

High Frequency of 16S Ribosomal RNA Methyltransferases among *Klebsiella pneumoniae* Isolates: First Report of *rmtA*, *rmtD*, *rmtE* and *rmtF* Resistance Genes in Iran

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ABSTRACT

Aims: 16S ribosomal RNA methyltransferases (RMTases) confer high-level resistance to aminoglycosides and are increasingly reported among Gram-negative bacilli, especially *Klebsiella pneumoniae* isolates. The objectives of the present study were to assess the resistance to aminoglycosides, the presence of RMTase genes, and the multilocus sequence typing (MLST) in urinary *K. pneumoniae* isolates.

Materials & Methods: In this study, 100 *K. pneumoniae* isolates were tested for susceptibility to amikacin and gentamicin by broth microdilution test according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Then the prevalence of RMTase genes was determined, and alleles and ST type of two selected isolates were identified by MLST. Finally, the isolates were genetically typed using Enterobacterial Repetitive Intergenic Consensus (ERIC) method.

Findings: Broth microdilution assay showed that resistance to amikacin and gentamicin was 70 and 52%, respectively. In addition, 40% of the strains were resistant to both aminoglycosides. Also, *rmtC* (59.8%) gene was the most common type of RMTase genes investigated, followed by *rmtA* (51.2%), *rmtD* (47.6%), *rmtF* (43.9%), *rmtE* (41.5%), *armA* (41.5%), and *rmtB* (7.3%). *K. pneumoniae* isolates were assigned into two sequence types: ST51 and ST149. Using ERIC-PCR method, 3-7 different bands and 21 ERIC-PCR profiles were detected among the studied isolates.

Conclusion: The high frequency of aminoglycoside resistance and the increased presence of 16S RMTases in *K. pneumoniae* strains are of great concern in Iran. Molecular typing showed high genetic diversity among the studied isolates. However, ST51 and ST149 were reported for the first time in Iran and could be considered as emerging strains.

Keywords: *Klebsiella pneumoniae*, Aminoglycoside, Multilocus sequence typing, Antibacterial agent.

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Introduction

Klebsiella pneumoniae is one of the most momentous causes of nosocomial infections, which could cause different types of infections including bloodstream infections, urinary tract infections, meningitis, pneumonia, and wound or surgical site infections^[1]. Despite the development of new antimicrobial agents, aminoglycosides are one of the main drugs used in the treatment of *Klebsiella* infections. However, in recent years, resistance to these antibiotics has also increased in this bacterium^[2].

Several mechanisms have been proposed for *K. pneumoniae* resistance to aminoglycosides. These mechanisms include: 1) the presence of aminoglycoside modifying enzymes such as aminoglycoside acetyltransferases (AACs), phosphotransferases (APHs), and nucleotidyltransferases (ANTs), which inactivate aminoglycosides; 2) decrease in membrane permeability, 3) the presence of 16S ribosomal RNA methyltransferases (RMTases), and 4) overexpression of the resistance-nodulation-division (RND)-type efflux pump (AdeABC) and multi-antimicrobial extrusion protein (MATE)-type efflux pump (AbeM). However, none of which alone could cause resistance to all aminoglycosides^[3].

In recent years, RMTases have emerged as a novel and worrying source of conferring aminoglycoside resistance. RMTases contribute to antibiotic resistance by methylation of nucleotides at the AG junction of 16S rRNA (location A) using S-adenosyl-L-methionine as a co-substrate. RMTases are classified into two groups: those producing an N1-methyl A1408 (NpmA) and those producing an N7-methyl G1405 (RmtA, RmtB, RmtC, RmtD1, RmtD2, RmtE, RmtF, RmtG, RmtH, and ArmA)^[3-4].

The first 16S RMTase genes (*rmtA* and *armA*) were found in *Pseudomonas aeruginosa* and *K. pneumoniae* strains, respectively^[5].

Thereafter, other 16S RMTase genes (*rmtB* through *rmtH* and *npmA*) were detected in clinical isolates. Among 16S RMTase genes, *armA* and *rmtB* have been detected in various species of Gram-negative bacilli in Asia^[6].

