

## High Prevalence of CTXM-15 Type Extended-Spectrum Beta-Lactamase Among Clinical Isolates of *Klebsiella Pneumoniae*

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### Abstract

**Background:** Production of  $\beta$ -lactamases by enterobacteriaceae, especially *Klebsiella pneumoniae*, is one of the emerging health problems in the world. The purpose of this study was to assess the frequency of *bla*<sub>CTX-M15</sub> gene in *K. pneumoniae* isolates and determine the molecular diversity of CTXM producing isolates.

**Methods:** In this descriptive cross-sectional study, 100 *K. pneumoniae* strains were tested for susceptibility to cephalosporins category by using disk diffusion method and the prevalence of *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-2</sub>, *bla*<sub>CTX-M-9</sub> and *bla*<sub>CTX-M-15</sub> genes in these isolates were determined by PCR method. Eventually, a number of isolates were sequenced and typed using single locus sequence typing (SLST) of *bla*<sub>CTX-M<sub>group1</sub></sub> gene. The phylogenetic relatedness of all CTX-M producing isolates was determined using enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR).

**Results:** The rates of resistance to cephalosporins category were in the following order: cefepime (57%), ceftazidime (54%), cefotaxime (36%), cefoxitine (32%), and ceftriaxone (31%). Of all 100 *K. pneumoniae* isolates, 31 were harboring CTX-M genes, and the *bla*<sub>CTX-M-15</sub> (77.5%) was the most common of the genes investigated. The results of SLST showed that all of the sequenced isolates were divided into two groups. ERIC-PCR method represented ten different genotypes in CTX-M positive isolates.

**Conclusion:** The results of this study showed that one of the most important reasons for resistance to cephalosporins is the presence of *bla*<sub>CTX-M</sub> genes. In addition, there was a high genetic variation among *bla*<sub>CTX-M</sub> genes positive isolates.

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### Introduction

*Klebsiella pneumoniae* is one of the opportunistic pathogens which is associated with both hospital and community infections. This bacterium causes many diseases including septicemia, bacteremia, arteritis, meningitis, urinary tract and soft tissue infections in people with weakened

immune systems (1).  $\beta$ -Lactamases are major drugs in the treatment of *K. pneumoniae* infection. These antibiotics inhibit bacterial cell wall synthesis by binding covalently to penicillin binding proteins (PBPs) in the cytoplasmic membrane. However, over the past 10 years, extended spectrum beta-lactamase (ESBL)-producing *K. pneumoniae* have

increasingly been reported worldwide, and beta-lactam drugs gradually lose their effectiveness (2).

One of the reasons for resistance to beta-lactams in enterobacteriaceae is the outbreak of extended-spectrum  $\beta$ -lactamase producers. Beta-lactamase enzymes, by destroying the  $\beta$ -lactam ring, inhibit the effect of  $\beta$ -lactam antibiotics on the bacterial cell wall (3). Based on function,  $\beta$ -lactamase enzymes are classified into four groups or four main classes including A, B, C, and D Ambler. The TEM, SHV and CTX-M-type beta-lactamases are placed in class A Ambler. CTX-M  $\beta$ -lactamases have little genetic linkage with TEM and SHV beta-lactamases types, but are very similar to the chromosomal AmpC enzyme (4). The CTX-M-type beta-lactamases based on their amino acid are divided into five main groups that include CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (4,5).

The prevalence of CTX-M is increasing in all continents; however, the abundance of these genes in the three geographic regions of South Africa, Eastern Europe and South America is very high (6). In sum, the high prevalence of CTX-M in India and China shows that these genes are the most abundant beta-lactamase genes in the world (7). In particular, a rapid increase of CTX-M-15, which belongs to the CTX-M-1 group has been widely reported, and CTX-M-15 is now the most common ESBL in many parts of the world (6).

The increased prevalence of extended-spectrum  $\beta$ -lactamase gene in *K. pneumoniae* is a serious global health issue for healthcare professionals and the importance of the disease makes new demands on researchers to perform ESBL typing. In fact, typing of ESBL isolates is necessary to prevent and control infections caused by these bacteria. Several phenotypic and genotypic methods are available for this

purpose. However, PCR-based methods such as enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and single locus sequence typing (SLST) are simple, rapid, cheap and useful techniques for epidemiological investigations of enterobacteriaceae (8).

