

Presence of *bla-AmpC (FOX)* Gene in *Klebsiella pneumoniae* Isolates Collected From Different Clinical Specimens of Hospitalized Patients in North of Iran



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ABSTRACT

Background: *Klebsiella pneumoniae* (Kp) is a major opportunistic pathogen responsible for diverse clinical infections. Research on *bla_{AmpC}* genes is widespread; however, data on the prevalence of *bla_{AmpC} (FOX)* and its impact on pneumonia isolates from hospitalized patients are limited. This study aims to evaluate the antimicrobial resistance profiles and presence of *blaFOX* in Kp strains recovered from hospitalized patients.

Materials and Methods: The Kp isolates were collected from clinical specimens (urine, blood, sputum, wound, and cerebrospinal fluid) of 100 hospitalized patients in Mazandaran Province, north of Iran. Antibiotic susceptibility test was performed to assess resistance patterns. The combined disk test (CDT) was used to identify AmpC producers. PCR analysis was conducted to detect the presence of the *blaFOX* gene among cefoxitin-resistant isolates.

Results: Resistance rates were highest for aztreonam (77%), cefotaxime (69%), and piperacillin (68%). Base on antibiogram results, 61 cefoxitin-resistant isolates were detected among the tested isolates, of which 26 were CDT positive. PCR data indicated that 22.95% of strains harboring the *blaFOX* gene.

Conclusion: The elevated antibiotic resistance in Kp strains underscores the need for effective antimicrobial stewardship and surveillance to manage and prevent the spread of resistant isolates.

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Introduction

Klebsiella pneumoniae (*Kp*), a gram-negative opportunistic pathogen belonging to the Enterobacterales family, is a significant cause of various clinical infections, including pneumonia, urinary tract infections (UTIs), wound infections, and sepsis [1-3]. Because these bacteria may cause pneumonia in those with compromised immune systems, this is especially alarming because pneumonia can progress to more serious ailments. Due to its multifaceted pathogenicity, *Kp* can evade the immune system and spread infection due to virulence factors such as lipopolysaccharides (LPS), capsules, and biofilms [4]. The clinical problem caused by *Kp* is increased by the frequent emergence of multidrug-resistant (MDR) strains. These isolates produce carbapenemases or extended-spectrum beta-lactamases (ESBL), which significantly limit the available therapeutic options [5]. *AmpC* β -lactamases are clinically relevant enzymes related to resistance to many different types of β -lactam antibiotics. *AmpC* β -lactamases, classified as class C enzymes under the Ambler classification scheme, are frequently detected in gram-negative microorganism [6]. The biosynthesis of *AmpC* β -lactamase by plasmid-mediated pathways in *Escherichia coli* and *Kp* can result in colonization and transmission of infection within healthcare facilities [7, 8]. *Kp* bacteria develop resistance to antibiotics, including beta-lactams, cephalosporins, and carbapenems, through a variety of mechanisms. These include the synthesis of enzymes such as beta-lactamases, changes in the target location of antimicrobial agents, reduced cell permeability due to the loss of outer membrane proteins, and overexpression of efflux pumps [9-13]. The co-existence of these mechanisms is often correlated with the emergence of MDR and extensively drug-resistant (XDR) strains, for which limited antibiotic treatment options are available [14]. Based on sequence similarity, the *ampC* genotypes are divided into CIT, EBC, DHA, ACC, FOX and MOX groups. The *bla*_{FOX} gene refers to a specific genotype of the *AmpC* β -lactamase present in *Kp* isolates. This genotype is particularly notable because of its association with resistance to third- and fourth-generation cephalosporins, a commonly used class of antibiotics. *AmpC* β -lactamase is encoded by the *bla*_{AmpC} gene, which indicates resistance mechanisms that make decisions about therapy more challenging [15, 16]. Research on *AmpC* genes is widespread; however, data on the prevalence of *bla*_{FOX} and its impact on *Kp* isolates from hospitalized patients are limited [17]. Therefore, this study aimed to evaluate the antimicrobial resistance profiles and the presence of *bla*_{FOX} in *Kp* strains recovered from hospitalized patients in Mazandaran Province, north of Iran.

