Coexistence of aminoglycoside resistance genes in CTX-M-producing isolates of *Klebsiella pneumoniae* in Bushehr province, Iran

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**ABSTRACT**

**Background and Objectives:** Increasing the rate of extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* has given rise to a major healthcare issue in clinical settings over the past few years. Treatment of these strains is hardly effective since the plasmid encoding ESBL may also carry other resistance genes including aminoglycosides. The current study aimed to evaluate the prevalence of ESBL-producing *K. pneumoniae* and investigate the coexistence of Cefotaximase-Munich (*bla*<sub>CTX-M</sub>) with aminoglycoside-modifying enzyme (AME) genes, *aac*(3)*Ia* as well as *aac*(6)*Ib*, in CTX-M-producing *K. pneumoniae* isolated from patients in Bushehr province, Iran.

**Materials and Methods:** A total of 212 *K. pneumoniae* isolates were collected and confirmed using polymerase chain reaction (PCR) of the malate dehydrogenase gene. Isolates were screened for production of ESBL. Phenotypic confirmatory test was performed using combined disk test. The genes encoding CTX-M groups and AME genes, *aac*(3)*Ia* and *aac*(6)*Ib*, were investigated by PCR.

**Results:** The ESBL phenotype was detected in 56 (26.4%) *K. pneumoniae* isolates. Moreover, 83.9% of ESBL-producing isolates carried the genes for CTX-M type β-lactamases, which were distributed into the two genetic groups of CTX-M-1 (97.8%-), and CTX-M-2 (2.1%)-related enzymes. Notably, among *K. pneumoniae* isolates containing the *bla*<sub>CTX-M</sub> gene, 68.08% of isolates harbored AME genes. In addition, the coexistence of *bla*<sub>CTX-M</sub> with *aac*(3)*Ia* and *aac*(6)*Ib* was observed in 46.8% of CTX-M-producing *K. pneumoniae* isolates.

**Conclusion:** This study provides evidence of a high prevalence of AME genes in CTX-M-producing *K. pneumoniae* isolates; therefore, in the initial empirical treatment of infections caused by ESBL-KP in regions with such antibiotic resistance patterns, aminoglycoside combination therapy should be undertaken carefully.

**Keywords:** Aminoglycoside-modifying enzymes; Cefotaximase-Munich; *Klebsiella pneumoniae*; Extended spectrum β-lactamases; Iran

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INTRODUCTION

Cefotaxime-Munich (CTX-M)-producing Klebsiella pneumoniae which is becoming growingly widespread in clinical and nosocomial settings (1) poses a worldwide growing threat to public health (2). CTX-M-type enzymes are defined as a group of non-TEM and SHV class A extended-spectrum β-lactamases (ESBLs) with a prompt ability to spread amongst Gram-negative bacteria (1, 3). In the 1980s and 1990s, TEM-type and SHV-type ESBLs were dominant. Nevertheless, a swift and massive spread of organisms producing CTX-M-type enzymes has come to light over the recent decade, resulting in CTX-M-β-lactamases becoming the most prevalent ESBLs throughout the world (4). The spread of CTX-Ms on such a large scale around the world has been referred to as the “CTX-M pandemic” because of their rising description across the world (5). The phylogenetic study demonstrates five main groups of acquired CTX-M enzymes based on their amino acid sequence similarities (6): the CTX-M-1 group (CTX-M-1, -3, -10, -11, -12, -15, -22, -23, -29, -30, -32, -33, -36, -54 and UOE-1), the CTX-M-2 group (CTX-M-2, -4, -6, -7, -20, -31, -44) (previously TOHO-1), the CTX-M-8 group (CTX-M-8 and CTX-M-40), the CTX-M-9 group (CTX-M-9, -13, -14, -16, -17, -18, -19, -24, -27, -45) (previously TOHO-2), (-46, -47, -48, -49, -50) and the CTX-M-25 group (CTX-M, -26, -25, -39, -41) (7), among which the groups CTX-M-1, -M-2 and -M-9 seem to be the most widespread globally, while many of the other CTX-M ESBLs tend to be more limited in their distribution (8).