The present study was conducted to investigate the distribution of  $\beta$ -lactamase genes (*bla*<sub>CTX-M</sub>) and genotyping based on the sequence of *bla*<sub>CTX-M</sub> genes and intergenic consensus in clinical isolates of *K. pneumoniae*, Tehran, Iran.

## Material and Methods

### Sample collection and bacterial isolation

This descriptive cross-sectional study was conducted during May 2016 to April 2017. In this study, 100 urinary isolates of *K. pneumoniae* were collected from Milad hospital and identified using standard microbiological techniques. In the following, DNA of all isolates was extracted by the boiling method and stored at  $-20\text{ }^{\circ}\text{C}$  prior to PCR assay (9).

### Antimicrobial susceptibility testing

The antibiotic susceptibility test was carried out by agar disk diffusion method, according to the guidelines of the National Committee for Clinical Laboratory Standards (CLSI) (2017), using 5 antibiotics from cephalosporins category including: ceftriaxone (30  $\mu\text{g}$ ), cefotaxime (30  $\mu\text{g}$ ), ceftazidime (30  $\mu\text{g}$ ), cefepime (30  $\mu\text{g}$ ) and ceftazidime (30  $\mu\text{g}$ ) (10).

### Detection of *bla*<sub>CTX-M</sub> genes

At first, all 100 *K. pneumoniae* isolates were assessed for the detection of *bla*<sub>CTX-M</sub> gene and then positive isolates were subjected to identify *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-2</sub>, *bla*<sub>CTX-M-9</sub> and *bla*<sub>CTX-M-15</sub> genes by specific primers (Table 1). The PCR reaction was carried out in a final volume of 25  $\mu\text{l}$  containing 12.5  $\mu\text{l}$  of

mastermix (Amplicon, Denmark), 10 pmol of each primer, 450 ng of extracted DNA and 9 µl of deionized water. The PCR amplification was performed under the following conditions: initial denaturation at 94°C for 7 min followed by denaturation at 95°C for 50 sec, annealing at 55°C for 50 sec and extension

at 72°C for 1:20 min (35 cycles) and a final extension at 72°C for 12 min. Then, the amplified products were visualized with UV light after electrophoresis on 1% agarose gel containing DNA Safe Stain Dye (3 µl in 100 ml) (Cinaclone, Iran).

**Table 1.** Primers used to detect bla<sub>CTX-M</sub> genes

Target gene	Sequence 5'-3'	Product size (bp)	Reference
<i>bla</i> <sub>CTX-M</sub>	ATGTGCAGCACCAGTAAAGTGATGGC TGGGTAAAGTAAGTGACCAGAATCAGCGG	593	(11)
<i>bla</i> <sub>CTX-M1</sub>	GGTTAAAAAATCACTGCGTC TTGGTGACGATTTTAGCCGC	863	(12)
<i>bla</i> <sub>CTX-M2</sub>	ATGATGACTCAGAGCATTTCG TGGGTTACGATTTTCGCCGC	865	(12)
<i>bla</i> <sub>CTX-M9</sub>	ATGGTGACAAAGAGAGTGCA CCCTTCGGCGATGATCTC	869	(12)
<i>bla</i> <sub>CTX-M15</sub>	CACACGTGGAATTTAGGGACT GCCGTCTAAGGCGATAAACA	996	(13)

### ERIC-PCR

ERIC-PCR typing was performed using the primer ERIC-1 (5-AAGTAAGTGACTGGGG TGAGCG-3). This has been previously reported by Wasfi et al. (14). The ERIC-PCR reaction (20 µl) contained 12.5 µl of Master Mix 2x (Amplicon, Denmark), 300 ng of template DNA, 10 pmol of primer and 9.5 µl distilled water. The amplification was carried out using a similar PCR program to that of the *bla*<sub>CTX-M</sub> genes, except for an annealing temperature of 37 °C.