Knowing the resistant types of *K. pneumoniae* isolates is a crucial step for epidemiological surveillance and controlling infections caused by this bacterium. Numerous biochemical and molecular methods could be used to characterize *K. pneumoniae* strains. Molecular methods such as multilocus sequence typing (MLST) and enterobacterial repetitive intergenic consensus (ERIC) are more preferable than phenotypic methods. MLST is an unambiguous method used for characterizing isolates using the sequences of seven housekeeping genes^[7]. However, ERIC-PCR procedure is based on the amplification of random distribution of inter-genome parts, by which various organisms could be recognized from one another^[8].

Objectives: The emergence of aminoglycoside resistance among Gram-negative bacilli has challenged therapeutic approaches. The present study aimed to assess phenotype (determination of the MIC of the aminoglycosides) and genotype-based (identification of RMTases genes) aminoglycoside resistance in *K. pneumoniae* isolates. In addition, the isolates were typed using ERIC-PCR and MLST methods to understand the genetic relationship and diversity of the resistant isolates.

Materials and Methods

Isolation and identification of *K. pneumoniae*: From September 2018 to August 2019, a total of 300 urine samples were collected from patients at Milad hospital in Tehran, Iran. The samples were cultured on macConkey agar and blood agar and incubated at 37 °C for 24 hrs.

Identification of *K. pneumoniae* isolates was carried out by common biochemical tests (Gram staining, growth on MacConkey agar, catalase, urease, and MRVP tests) [9].

Minimum inhibitory concentrations:

The minimum inhibitory concentration (MIC) was determined for gentamicin and amikacin by broth microdilution tests according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [10]. In brief, 100 µL of bacterial suspension (OD=0.8 to 1 at 625 nm) and 100 µL of gentamicin or amikacin at different concentrations (0.25 to 256 µL/mL) were added into each well of ELISA microplates and incubated at 37 °C for 24 hrs. MIC was defined as the lowest concentration of antibiotics, inhibiting the visible growth of bacteria.

Identification of 16S rRNA methyltransferase genes: DNA was extracted using the phenol-chloroform procedure, and its concentration and purity was quantified using a Nanodrop

spectrophotometer (Thermo Scientific). The targeted sequences of seven RMTase genes were amplified using the previously reported primers (Table 1). The total reaction volume (25 µL) contained 12.5 µL of 2X Master Mix RED (Amplicon), 0.5 µL (25 pmol) of each primer, 2.5 µL (300 ng) of extracted DNA, and 9 µL of deionized water (SinaClon, Iran). Polymerase chain reaction (PCR) was performed under the following conditions: an initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 60 s, 55 °C for 45 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. Following amplification, 8 µL of each microtube was analyzed with 1% agarose gel electrophoresis to detect positive samples.

Multilocus Sequence Typing: *K. pneumoniae* genomic DNA was extracted using DNA extraction kit (CinnaGen, Iran). The internal fragments of seven

Table 1) Primers used for the amplification of RMTase genes and ERIC-PCR

Gene	Primer Sequence	Amplicon Size(bp)	Reference
<i>armA</i>	ATTCTGCCTATCCTAATTGG ACCTATACTTTATCGTCGTC	315	[6]
<i>rmtA</i>	ATGAGCTTTGACGATGCCCTA TCACTTATTCCCTTTTATCATG	756	[11]
<i>rmtB</i>	GCTTTCTGCGGGCGATGTAA ATGCAATGCCGCGCTCGTAT	173	[6]
<i>rmtC</i>	CGAAGAAGTAACAGCCAAAG ATCCCAACATCTCTCCCACT	711	[12]
<i>rmtD</i>	CGGCACGCGATTGGGAAGC CGGAAACGATGCGACGAT	401	[12]
<i>rmtE</i>	ATGAATATTGATGAAATGGTTGC TGATTGATTTCTCCGTTTTTG	818	[13]
<i>rmtF</i>	GCGATACAGAAAACCGAAGG ACCAGTCGGCATAGTGCTTT	589	[14]
ERIC	AAGTAAGTGACTGGGGTGAGCG	100-1500	[15]

