ST, sequence type; IMP, imipenem; ERT, ertapenem; Tri/Sul; trimethoprim/sulfamethoxazole; Gentamicin, GEN; Tigecycline, TIG; Amikacin, AMK; Ciprofloxacin, CIP; Levofloxacin, LEV; Nitrofurantoin, NIT; ND, Not Determined.

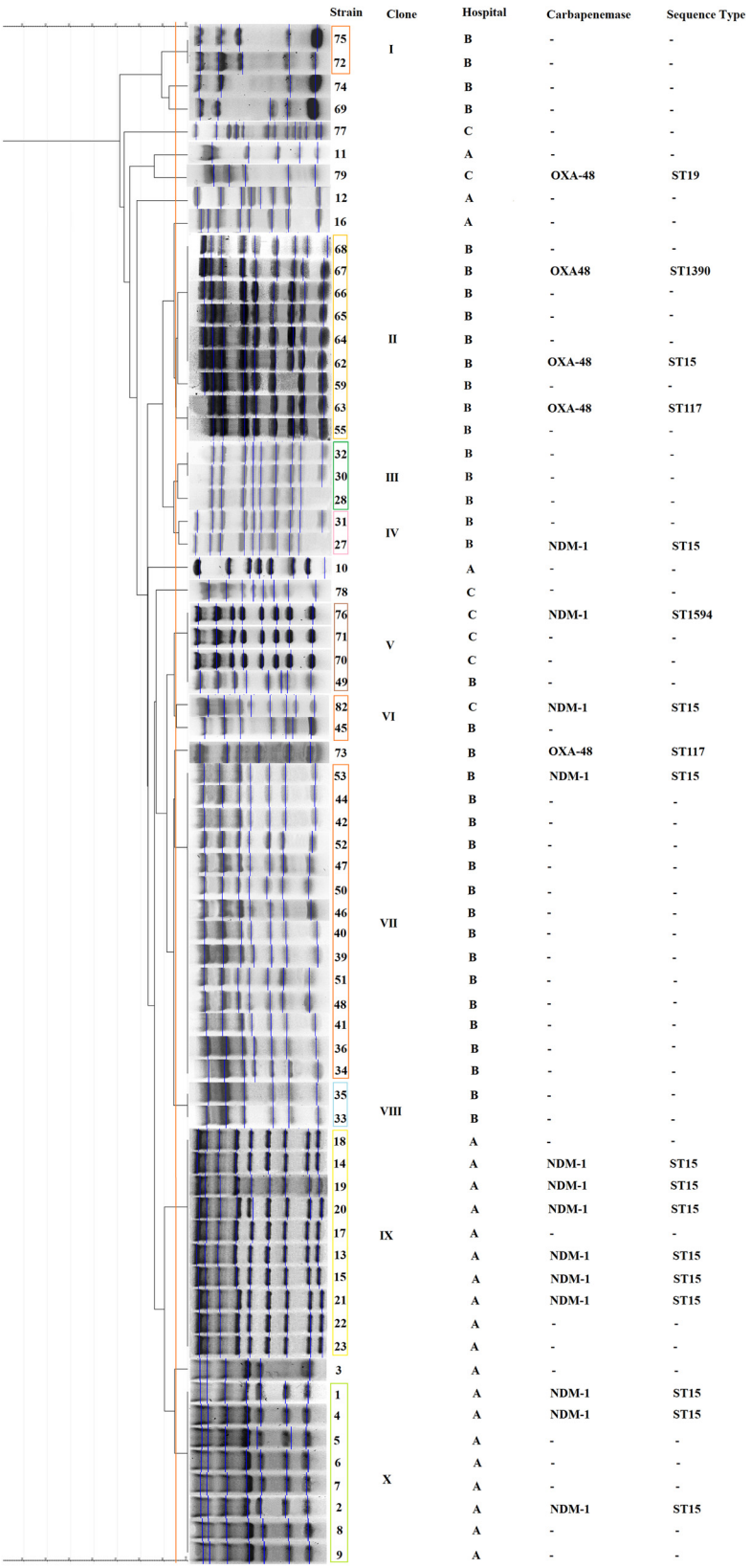

Fig. 1. Dendrogram of REP-PCR analysis of *K. pneumoniae* isolates drew with tolerance 2. Clones are shown in color boxes

In addition to ST15, *K. pneumoniae* ST1594 NDM-1 carrier was also identified in our study. This ST has been reported only once in Kuwait [44] and, to the best of our knowledge, no report was available elsewhere. Iranian traveling to Kuwait may justify ST1594 outbreaks in these two areas. The main limitation of this study was information

regarding the sequence of plasmids as well as transposons that carry bla_{OXA-48} and bla_{NDM}. On the other hand, metadata regarding mortality of isolates regarding carbapenemase producer and non-carbapenemase isolates, were not available. Such informative data helps policy makers to focus the funding and manpower to control emerging outbreaks.

5. CONCLUSION

The present study reported the distribution of CRKP corresponding to high-risk ST15 clone in the west of Iran. ColE1 as bla_{NDM-1} and IncL/M as bla_{OXA-48} carriers were highlighted in this study.

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