In terms of antibiotics, aminoglycosides are potent and broad-spectrum antibiotics in the clinical setting (9). These agents are characteristically bactericidal and display synergy with other antimicrobials, most notably β-lactams (10). Since investigating carbapenem-sparing regimens for infections caused by ESBL is medically required, we can observe a renewed interest in aminoglycosides as a potential alternative (11). Moreover, non-carbapenem antibiotic therapy has demonstrated favorable therapeutic effects on UTIs because of ESBL-producing strains in adults as well as children. In such cases aminoglycosides can be a substitute to carbapenems and should be included in initial empirical treatment. In addition, the effectiveness of aminoglycoside combination therapy has also been observed in bacteremia caused by ESBL-producing strains (12).

Unfortunately, ESBL-producing organisms also confer cross-resistance to other families of antimicrobial agents, including fluoroquinolones and aminoglycosides (13).

Enzymatic inactivation through the production of aminoglycoside-modifying enzymes (AMEs) is the main mechanism conferring resistance to aminoglycosides in Enterobacteriaceae (9, 14). These enzymes are categorized into three families: aminoglycoside acetyltransferases (encoded by aac genes), aminoglycoside nucleotidyltransferases (encoded by ant genes), and aminoglycoside phosphoryltransferases (encoded by aph genes) (9, 10, 13).

The subclass AAC(3)-II, which is known for its resistance to gentamicin, netilmicin and tobramycin, includes three enzymes: AAC(3)-IIa, AAC(3)-IIb, and AAC(3)-Iic. Among them, 3-N-acetyltransferases type IIa (aac(3)-IIa) is widely observed among the members of Enterobacteriaceae, including K. pneumoniae. Also, 6'-N-acetyltransferase type Ib (aac(6')-Ib) is likely to be the most clinically related acetyltransferase responsible for the resistance to amikacin and other aminoglycosides found in Enterobacteriaceae (14).

Regarding the point that CTX-M-β-lactamases are the most prevalent ESBLs around the world, we focused our study on these β-lactamases. A large number of publications have provided in-depth information on microbiological, clinical, and epidemiological aspects of β-lactam resistance in ESBL-producing enterobacteria; however, there is a lack of sufficient data when considering aminoglycosides. So we evaluated the prevalence of common aminoglycoside-modifying genes, aac(3)-IIa and aac(6')-Ib, conferring resistance to aminoglycosides among CTX-M-producing K. pneumoniae isolates to predict the efficacy of β-lactam/aminoglycoside combination therapy.

MATERIALS AND METHODS

Strains and identification tests. This project was approved by the Ethical Committee of Bushehr University of Medical Sciences with reference number IR.BPUMS.REC.1398.051. A total of 212 K. pneumoniae were collected from eight hospitals and two medical diagnostic laboratories in Bushehr province, Iran, between December 2017 and November 2018.
The isolates were recovered from urine (n = 170), tracheal aspirate (n = 15), wound swab (n = 8), blood (n = 8), burn (n = 3), sputum (n = 2), feces (n = 2), abscess (n = 2), Ascites fluid (n = 1) and Shaldon catheter (n = 1). All the strains were identified according to standard microbiological procedures and stored at -70°C. Molecular identification of *K. pneumoniae* isolates was done to target the malate dehydrogenase (*mdh*) housekeeping gene by PCR (15).

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility was determined using disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (16). In doing so, the following antibiotic disks (MastGroup Ltd., Merseyside, United Kingdom) were used: ampicillin (25 µg), amoxicillin–clavulanic acid (20/10 µg), piperacillin-tazobactam (100/10 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), aztreonam (30 µg), amikacin (30 µg), gentamicin (10 µg), and tobramycin (30 µg). Likewise, MICs of amikacin and gentamicin were determined using E-test (Liofilchem, Italy) on Muller-Hinton agar (Biolife, Italy). *E. coli* strains ATCC 25922 and ATCC 35218 were used as controls.