mhousekeeping genes (*gapA*, *rpoB*, *tonB*, *pgi*, *mdh*, *infB*, *phoE*) of *K. pneumoniae* isolates were amplified using specific primers (Table 2) by referring to the online MLST database (<http://mlst.warwick.ac.uk/mlst/dbs/Senterica>). PCR amplification was performed as follows: an initial denaturation at 95 °C for 2 min, followed by denaturation at 94 °C for 2 min, annealing at 45-60°C for 20 s (based on each gene), extension at 72 °C for 30 s (35 cycles), and a final extension at 72 °C for 5 min. PCR products were sent to MicroGen Company in South Korea for sequencing. Alleles number and STs were determined based on the instructions provided in the online MLST databases [<https://bigsd.pasteur.fr/klebsiella/klebsiella.html>].

ERIC-PCR typing: ERIC-PCR reactions were performed in 24 µL volumes containing 1 µL of template DNA (400 ng/µl), 12 µL of 2X master mix (Amplicon), 1 µL of primer (10 pmol) (Table 1), and 9 µL of distilled water. ERIC-PCR reaction was carried out under the following conditions: an initial denaturation at 94 °C for 15 min, denaturation at 94 °C for 60 s, annealing at 37 °C for 60 s, extension at 72 °C for 4 min (32 cycles), and a final extension at 72 °C for 5 min. Eventually, 10 µL of PCR product was electrophoresed on 2% agarose gel and photographed under UV light in a gel documentation system [15]. The zero-one manual procedure was applied to count the bands. Thereafter, data were entered in NTSYS pc 2.02 software to draw a dendrogram.

Table 2) Primers used for MLST typing [7]

Locus	Primer Sequence	Size (bp)	Annealing Temp. (°C)	No. of Alleles
<i>rpoB</i>	GGCGAAATGGCWGAGAACCA GAGTCTTCGAAGTTGTAACC	501	50	8
<i>gapA</i>	TGAAATATGACTCCACTCACGG CTTCAGAAGCGGCTTTGATGGCTT	450	60	6
<i>mdh</i>	CCCAACTCGCTTCAGGTTTCAG CCGTTTTTCCCCAGCAGCAG	477	50	10
<i>pgi</i>	CTGCTGGCGCTGATCGGCAT TTATAGCGGTTAATCAGGCCGT	432	50	6
<i>phoE</i>	ACCTACCGCAACACCGACTCTTCGG TGATCAGAAGTGGTAGGTGAT	420	50	14
<i>infB</i>	ACTAAGGTTGCCTCCGGCGAAGC CGCTTTCAGCTCAAGAACTTC	318	50	10
<i>tonB</i>	CTTTATACCTCGGTACATCAG GTT ATTCCCGGCTGRGCRGAGAG	414	45	21

Findings

MIC: Totally, antibiotic susceptibility testing was performed for 100 confirmed *K. pneumoniae* isolates recovered from urine samples. Antibiotic sensitivity was reported according to data presented by CLSI 2017 [10]. Broth microdilution test results showed that 70% of the isolates were resistant to amikacin, and 30% were susceptible. In addition, 52% of the isolates were resistant to gentamicin, 45% were susceptible, and 3% were intermediate. Overall, 40% of the strains were resistant to both aminoglycosides.

Detection of 16S rRNA methyltransferase genes: All 82 *K. pneumoniae* isolates that were phenotypically resistant to at least one tested aminoglycoside, were screened for the presence of seven genes encoding RMTases (Figure 1). The electrophoresis of PCR product of RMTase genes among the aminoglycoside-resistant isolates revealed that *rmtC* (59.8%) was the most prevalent type of RMTases, followed by *rmtA* (51.2%),

rmtD (47.6%), *rmtF* (43.9%), *rmtE* (41.5%), *armA* (41.5%), and *rmtB* (7.3%).