### Analysis of ERIC-PCR data

For this purpose, the results of the ERIC-PCR electrophoresis were scored 1 and 0 (the presence of band = 1, the absence of band = 0), and then the data were entered into the NTSYS-PC software (version 2.1, USA). First, Jaccard's

coefficient was calculated among the isolates, then the clustering of isolates and construction of a dendrogram was performed using unweighted pair group method with arithmetic averages (UPGMA) (15).

### Power of discrimination

Numerical index of the discriminatory ability of ERIC-PCR method was calculated by applying Simpson's Index of Diversity equation which is given as follows:

$$D = \frac{1}{N(N-1)} \sum_{j=1}^S nj(nj - 1)$$

N = Total number of isolates

S = Total number of types

N<sub>j</sub> = The number of strains belonging to the jth type (16).

### SLST typing

Twelve isolates were selected for sequencing the *bla*<sub>CTX-M group-1</sub> gene. This gene was amplified using primers described above in a volume of 100 µl. Sequences were determined with an ABI 3730XL DNA Analyzer by BIONEER Company in Korea. The nucleotide sequences were analyzed using the DNASTAR's Lasergene sequence analysis software (Version 14.1), and dendrogram was designed using the Mega software (Version 6.0) by comparing the sequences recorded in the NCBI database.

### Statistical analysis

The results were compared by Spearman correlation test and Chi square test using SPSS software (Version 19.0), and  $P < 0.05$  was considered statistically significant.

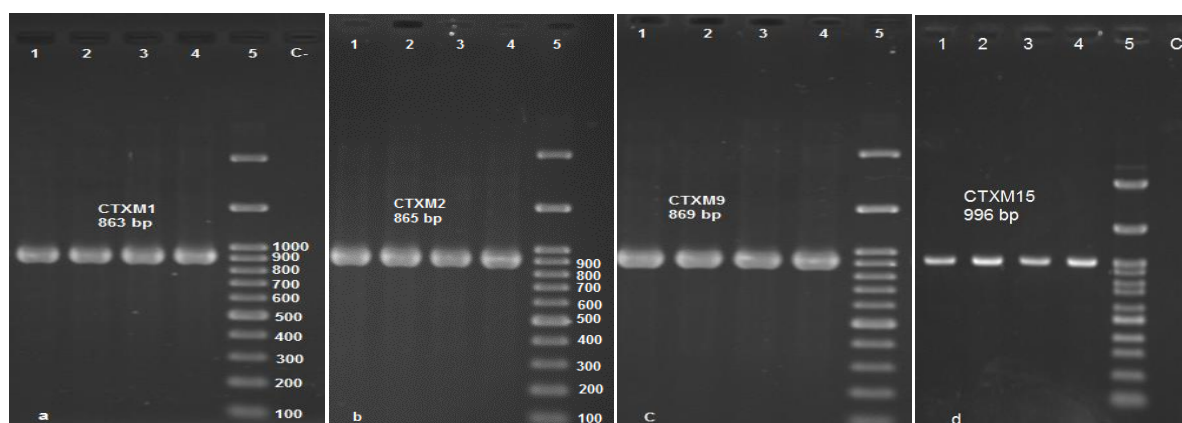
## Results

### Antimicrobial susceptibility test

Antimicrobial susceptibility of 100 *K. pneumoniae* isolates against 5 agents from cephalosporin category was performed by disk diffusion test and results showed that the percentage of resistance to cefepime, ceftazidime, cefotaxime, ceftioxin and ceftriaxone was 57%, 54%, 36%, 32% and 31%, respectively.

### Detection of *bla*<sub>CTX-M</sub> genes

In the current study, the amplification results of beta-lactamase genes demonstrated that out of the 100 isolates, 31 strains (31%) were positive for *bla*<sub>CTX-M</sub> gene. Of all 31 isolates, 87%, 77.5%, 25.8% and 16.1% of isolates were harboring *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>CTX-M-9</sub> and *bla*<sub>CTX-M-2</sub> genes, respectively. In addition, the simultaneous presence of *bla*<sub>CTX-M-1</sub>/*bla*<sub>CTX-M-2</sub> and *bla*<sub>CTX-M-1</sub>/*bla*<sub>CTX-M-9</sub> was detected in five and six isolates, respectively. Figure 1 represents the electrophoretic patterns of the *bla*<sub>CTX-M</sub> genes.



