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 β-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 β-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.

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

Keywords: Klebsiella pneumoniae, AmpC β-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 β-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-
cally 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.)
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. pneumoniae* 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 H₂O. 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 gentamicin, 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%
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(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/ceftazidime/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: GCTGCTCAAGGACACAGGAT                                  | MOX-1, MOX-2, CMOY-1, CMOY-8 to CMOY-11 | 520       | 16        |
| MOXMR   | R: CACATTGACATAGTGTTGATGC                                 |              |                      |           |
| CITMF   | F: TGCCAGAAACTGACAGGCAA                                   | LAT-1 to LAT-4, CMOY-2 to CMOY-7, BIL-1 | 462       | 16        |
| CITMR   | R: TTCTCTGAAACGTGGCTGGC                                   |              |                      |           |
| DHAMF   | F: AACATTCACAGGTGCTGGT                                     | DHA-1, DHA-2 | 405       | 16        |
| DHAMR   | R: CCGTACGCATACTGGCCTGGC                                  |              |                      |           |
| ACCMF   | F: AACAGCCCTCAGCAGCGGTTA                                  | ACC          | 346       | 16        |
| ACCMR   | R: TGCAGCCGAATCATCCCTAGC                                  |              |                      |           |
| EBCMCF  | F: TGGTAAAGCGGATGTGGGGG                                    | MIR-1T ACT-1 | 302       | 16        |
| EBCMR   | R: CTCCACTGGCGCTTCATTAGG                                  |              |                      |           |
| FOXMF   | F: AACATGGGGGATCAGGGAGATG                                 | FOX-1 to FOX-5b | 190       | 16        |
| FOXMR   | R: CAAAGCGGTAACCGGATG                                     |              |                      |           |
| ERIC-2  | AAGTAAGTGAACGCCGGTGAGG                                      |              |                      | 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%)               |
**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-MEP³-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-MEM³-CPM-CPM-LEV-PRL     | CIT, ACC    |
| Kp10   | GM-CPM-CZA-CPM-LEV-PRL        | CIT, MOX    |
| Kp11   | GM-AK-IMI-CPM-CZA-CPM-LEV-PRL-CRO-FOX | ACC⁴       |
| Kp12   | GM-AK-IMI-CPM-CZA-CPM-LEV-PRL  | CIT, ACC    |
| Kp17   | GM-AK-IMI³-CPM-CPM-LEV-PRL     | CIT, MOX    |
| Kp19   | GM-CPM-CZA-CPM-LEV-PRL-CRO-FOX | EBC         |
| Kp20   | GM-AK-CPM-CZA-CPM-LEV-PRL     | ACC⁵        |
| Kp29   | GM-AK-CPM-CZA-CPM-LEV-PRL     | MOX, ACC    |
| Kp33   | GM-AK-IMI-CPM-CZA-CPM-LEV-PRL  | MOX         |
| Kp34   | GM-CPM³-CZA-CPM-LEV-PRL-CRO-FOX | MOX         |
| Kp46   | GM-CPM³-CZA-CPM-LEV-PRL-CRO-FOX | MOX         |
| Kp49   | AK-MER-CPM-CZA-CPM-LEV-PRL-CRO-FOX | ACC⁶       |
| Kp51   | AK-CPM-CZA-CPM-LEV-PRL-CRO-FOX | FOX         |
| Kp57   | AK-CPM³-CZA-CPM-LEV-PRL-CRO-FOX | ACC⁵        |
| Kp68   | AK-CPM³-CZA-CPM-LEV-PRL-CRO-FOX | ACC         |
| Kp75   | GM-MER³-CPM-CPM-LEV-PRL        | MOX         |
| Kp76   | AK-CPM-CZA-CPM-LEV-PRL        | ACC         |
| Kp79   | AK-CPM-CZA-CPM-LEV-PRL        | FOX         |
| Kp81   | AK-CPM-CZA-CPM-LEV-PRL        | EBC         |
| Kp82   | GM-AK-CPM-CZA-CPM-LEV-PRL     | MOX         |
| Kp83   | GM-AK-CPM-CZA-CPM-LEV-PRL     | MOX         |
| Kp89   | GM-AK-CPM-CZA-CPM-LEV-PRL-CRO-FOX³ | DHA, CIT     |
| Kp92   | AK-MER-CPM-CZA-CPM-LEV-PRL     | DHA, MOX    |
| Kp93   | AK-CPM-CZA-CPM-LEV-PRL        | MOX         |
| Kp94   | GM-AK-CPM-CZA-CPM-LEV-PRL     | EBC, ACC    |
| Kp95   | GM-AK-CPM-CZA-CPM-LEV-PRL     | MOX         |
| Kp98   | GM-AK-CPM-CZA-CPM-LEV-PRL     | CIT         |

Abbreviations: CAZ: Cefazidime; 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. pneumoniae 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 levofloxacina. 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, Shafigh et al. found that the rate of AmpC-producing K. pneumoniae 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 PMABLS-producing MDR K. pneumoniae 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. pneumoniae isolates in Iran. The emergence of the polyclonal MDR and blaAmpC-gene-carrying K. pneumoniae 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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