Molecular Epidemiology of *bla$_{CMY-1}$*, *bla$_{CMY-2}$*, *bla$_{FOX}$* Genes in *K. pneumoniae* From Elderly Patients in Tehran, Iran

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Abstract- The growth rate of the population aging is increasing worldwide. To assess antimicrobial susceptibility of extended-spectrum β-lactamase- (ESBL-) producing *Klebsiella pneumoniae* Isolated from Patients aging in Rasool Akram, Hospital, as well as to identify AmpC genes. 100 K. pneumoniae strain isolated from different clinical samples. Isolates resistant to oxyimino-cephalosporins and to cefoxitin evaluated to phenotypic ESBL production and to phenotypic AmpC production, respectively. Detection of resistance genes was then performed using primers specific for AmpC genes. Piperacillin/tazobactam and carbapenems remained the active β-lactam antibiotic against *K. pneumoniae*. ESBLs were detected among 40 (40%) of *K. pneumoniae* isolates. *CMY*-1 gene was detected in 34.3% of all AmpC-positive isolates, whereas *CMY-2* and *FOX* genes were 14.2% and 28.6%, respectively. The consumption of Carbapenem family drugs is high in Iranian hospitals which are used as a first line of treatment without antibiotic susceptibility testing. Therefore, increase in antibiotic resistance to this family drugs is unavoidable in the near future. Therefore, it is necessary to take the necessary measures to modify the administration and use of antibiotics.

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Introduction

Klebsiella pneumoniae is a gram-negative bacterium in the family Enterobacteriaceae, which is an important cause of nosocomial infections (1). *Klebsiella* species colonize in the human gut, bladder, upper respiratory tract, and skin (2). They persist in hard environmental conditions, including wet sites of hospitals, and increasing hospital outbreak of extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* isolates is of particular concern (3).

ESBL enzymes cause resistance to penicillins and cephalosporins, including the sulbactam and clavulanic acid combinations and monobactams such as aztreonam AmpC β-lactamase as well, in *k. pneumoniae* ESBL enzymes show activity against cephalosporin (first-through-third-generation), penicillins and monobactams such as aztreonam in *K. pneumonia* antimicrobial-resistant isolates (4). The evolving of multidrug-resistant pathogens and increased chance for community-associated infection would be expected due to the fast dissemination of AmpC lactamase- and ESBL-producing bacteria (5,6).

However, the true diagnosis of AmpC-overexpressed *k. pneumonia* strains is significant for clinical management, as the administration of beta-lactam antibiotics frequently results in therapeutic failure. To date, New Delhi metallobeta-lactamase 1(NDM-1) (7), NDM-7, and oxacillinases (OXA-48) Carbapenemases identified by Shahcheraghi *et al.*, in *K. pneumoniae* isolated in Iran (8). Polymerase chain reaction (PCR) is considered as the method of choice for the detection of

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AmpC β-lactamases (9).

Microorganisms showing resistance to multiple drugs (MDR) are those with resistance to three or more categories of drugs such as Cephalosporins, aminoglycosides and Fluoroquinolones. *K. pneumonia* is an important agent of nosocomial infections. Multiple drug resistance *K. pneumonia* is developed through ESBLs and carbapenemase enzymes (10). In a study conducted in a children's hospital, the emergence of a plasmid-mediated β-lactamase gene, Beta-lactamase CMY-2 (bla_{CMY-2}), among Enterobacteriaceae isolates has been increased over a 3-year period. Therefore, the detection of pAmpC β-lactamases has a significant effect on the clinical use of antibiotics (11). The aim of the current study was to detect ESBL among elderly patients admitted to a tertiary hospital in Tehran, Iran.

Materials and Methods

Strain source

The clinical samples including 100 isolates were obtained from patients more than 85-year-old at Rasool Akram hospital from March 2015 to November 2017.

Antibiotic Susceptibility Testing

Disk diffusion method was mainly used to test antibiotic susceptibility of bacteria using CLSI guidelines. The antibiotics included Imipenem (10 µg), Meropenem (10 µg), Cefotaxime (30 µg), Piperacillin (100 µg), Piperacillin/ Tazobactam (100/10 µg), Ceftazidime (30 µg), Cefepime (30 µg), Ceftriaxone (30 µg), Gentamicin (10 µg), Amikacin (30 µg), Trimethoroprim/sulfamethozaxole (SXT) (25 µg) and, Ciprofloxacin (5 µg), were placed on the Mueller Hinton Agar. The plates were observed after 18hrs post-inoculation at 35° C. All of the antibiotic discs were manufactured by Mast (UK).

