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AmpC β-lactamases in Urinary *Klebsiella pneumoniae* Isolates: First Report of ACC Type AmpC β-lactamase Resistance in Iran

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ABSTRACT

Background & Objective: The production of plasmid-mediated AmpC beta-lactamases (PMABLs) among urinary *Klebsiella pneumoniae* isolates causes a severe problem to the successful treatment of urinary tract infections (UTIs). This study was designed to evaluate antimicrobial resistance, the presence of AmpC beta-lactamase genes, and the genetic relatedness among *K. pneumoniae* strains separated from patients with UTI.

Materials & Methods: In this cross-sectional descriptive study, a total of 100 *K. pneumoniae* isolates were collected from UTI cases in Milad Hospital, Tehran, Iran. The sensitivity of the isolates to 12 antibiotics was tested using the Kirby-Bauer disk diffusion method. AmpC production was determined using a boronic acid combined-disk test. Polymerase chain reaction (PCR) was carried out to screen all isolates with family-specific *PMABL* genes. The genetic relatedness of AmpC-producing isolates was determined by an enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR).

Results: Over a period of 11 months, PMABLs were detected in 49 isolates (49%) of *K. pneumoniae*. Resistance to at least three classes of antimicrobials was detected in 30 (61.2%) PMABL producers. Among AmpC producers, 34 isolates harbored only one *AmpC* gene group, including MOX (n=11), EBC (n=8), ACC (n=7), CIT (n=4), FOX (n=2), and DHA (n=2). Multiple *AmpC* gene groups were detected in 15 isolates. The ERIC-PCR showed the polyclonal distribution of AmpC-producing isolates.

Conclusion: In our study, a high frequency of AmpC-producing *K. pneumoniae* was observed. This is the first report of ACC type AmpC beta-lactamase in Iran. Strategies to minimize the spread of AmpC beta-lactamase-producing isolates should be implemented.

Keywords: *Klebsiella pneumoniae*, AmpC beta-lactamases, Urinary tract infections

Introduction

Urinary tract infections (UTIs) are a problematic health issue that can cause severe clinical complications and create substantial economic costs (1). *Klebsiella pneumoniae* is among the most frequently isolated bacteria from UTIs. It is responsible for a significant proportion of hospital-acquired and healthcare-associated infections worldwide (2). In recent decades, the drug resistance of *K. pneumoniae* has rendered the efficacy of beta-lactam antibiotics insufficient (3). The emergence of resistance against beta-lactam drugs due to AmpC cephalosporinases and extended spectrum beta lactamases (ESBLs) is a global public health problem (4).

AmpC beta lactamases are important cephalosporinases whose genes are located on the chromosomes of microorganisms such as *Citrobacter* spp., *Enterobacter* spp., *Morganella* spp., *Hafnia* spp., *Providencia* spp., *Serratia* spp., and *Shigella* spp. (5). They are active against penicillins, monobactams, cephalosporins, oxyiminocephalosporins, and cephamycins. These enzymes, unlike ESBLs, are not impeded by clavulanic acid (5).

Plasmid-mediated AmpC cephalosporinases were first identified in 1989 and are thought to be a derivative of chromosomal *AmpC* genes (6). The presence of such genes in transmissible plasmids facilitates their distribution to the other hospital microorganisms. Plasmid-mediated AmpC beta-lactamases (PMABLs) are most commonly found in nosocomial *K. pneumoniae* and *Escherichia coli* isolates (7-9), and their presence has been reported in other members of the Enterobacteriaceae family (9). This has increased the spread of PMABLs worldwide (5).

Infections caused by AmpC beta-lactamase-producing isolates are clinically and epidemiolog-

ically important and may increase morbidity and mortality (10,11).

To the best of our knowledge, few data are available concerning the frequency of PMABLs in urinary *K. pneumoniae* isolates in Iran. Therefore, the main goal of the present study was to assess the frequency of *AmpC* genes and their variants in urinary *K. pneumoniae* isolates. In addition, the enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) was used to specify the genetic relatedness of AmpC-producing isolates.

Materials and Methods

Bacterial Isolates

In this descriptive cross-sectional study, 100 urinary *K. pneumoniae* isolates were obtained from hospitalized patients in Milad Hospital, Tehran, Iran, from December 2016 to October 2017. The isolates were identified as *K. pneumoniae* by colony morphology, gram staining and standard biochemical tests (12). The ethical approval of the present study was provided by the Ethics Committee of Islamic Azad University of Tehran Medical Branch (No: IR.IAU.TMU.REC.1396.278).