**Figure 1.** PCR amplification of *bla*<sub>CTX-M</sub> genes. Lane 1-4 positive results for *bla*<sub>CTX-M-1</sub> (a), *bla*<sub>CTX-M-2</sub> (b), *bla*<sub>CTX-M-9</sub> (c) and *bla*<sub>CTX-M-15</sub> (d) genes, Lane 5 100bp DNA size marker.

Our findings indicated that resistance to cephalosporins category among *bla*<sub>CTX-M</sub> positive isolates was higher than negative isolates ( $p < 0.05$ ). The results also showed that resistance to ceftriaxone, cefotaxime and ceftazidime was significantly correlated with the presence of *bla*<sub>CTX-M</sub> genes.

As shown in Table 2, most of the CTXM positive isolates belonged to phenotypic group a. This group harbored CTX-M-1 as predominate gene group followed by CTX-M-9. CTX-M-15 was found to be the most frequent gene in CTXM group 1.

**Table 2.** Phenotypic and genotypic characteristics of urinary isolates of *K. pneumoniae*

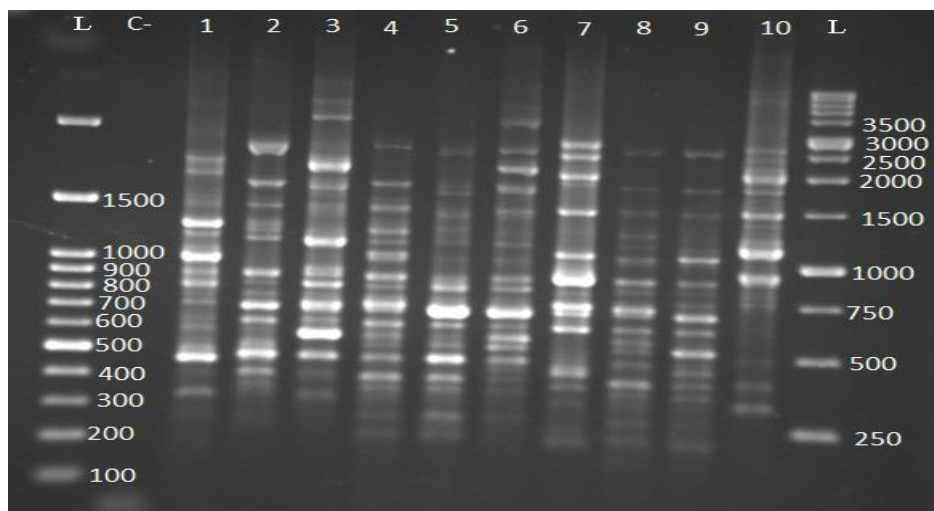
Phenotypic group	No. of isolates (31)	Antibiotics					No. of CTX-M genes positive isolates				ERIC-PCR cluster
		CRO	CTX	FOX	FEP	CAZ	CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-15	
a	17	R	R	R	R	R	16	1	4	15	I-VI, X
b	7	R	R	R	S/I	S/I	6	1	3	6	I, II, V, VII, VIII
c	3	R	R	R	R	S/I	3	2	1	2	VI, VIII
d	1	R	S	S	I	R	1	0	0	0	VI
e	1	S	S	S	I	R	0	1	0	0	IX
f	1	S	I	S	R	I	1	0	0	1	VI
g	1	S	S	S	R	S	0	0	0	0	IX

CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, ceftazidime; CRO, ceftriaxone, Susceptibility results were interpreted according to the CLSI criteria: R, resistant; I, intermediate; S, susceptible.

