Original Article

Molecular Epidemiology of AmpC-Producing Klebsiella Pneumoniae Isolated from Perioperative Patients in a Tertiary Hospital

Roghayeh Gholizadeh Doran Mahalleh[1], Nadia Kazemi Pour[1], Farokh Rokhbakhsh-Zamin[1]

Abstract

Background: Extended-spectrum cephalosporin-resistant organisms are the sources of AmpC- type β-lactamase isolates. AmpC-producing Klebsiella pneumoniae isolates are a major challenge in clinics due to their high rate of antibiotic resistance. This study aimed to detect AmpC β-lactamases in K. pneumoniae isolated from hospitalized patients with a surgical history.

Materials and Methods: This cross-sectional study was performed on 120 urine catheter samples. After antibiotic susceptibility testing, plasmid-mediated AmpC was detected by double-disc plus combined disc test (DCDT) and three-dimensional extract test (TDET). The genotypic detection of plasmid-mediated AmpC was carried out using multiplex-polymerase chain reaction (PCR).

Results: Of 120 samples, 60 (50%) instances of K. pneumoniae were isolated. The highest and lowest resistance rates were related to cefotaxime (CTX) (50%) and ciprofloxacin (CIP) (23.3%), respectively. 18.3% (n: 11) K. pneumoniae showed an AmpC phenotype in DCDT and TDET tests. AmpC genotyping showed that the prevalence of CIT, DHA, EBC, and ACC genotypes were 47.1%, 35.3%, 29.5%, and 23.6%, respectively. MOX and FOX genotypes were not identified in the isolates of interest.

Conclusion: AmpC β-lactamase identification tests should be considered a routine microbiology workup for gram-negative microorganisms. Multiplex-PCR can identify the plasmid AmpC genotypes.

Keywords: AmpC, *Klebsiella pneumoniae*, Double-disc plus combined test, Three-dimensional extract test

1. Department of Microbiology, School of Medicine, Kerman Branch, Islamic Azad University, Kerman, Iran

Corresponding Author: Nadia Kazemi Pour, Islamic Azad University Kerman Branch University Blvd, Emam Ali, Kerman, Iran
Tel: (+98)-34-33210043-50
Fax: (+98)-34-31320051
Postal Code: 7635131167

Email: na.kazemipour97@gmail.com

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Introduction

Perioperative hospital infection is a serious medical challenge with a difficult and sophisticated management process. Bacterial agents can affect patients' treatment process and hospitalization time (1). Several bacterial agents can cause hospital infections; among them, *Klebsiella pneumoniae*, a rod-shaped gram-negative bacterium, is a common cause (2-4). Treatment of infections caused by drug-resistant *K. pneumoniae* is a major challenge because of its intrinsic and acquired resistance to a broad spectrum of antimicrobials (4). The main mechanism for resistance to β-lactam-class antibiotics is synthesizing b-lactamases, one of the most important beings AmpC

(5). This enzyme confers resistance to most β-lactam drugs except cefepime and carbapenems. AmpC β-lactamases are efficient at destroying penicillins, cephalosporins, and, in particular, cephamycins (6). According to the Ambler structural and Bush functional classifications, AmpC enzymes belong to class C and group III, respectively (7). AmpC-encoding genes are located on both the chromosome and the plasmid. AmpC plasmid-encoded genes originate in the chromosome and can carry resistance genes against such antibiotics as aminoglycosides, quinolones, chloramphenicol, tetracycline, trimethoprim, and other categories of β-lactamases (8). Multidrug-Resistant-*K* (MDR-K) *pneumoniae* isolates that produce AmpC-resistance elements have become a major challenge to human health. This study aims to determine the antibiotic resistance profile and frequency of AmpC β-lactamases in *K. pneumoniae* isolated from hospitalized patients in territory hospitals.

Methods

Patients and bacterial strains: This cross-sectional study was carried out in 2017, for the year, in three teaching hospitals in Zahedan City. The ethical committee approved the study of Islamic Azad University Kerman Branch University. In total, urine catheter 120 samples were gathered from hospitalized patients with a history of a recent operation. Samples were streaked on MacConkey agar (Merck Co., Germany), then incubated at 37°C for 24 hours. All colonies were identified as *K. pneumoniae* by standard microbiological and biochemical tests, e.g. oxidase, gram stain, citrate utilization, motility, urease production, Triple Sugar Iron agar (TSI), Methyl Red, and Vogue Proskauer (MR-VP), and Sulfur, Indole, Motility (SIM). The Analytical Profile Index (API) 20E system (Analytab, Inc., New York) confirmed the identification.