**Phenotypic ESBL detection.** Reduced zones of inhibition around 3rd generation beta-lactam disks on Mueller-Hinton agar recommended ESBL production (17). Subsequently, ESBL production was confirmed by combined disk test (CDT) using disks of ceftazidime (30 µg) and cefotaxime (30 µg) with and without clavulanic acid (10 µg). A positive test result was defined as a ≥ 5 mm increase in the zone diameter compared with a disk without clavulanic acid. Also, *E. coli* ATCC 25922 (β-lactamase negative) and *K. pneumoniae* ATCC 700603 (ESBL positive) were used for controlling ESBL detection.

**Detection of drug resistance genes.** Detection of *blaCTX-M* genes and aminoglycoside resistance encoding genes, *aac(6’)-Ib* and *aac(3)-IIa*, was carried out by PCR method using specific oligonucleotide primers (Table 1). *K. pneumoniae* was cultured in Muller Hinton broth (Merck, Germany) at 37°C overnight and then the total DNA was extracted using an extraction kit (GeneAll, Korea) as recommended by the manufacturer.

Amplification reactions were conducted in a T100 Thermocycler (BIO-RAD, USA) under the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at a specific temperature (for each primer) for 30 s as shown in Table 1, and elongation at 72°C for 1 min. A final elongation step was extended to 5 min. Amplification reactions were prepared in a total volume of 25 µl including 12.5 µl Taq DNA polymerase 2× Master Mix with 1.5 mM MgCl₂ (Amplicon, Odense, Denmark), 1 µM forward primer, 1 µM reverse primer, 9.5 µl nuclease-free water, and 1 µl DNA template (50pg concentration). PCR products were electrophoresed on a 1.5% agarose gel at 80V, stained with safe dye (Yekta Tajhiz Azma, Iran), and finally visualized with a gel documentation system (Upland, CA, USA). To confirm the PCR results, randomly selected amplicons were purified and sequenced by the Bioneer Company (Seoul, Korea). The nucleotides and deduced protein sequences alignment and analysis were also done online applying the basic local alignment search tool (BLAST) program of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**RESULTS**

**Antimicrobial susceptibilities of *K. pneumoniae* isolates.** The combined disk test (CDT) confirmed that 56 (26.4%) of *K. pneumoniae* isolates were ESBL producers. The data on the activities of different antimicrobial agents against ESBL–KP and non-ESBL *K. pneumoniae* isolates is summarized in Table 2. As expected, all isolates were resistant to ampicillin (100%). Generally, the resistance to the other tested antimicrobial agents was significantly higher in ESBL–KP than in non-ESBL–KP (*p < 0.0001*) (Table 2). The most effective antimicrobial agent against ESBL–KP was amikacin (78.3%). Among penicillin–β-lactamase inhibitor combinations, piperacillin–tazobactam was slightly more active (40.9%) than amoxicillin–clavulanicacid (23.1%) against ESBL–KP isolates.

As shown in Table 2, the resistance to (one or more) aminoglycosides was significantly higher in ESBL–KP than in non-ESBL–KP (*p < 0.0001*). Out of a total of 212 isolates, 38 isolates were resistant to tobramycin, 28 isolates were resistant to gentamicin and 11 isolates were resistant to amikacin, of which 34 (89.4%), 27 (96.4%) and 11 (100%) isolates were ESBL producers, respectively. Notably, among ES-
Table 1. The sequences of primers and annealing temperatures used in PCR amplification of diverse CTX-M groups as well as \( \text{aac}(3)\text{Il} \) and \( \text{aac}(6')\text{Ib} \) genes.