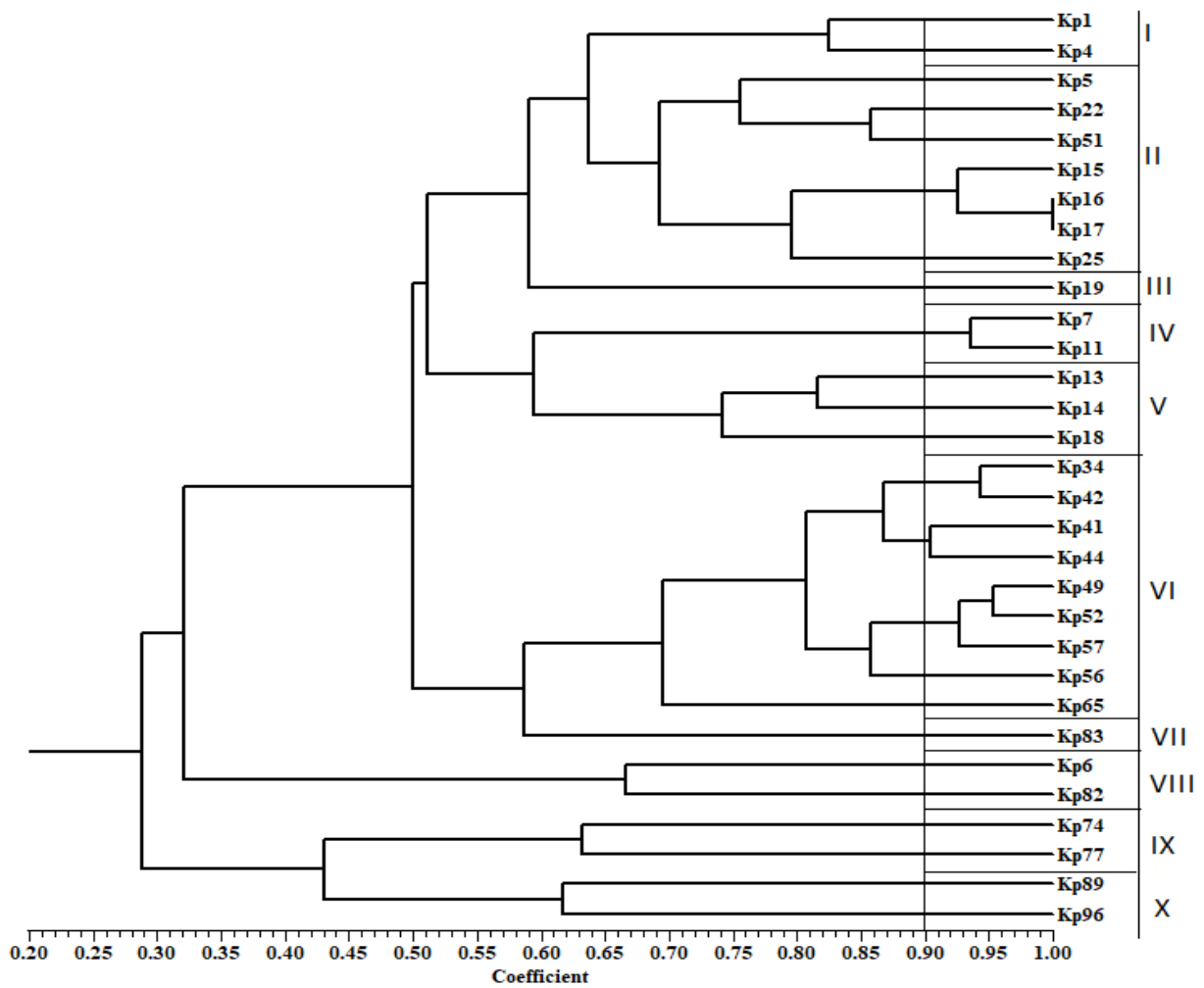
### ERIC-PCR

Of the total 31 isolates subjected to ERIC-PCR method, 31 PCR patterns were observed. In total, 5–12 bands were generated by ERIC primer with molecular weight ranging from

100< to >3000 bp (Figure 2). The generated dendrogram showed that all of the isolates were clustered into ten groups (I–X). In addition, the discriminatory index of this method involving 31 strains was 0.9921 (Figure 3).



**Figure 2.** Example of ERIC-PCR patterns among *bla*<sub>CTXM</sub> positive *K. pneumoniae* isolates. L: 100 and 250 bp DNA ladder, lane C- (Negative control), lane 1-10: tested isolates.