Among the resistant isolates, 11 (13.4%) carried only one RTMase gene. The simultaneous presence of 2, 3, 4, 5, and 6 genes was observed in 14 (17.1%), 12 (14.6%), 16 (19.5%), 11 (13.4%), and 7 (8.5%) isolates, respectively. Also, 11 (13.4%) isolates carried none of the RMTase genes.

MLST typing: In the present study, two isolates of *K. pneumoniae* were studied using MLST method. After sequencing of seven housekeeping genes, the number of alleles and ST of the isolates were identified. The allelic profiles of *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB* genes were 1, 2, 13, 10, 17, 1, and 19 in ST51 and 4, 2, 2, 1, 4, 25, and 8 in ST149, respectively. ST51 and ST149 were reported for the first time in Iran, and both strains carried at least four RMTase genes.

ERIC-PCR typing: To investigate the similarity of aminoglycoside resistant isolates, ERIC-PCR electrophoresis results

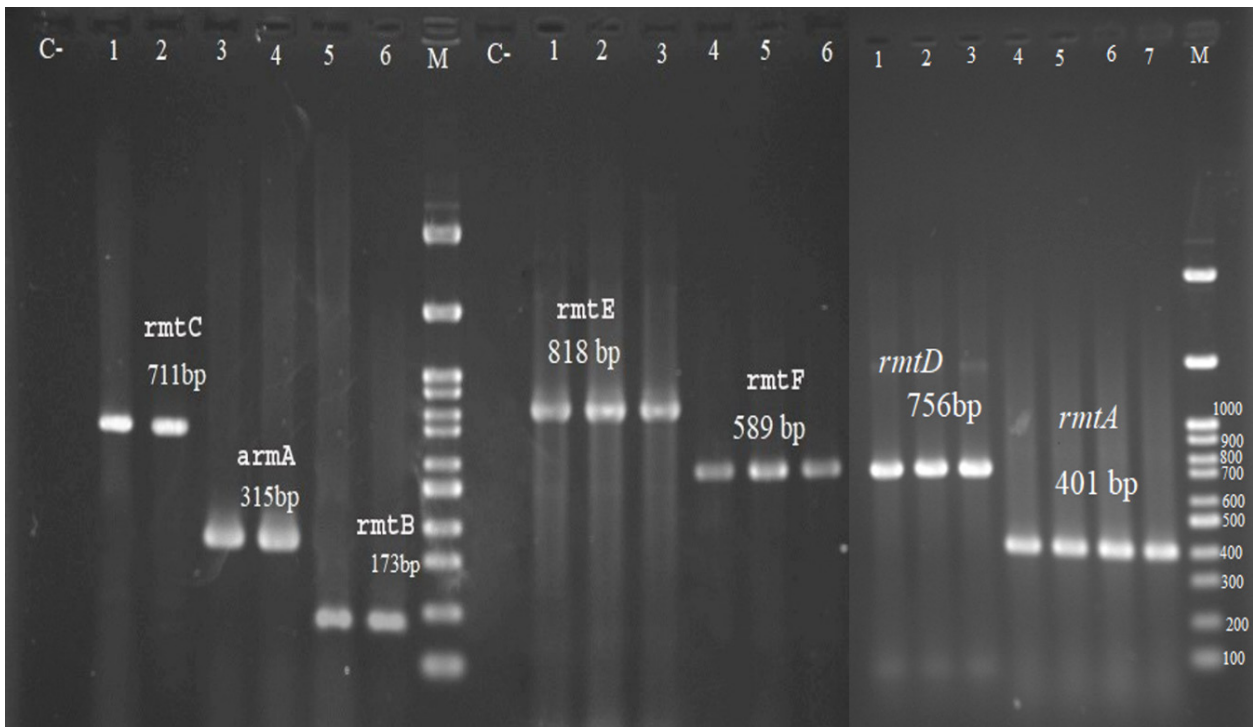


Figure 1) Electrophoresis of PCR products of 16S rRNA methyltransferase genes. M: 100 bp DNA size marker, Lane 1-7: PCR product of RMTase genes, Lane C- (Negative control).