| Primer     | Sequence (5’ to 3’)                                                                 | Size (bp) | Annealing temperature | Gene       | Reference |
|------------|-------------------------------------------------------------------------------------|-----------|------------------------|------------|-----------|
| mdh F      | GCGTGGCGTGTATCTAAGTCATA                                                            | 364       | 55                     | mdh        | (15)      |
| mdh R      | TTCAGCTCCGCCACAAAGGTA                                                              |           |                        |            |           |
| CTX-M-1 F  | CCCATGTTAAAAAAATCCTG                                                              | 891       | 57                     | \( \text{bla}_{\text{CTX-M-1}} \) | (18)      |
| CTX-M-1 R  | CGTTTCCGCTATCACAAC                                                                |           |                        |            |           |
| CTX-M-2 F  | ATGATGACCTAGCAGCTCG                                                                | 866       | 55                     | \( \text{bla}_{\text{CTX-M-2}} \) | (19)      |
| CTX-M-2 R  | TGGGTACAGTGGTTCGCC                                                                 |           |                        |            |           |
| CTX-M-8 F  | ATGTTAATGCGACAGCCTGTG                                                              | 689       | 57                     | \( \text{bla}_{\text{CTX-M-8}} \) | (18)      |
| CTX-M-8 R  | CCGGTTTTATCCCCGACA                                                                |           |                        |            |           |
| CTX-M-9 F  | ATGTGACACAAAGAGAGTGCA                                                              | 870       | 55                     | \( \text{bla}_{\text{CTX-M-9}} \) | (19)      |
| CTX-M-9 R  | CCCTCAGCGATGGATCTC                                                              |           |                        |            |           |
| \( \text{aac}(3)\text{Il} \) F | ATATCGGGATGCATACGGCGG                                                          | 877       | 56                     | \( \text{aac}(3)\text{Il} \)   | (20)      |
| \( \text{aac}(3)\text{Il} \) R   | GACGGCCTAATACCGGAAGG                                                             |           |                        |            |           |
| \( \text{aac}(6')\text{Ib} \) F | TCGCATGCTCTAGTAGTGCGTA                                                        | 482       | 57                     | \( \text{aac}(6')\text{Ib} \) | (21)      |
| \( \text{aac}(6')\text{Ib} \) R   | CTCGAATGCTTCCGCGTTTT                                                          |           |                        |            |           |

Table 2. Comparison of antimicrobial susceptibility profiles of 56 ESBL-KP and 156 non ESBL-KP isolates.

| Antimicrobial Agent | ESBL-KP (n=56) | Non ESBL-KP (n=156) |
|---------------------|---------------|---------------------|
|                     | R (%)         | I (%)               | S (%)               | R (%)         | I (%)               | S (%)               | p value* |
| Cefepime            | 45 (80.1)     | 5 (8.9)             | 6 (10.6)            | 0             | 2 (1.2)             | 154 (98.5)          | p < 0.0001 |
| Ceftriaxone         | 53 (94.3)     | 0 (0)               | 3 (5.3)             | 5 (3.2)       | 1 (0.6)             | 150 (96)            | p < 0.0001 |
| Cefotaxime          | 54 (96.1)     | 0 (0)               | 2 (3.5)             | 7 (4.4)       | 2 (1.2)             | 147 (94)            | p < 0.0001 |
| Cefoxitin           | 28 (49.8)     | 5 (8.9)             | 23 (40.9)           | 7 (4.4)       | 6 (3.8)             | 143 (91.5)          | p < 0.0001 |
| Aztreonam           | 51 (90.7)     | 2 (3.5)             | 3 (5.3)             | 3 (1.9)       | 2 (1.2)             | 151 (96.6)          | p < 0.0001 |
| Ceftazidime         | 49 (87.2)     | 6 (10.6)            | 1 (1.7)             | 6 (3.8)       | 2 (1.2)             | 148 (94.7)          | p < 0.0001 |
| TZP                 | 20 (35.6)     | 13 (23.1)           | 23 (40.9)           | 1 (0.6)       | 9 (5.7)             | 146 (93.4)          | p < 0.0001 |
| AMC                 | 35 (62.3)     | 8 (14.2)            | 13 (23.1)           | 5 (3.2)       | 10 (6.4)            | 141 (90.2)          | p < 0.0001 |
| Tobramycin          | 34 (60.5)     | 19 (33.8)           | 3 (5.3)             | 4 (2.5)       | 8 (5.1)             | 144 (92.1)          | p < 0.0001 |
| Amikacin            | 11 (19.6)     | 1 (1.7)             | 44 (78.3)           | 0 (0)         | 1 (0.6)             | 155 (99.2)          | p < 0.0001 |
| Gentamicin          | 27 (48.2)     | 1 (1.7)             | 29 (51.6)           | 1 (0.6)       | 7 (4.4)             | 147 (94)            | p < 0.0001 |

Note: TZP: Piperacillin-tazobactam, AMC, Aminoglycoside-clavulanic acid. *: Fisher’s exact.