Antimicrobial Susceptibility Testing

All *K. pneumoniae* isolates were examined for their antibiotic resistance profile using Kirby Bauer's disk diffusion method according to the instructions of the Clinical and Laboratory Standard Institute (CLSI) (13).

The antibiotic disks used were ceftriaxone (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), cefepime (30 µg), gentamicin (30 µg), ciprofloxacin (30 µg), levofloxacin (5 µg), amikacin (30 µg), imipenem (10 µg), meropenem (30 µg), piperacillin (30 µg) and aztreonam (30 µg) (Mast Diagnostics, UK). *E. coli* ATCC 25922 was used as a reference (13).

Multidrug resistant (MDR) was estimated according to previously described definitions (14).

Screening of AmpC beta-lactamase-producing Strains

All the isolates were tested for AmpC beta-lactamase production using discs of cefoxitin (30 µg) alone and in combination with boronic acid (400 µg). For this purpose, each isolate was inoculated on a Mueller–Hinton agar plate (Himedia, India). The discs were then placed on the surface of the plate and incubated overnight at 37°C. An increase of ≥ 5 mm in zone diameter around the cefoxitin disc in combination with boronic acid compare to that of cefoxitin disc alone was considered positive (15).

DNA Extraction and PCR Assay

The DNA extraction was carried out by the boiling method as explained by Perez-Perez and Hanson (16). Six families of plasmid-mediated AmpC beta-lactamases, including DHA, MOX, ACC, EBC, CIT and FOX were amplified by a polymerase chain reaction (PCR) using the primers shown in Table 1. PCR reaction (50 µL) contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl2, 0.2 mM each deoxynucleoside triphosphate, 0.5 µM of each primers, 100 ng of extracted DNA and 1.25 U of Taq DNA polymerase (Ampliqon, Denmark). PCR reaction was

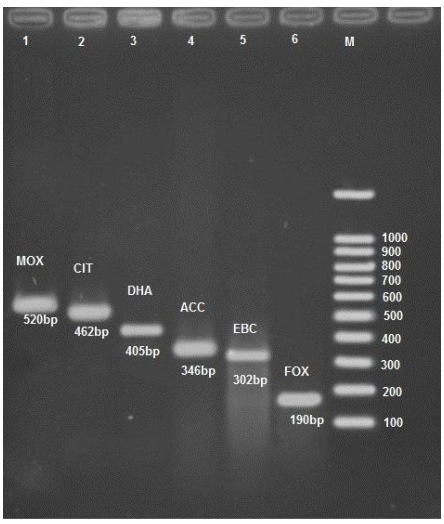

Figure 1. PCR amplification of the *AmpC* genes. Lane 1-6 positive results for *AmpC* genes, M: 100 bp DNA ladder.

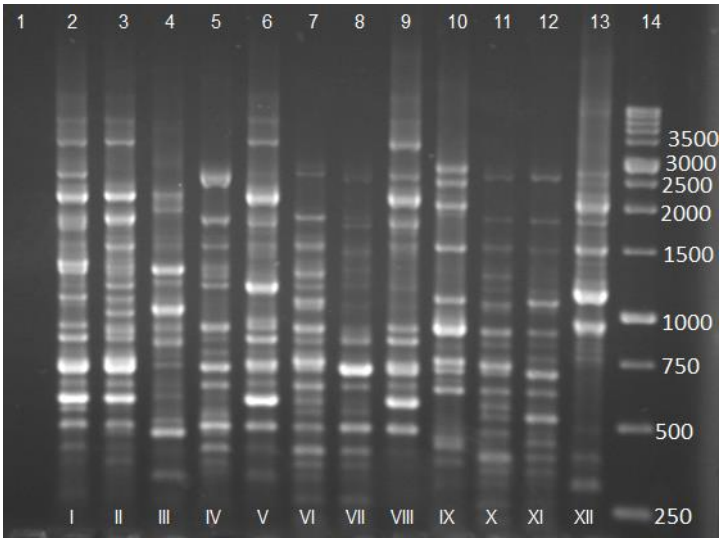

Figure 2. Example of DNA banding patterns obtained for AmpC producing *K. pneumoniae* isolates by ERIC-PCR fingerprinting. 1: Negative Control, 2-13: Twelve clonal types of *K. pneumoniae* isolates, 14: 1Kb Ladder).

