Original Research Article

Frequency of AmpC β-lactamase Resistance in *Escherichia coli* Isolates from Urinary Tract Infections in Gorgan, Iran

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ABSTRACT

Introduction: AmpC β-lactamases are among the most important cephalosporinases. Production of AmpC β-lactamases in microorganisms is often associated with multidrug resistance and limited treatment options. This study aimed to determine antimicrobial susceptibility pattern and frequency of AmpC β-lactamase genes in *Escherichia coli* strains isolated from urinary tract infections. **Materials and Methods:** The study included 154 *E. coli* isolates from urine samples of patients in medical centers of Gorgan, Iran. Cefoxitin-resistance was evaluated by the Kirby-Bauer method. Cefoxitin-resistant strains were evaluated using cefoxitin disks alone and combined with boronic acid to confirm production of AmpC β-lactamase. Presence of *MOX* gene was investigated by polymerase chain reaction. **Results:** Among the 154 *E. coli* isolates, 37 (24%) were cefoxitin-resistant, 16 of which were found to be AmpC β-lactamase-positive in the phenotypic test. In addition, the *MOX* gene was found in two isolates. **Conclusions:** The frequency of AmpC β-lactamase-producing *E. coli* and *MOX* gene in Gorgan is close to the regional and national average.

KEYWORDS: *Escherichia coli*, urinary tract infection, AmpC β-lactamase

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INTRODUCTION

Urinary tract infection (UTI) is one of the most common bacterial infections in humans, which can cause morbidity and mortality. Uropathogenic *Escherichia coli* is the causative agent for 90% of community-acquired UTIs and more than 50% of hospital-acquired UTIs [1]. β-lactam antibiotics are commonly used in the treatment of hospital-acquired UTIs. Widespread use of these antibiotics has led to increased prevalence of resistance among these bacteria. Hydrolysis of β-lactams by β-lactamases is the most common mechanism of resistance in Gram-negative bacteria. So far, more than 200 types of β-lactamase enzymes have been identified [2, 3]. According to the Ambler classification, β-lactamases are divided into four main groups (A-D) based on their amino acid sequence. In the Bush-Jacoby classification, the enzymes are divided into three groups based on their functional properties. Cephalosporins, penicillin and monobactam, and carbapenem are substrates for β-lactamases group 1, 2 and 3, respectively [4]. AmpC β-lactamases are among the most important cephalosporinases, which can cause resistance to several antibiotics such as penicillin, amoxicillin, broad-spectrum cephalosporins and carbapenem [5,6]. Plasmid AmpC β-lactamases have arisen through the transfer of chromosomal genes to plasmid, and are divided into six families (EBC, FOX, CIT, ACC, DHA, MOX) [4, 5, 7]. Plasmid AmpC β-lactamases have been studied by several phenotypic methods such as AmpC disc test [5]. However, genotypic methods such as molecular detection of resistance genes could also be useful. AmpC β-lactamase production in microorganisms

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is often associated with multidrug resistance and limited treatment options. Due to excessive consumption of β-lactam antibiotics in Iran, especially cephalosporins, and lack of enough studies on the prevalence of resistance against these antibiotics, the present study was aimed to evaluate the prevalence of AmpC β-lactamase in *E. coli* isolates from urinary tract infections using both phenotypic and genotypic methods.

MATERIALS AND METHODS

Bacterial isolates

This descriptive study was performed on 154 *E. coli* isolates from urine samples of patients referred to medical centers in Gorgan between 2012 and 2013. The isolates were identified by culture on blood agar, MacConkey agar and eosin methylene blue agar. Biochemical tests such as simon citrate, TSI, MR, VP and SIM were also used in the study [8].

Phenotypic detection of AmpC β-lactamase-producing strains

Antimicrobial susceptibility test was performed by disk diffusion method according to the CLSI guidelines for primary screening of isolates producing AmpC β-lactamases. First, bacterial suspension (equal half of McFarland standard) was prepared. After placing a cefoxitin disk (30µg, ROSCO Co.) on the medium and incubation at 37 ^{o}C for 24hours, growth inhibition zone around the disk was measured. Phenotypic confirmatory combined-disk test (cefoxitin alone and combined with broronic acid) was performed for isolates resistant to cefoxitin. To prepare the disks, 120mg of phenylboronic acid were added to 3 ml of dimethyl sulfoxide. The mixture was diluted with an equal volume of distilled water, and 20ml of this solution were added to the cefoxitin disk. The disks were stored at room temperature for one hour. Boronic acid-cefoxitin and cefoxitin discs were placed on Mueller Hinton agar inoculated with bacteria. Diameter of inhibition zone around the disks were measured. According to the CLSI guidelines, difference in inhibition zones of cefoxitin and boronic-cefoxitin of >5 mm was considered as positive for AmpC β-lactamase production [9].