BL-KP, 37 (66%) isolates were non-susceptible to at least one aminoglycoside.

In this study to determine the level of resistance to aminoglycoside, MICs of amikacin and gentamicin in non-susceptible isolates were determined using Etest (Table 3). Among isolates resistant to amikacin, 72.7%, of isolates represented MIC > 256. A high level of resistance to gentamicin (MIC > 1024) was also seen in 18.5% of isolates.

Detection of resistance genes. All isolates were confirmed as \( K. \) pneumoniae in PCR assay targeting the \( \text{mdh} \) (Fig. 1A). As shown in Table 3, 83.9% of ESBL-producing isolates harbored the genes for CTX-M type \( \beta \)-lactamases, which were distributed into the two genetic groups of CTX-M-1 (97.8%) and CTX-M-2 (2.1%)–related enzymes (Fig. 1B).

Among all 212 isolates, \( \text{aac}(3)\text{Il} \) and \( \text{aac}(6')\text{Ib} \) were found in 45 (21.2%) and 42 (19.8%) isolates,
Table 3. *In vitro* activity of aminoglycosides against 56 ESBL-producing *K. pneumoniae* isolates and the presence of CTX-M and AME genes.

| Sample ID | G/A | Source       | ESBL   | AME genes | Disk Diffusion | MIC (µg/ml) |
|-----------|-----|--------------|--------|-----------|----------------|-------------|
|           |     |              | CTX-M  | Non-CTX-M | aac(3)-Ia     | aac(6’)-Ib  | TN | AK | GN | AK ≥ 64 | GN ≥ 16 |
| KP-5      | F/82| Urine        | +      | -         | -              | +           | R  | S  | S  | -       | -       |
| KP-9      | F/62| Urine        | +      | -         | -              | -           | I  | S  | S  | -       | -       |
| KP-11     | F/49| Urine        | +      | -         | +              | +           | R  | R  | R  | ≥256    | 512     |
| KP-12     | F/80| Pleural fluid| +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-21     | F/49| Urine        | +      | -         | +              | +           | I  | R  | R  | ≥256    | 256     |
| KP-28     | M/22| Urine        | +      | -         | -              | +           | R  | S  | S  | -       | -       |
| KP-43     | M/65| Urine        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-65     | M/59| ETT          | +      | -         | +              | +           | R  | S  | R  | -       | 96      |
| KP-69     | F/20| Urine        | +      | -         | -              | +           | R  | 1  | S  | 24      | -       |
| KP-71     | M/52| Urine        | +      | -         | -              | +           | R  | R  | R  | ≥256    | ≥1024   |
| KP-75     | F/17| Urine        | +      | -         | -              | +           | R  | S  | S  | -       | -       |
| KP-79     | F/34| Urine        | +      | -         | +              | +           | R  | S  | R  | -       | 96      |
| KP-81     | F/68| Urine        | +      | -         | +              | +           | S  | S  | I  | -       | 4       |
| KP-83     | F/5  | Urine        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-84     | M/50| Urine        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-85     | F/64| Urine        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-89     | M/48| Blood        | +      | -         | +              | +           | R  | S  | R  | -       | 64      |
| KP-96     | M/64| ETT          | +      | -         | -              | +           | R  | S  | S  | -       | -       |
| KP-97     | M/52| Urine        | +      | -         | +              | +           | R  | S  | R  | -       | 48      |
| KP-98     | F/63| ETT          | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-100    | F/56| Urine        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-101    | F/58| Blood        | +      | -         | -              | +           | R  | S  | S  | -       | -       |
| KP-102    | M/51| ETT          | +      | -         | +              | +           | R  | S  | R  | -       | 48      |
| KP-104    | M/38| Blood        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-107    | */* | Urine        | +      | -         | +              | +           | R  | R  | R  | 96      | 96      |
| KP-110    | */* | Urine        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-116    | F/36| Urine        | +      | -         | +              | -           | R  | S  | R  | -       | 192     |
| KP-117    | M/1 | Urine        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-121    | M/52| Wound        | +      | -         | -              | +           | R  | R  | S  | ≥256    | -       |
| KP-122    | F/54| Shaldon      | +      | -         | +              | +           | R  | S  | R  | -       | 192     |
| KP-128    | F/23d| Urine       | +      | -         | +              | +           | R  | S  | R  | -       | 48      |
| KP-130    | M/67| ETT          | +      | -         | +              | +           | R  | S  | R  | -       | 48      |
| KP-132    | M/74| Urine        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-133    | F/66| Urine        | +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-136    | M/87| Ascites fluid| +      | -         | -              | -           | S  | S  | S  | -       | -       |
| KP-144    | F/25| Urine        | +      | -         | -              | -           | R  | R  | R  | ≥256    | 48      |
| KP-147    | M/40| Urine        | +      | -         | +              | +           | R  | S  | R  | -       | 48      |
| KP-150    | F/27| Urine        | +      | -         | +              | +           | R  | S  | R  | -       | 64      |
| KP-153    | F/28| Urine        | +      | -         | +              | +           | R  | S  | R  | -       | 1024    |
| KP-162    | M/42| Urine        | +      | -         | +              | +           | R  | S  | R  | -       | 64      |
| KP-165    | F/41| Urine        | +      | -         | +              | +           | R  | S  | R  | -       | 64      |
| KP-177    | F/45| Urine        | +      | -         | +              | +           | R  | S  | R  | -       | 32      |
| KP-184    | F/17| Urine        | +      | -         | +              | +           | R  | S  | R  | -       | 48      |
| KP-188    | M/38| Urine        | +      | -         | +              | +           | R  | R  | R  | 32      | 128     |
| KP-191    | M/24| Urine        | +      | -         | +              | +           | R  | R  | R  | 96      | 96      |
Table 3. Continuing...