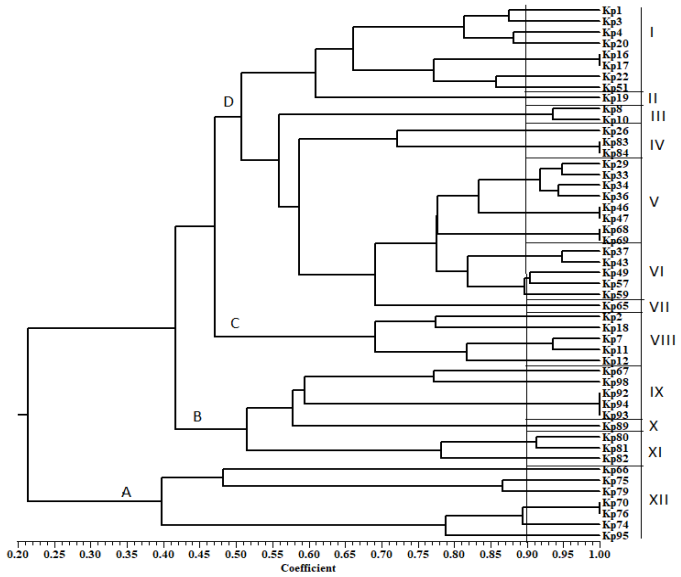

Figure 3. Dendrogram generated by NTSYS software of ERIC-PCR patterns from the 49 AmpC producing isolates. The vertical line represents the similarity cut-off level of 90%.

carried out as follows: initial denaturation at 94°C for 3 min followed by denaturation at 94°C for 30s, annealing at 64°C for 30s, extension at 72°C for 1 min (25 cycles) and a final extension at 72°C for 7 min.

Molecular Typing of AmpC-producing Isolates by ERIC-PCR

The clonal relationships among the AmpC-producing *K. pnumoniae* isolates were determined by ERIC-PCR using the ERIC2 primer as previously described **(17)**.

Briefly, 2 µL of the DNA template was added to 12.5 µL master mix (Ampliqon, Denmark), 1 µL primer (10 pmol), and 9.5 µL H_2O. A PCR reaction was performed under the following conditions: initial denaturation at 94°C for 15 min followed by denaturation at 94°C for 1 min, annealing at 37°C for 1 min, extension at 72°C for 1 min (40 cycles), and a final extension at 72°C for 8 min. The resulting products were analyzed on 1.5% agarose gels. Then, the presence and absence of the bands were scored as 1 and 0, respectively, and the data were analyzed by the NTSYS program (NTSYSpc version 2.10e). Finally, a cluster analysis was performed, and a dendrogram was constructed using an unweighted pair group method with arithmetic averages (UPGMA). To identify clonally related isolates, the similarity cut-off level was set at 90% **(18)**.

Statistical Analysis

Data were analyzed with SPSS 20 (SPSS Inc., Chicago, Ill., USA). Differences between antibiotic resistance among AmpC-positive and negative isolates were statistically analyzed by Chi-square tests. A P-value<0.05 was considered significant.

Results

One hundred *K. pneumoniae* isolates were obtained from the urine samples of patients with UTIs at Milad Hospital during the aforementioned study period. Of these, 50 isolates (50%) were obtained from females, and 50 (50%) were obtained from males. The mean age of patients was 46.95 ± 23 years. The highest rates of resistance were observed against amikacin and levofloxacin (65% and 64%, respectively). Moreover, the highest susceptibility was demonstrated in relation to aztreonam and imipenem (97% and 83%, respectively). In this study, more than 50% of the isolates were resistant to gentimicin, cefepime, ceftazidime, and piperacillin. Among the 100 *K. pneumoniae* isolates, 49 (49%) produced AmpC beta-lactamases. The results of antibiotic susceptibility testing are shown in Table 2.

Among the 100 *K. pneumoniae* isolates studied, the PCR revealed that *PMABL* genes were present in 49 (49%) isolates. Of these, 34 isolates harbored only one *AmpC* gene group, including MOX (n=11), EBC (n=8), ACC (n=7), CIT (n=4), FOX (n=2), and DHA (n=2). The 15 remaining PMABL-containing isolates harbored at least two *AmpC* gene groups as follows: DHA, CIT, and MOX in 1 isolate; CIT and MOX in 2 isolates; CIT and ACC in 2 isolates; MOX and ACC in 2 isolates; FOX and DHA in 1 isolate; DHA and ACC in 2 isolates; DHA and MOX in 2 isolates; DHA and CIT in 2 isolates; and EBC and ACC in 1 isolate. Figure 1 displays the electrophoretic pattern of the *AmpC* genes.