DNA extraction

DNA was extracted using the phenol-chloroform method. Bacteria were cultured in TSB and then incubated at 37°C in a shaking incubator for 24 hours. Later, 2 ml of the bacterial suspension were centrifuged at 7000 rpm for 10 minutes. Sediment was dissolved in a solution of 600 ml lysis buffer, 20 µl 25% SDS and 3 ml proteinase-K. Microtubes were incubated in water bath at 60°C for one hour. Then, 620 ml of the phenol-chloroform mixture and isoamyl alcohol were added to the cell lysate. This suspension was centrifuged at 10000 rpm for 10 min at 4°C. Supernatant was transferred to a 2 ml microtube and mixed with 620ml of the phenol-chloroform mixture and isoamyl alcohol. The suspension was centrifuged at 10000 rpm for 10 min at 4 °C. Supernatant was transferred to a 2 ml microtube and then slowly mixed with 1.5 ml cold ethanol. After centrifugation at 9000 rpm for 10 minutes, supernatant was removed. DNA precipitated in the microtube was air-dried at room temperature and then dissolved in 50-200ml of TE buffer containing RNAase [10].

Detection of MOX gene using polymerase chain reaction (PCR)

PCR was performed using a 25µl reaction solution containing 3 µl of DNA template, 1 µl of each primer, 0.5 µl of dNTP (10mM), 0.8 µl of $MgCl_{۲}$ (25 mM), 2.5 µl of buffer (10X), 0.2 µl of Taq polymerase (5U/µl) and 16 µl of distilled water. A strain containing the *MOX* gene was used as positive control

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and sterile water was used as negative control. Nucleotide sequence of the primers used is shown in table 1 [10,11]. PCR products were electrophoresed on 1.5% agarose gel. The resulting bands were visualized in a gel documentation system.

Table 1. Sequence of specific primers used in the study

Gene	Oligonucleotide sequence (5'3')	Nucleotide position	Product size (bp)
Mox	5'- GCT GCT CAA GGA GCA CAG GAT-3'	358–378	520
	5'- CAC ATT GAC ATA GGT GTG GTG C -3'		

Data collected were analyzed in SPSS software (version 16). The data were reported as frequency and percentage. P-values less than 0.05 were considered as statistically significant.

RESULTS

Of the 154 isolates, 35 (22.7%) isolates were from men and 50(32.5%) isolates were from inpatients. In addition, 52(33.8%) isolates were from patients with history of catheterization. Moreover, 63(40.9%) isolates were extended spectrum β-lactamase-positive. Furthermore, 18(11.7%), 13 (8.4%), 53 (34.4%), 40 (26%) and 30 (19.5%) isolates were collected from children, adolescents, young, middle-aged and elderly patients, respectively. In addition, 37 (24%) isolates were resistant to cefoxitin and therefore were used in the phenotypic confirmatory test (Figure 1).

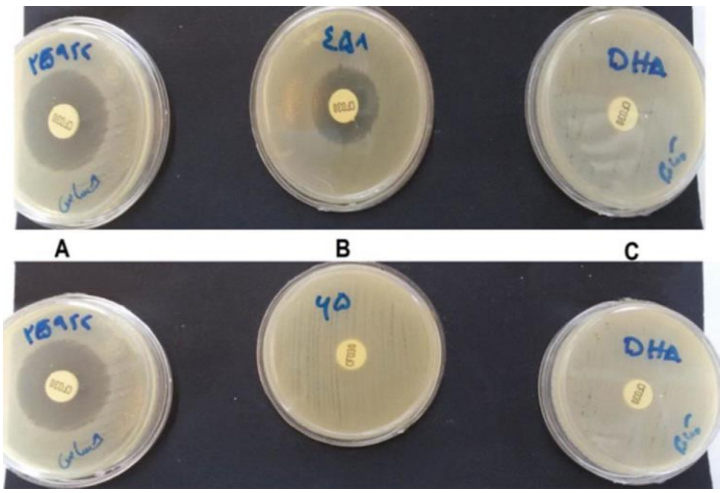

Figure 1. Results of the antibiogram test with cefoxitin (A = *E. coli* ATCC 25922 strain susceptible to cefoxitin, B = sample, C = control strain resistant to cefoxitin)

In the phenotypic confirmatory combined-disk test, 4.1% of all isolates and 43.24% of isolates resistant to cefoxitin were positive for AmpC β-lactamase production (Figure2).