|    |    |    |    |    |    |    |    |    |    |
|---|---|---|---|---|---|---|---|---|---|
| KP-205 | F/40 | Wound | + | - | + | + | R | S | R | - | 48 |
| KP-206 | M/53 | Urine | + | - | + | - | S | S | S | - | - |
| KP-6 | F/39 | Urine | - | + | - | + | R | S | S | - | - |
| KP-34 | M/22 | Urine | - | + | - | - | S | S | S | - | - |
| KP-73 | M/38 | Urine | - | + | - | - | S | S | S | - | - |
| KP-74 | F/41 | Urine | - | + | - | - | S | S | S | - | - |
| KP-91 | M/49 | Urine | - | + | - | - | S | S | S | - | - |
| KP-92 | M/60 | Wound | - | + | - | + | R | R | R | ≥256 | ≥1024 |
| KP-94 | M/32 | Blood | - | + | - | + | R | R | R | ≥256 | ≥1024 |
| KP-106 | */* | Blood | - | + | - | + | R | R | R | ≥256 | ≥1024 |
| KP-134 | F/58 | Urine | - | + | - | - | S | S | S | - | - |

Note: G: Gender, A: age, ETT: Endotracheal tube, *: Not defined, d: Days. AK: Amikacin, TN: Tobramycin, GN: Gentamicin, ESBL: Extended spectrum beta-lactamase, AME: Aminoglycoside-modifying enzyme, MIC: minimum inhibitory concentration. a: CLSI clinical breakpoints.