The antimicrobial susceptibility pattern of the 49 AmpC-producers showed resistance levels of 49%

(n=24) to piperacillin and gentamicin, 69.4% (n=34) to amikacin, 63.3% (n=31) to levofloxacin, 44.9% (n=22) to ciprofloxacin, 55% (n=27) to ceftazidime, 46.9% (n=23) to cefepime, 38.8% (n=19) to ceftriaxone, 28.6% (n=14) to meropenem, and 10.2% (n=5) to imipenem. There was a significant association (P<0.05) between *AmpC* gene carriage and resistance to cefoxitin and levofloxacin. In this study, aztreonam (98% susceptibility) was found to be the most active antibiotic against AmpC-producing isolates.

Multidrug drug resistance was detected in 30 (61.2%) of the AmpC beta-lactamase producers. These isolates were distributed into 24 antimicrobial resistance patterns, dominated by resistance to gentamicin/amikacin/meropenem/ceftriaxone/cefoxitin (GM/AK/MER/CRO/FOX; 3/30, 10%), followed by amikacin/cefepime/ceftazidime/ciprofloxacin/levofloxacin/piperacillin (AK/CPM/CZA/CIP/LEV/PRL; 2/30, 6.7%). The profile of antimicrobial sensitivity in MDR isolates and the prevalence of *AmpC* beta-lactamase genes are reported in Table 3.

Enterobacterial repetitive intergenic consensus analyses revealed 12 distinct patterns of AmpC-producing *K. pneumoniae* isolates with a similarity of above 90% (Figures 2 and 3). The 49 AmpC-producing isolates were divided into four groups (A, B, C, and D), among which group D, with 7 clonal types and 28 isolates, was the most dominant. As shown in the dendrogram, among 12 clonal types, types I, V, and XII were the predominant types, with 8, 8, and 7 isolates, respectively (Figure 3).

Table 1. **List of used primers in the present study.**

Primer	Oligonucleotide sequence (5’ to 3’)	Target genes	Fragment length (bp)	Reference
MOXMF	F: GCTGCTCAAGGAGCACAGGAT	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	520	16
MOXMR	R: CACATTGACATAGGTGTGGTGC			
CITMF	F: TGGCCAGAACTGACAGGCAAA	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	462	16
CITMR	R: TTTCTCCTGAACGTGGCTGGC			
DHAMF	F: AACTTTCACAGGTGTGCTGGGT	DHA-1, DHA-2	405	16
DHAMR	R: CCGTACGCATACTGGCTTTGC			
ACCMF	F: AACAGCCTCAGCAGCCGGTTA	ACC	346	16
ACCMR	R: TTCGCCGCAATCATCCCTAGC			
EBCMF	F: TCGGTAAAGCCGATGTTGCGG	MIR-1T ACT-1	302	16
EBCMR	R: CTTCCACTGCGGCTGCCAGTT			
FOXMF	F: AACATGGGGTATCAGGGAGATG	FOX-1 to FOX-5b	190	16
FOXMR	R: CAAAGCGCGTAACCGGATTGG			
ERIC-2	AAGTAAGTGACTGGGGTGAGCG			17

Table 2. **Antimicrobial susceptibilities of the *K. pneumoniae* isolates (n=100).**

Antimicrobial agents	Susceptible, No. (%)	Resistant, No. (%)	Intermediate, No. (%)
Gentamicin	44 (44%)	52 (52%)	4 (4%)
Amikacin	29 (29%)	65 (65%)	6 (6%)
Imipenem	83 (83%)	11 (11%)	6 (6%)
Meropenem	59 (59%)	35 (35%)	6 (6%)

Antimicrobial agents	Susceptible, No. (%)	Resistant, No. (%)	Intermediate, No. (%)
Ciprofloxacin	27 (27%)	47 (47%)	17 (17%)
Levofloxacin	31 (31%)	64 (64%)	5 (5%)
Aztreonam	97 (97%)	0 (0%)	3 (3%)
Cefepime	36 (36%)	57 (57%)	7 (7%)
Ceftazidime	41 (41%)	54 (54%)	5 (5%)
Cefoxitin	65 (65%)	32 (32%)	3 (3%)
Ceftriaxone	67 (67%)	31 (31%)	2 (2%)
Piperacillin	41 (41%)	55 (55%)	4 (4%)

Table 3. Antimicrobial resistance patterns of multidrug resistant *K. pneumoniae* isolates and frequency of genes coding for MDR AmpC beta-lactamase.