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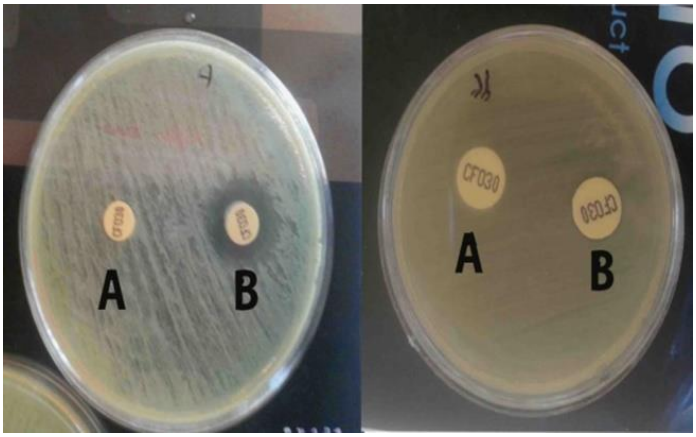

Figure 2. Results of the phenotypic confirmatory combined-disk test (A = cefoxitin, B = cefoxitin combined with boronic acid)

The frequency of Ampc β-lactamase resistance in E. coli was significantly associated with some of the demographic features like male gender (p =0/034), history of Catheterization (p =0/010), dysuria (p =0/032) and admission (p =0/032) also it was significantly associated with previous use of antibiotics (p = <0/001) and resistance to gentamicin (p =0/028), ciprofloxacin (p =0/011), aztreonam (p =0/005), cefepime (p =0/008) and levofloxacin (p =0/017).

Frequency of the *MOX* gene was evaluated by PCR and the results showed that only two (1.3%) isolates contained the gene (Figure 3).

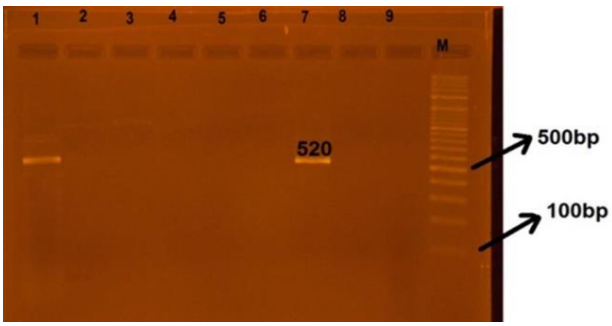

Figure 3. PCR product gel electrophoresis for detection of the *MOX* gene (M = marker, 1 = positive control, 2 = negative control, 3-6 and 8, 9 = negative samples, 7 = positive sample)

Characterization of the isolates containing the MOX gene

The two samples that contained the *MOX* gene were isolated from Persian, married subjects living in urban areas. In addition, both isolates were resistant to nalidixic acid, clindamycin, ciprofloxacin and levofloxacin but susceptible to gentamicin, imipenem and tazobactam.

DISCUSSION

β-lactamase production is an important mechanism for antibiotic resistance, that include Ampc, β-lactamases enzymes and carbapenems [12].in this study, AmpC β-lactamase resistance and MOX gene was studied. Using combination of Cefoxitin disk and phenyl Boronic acid, AmpC β-lactamase producing isolates were detected. This method is simple and furthermore its results is in accordance of the of the PCR test results. [13]. Similar findings was reported in study published by Coudron and colleagues [9].

Based on the results of our study, the number of 16 isolates (4/10% of total isolates and 43/24% of cefoxitin resistant strains) were considered as positive for AmpC β-lactamase. Different reports are available for the Ampc beta Lactamase resistance statistics in Escherichia coli in