Materials and Methods

Strain source

In this study, 100 clinical isolates of *Kp* were collected during a prospective study conducted in Sari City from March to September 2023. The suspected *Kp* strains were obtained from various clinical specimens, including urine (n=49), blood (n=27), sputum (n=3), wound (n=5), cerebrospinal fluid (CSF) (n=2), and other specimens (n=4). The isolates were cultured on blood agar and MacConkey agar, and identified using gram staining and standard biochemical tests, including citrate utilization, urease activity, triple sugar iron (TSI), and methyl red/Voges-Proskauer (MR/VP) tests [18].

Antibiotic susceptibility testing

According to the Clinical and Laboratory Standards Institute (CLSI) 2024 guidelines [19], the disk diffusion susceptibility test was performed to identify the patterns of antimicrobial resistance for all confirmed *Kp* isolates against 12 antimicrobial agents, including aztreonam (30 μ g), cefotaxime (30 μ g), cefepime (30 μ g), Imipenem (10 μ g), meropenem (10 μ g), amikacin (30 μ g), ceftriaxone (30 μ g), gentamicin (10 μ g), ceftazidime (30 μ g), piperacillin (10 μ g), ciprofloxacin (5 μ g), and ceftiofur (30 μ g). Disks were placed on Mueller-Hinton Agar (MHA) with a 0.5 McFarland standard. Plates were observed 18 hours after inoculation at 35 °C. All antibiotic discs were manufactured by Padtan Teb Company in Iran. The *E. coli* ATCC 25922 strain was used as a quality control.

Phenotypic CDT to confirm the presence of *AmpC*

To create the boronic acid (BA) solution, 12 mg of 3-aminophenylboronic acid was dissolved in 3 mL of dimethyl sulfoxide. Subsequently, 3 mL of sterile distilled water was introduced to this mixture. The BA solution (400 μ g in 20 μ L) was applied to disks containing ceftiofur (i.e., FOX). Following the CLSI 2024 guidelines, a test strain was cultured on the MHA plates. The plates were then fitted with disks containing ceftiofur alone (FOX) and both ceftiofur and BA (FOX/BA), followed by overnight incubation at 37 °C. A positive result was indicated by an expansion $\geq$ 5 mm in the inhibition zone around the ceftiofur when BA was present, compared to ceftiofur alone [20, 21].

Polymerase chain reaction (PCR)

The PCR method was used to screen the *bla*_{FOX} gene, as a transferable *AmpC* β -lactamases in bacteria [22]. DNA was extracted from the samples using the boiling method. A PCR based on the specific oligonucleotide primers F-CAAAGCGCGTAACCGGATTGG and R-AACATGGGGTATCAGGGAGATG was performed using the gene sequence [23]. In a total volume of 15 μ L, the final mixture for the reaction was mixed with 1 μ L of each primer (10 picomoles), 7.5 μ L of Taq DNA polymerase master mix red (Ampliqon Co.), 3 μ L of template DNA, and 2.5 μ L of double-distilled water. The PCR process consisted of an initial denaturation at 94 °C for 4 minutes, followed by 35 cycles of denaturation (94 °C for 45 seconds), primer annealing (56 °C for 40 seconds), and extension (72 °C for 50 seconds). The last extension stage was carried out for 5 min at 72 °C. Following amplification, the PCR products were separated on a 1% agarose gel containing DNA safe stain using electrophoresis (90 V, 1 h), and the bands were visualized under UV illumination.

Statistical data analysis

Statistical analysis was performed in SPSS software, version. Pearson's chi-square test was employed to assess the statistical significance of differences. The significance level was set at 0.05.