Strain	Antimicrobial Resistance profile	AmpC groups
Kp2	GM-MEP$^{\Delta}$-CPM-CZA-CIP-LEV	EBC
Kp4	GM-AK-MEM-CPM-CZA-CIP- LEV-PPL-CRO	CIT
Kp7	AK-CPM-CZA-LEV-CRO-FOX	CIT, MOX
Kp8	GM-AK-MEM$^{\Delta}$-CPM- CZA-LEV-PRL$^{\Delta}$	CIT, ACC
Kp10	GM-CPM-CZA-CIP-LEV-PRL	CIT, MOX
Kp11	GM-AK-IMI-MER-CPM-CZA-CIP-LEV-PRL-CRO-FOX	ACC
Kp12	GM-AK-MER-CPM-CZA-CIP-LEV-PRL	CIT, ACC
Kp17	GM-AK-IMI$^{\Delta}$-CPM$^{\Delta}$-CZA-CIP-LEV-FOX	CIT,
Kp19	GM-CPM-CZA-CIP-PRL-CRO-FOX	EBC
Kp20	GM-AK- CPM-CZA-CIP-LEV-PRL	ACC
Kp29	GM-AK-MER$^{\Delta}$-CPM-CZA-LEV-PRL	MOX, ACC
Kp33	GM-AK-IMI-MER-CPM-CZA-CIP-LEV-PRL	MOX
Kp34	GM-AK-CPM$^{\Delta}$-CZA-CIP-LEV-PRL-CRO	MOX
Kp46	GM-AK-IMI-CPM-CZA-CIP-LEV-PRL	MOX
Kp49	AK-MER-CPM-CZA-LEV-PRL-CRO-FOX	ACC
Kp51	AK-CPM-CZA- LEV-PRL-CRO-FOX	FOX
Kp57	AK-CPM$^{\Delta}$-CIP-LEV-PRL-CRO-FOX	ACC
Kp68	AK-IMI-CPM-CZA-LEV-PRL	DHA, ACC
Kp75	GM-MER$^{\Delta}$-CPM-CZA-CIP-LEV-PRL	MOX
Kp76	AK-CPM-CZA-CIP-LEV-PRL	ACC
Kp79	AK-CPM-CZA-CIP-LEV-PRL	FOX
Kp81	AK-CPM-CZA-CIP-LEV-PRL	EBC
Kp82	GM-AK-MER-CRO-FOX	MOX
Kp83	GM-AK-MER-CRO-FOX	MOX
Kp89	GM-AK-CPM-CZA-CIP-PRL-CRO-FOX$^{\Delta}$	DHA, CIT
Kp92	AK-MER-CPM-CZA-CIP$^{\Delta}$-LEV-PRL	DHA, MOX
Kp93	AK-CPM-CZA-CIP-PRL	MOX
Kp94	GM-AK-CIP-LEV-CRO-FOX	EBC, ACC
Kp95	GM-AK-CPM-CZA-CIP-LEV-PRL-FOX	MOX
Kp98	GM-AK-MER-CPM-CZA-CIP$^{\Delta}$-LEV-PRL	CIT

Abbreviations: CAZ: Ceftazidime; CRO: Ceftriaxone; IMI: Imipenem; MEM: Meropenem; AK: Amikacin; GM: Gentamicin; CPM: Cefepime; LEV: Levofloxacin; CIP: Ciprofloxacin; PRL: Piperacillin; FOX: Cefoxitin; Δ Intermediate sensitivity.

Discussion

The resistance of *K. pneumoniae* to third- and fourth-generation cephalosporins due to PMABLs has become a global health threat. The unnecessary or inappropriate use of antibiotics particularly beta-lactams and long-term hospitalization are two possible important causes of the isolation of cephalosporinase-producing *K. pneumoniae* strains in patients. An awareness of the prevalence of PMABLs will be beneficial in terms of epidemiological studies and infection control, as these genes can be transmitted to other microorganisms in hospital settings **(19)**.