Genomic DNA extraction and ESBL phenotypic diagnosis

The bacterial DNA was extracted using nucleic acid extraction kit (Sinagen, Iran). Double-disk synergy test (DDST) was used according to the CLSI guidelines to study ESBL production by Piperacillin disc with tazobactam (10 µg) or without tazobactam (10 µg). Enhancement of more than 5 mm in the area of inhibition of both cephalosporin in combination with tazobactam in the evaluation with cephalosporin alone was considered as an ESBL maker (3). *E. coli* (ATCC 25922) was used as a non-ESBL-producing strain.

Phenotypic detection of AmpC beta-lactamases

Screening of AmpC-producing strains was performed by Cefoxitin disks (30 µg). Bacterial strains showing inhibition zones with a diameter ≤18 mm were considered positive for AmpC screening. The isolates were further subjected to testing by AmpC detection set, cefoxitin-cloxacillin double-disk synergy test (CC-DDS) (12).

Amplification of the AmpC genes

Detection of *AmpC* genes was performed using primers illustrated in (Table 1) (13).

Table 1. The primer sets used to detect *AmpC* genes in *K.pneumoniae* from patients hospitalized at Rasool Akram Hospital

Gene type	primer Sequence	PCR product (bp)	Annealing temperature
blaCMY-1	F:GCTGACAGCCTCTTTCTCCAC R:CCTCGACACGGRCAGGGTTA	1080	56^{O} c
blaCMY-2	F:GGTCTGGCCCATGCAGGTGA R:GGTCGAGCCGGTCTTGTTGA	960	56^{O} c
blaFOX	F:CACCACGAGAATAACC R:GCCTTGAACTCGACCG	1180	50^{O} c

F: Forward; R: Reverse

Results

Collectively, 100 isolates of *K. pneumoniae* were detected in the current study. Different types of clinical specimens including Urine50 (50 %), Blood 16 (16%), Trachea tube15 (15 %), Sputum12 (12 %), Diabetes Score, 7 (7%) were targeted in this study. The most of patients with *K. pneumoniae* infection were observed in ICU (30%; 30/100), Urology (21%; 21/100), Lung (20%; 20/100), Infectious (15%; 15/100), Internal (8%; 8/100) and surgical section (6%; 6/100).

A hundred strains were tested for antibiotic susceptibility according to CLSI rules. 70% of strains were resistant to ceftazidime. The summary of antimicrobial resistance pattern is shown in Table 2. Of all strains 40% (40 isolates) were phenotypically ESBL producer.

All cefoxitin-non susceptible *k. pneumoniae* (n=35),

as putative AmpC producers, were tested by PCR assay to identify three specific AmpC genes, including 12/35(34.3%) Beta-lactamase CMY-1 (*bla* $_{CMY1}$), 5/35 (14.2 %), Beta-lactamase CMY-2 (*bla* $_{CMY2}$), 10/35 (28.6 %) Beta-lactamase-Cefoxitin (*bla* $_{FOX}$). Also, 25.7% (9/35) of isolates were negative for AmpC gene.

Table 2. Antibiotic susceptibilities of K.pneumoniea isolated from clinical samples at a tertiary care hospital in Tehran, Iran

Type of antibiotic	Sensitive N (%)	Intermediate N (%)	Resistance N (%)
Imipenem	60(60%)	2(2%)	38(28%)
Meropenem	70(70%)	2(2%)	28(38%)
Cefotaxime	60(60%)	2(2%)	38(38%)
Piperacillin	35(35%)	5(5%)	60(60%)
Piperacillin/ Tazobactam	60(50%)	1(1%)	39(39%)
Ceftazidime.	25(25%)	5(5%)	70(70%)
Cefepime	40(40%)	4(4%)	56(56%)
Ceftriaxone	40(40%)	0	60(60%)
Gentamicin	35(35%)	5(5%)	60(60%)
Amikacin	40(40%)	5(5%)	55(55%)
SXT	35(35%)	0	65(65%)
Ciprofloxacin	40(40%)	0	60(60%)
Cefoxitin	55(55%)	10(10%)	35(35%)

Discussion

AmpC beta-lactamases hydrolyze Cephalosporins. It is unsusceptible to clavulanic acid (14). Although AmpC is expressed at a low level in *Enterobacteriaceae*, it can be induced in response to beta-lactam (15). The indiscriminate use of beta-lactam antibiotics stimulates the production of Ampc Genes in the intestinal flora. Isolates harboring acquired ampCs are usually multi-resistant (15). The results of *K. pneumonia* producing AmpC enzyme-mediated by plasmid were resistant to imipenem (10%) with high resistance to cefepime (60%), amikacin (55%), ceftazidime (70%), and SXT (65%).