Fig. 1. A: Agarose gel electrophoresis of mdh gene (364bp) of K. pneumoniae, lanes 1-5: K. pneumoniae carrying mdh gene, C+: K. pneumoniae ATCC 700603, C-: Escherichia coli ATCC 25922, M: 100 bp DNA ladder. B: Agarose gel electrophoresis of PCR products using primers for detection bla<sub>CTX-M-1</sub> (891 bp) and bla<sub>CTX-M-9</sub> (870 pb) genes. M: 100 bp DNA ladder, lane 1 and 3: K. pneumoniae carrying bla<sub>CTX-M-1</sub> gene, C+: E. coli ATCC 25922, lane 4: K. pneumoniae carrying bla<sub>CTX-M-9</sub> gene. C: Agarose gel electrophoresis of PCR products using primers for detection of aac(3)-IIa (877 bp) gene, lanes 1and 2 K. pneumoniae carrying aac(3)-IIa gene, lane 4 sequenced aac(3)-IIa gene used as positive control, M: 100 bp DNA ladder. D: Agarose gel electrophoresis of PCR products using primers for the detection of aac(6')-Ib (482 pb) gene, M: 100 bp DNA ladder, lane 1 sequenced aac(6')-Ib gene used as positive control, lanes 3 and 4 K. pneumoniae carrying aac(6')-Ib gene.
respectively (Fig 1C and 1D). In ESBL-KP isolates, aac(3)-Ila was detected in 61.7%, 72.7% and 81.4% of isolates resistant to tobramycin, amikacin and gentamicin, respectively. In addition, 96.1%, 94.1% and 90.9% of ESBL-KP isolates resistant to gentamicin, tobramycin and amikacin harbored aac(6')-Ib respectively (Table 4). Notably as shown in Table 3, the coexistence of aminoglycoside resistance genes, aac(3)-Ila and aac(6')-Ib, with CTX-M was detected in 32 (68%) ESBL-KP isolates ($p < 0.0001$).

**DISCUSSION**

*K. pneumoniae* as a member of Enterobacteriaceae family is one of the main pathogens triggering nosocomial and community-acquired infections. Correspondingly, the increasing rate of ESBL-KP has given rise to a serious healthcare problem in clinical settings in recent years. Indeed, an increased risk of treatment failure awaits patients infected by an ESBL-producing organism. Even worse, the plasmid coding ESBL may also carry other resistance genes including aminoglycosides. Thus, the treatment of such strains may be costly, long, and hardly effective (22).

The current study was done not only to evaluate the prevalence of ESBL-KP but also to investigate the coexistence of the bla<sub>CTX-M</sub> gene with AME genes, aac(3)Ila as well as aac(6')Ib, in CTX-M-producing *K. pneumoniae* in clinical samples.

It is notable that resistance to all tested antimicrobial agents was higher in ESBL-KP than in non ESBL-KP isolates ($p < 0.0001$). Antimicrobial susceptibility test results via disk diffusion of ESBL-KP isolates revealed amikacin as the most effective (78.3%), while the least effective of all tested antimicrobial agents following ampicillin (0%) were observed in ceftazidime (1.7%) and cefotaxime (3.5%). A similar study on this bacterium carried out in Bandar Abbas demonstrated the same findings, as shown in Table 2, wherein amikacin (84.1%) and cefotaxime (42.4%) were the most and least effective antibiotics, respectively (23). Moreover, in another similar study conducted in Shiraz, following ampicillin, it was ceftazidime which observed the most resistance (66.8%), and amikacin (37%) proved to be the most effective (24). In fact, the results of the just mentioned study were also consistent with those of the current study, which represents a similar resistance pattern in the south of Iran. The prevalence of ESBL-KP varies in different countries. In our study, the prevalence of ESBL-KP isolates was 26.4%, whereas that of ESBL-KP isolates in Iran ranges from 28% in Kerman (25) to 74% in Tehran (17). In the systematic study of Bialvaei, the average of ESBL-KP isolates reached 45.1% from 2010 to 2018 (26). Comparing the prevalence of ESBL-KP isolates in Bushehr with other regions of Iran, it can be concluded that the prevalence of ESBLs in Bushehr is lower than that of the nation average. The prevalence of ESBL-KP differs in other countries, as well. For instance, Sweden, Canada, and Spain reported the least amount of extended spectrum of β-Lactams with 2% (27), 3.6% (28), 7.2% (29), respectively, while the highest prevalence of ESBLs has been reported in India (69.4%), Thailand (45.5%), and the Philippines (40%) (30).

Over the past decade, CTX-M-15 (group 1), CTX-M-14 (group 9), and CTX-M-2 (group 2) have been the most prevalent CTX-M enzymes in different European countries as well as Iran (13). Indeed, the present study further showed that the CTX-M1 group had a high (81.7%) prevalence in clinical ESBL-KP isolates. However, the prevalence of CTX-M1 group in ESBL-KP isolates varied from 7.7% in Tabriz (17) to 100% in Tehran (31) and Zahedan (32). Considering other countries, Iraq (33) and Bahrain (34) harbored the least prevalence by 26%, and 10%, respectively, while the highest prevalence has been reported in countries such as Kuwait 100% (35), Pakistan 93.84% (36), and UAE 64.4% (37).