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Iran. For other regions of Iran, Zahedan [14] and Tehran [15] that the percentage of Ampc beta lactamase resistance in Escherichia Coli were 1/3% and 5/7% respectively. More Similar to our findings, Soltan Dallal and colleagues found that/10/ 2 % are positive for AmpC β-lactamases [3]. However, Shahla Mansouri and colleagues showed more Ampc β-lactamase resistance in Kerman (39/3 %) [16]. According to the available reports, Ampc beta lactamase resistance in E. coli in Gorgan is in moderate of country. Also for the other parts of the world, various statistics is reported for the Ampc of beta lactamase resistance in E. coli. Ding and colleagues reported 3/7% in China [17], Reuland and colleagues showed 6/5% in Netherland [18] and research performed by Polsfuss and colleagues in Switzerland revealed 2/3% resistance [19]. Different factors can cause less resistance like higher health and appropriate use of antibiotics in these areas is of the world. Same to our results, Barua et al. used various diagnostic methods to detect resistance including three dimensional test (11/6 %), Ampc disc (11/6 %), Ampc disc with Tris EDTA (7/6 %) and a cefoxitin-boronic combined disc (11/6%) [20]. In study of Helmy et al. Prevalence of Ampc β-lactamase resistance in Egypt using Ampc disc, combined with cloxacillin and combined disks with Boronic was15/6 %,15/6 % and 13/7%, respectively [21]. Shafiq in Pakistan reported 7/97 % of Escherichia coli and 12/37% of K. pneumoniae strains have AmpC enzymes [22]. KAUR studied the resistance with the combined disc and revealed 10/5% was Ampc positive [23]. Sen and colleagues using cefoxitin-Boronic acid, ceftazidime and cefotaxime acid - Boronic acid reported 23/7 %, 47/5 % and 56/3 % were positive respectively [24]. Different statistics with higher rate of resistance were also reported by Tan (49/8 %) from Singapore [25] and by Parveen (63/6 %) in India [5] using Amp-C three dimensional test which could be due to inappropriate use of antibiotics. Altogether, it can be concluded that the variation reported for the Ampc β-lactamase resistance worldwide may be for reasons such as variation in location, time of the study, different used methods, different population characteristics such as hospitalization history and period, age and sex of the patients. We found some of the characteristics of population including gender is significantly associated with Ampc beta lactamase resistance (p=0/034). The rate of resistance was higher in men. However, due to anatomical features of men, usually urinary tract infection in men is less, but this increased level of resistance in men may be due to uncontrolled antibiotic use. Similar results were also reported i[26]. The rate of resistance was higher in patients with history of catheterization (p =0/010) which may be related to inoculation of bacteria into the urinary tract upon catheterization. We found that the rate of resistance was higher in those with a history of hospitalization (p = 0/032) that was in accordance to findings of Manchanda et al [27]. On the other hand the conflict with invasive diagnostic and therapeutic tools may escalate the bacterial colonization with urinary tract. The resistance was also higher in patients with dysuria (p =0/032) that can cause faster disease spread faster. Among the patterns of antibiotic resistance, the Ampc beta lactamase resistance in patients with a history of antibiotic use was more. It can be explained by the effect of taking antibiotics in flora microbiome change in terms of strengthening and selecting the resistant bacteria. The Ampc beta lactamase resistance was more in resistant strains to antibiotics including gentamicin, ciprofloxacin, aztreonam cefepime, and

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levofloxacin since most of the antibiotics have the same mechanism of action. Along with phenotypic methods using molecular detection Ampc beta lactamase resistance is also very necessary and useful and can be showed genetic patterns of this type of resistance in each region. In our study, based on the results of PCR, two isolates (3.1%) were positive for the presence of *MOX* gene. The two isolates both were taken from two married Fars individuals, living in the city, with dysuria and history of hospitalization, resistant to nalidixic acid, clindamycin, levofloxacin and ciprofloxacin and sensitive to gentamicin, imipenem and tazobactam. Researches on the beta lactamase resistance genes in E. coli in Iran are limited and there are no enough statistical reports available in this field. Therefore it indicates that there is a need to conduct such studies. In a study Soltan Dallal at Tehran reported that 38/5 % of the positive Ampc strains, have Ampc beta lactamase resistance genes, including genes of CMY and DHA but no positive strains for *MOX* gene was detected [3]. In different parts of the world also various frequencies of this gene has been reported. In a study in Egypt Helmy et al, reported 22/5 % were positive by PCR and *MOX* gene was found in 2/9 % of the samples; that same to our findings [21]. Reuland in the Netherlands reported fewer values, with only 2/6 % in Enterobacteriaceae samples detected positive by PCR, with *CMY, DHA, ACC* genes [18]. In contrast, Parveen and colleagues in India reported higher percentages with ٤٥/٤ % of eligible resistance with *DHA, CIT, MOX* and *ACC* genes, respectively, in 18/1%,18/1 %, 8/3 % and 0/7 % of samples, however, family of the *FOX, EBC* genes were not in any of the samples [5]. In another study Hussain in Pakistan from the 48 samples that were positive by phenotypic methods of Ampc betalactamase, PCR method revealed that *CIT* and *MOX* genes in 16samples were the most frequent resistant gene, *CMY* and *EBC* in seven and *FOX* in two samples were detected [7].Wassef study in Egypt, from 62 isolates positive for Ampc phenotype, 16 (25/8%) isolates were Ampc lactamase gene positive, the highest frequency was reported for *FOX, MOX* and *EBC* genes [28]. Shanthi and colleagues in a study in India, the number of 16 Ampc and MBL enzymes producing strains were isolated, meanwhile25% of the samples contained resistance genes including *MOX, FOX, ACT*[29].

CONCLUSIONS

Our results showed that the frequency of Ampc β-lactamase and *MOX* gene were at the average of the country and regional level. There was significant association between Ampc β-lactamase resistance with features such as gender, history of catheterization, hospitalization, dysuria, previous use of antibiotics, and resistance to antibiotics gentamicin, ciprofloxacin, aztreonam, cefepime and levofloxacin. Broad-spectrum of cephalosporin can be used for strains resistant to cefoxitin and non Ampc, non-extended spectrum β-lactamases-positive strains and carbapenems could be used for cefoxitin resistant and Ampc Positive strains. Because of the limitations of this study, we only investigated one genetic group responsible for this type of resistance; however it is necessary to check other resistance gene groups.

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