Out of the 130 *K. pneumoniae* strains that were screened, 62 strains were identified intermediate or unsusceptible to cefoxitin as AmpC β-lactamase-producers, consistent with a positive rate of 47.7% (16). In Sonia Younas's (17) study on 585 *K. pneumoniae* were determined that 21.53% of isolates were AmpC b-lactamase producers. Another study conducted on 100 isolates of *K. pneumoniae* reported 32 (32%) isolates of AmpC b-lactamase producers (17). Plasmid-mediated AmpC beta-lactamase-producing Enterobacteriaceae can confer resistance to many antibiotics, such as penicillin, cefotaxime, ceftazidime, and ceftriaxone, and cefoxitin (17).

In Turkey, 10.9% of E. coli and 3.6% of *K. pneumoniae* isolates were AmpC producers (18). While In Egypt, 28.3% of *E. coli, Klebsiella spp*. and *Proteus mirabilis* isolates were positive for pAmpC genes (19). In the current study, the highest prevalence rate of AmpC *bla*$_{CMY1}$ (34.3%), *bla*$_{Fox}$ (28.6%), and its lowest *bla*$_{CMY2}$ (14.2%) was observed. *bla*$_{CMY-2}$ has a worldwide distribution. In a study by Fam *et al.*, it was the predominant enzyme (66.7%), followed by *Fox* (25.6%) (19). Bla$_{CMY-2}$ is observed on large IncA/C plasmids (bla$_{CMY-2}$ plasmids) from a diverse bacterial hosts. The prevalence of the *Fox* gene was similar to our study. But there is a significant difference in the prevalence of bla $_{CMY1}$ and bla $_{CMY2}$ gene. This difference is due to the diversity of studied strains. In the study of Fam *et al*., in addition to *Klebsiella spp, E. coli* and *Proteus* spp and *Salmonella* spp and *Citrobacter* spp strains were studied, but bla $_{CMY1}$ gene is more commonly found in *Klebsiella* spp (19).

The limitation of our study was that cefoxitin resistance in *K. pneumonia* might not only be due to AmpC production, but it could also be due to carbapenemases, a few Class A β-lactamases, and diminished levels of production of outer membrane porins. However, 38% of our isolates were carbapenem-resistant. Eighty percent of MBL isolates were detected from urine specimen of admitted patients, and 20% of MBL strains were isolated from the urology section. AmpC-producing *K. pneumoniae* were isolated from different wards in current study. The highest numbers of the strains were isolated from ICU (50%). But in the study of Mata *et al.*, the prevalence rate of AmpC-producing *k. pneumoniae* from ICU and the burn unit was 45% and 22%, respectively (20). Another study reported the rate of AmpC -beta-lactamases in various wards, including (60%) from outpatients, (28%) from admitted patients,

and (12%) from ICU (21).

Hospital outbreaks due to multi-resistant *K. pneumoniae* strains have been reported all over the world (22). Ninety percent of isolates were MDR, the higher rate compared with other studies. All of the AmpC-producing *K. pneumonia* were unsusceptible to tazobactam and cephalosporins. There were 38(38%) strains resistant to imipenem.

Veev *et al*., showed that the unsusceptible pattern of AmpC-producing isolates to piperacillin-tazobactam (36.73%), amikacin (73.46%), ciprofloxacin (53.06%), and gentamicin (69.38%). All of the organisms were susceptible to imipenem (23). Resistance of 81.0% to cefotaxime, 67.5% to cefuroxime, 63.0% to ceftazidime, 40.5% to azithromycin, 27.0% ciprofloxacin and 18.2% to co-amoxiclav was found among AmpC producing isolates in other studies (24,25).

Imipenem and Meropenem were found most effective antibiotics against *K.pneumonia* from hospitalized patients in our study (26). In the similar study by Ishii in Japan, the *k. pneumonia* infections were effectively treated using Imipenem (26).

In our study, tazobactam demonstrates to be the best β-lactamase inhibitor in detecting ESBL production, followed by sulbactam but clavulanic acid being the poorest. As it has been known that in organisms producing both ESBL and AmpC together, clavulanic acid may induce the expression of high-level AmpC production. Liu *et al*., (27,28) have noticed that the enzyme may be connected with the widely used third-generation antibiotics in this region. The presence of *Enterobacteriaceae* strains with broad-spectrum beta-lactamases in clinical isolates from inpatients, further emphasizes to equipping laboratories with molecular diagnostic techniques to investigate genetic resistance and the type of resistance. The outcome of the results could be implemented to treat patients effectively and to control the spread of resistance bacteria.

The consumption of Carbapenem family drugs is high in Iranian hospitals which are used as a first line of treatment without antibiotic susceptibility testing. Therefore, increase in antibiotic resistance to this family drugs is unavoidable in the near future. Therefore, it is necessary to take the necessary measures to modify the administration and use of antibiotics.

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