were entered into NTSYS software, and the amount of genetic distance was measured and compared. Cluster analysis and construction of a dendrogram were carried out using unweighted pair group method with arithmetic averages (UPGMA) to determine the relatedness of *K. pneumoniae* isolates

carrying RMTase genes (Figure 2). Out of 82 isolates subjected to ERIC-PCR method, 74 PCR patterns were obtained. In total, 5–10 bands were generated by ERIC primer with molecular weights ranging from 100 < to >1500 bp. The discriminatory index of this method involving 82 strains was 0.981.

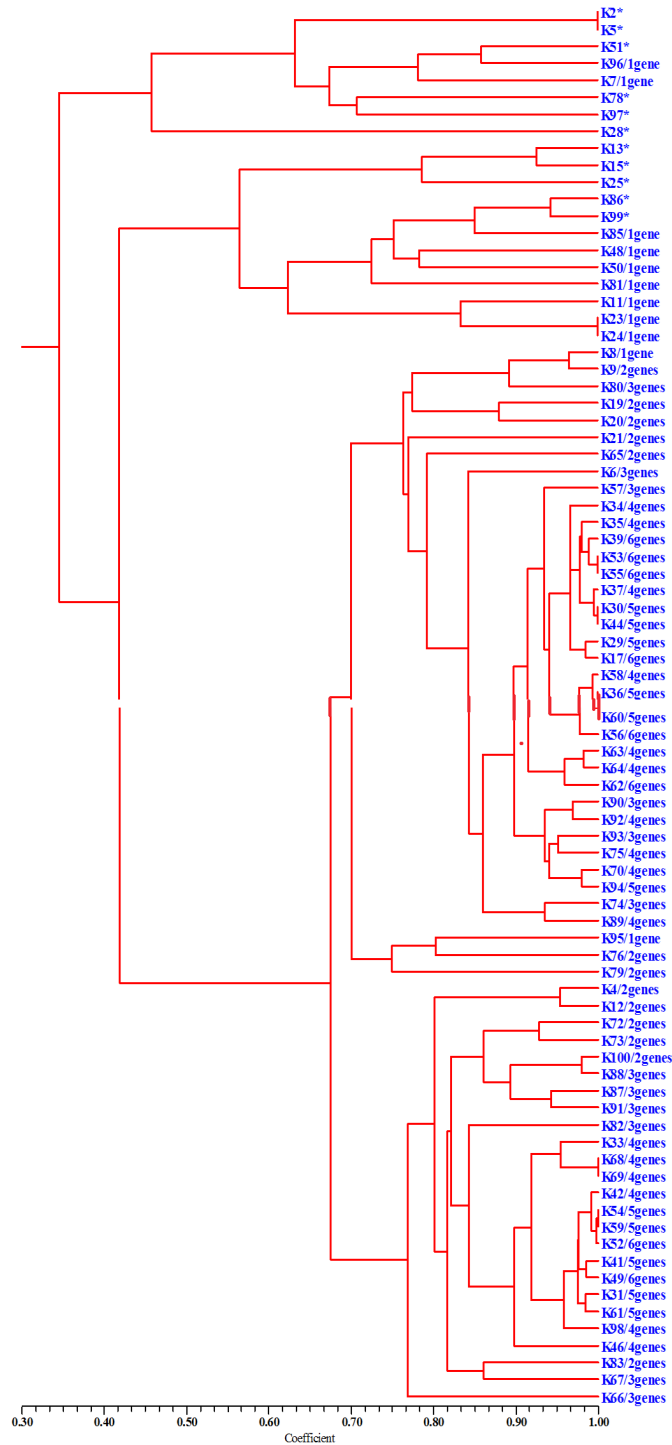


Figure 2) Dendrogram of 82 aminoglycoside resistant *K. pneumoniae* isolates based on the results of ERIC-PCR method.
*:RMTase-free isolates