In our study aac(3)-Ila and aac(6')-Ib genes re-

| Drug            | Phenotypic Resistance | Aminoglycoside-modifying enzyme genes |
|-----------------|-----------------------|--------------------------------------|
|                 | aac(3)-Ila | aac(6')-Ib | aac(3)-Ila / aac(6')-Ib |
| Tobramycin      | 34 (60.7%) | 21 (61.7%) | 32 (94.1%) | 20 (58.8%) |
| Amikacin        | 11 (19.6%) | 5 (45.4%)  | 10 (90.9%)  | 5 (45.4%)  |
| Gentamicin      | 27 (48.2%) | 22 (81.4%) | 25 (96.1%)  | 21 (80.7%) |
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respectively displayed a prevalence of 21.2% and 19.8% among our isolates. Similarly, a prevalence of 20.75% and 21.69% for aac(3)-IIa and aac(6’)-Ib genes has been reported by Lotfollahi et al. (38). In another study conducted in Tehran by Eftekhar et al. the prevalence of aac(6’)-Ib gene was 53.16% (39). Finally, in another study conducted in Qazvin and Tehran by Nasiri et al. aac(6’)-Ib and aac(3)-IIa genes showed a prevalence of 91.5% and 78.5%, which is the highest reported prevalence ever in Iran (40). Similarly, the most common AME gene detected in ESBL-KP by Fernandez-Martinez in Spain was aac(6’)-Ib (44.6%) followed by aac(3)-IIa (43.1%) (11).

Associated resistance to aminoglycosides was often seen among ESBL-KP. Many studies reported the association between the presence of CTX-M enzymes and resistance to other antibiotics, especially aminoglycosides. Genes encoding AMEs can be located on integrons or transposons carried by different kinds of conjugative plasmids also coding for ESBLs or carbapenemases. Efficient mobile elements may have caused the acceleration of the simultaneous and quick spread of both ESBL and AME genes (11).

The comparison drawn between the results of this current study and those of other studies conducted by other researchers indicates a low prevalence of aac(6’)-Ib, and aac(3)-IIa genes in Bushehr province. Notably, 66% of ESBL-KP isolates were resistant to at least one aminoglycoside. Among K. pneumoniae isolates harboring blaCTX-M AME genes were found in 68.08% of isolates and 46.8% of these isolates which contained both aac(6’)-Ib and aac(3)-IIa genes were resistant to at least one aminoglycoside. Notably, resistance to all β-lactams, except penicillin-β-lactamase inhibitor combinations, was higher in CTX-M type ESBL than in non-CTX-M type ESBL (data not shown). The coexistence of blaCTX-M with AME genes in our isolates also led to simultaneous resistance to aminoglycosides. Thus, such an incidence renders treatment extremely difficult. Similar to our study, a high rate of AME genes 71% among blaCTX-M carrying isolates was reported by Peerayeh et al. (13).

In conclusion, the present study demonstrated that the prevalence of ESBL-KP in Bushehr is lower than not only the average but also, to the best of our knowledge, the least prevalent reported in Iran, however, our study further showed that the CTX-M1 group had a high (81.7%) prevalence in clinical ESBL-KP isolates. Unfortunately, the coexistence of blaCTX-M with aac(3)-IIa and aac(6’)-Ib genes among isolates probably indicates the presence of multi drug resistant K. pneumoniae; therefore, making the treatment of these strains more difficult due to the limitation of effective antibiotics. Although imipenem is an effective drug against the so-called strains, it should be noted that the use of imipenem increases the risk of resistance to carbapenems, which is the last line of treatment. The alarming global dissemination of K. pneumoniae strains carrying AMEs and blaCTX-M genes simultaneously emphasizes the need for a careful undertaking of aminoglycoside combination therapy in the initial empirical treatment of infections caused by ESBL-producing K. pneumoniae.

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