Antimicrobial susceptibility testing: The susceptibility profile was determined using Mueller-Hinton Agar (Merck Co., Germany). The disc diffusion method, based on the Clinical and Laboratory Standards Institute recommendation, was used for some antibiotics including cefotaxime (CTX: 30µg), ceftazidime (CAZ: 30µg), ceftriaxone (CRO: 30µg), ciprofloxacin (CIP: 5µg), and cefpodoxime (CPD, 10µg) (Mast, Merseyside, UK). Fresh cultures were used in formulating a microbial suspension. The opacity of each suspension was adjusted equivalent to a 0.5 McFarland standard. Zone diameters were recorded after incubation at 37ºC for 18-24 hours. The results were reported as resistant, intermediate, and susceptible. The standard strain of *K. pneumoniae* ATCC 1029 was used as quality control.

Identification of plasmid-mediated AmpC production: A disc impregnated with cefoxitin (FOX, 30 µg) and another disc plus clavulanic acid/ ceftazidime (CAZ/CLA) were included in double-disc plus combined discs (DCDT) identification of AmpC-

Table 1: Primer oligonucleotide sequencing used in this study.

Target	Primer	Sequence (5'→3')	Product size (base-pair)
MOX	Forward	GCTGCTCAAGGAGCACAGGAT	385
	Reverse	CACATTGACATAGGTGTGGTGC	
CIT	Forward	TGGCCAGAACTGACAGGCAAA	462
	Reverse	TTTCTCCTGAACGTGGCTGGC	
DHA	Forward	AACTTTCACAGGTGTGCTGGGT	405
	Reverse	CCGTACGCATACTGGCTTTGC	
ACC	Forward	AACAGCCTCAGCAGCCGGTTA	346
	Reverse	TTCGCCGCAATCATCCCTAGC	
EBC	Forward	TCGGTAAAGCCGATGTTGCGG	302
	Reverse	CTTCCACTGCGGCTGCCAGTT	
FOX	Forward	AACATGGGGTATCAGGGAGATG	412
	Reverse	CAAAGCGCGTAACCGGATTGG	

type enzymes. An empty disc moistened with sterile saline was inoculated with a few colonies of the test organisms, placed next to a 30µg FOX disc on a Mueller-Hinton agar plate previously impregnated with a lawn of Escherichia coli ATCC 25922, and incubated at 37ºC overnight. Indentation of the FOX inhibition zone was an indicator of AmpC β-lactamase production. AmpC production was also confirmed by a modified three-dimensional extract test (TDET) (9). The colonies from overnight bacterial cultures on the Mueller-Hinton agar were moved to a sterile microcentrifuge tube, accommodating 10-15 mg of bacterial wet weight per sample. The bacteria were suspended in peptone water and centrifuged at 3000 rpm (Sigma 1-14 Microfuge, St. Louis, United States) for 15 minutes. Crude cell extracts were obtained using ten cycles of alternate freeze-thawing at -78ºC (dry ice/ethanol bath) and 37ºC. Linear slits 3 cm long were cut into plates inoculated with each test bacterium using a sterile scalpel blade, 3 mm away from a 30 µg FOX disc, in an external radial direction. About 40-50µL of the prepared extract was loaded into each split, and the plates were kept upright for 5 to 10 minutes, followed by incubation at 37ºC for 24 hours. AmpC production was verified when clear inhibition zones around the FOX disk were merged (10).