Discussion

The prevalence of antibiotic resistance among pathogenic bacteria has become a serious matter worldwide. *K. pneumoniae* is an opportunistic pathogen that causes community and hospital acquired infections [2]. Effective use of microbiological laboratories to correctly and timely diagnose and prevent the spread of resistant pathogens reduces the need for drug. Controlling infection is very beneficial for patients and reduces mortality worldwide. Aminoglycosides are applied to treat severe infections caused by Gram-positive and negative bacteria such as *Enterobacteriaceae*. Therefore, resistance to aminoglycosides has caused widespread concern in medicine [16].

In this study, resistance of 100 *K. pneumoniae* isolates to amikacin and gentamicin was measured by broth microdilution method. According to the results, 82 isolates (82 %) displayed resistance to amikacin and/or gentamicin. Among which, 40 isolates were resistant to both aminoglycosides. These results indicated the expansion of resistance to aminoglycosides among the studied isolates. Therefore, these drugs may not be as effective as first-line agents in treating infections caused by *K. pneumoniae*. In this case, Kiani et al. (2015) reported that 36.8 and 22.3% of *K. pneumoniae* clinical isolates recovered from three hospitals in Tehran were resistant to gentamicin and amikacin, respectively [17]. In our previous study conducted in 2017, resistance to gentamicin and amikacin was found to be 52 and 65%, respectively [18]. In another study, Liang et al. (2015) showed that the resistance rate of ESBL-producing and ESBL-negative *K. pneumoniae* isolates to amikacin was 22.2 and 12.1%, respectively [19]. The rate of resistance to amikacin and gentamicin in this study was higher than that reported by Liang et al. (2015) and Kiani et al. (2015). The pattern of antibiotic resistance usually

varies from region to region or from hospital to hospital, which could be due to the treatment regimen used in each area and the difference in access to antibiotics [20].

In the present study, all the resistant isolates to either amikacin or gentamicin were screened for the presence of seven genes encoding the aminoglycoside resistance protein. The results showed that 86% of the isolates carried at least one of the genes screened, indicating the important role of these genes, especially *rmtC*, in conferring resistance to amikacin and gentamicin among *K. pneumoniae* isolates. It was documented that RMTase genes were first found in *K. pneumoniae* strains in 2003 [21]. Also, *rmtB* gene was first identified in *Serratia marcescens* strains in Japan in 2004 and subsequently in *Escherichia coli* and *K. pneumoniae* isolates in Belgium, Korea, and Taiwan [22-23].

Very few studies have been conducted in our country to detect RMTase genes in *K. pneumoniae* isolates. In a study by Ashrafian et al. (2015), examination of genes encoding RMTase proteins in *K. pneumoniae* clinical isolates demonstrated that *armA* and *rmtC* were present in 36.3 and 13.6% of the isolates, respectively. However, *rmtB* and *rmtD* genes were detected in none of the isolates [24]. Wu et al. (2009) showed that among the 202 *K. pneumoniae* isolates, the prevalence of *armA* and *armB* genes was 3.0 and 2.8%, respectively. Also, none of the 16S rRNA methylase genes were observed in the strains susceptible to amikacin and/or gentamicin [25]. In the present study, all seven RMTase genes under study were detected in *K. pneumoniae* isolates, most of which had not been previously reported. To the best of our knowledge, this is the first report concerning the presence of *rmtA*, *rmtD*, *rmtE*, and *rmtF* genes among *K. pneumoniae* strains in Iran. Overall, in this study, the prevalence rate of 16S rRNA methylase determinants (*rmtC*,

rmtE, *rmtF*, *rmtD*, *rmtA*, and *armA*) was more than 41%, which is very high compared to the results reported from Japan (0.03%) [6], Turkey (0.7%) [26], and China (10%) [25].

Difference in the reported frequencies of resistance genes could be due to the difference in study method, technician experience, and study area. For example, Guo et al. (2014) demonstrated that using qPCR, it is possible to rapidly detect 16S methyltransferase activity in *Enterobacteriaceae* [27].