Results

The *Kp* isolates were collected from 100 patients, including 39% females and 61% males. The samples were obtained from various sources, including 41% urine, 27% blood, 13% sputum, 5% wounds, 4% other specimens, and 2% cerebrospinal fluid. The age ranges of the *Kp* isolates are shown in Table 1. Aztreonam (77%), cefotaxime (69%), and piperacillin (68%) showed the highest resistance rates in antibiogram examination (Table 2). By employing the CDT, 26 isolates out of 61 cefoxitin-resistant strains were identified as *AmpC* producer. Additionally, PCR analysis showed that 22.95% (14 out of 61 cefoxitin-resistant strains) of isolates were found to carry the *bla*_{FOX} gene.

Discussion

Antimicrobial resistance is facilitated by the predominate antimicrobial resistance is facilitated by the predominance of *bla*_{FOX} gene, resulting in more complicated treatment approaches [24]. This gene caused up to 60.8% of *Kp* isolated from patients to be resistant to cephalosporin

substances such as cefoxitin [25]. Clinical laboratory technicians encounter a significant issue when identifying *AmpC* β -lactamases in *Kp*. Although molecular methods, such as PCR and DNA sequencing, provide more number and precise characteristics of these enzymes, lack of resources sometimes limit their routine use [26]. The primary purpose of this study was to identify bacterial strains that might produce β -lactamase and *AmpC* enzymes. As reported by Heidary et al, *Kp* strains in Iran showed the highest percentage of ampicillin resistance (82.2%). Resistance rates to ceftazidime, aztreonam, imipenem, and nitrofurantoin were 55.7%, 55.4%, 54.5%, and 3.2%, respectively [27]. In 2018, Vaez et al., in a meta-analysis and systematic review study, assessed the prevalence of carbapenem-resistant *Kp* strains in different cities of Iran. The lowest prevalence (<1%) was found in Tehran city (0.04%), whereas Isfahan city showed the highest prevalence (58%). Furthermore, aztreonam (55%) and amikacin (23%) demonstrated the highest and lowest levels of resistance to medications, respectively [28].

Compared to previous studies, our results showed a significant increase in drug resistance rates, particularly for imipenem and aztreonam, with resistance rates of 59% and 77%, respectively. Moghaddas et al. in a study conducted in 2018, evaluated the resistance of *Kp* to many antibiotics in Semnan, Iran. Imipenem had the lowest resistance rate (7.5%), while ceftazidime had the highest (42.7%) [29]. In a study conducted in 2019 in Iran, 20% of *Kp* isolates produced *AmpC*, and 40% showed ESBL production by phenotypic testing. These results were confirmed by molecular analysis, which showed that *AmpC* was found in 24.4% of isolates and ESBL was positive in 40% [17]. Each isolate in the current study tested negative for the *bla*_{FOX} gene.

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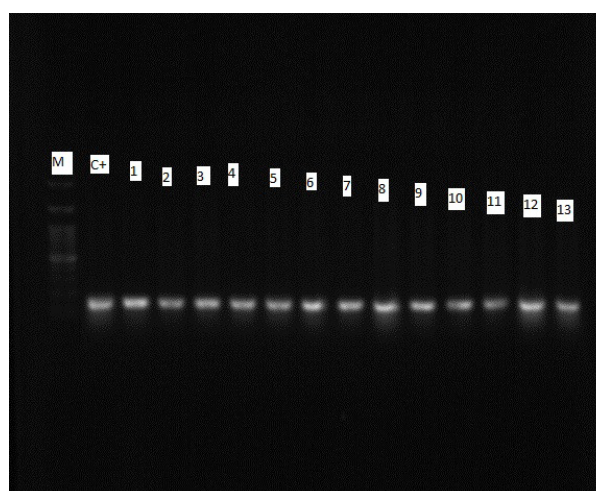


Figure 1. Agarose gel electrophoresis of the *AmpC* gene of clinical *Kp* isolates
Lanes 1-13: *Kp* carrying the *AmpC* gene (190bp), C+: *E. coli* ATCC 25922, M: 100 bp DNA ladder.