Polymerase chain reaction: AmpC-encoding genes were characterized via polymerase chain reaction (PCR). Template DNA was extracted from colonies grown overnight on the brain-heart infusion agar (Merck Co., Germany) plates provided by the QIAprep Spin miniprep kit (Qiagen, Hilden, Germany). This product is stored at -20ºC until required. The quality and concentration of the extracted genomic DNA were assessed by a Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE, USA). Genomic DNA with a concentration of 0.1 ng/µl was used as the template. Amplification reactions were carried out in a total volume of 25 µl, containing 0.8 µl of extracted DNA, 2.0µl of 10× PCR buffer, 1.2mmol/l MgCl2, 0.6µl (each) of dATP, dGTP, dCTP, and dTTP, 0.7µl of each primer, 0.9µl of Taq DNA polymerase (5 U/µl) (Ampliqon, Odense, Denmark) and 18.1µl ddH2O (Table 1). The samples were amplified in a Techne TC-512 thermocycler (Eppendorf, Hamburg, Germany), following initial denaturation at 94ºC for 5 minutes, 35 cycles of denaturation for 35 seconds at 94ºC, annealing for 35 seconds at 56ºC and extension for 35 minutes at 72ºC, with a final extension for 5 minutes at 72ºC. The PCR product was subjected to electrophoresis in a 1.0% agarose gel, stained by safe stain (Sina Genes, Iran), and photographed under ultraviolet illumination (Bio-Rad, Hercules, CA, USA).

Amplicon sequences: Sanger dideoxy chain termination technique in both directions was performed by an ABI3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). Similarity searches for the nucleotide sequences were conducted by special software called the "BLAST algorithm" (available at http://www.ncbi.nlm.nih.gov/blast).

Statistical data analysis: The data were analyzed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Pearson's chi-square tests were used in this study. $P<0.05$ was considered statistically significant.

Results

Out of 120 samples, 60 (50%) containing *K. pneumoniae* were obtained. The mean patient age was 48.00±1.60 years (ranging from 15 to 75 years). The

Table 2: Antibiogram of clinical isolates of *K. pneumoniae*.

Antibiotic	S#	I†	R*
Ciprofloxacin (CIP)	**46 (76.6%)**	**0 (0%)**	14 (23.3%)
Cefotaxime (CTX)	**23 (38.3%)**	**7 (11.7%)**	30 (50%)
Ceftriaxone (CRO)	27 (45%)	4 (6.6%)	27 (45%)
Cefpodoxime (CPD)	25 (41.7%)	6 (10%)	29 (48.3%)
Ceftazidime (CAZ)	31 (51.7%)	10 (16.6%)	19 (31.7%)

*Resistant, †Intermediate, #Susceptible

studied strains belonged to different age groups: 15-25, n: 8; 26-36, n: 17; 37-47, n: 22; 48-58, n: 11 and 59-75, n: 2. Forty-six (76.6%) patients were female and 14 (4.23%) patients were male. Table 2 shows that the majority of the isolates are resistant to CTX (50%), CPD (48.3%), CRO (45%), CAZ (31.7%), and CIP (23.3%).

In this study, 11 isolates of *K. pneumoniae* (18.3%) showed an AmpC-type phenotype in both DCDT and TDET tests. The AmpC gene amplification test showed that the prevalence of CIT, DHA, EBC and ACC genotypes was 47.1% (n: 8), 35.3% (n: 6), 29.5% (n: 5) and 23.6% (n: 4), respectively. MOX and FOX genes were not identified in the studied isolates (Figure 1).

Discussion

Effective use of microbiology laboratories to identify and prevent the spread of resistant microbial pathogens reduces the need for medication (11). Infection control is beneficial for patients and reduces the mortality rate (12). If microbial pathogens are misidentified as resistant or susceptible in laboratory tests, it may result in patients receiving inappropriate medication and transmitting the bacteria to other patients (13). Therefore, identifying human bacterial pathogens with hidden antibiotic resistance is essential, and a useful assay is needed to identify resistance mechanisms. This detection can be done by different methods, including molecular and microbial detection methods. Each of these methods has its advantages and disadvantages, but they are reliable with relatively consistent results. Among molecular methods, M-PCR can be used as a rapid and reliable procedure for detecting several antibiotic resistance genes at the same time.

Bacteria have used several mechanisms to protect against the harmful effects of antibiotics (14). They are stepping up the production of broad-spectrum β-lactamases to inactivate antibiotics, resulting in multi-drug-resistant infections that have spread rapidly worldwide since their discovery in 1980. The main mechanism used by gram-negative bacteria against β-lactam antibiotics is β-lactamase production. This type of enzyme inactivates antibiotics by hydrolyzing the β-lactam ring (15). New β-lactam antibiotics developed during the past two decades are specifically resistant to this function of β-lactamases. Despite this, new types of β-lactamases, such as extended-spectrum β-lactamases (ESBLs) and AmpC, have emerged. This