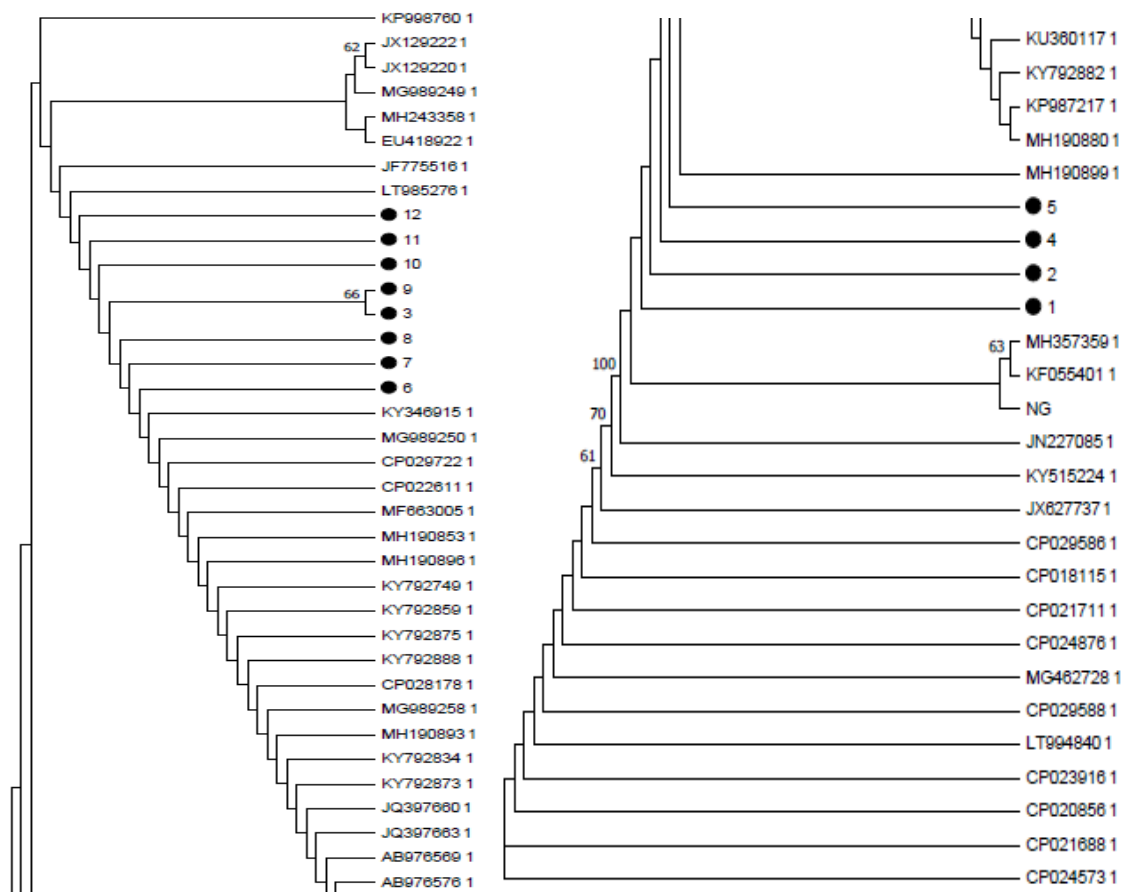


**Figure 3.** Dendrogram of 31 CTX-M positive strains based on ERIC-PCR. (ERIC-PCR cluster I-X).

### SLST typing

The consensus sequences of each isolate were obtained using DNASTar software, and the dendrogram was drawn using Mega software. In Figure 4, 12 isolates of *K. pneumoniae*, which are related to this study, are labeled with solid circles. As

it is shown, our isolates were divided into two groups, indicating that the two groups of the genes are predominant in Milad Hospital, Tehran.



**Figure 4.** Dendrogram of 12 isolates of *K. pneumoniae* carrying beta-lactamase genes based on *bla*<sub>CTX-M\_group1</sub> gene sequence

### Discussion

Multiple drug resistance and ESBL-producing enterobacteriaceae have significantly increased in recent years, and are now an emerging problem worldwide (2). The production of CTX-M  $\beta$ -lactamases is an important mechanism of resistance to cephalosporins category in *K. pneumoniae* isolates (17). The surveillances of phenotypic and genotypic antibiotic resistance in enterobacteriaceae can provide useful information regarding their epidemiology (8).