In our study, 39% of the isolates were resistant to aminoglycosides (amikacin), 69% to cefazolin, and 63% to meropenem. In Babazadeh et al.'s study on 100 *Kp* isolates, 12% (n=12), 27% (n=27), and 9% (n=9) of the isolates tested positive for the modified Hodge test (MHT), double-disc test (DDT), and disc potentiation test (DPT), respectively. The most prevalent genes that encoded carbapenemase were *blaOXA-48* (24%) and *blaIMP* (13%), whereas *blaKPC* and *blaGIM* were not found. The most common ESBL and *AmpC* β -lactamase genes were *blaTEM* (48%) and *blaCMY* (8%). Notably, the *Kp* isolates showed the

highest percentages of cefazolin (66%) and cefotaxime (66%). Meropenem and amikacin showed the strongest antibacterial activity against the isolates, with susceptibility rates of 76% and 69%, respectively [30]. Compared to previous studies, our study on investigating the presence of a *bla-Amp-C* gene showed that 14 out of 61 cefoxitin-resistant isolates (22.95%) contained the *bla_{FOX}* gene. Hadadi et al. found that 43.42% of *Kp* isolates tested positive for the *bla_{FOX}* gene, indicating its role in *AmpC* β -lactamase production, which contributes to antibiotic resistance in these strains, particularly against β -lactam antibiotics [31].

Table 2. Antibiotic sensitivity pattern of *K. pneumoniae* samples

Antibiotic	Resistance (%)	Intermediate (%)	Susceptible (%)
Aztreonam	77	3	20
Cefotaxime	69	4	27
Cefepim	55	9	36
Imipenem	59	2	39
Meropenem	63	7	30
Amikacin	39	6	55
Ceftriaxone	66	10	26
Gentamicin	35	4	61
Ceftazidime	67	10	23
Ciprofloxacin	59	6	35
Piperacillin	68	2	30
Cefoxitin	54	7	39



Table 1. Age range of *K. pneumonia* isolates

Age (y)	No. Isolates (% of Abundance)
0-10	23
11-20	9
21-30	3
31-40	7
41-50	11
51-60	16
>61	31



Conclusion

Our results suggest that *Kp* isolates in northern Iran have a high degree of antibiotic resistance, which poses serious problems in treating illnesses caused by these strains. Additional studies are needed to determine the antibiotic resistance profiles of these bacteria in patients stratified by the hospitalization ward or source of isolation. Furthermore, it is important to examine the frequency of other β -lactamase genes in *Kp* that are linked to *Amp-C* resistance mechanisms, such as the plasmid-mediated *DHA*, *MOX*, and *ACT* genes. Comprehensive research is also needed to clarify the fundamental mechanisms of resistance and to develop innovative treatment strategies to combat these diseases. Effective antimicrobial management and surveillance of resistant strains are crucial to manage infections and prevent the dissemination of drug-resistant isolates. We hope that this study can help healthcare professionals in selecting the most appropriate treatment for infections caused by this opportunistic and virulent pathogen.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of [Mazandaran University of Medical Sciences](#), Sari, Iran (Code: IR.MAZUMS.REC.1402.494).

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Authors contribution's

Conceptualization, study design, review and editing: Mehrdad Gholami; Sampling: Shima Keshavarzi; Data acquisition, and experiments: Shayan Edrisi; Data analysis and interpretation: Mehrdad Gholami and Mohammad Ahanjan; Statistical analysis and writing the original draft: Mohammad Karimbakhsh; Final approval: Mehrdad Gholami, Shima Keshavarzi, and Mohammad Ahanjan.

Conflict of interest

The authors declared no conflict of interest.

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