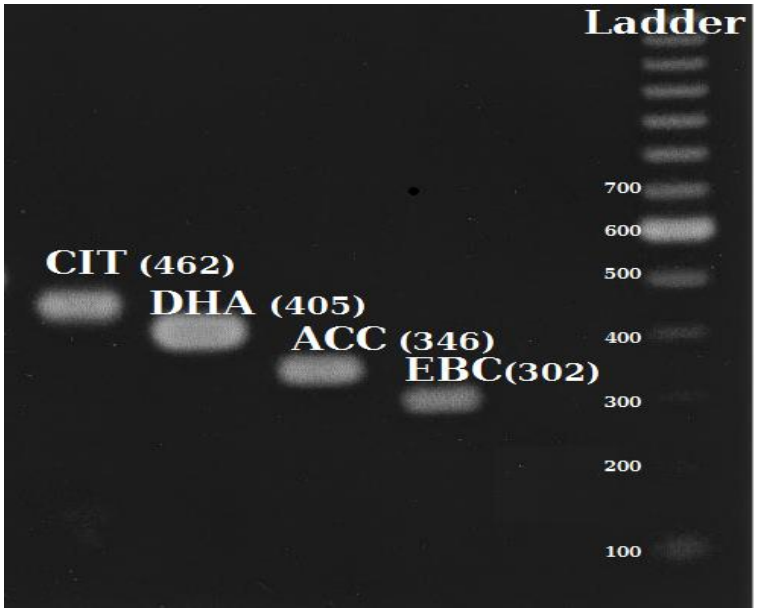

Figure 1. Amplification of AmpC using multiplex-polymerase chain reaction (M-PCR) among the K. pneumoniae isolates. The CIT (462 bp), DHA (405 bp), EBC (302 bp), and ACC (346 bp) PCR products were amplified, while MOX and FOX genes were not detected.

continues to present serious health challenges (16).

This study found that 50%, 48.3%, 45%, 31.7%, and 23.3% of isolates were resistant to cefotaxime, cefpodoxime, ceftriaxone, ceftazidime, and ciprofloxacin, respectively. Pourali Sheshblouki et al. reported that 62.2% of isolates were resistant to ceftazidime, 55.9% to cefoxitin, 33.3% to aminoglycosides (gentamicin and amikacin), and 33.3% to ciprofloxacin (17). Al-Marzooq et al. reported 71% of strains as being resistant to ciprofloxacin (18). In this study, 17 isolates (28.4%) were identified as ESBLs. Behrooozi et al. in Tehran and Gafourian et al. in Ilam found ESBL production in *K. pneumoniae* at 12% and 59.2%, respectively.[18] In Saudi Arabia, Shibl et al. reported ESBL production in *K. pneumoniae* at 65% (19). The reported prevalence of ESBL-production strains depends on some factors: the high use of antibiotics, variations in regional antibiotic use, prolonged hospitalization inwards such as internal medicine and ICU, use of contaminated medical devices such as venous and urine catheters, and the scale of nosocomial infections.

Third-generation cephalosporins are overprescribed. Antibiotic resistance thus has increased in many cities in Iran. Mardaneh et al., isolating blood cultures of patients in Shiraz, found that 21.5% of positive cultures belonged to Enterobacteriaceae (20). Among the family members, 28% were *Klebsiella* spp., which ranked second after *Escherichia coli*. Polymyxin B, colistin, and imipenem were the most effective drugs against *Klebsiella's* positive ESBL strains. Rostamzad et al., in Isfahan, showed that 17 isolates (32.7%) had the CTX-M-15 gene and nine isolates (17.3%) had the FOX gene.[21] These results parallel the results of the current study; our study does not show a significant decrease in resistance. Japoni-Nejad et al. reported that the frequency of the AmpC β-lactamase gene in Arak was 19%. As in our study, the FOX gene was not detected in any of the isolates.[22] While these statistics reflect AmpC resistance in Zahedan, they are representative of Iran in general and are similar to most other countries.

Conclusion

Antibiotic resistance is relatively common in southeast Iran, and the molecular detection method is a reliable and rapid method for detecting this kind of bacteria. AmpC β-lactamase identification tests, particularly molecular methods, should be considered a routine microbiology workup for gram-negative microorganisms. Multiplex-PCR can be used as a rapid and reliable method for the identification of different plasmid AmpC genotypes.

Acknowledgment

None.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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