In this study, the antimicrobial susceptibility of 100 *K. pneumoniae* isolates against 5 agents from cephalosporins categories was determined. The highest rates of resistance were reported for cefepime and ceftazidime. Therefore, these drugs may not be as effective as first-line agents in treating infections caused by *K. pneumoniae*. On the other hand, resistance to other cephalosporins was also a matter of consideration, which is a serious warning to the ineffectiveness of this antibiotic in recent years. This finding is largely in accordance with the

findings reported by other studies (7,18). In a study from Egypt in 2013, more than 63% of *K. pneumoniae* isolates were resistant to cefoxitin and ceftriaxone. The results obtained were higher in comparison to our findings (19). There are many reasons for the variability of bacterial drug resistance among the studies. The access to medical care, population demographics and illicit drug use are some of the variables explaining such differences (20).

The production of CTX-M  $\beta$ -lactamases causes resistance to a range of beta-lactam antibiotics including cephalosporins. In this study, 31% of isolates carried *bla*<sub>CTX-M</sub> genes which indicate the increasing prevalence of this  $\beta$ -lactamase type. In this case, a high prevalence and broad geographic distribution of CTXM- producing enterobacteriaceae have been reported in UK, France, India, Cambodia, Qatar, Russia and Canada, that has become the most prevalent ESBL-type world-wide (21). In a study from Sari, Ahanjan et al. showed that 45% of *K. pneumoniae* isolates harbored the *bla*<sub>CTX-M</sub> gene (22). Maleki et al. reported that 24% of clinical isolates of *K. pneumoniae* from Al-Zahra Hospital, Isfahan, were positive for *bla*<sub>CTX-M</sub> gene (23). In another study conducted by Torshizi et al., in Hajar hospital in Shahrekord, it was shown that 4% of *K. pneumoniae* isolates carried the *bla*<sub>CTX-M</sub> gene (24). It seems that the annual rate of infection with *bla*<sub>CTX-M</sub> positive of *K. pneumoniae* has been on a rise in different regions of Iran, and this could be due to the convenient transfer of these genes by carrier plasmids (25).

The use of rapid and accurate methods in typing of *K. pneumoniae* isolates is essential to investigate the genetic diversity and delineate geographical distribution among enterobacteriaceae. In this study, PCR-based methods such as SLST typing and ERIC-PCR were used for typing of *K.*

*pneumoniae* isolates. In SLST typing, the PCR products of *bla*<sub>CTX-M group1</sub> gene were sequenced and by using the Mega software, the genetic relationship of the isolates was compared with sequences recorded in the NCBI database. The results showed that our isolates are located in two groups. This indicates that the two groups will be compromised by environmental conditions and spread to the community. On the other hand, most of the isolates in the dendrogram were strains identified from neighboring countries (such as Turkey and Russia). This shows the probability of a specific type of outbreak in the region.

In the ERIC-PCR method, based on the generated dendrogram, all the CTXM producing isolates were grouped into ten clusters (I - X) at 90% similarity. This shows that the genetic differences among *K. pneumoniae* isolates were enough to be clustered separately. However, majority of isolates belonged to groups II and VI which were identified as outbreak isolates. This finding is in line with the reports by Seifi et al. and Chmielewski et al. (26,27).

In the dendrogram of ERIC-PCR, most of the isolates were closely related to each other (coefficient 50 %<). However, six isolates (No. 6, 82, 74, 77, 89, 96) were exceptionally distinct and had a different ERIC-PCR profile. Hence, these isolates are considered as the emerging strains, which emphasize the need to control their prevalence (28).

## Conclusion

Monitoring of *bla*<sub>CTX-M</sub> genes in this study showed that the prevalence of these genes were high among clinical isolates of *K. pneumoniae*. It should also be noted that due to the easy transfer of these genes via plasmid, the probability of their spread in the future is possible. On the other hand, due to the high resistance to cefepime and ceftazidime, the use of these



drugs in the treatment of *K. pneumoniae* infections is not recommended. Moreover, results of ERIC-PCR method demonstrated the presence of genetic variation among *bla*<sub>CTX-M</sub> positive isolates and help the differentiation of outbreak isolates from sporadic cases.

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## Conflicts of interest

The authors declare no conflict of interest.

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