MLST is a technique in molecular biology for the typing of multiple loci. It was first suggested in 1998 as a typing method for the classification of *Neisseria meningitidis*, and subsequently, a number of different bacteria and eukaryotes were classified by this method [28]. Molecular typing of multidrug-resistant isolates plays a substantial role in preventing and controlling infections by limiting the release of resistant isolates [29-30]. Therefore, this study was focused on the epidemiological investigation of MLST, and to do so, two amikacin and gentamicin-resistant isolates were selected. The *gapA* gene allele was similar between the two strains, and both carried the *gapA2* allele, while the other alleles were different between the two isolates. In general, the results of allele detection showed a diversity of allele types between the two studied isolates and therefore led to the presentation of two different ST types (ST51, ST149). Since these types were reported for the first time in Iran, they could be considered as emerging strains. ST51 could be more important than ST149 because it carries four RMTase genes (*armA*, *rmtC*, *rmtE* and *rmtF*) at the same time. In this regard, Ayad et al. (2016) showed that RmtB-producing isolates were genotypically related and belonged to ST405, while ArmA-producing isolates belonged to ST10, ST117, and ST167 [31]. It is necessary to take appropriate health measures to control such strains. The

study of two MLST types also showed that such sequence types were reported only in China. These results could indicate the geographical transfer of these STs between China and Iran. However, such a report requires further studies to be conducted in this field. In this case, Diancourt et al. (2005) reported that MLST types of *K. pneumoniae* strains are very diverse, and to study their epidemiology, a lot of isolation is required [7]. ERIC-PCR results showed that *K. pneumoniae* isolates had a very high genetic diversity, and a total of 74 different patterns were observed among 82 isolates. The results of other typing methods, such as the MLST method, showed that the genetic diversity of *K. pneumoniae* strains was greater than that of other *Enterobacteriaceae* family members (<http://www.pasteur.fr/mlst/>). One of the important findings of ERIC-PCR method was that isolates with no RMTase gene had a close genetic relationship with each other and were located in a common cluster in the dendrogram. These results suggest that a specific group of *K. pneumoniae* isolates may carry RMTase genes, or the spread of aminoglycoside resistance genes occurs between certain strains of *K. pneumoniae*. In this case, Amiri et al. (2019) reported a significant association between efflux pump genes and ERIC-PCR patterns in some clonal types [32]. Furthermore, Zhang et al. (2018) showed that isolates harboring similar virulence factors were generally genetically related [33], which is similar to the present study findings. However, in a number of studies, there was no relationship between ERIC-PCR and biofilm formation or the origin of isolates [34], suggesting that in addition to the ERIC-PCR method, other molecular methods such as MLST could be used.

Conclusion

The present study results indicated that the frequency of *K. pneumoniae* isolates resistant

to aminoglycosides was high; therefore, these antibiotics have lost their effectiveness in the treatment of urinary tract infections caused by *K. pneumoniae*. In addition, the results of this study showed that *rmtC* followed by *rmtA* gene had the highest frequency among the studied isolates. This is the first report about the presence of *rmtA*, *rmtD*, *rmtE*, and *rmtF* genes among *K. pneumoniae* isolates in Iran. Since resistance genes are often located on moving genetic elements such as transposons and plasmids, they are more likely to be transmitted to other members of the *Enterobacteriaceae* family. In general, due to the increase in antibiotic resistance and rapid proliferation of resistance genes among *Enterobacteriaceae*, rapid and timely detection of resistant strains and their typing by molecular methods (such as MLST and ERIC-PCR) are essential to prevent their proliferation. ERIC-PCR typing indicated high genetic diversity among aminoglycoside resistant *K. pneumoniae* isolates, and bacteria without RMTase genes were generally genetically related.

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Ethical Permission: The study was approved by the Ethics Committee of Islamic Azad University of Tehran Medical science (No: IR.IAU.PS.REC.1399.102).

Conflict of Interests: The authors declare that there is no conflict of interest.

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