Plasmids containing *AmpC* beta-lactamase genes often carry genes that are resistant to other classes of antimicrobial agents **(5)**. Our results showed that AmpC-producing *K. pneumoniae* isolates were resistant to third- and fourth-generation cephalosporins, including ceftazidime, ceftriaxone, and cefepime. The latter is generally used to treat infections caused by AmpC-producing bacteria because it can pass through the outer membrane rapidly **(20)**. However, in the present study, AmpC-producing *K. pnumoniae* isolates were found to be highly resistant to cefepime (46.9%). These isolates were also found to be highly resistant to aminoglycoside and quinolone antibiotics, including amikacin, gentamicin, ciprofloxacin, and levofloxacin. Carbapenems are usually prescribed to treat AmpC-producing bacteria **(5)**. However, we found a notable rate of carbapenem resistance among *K. pneumoniae* isolates.

The present study revealed a high frequency of AmpC beta-lactamase among clinical isolates of *K. pneumoniae (*49%). In a study conducted by Azimi *et al.* (2013) in Iran, the prevalence of AmpC beta-lactamase among clinical isolates of *K. pneumoniae* was reported to be 1.6% **(21)**. In 2014, this value increased to 19% **(7)**. Our data highlight the sharp rise in AmpC beta-lactamase incidences over the past five years.

The prevalence of *AmpC* genes observed in this study is higher than in reports from other countries. In Pakistan, Shafiq et al. found that the rate of AmpC-producing *K. pneuminiae* was 12% **(22)**. In China, the positive rate of plasmid-mediated AmpC-beta-lactamase-producing *K. pneumoniae* was 10.8% **(8)**. In India, 32 out of 109 (29.4%) *K. pneumoniae* were AmpC-positive **(23)**. However, a higher prevalence of *AmpC* beta-lactamase genes (77%) was reported in Korea **(24)**. The primary reasons for the high occurrence of PMABL in Iran may be due to the relatively high rates of self-medication and the indiscriminate consumption of extended-spectrum cephalosporins in hospitals.

Japoni-Nejad *et al*. showed that, among 100 clinical isolates of *K. pneumoniae*, 19 isolates harbored *AmpC* genes that belong to CIT (42.2%), MOX (36.8%), EBC (15.7%), and DHA (5.2%) cluster genes **(7)**. Ghanavati *et al.* (2016) discovered that 43.1% of *K. pneumoniae* isolates from burn patients in Iran harbor *AmpC* genes. Of these cases, 22.5% of the isolates carried the *CIT* gene, and 21.5% carried the *EBC* gene, whereas only 9.8% and 7.8% carried FOX and *DHA* genes, respectively **(25)**. According to our data, the most prevalent *AmpC* genes among *K. pneumoniae* isolates resulting from UTIs are MOX and ACC. This is the first study to describe the presence of the ACC gene cluster in *K. pneumoniae* isolates in Iran.

In the present study, ERIC-PCR typing revealed the polyclonal distribution of AmpC-producing *K. pneumoniae* isolates (Figure 2). The genetic heterogeneity among the isolates revealed that different subtypes of *K. pneumoniae* were involved in UTIs in patients at Milad Hospital. Our findings are consistent with those reported by Seifi *et al.* **(2)**.

In a study conducted by Ghasemian *et al.* in Tehran, a wide genetic diversity of *K. pneumoniae* isolates was reported **(26)**. Likewise, results from studies conducted in other countries revealed the genetic diversity of clinical isolates of *K. pneumoniae* **(27-29)**.

In our study, there was no relationship between ERIC type and antibiotic resistance patterns. In other words, strains of a specific ERIC type showed different antibiotic patterns. These results are in agreement with the findings of previous studies **(29,30)**.

Conclusion

The present study revealed a high frequency of PMABL-producing MDR *K. pnuemoniae* isolates in UTI patients at Milad Hospital. It also indicated the coexistence of *AmpC* cluster genes in some isolates. This is the first study to describe the presence of the *ACC* cluster gene in *K. pnuemoniae* isolates in Iran. The emergence of the polyclonal MDR and *bla*AmpC-gene-carrying *K. pnuemoniae* isolates indicate that surveillance policies are needed for the detection and control of the dissemination of such organisms.

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Conflict of Interest

Authors declared no conflict of interests.

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