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Multiplex PCR Study of Plasmid-Mediated *AmpC* Beta-Lactamase Genes in Clinical Isolates of *Escherichia coli*

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ABSTRACT

***Background*:** AmpC β-lactamases are important cephalosporinases chromosomally encoded in many of *Enterobacteriaceae* and a few other organisms where they mediate resistance to cephalothin, cefazolin, cefoxitin and penicillins. The six different families of plasmid-mediated AmpC β-lactamases have been described, but no phenotypic test can discriminate among them. AmpC multiplex PCR has been successfully used to discriminate plasmid-mediated ampC specific families in organisms such as *Klebsiella pneumonia* and *Escherichia coli*. The aim of this study was to indicate the prevalence of AmpC β-lactamase genes by specifically designed primers through PCR test.

***Methods*:** 243 total clinical urine samples were collected, and 227 isolates were identified as *Escherichia coli* based on standard biochemical tests. Subsequently, the isolates were screened by disc diffusion and combined disc test for β-lactamase production. Resistant isolates were evaluated by PCR for ampC family determination.

***Results*:** Antibiotic resistance pattern were observed as follows: cefepime (30%), ceftazidime (36%), ceftriaxone (42%), cefotaxime (47%). The ratio of isolates was detected as ESBLs and AmpC producers were 34% and 5.2%, respectively. PCR performed on 12 selected isolates via phenotypic tests and the results revealed that among 12 isolates, 11 contained bla_{CMY-42}.

***Conclusion*:** Unfortunately, antibiotic resistance has become an increasingly critical problem in many countries like Iran and occurrence of isolates co-expressing AmpC-β-lactamases and ESBLs can create serious problems in the future. As antibiotic options in the treatment of AmpC β-lactamases and ESBLs producing organisms are extremely limited, molecular screening by laboratories is suggested to reduce the risk of therapeutic defeat.

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Introduction

The most common mechanism of resistance to beta-lactam antibiotics in clinically significant Gram negative bacteria is hydrolysis of this family of antibiotics by β-lactamases (1). A major public health problem is infection with resistant bacteria. The spread of resistance to beta lactam antibiotics in Gram negative organisms, in particular *Escherichia coli*, regularly results from the production of β-lactamase enzymes which are able to hydrolyze β-lactam ring (2). AmpC β-lactamases are typically encoded on the chromosomes of many of the *Enterobacteriaceae* and a few other organisms. AmpC enzymes belong to class C according to the Ambler structural classification of β-lactamases, while in the functional classification scheme of Bush et al., they were related to the group 1 (3). AmpC β-lactamases genes may also be carried in plasmids which show a new threat of spread to other organisms within a hospital or geographic district (2). Plasmid-encoded *ampC* genes have been distinguished since 1989 (3). Most acquired *ampC* genes are originated from chromosomal genes, which are mobilized by plasmids that has resulted in their wide spread (4) but are less common than extended-spectrum β-lactamases (ESBLs).The CMY-type enzymes are the most common reported plasmid mediated AmpC β-lactamases (3). The inducible chromosomal *AmpC* genes were discovered on plasmids of *Klebsiella* spp., *Escherichia coli*, and *Salmonella* spp. In *E. coli* AmpC is rarely expressed, while in *Klebsiella* and *Salmonella* species the *AmpC* gene is missing from the chromosome and found on the plasmids (5). As there are multiple β-lactamases within one organism phenotypic recognition of the β-lactamases is difficult. Many phenotypic tests can recognize these two resistance mechanisms but are not able to discriminate the different family types of plasmid-mediated AmpC β-lactamases (6). They can be discriminated from extended-spectrum β- lactamases by their ability to hydrolyze cephamycins and not inhibited by clavulanic acid (7). Multiplex PCR was used to discriminate the six plasmid-mediated AmpC specific families (MOX, CIT, DHA, EBC, FOX and ACC-1) in microorganisms (2).

Unfortunately, reliable data from most of the countries on ESBL/AmpC prevalence that could give more information about the spread of these isolates are still lacking. As far as the current literature is concerned, this is the first study that demonstrated the presence of the AmpC β-lactamases in clinical *E. coli* isolates in the immigration friendly city of Karaj, Iran. This knowledge may help physicians to prescribe more effective antibiotics and prevent the spread of antibiotics resistance among clinically important strains within the province.

Materials and methods

Bacterial isolates

A total of 227 non-duplicate *E. coli* isolates were collected from some hospitals and private clinical laboratories of Alborz province Karaj city, from May to July 2015. Isolates were identified as *E. coli* based on clinical laboratory standards institute document M100-S22.

Antimicrobial Susceptibility assay

The antibiotic susceptibility test was done by Kirby Bauer disc diffusion method on Mueller-Hinton agar according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). The inhibition zone of each isolate was tested on Muller–Hinton agar medium with commercial antimicrobial discs purchased from Padtan Teb Co., Tehran, Iran. The antibiotic discs utilized in this study were cefepime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg) and cefotaxime (30 µg). After incubation for 18-24h at 37 °C, the inhibition zone around each disc was recorded and weighed with standards of CLSI; the results were illustrated as either resistant, intermediate or sensitive. *E. coli* ATCC 25922 was used as the reference strain for antibiotic susceptibility tests.

Detection of ESBLs and AmpC β-lactamases

Escherichia coli isolates showing resistance to third generation cephalosporins, were screened to detect ESBL and AmpC producers. ESBL production was initially evaluated with the CLSI confirmatory test using both cefotaxime (30 mg) and ceftazidime (30 mg) discs alone and in combination with clavulanic acid (CA) (10 mg). The test organisms were considered to produce ESBL if the growth-inhibitory zone around either the cefotaxime (CTX) or the ceftazidime (CAZ) disc with CA was 5 mm or greater of the diameter around the disc containing CTX or CAZ alone (7). Non-ESBL producer isolates according to the above test were considered as presumptive AmpC producers.

Molecular Detection of AmpC

As phenotypic tests cannot distinguish among the different families of plasmid-mediated AmpC enzymes and may also overlook chromosomally determined AmpC β-lactamases with an extended spectrum, and also as the current "gold standard" for plasmid-mediated AmpC β-lactamase detection, multiplex PCR has been developed by utilizing six primer pairs (3).

For DNA preparation, a single colony of each organism was inoculated from a blood agar plate into 100 µl of distilled water. The cells were lysed by heating at 95 ºC for 10 minutes and cellular debris was separated by centrifugation at 14000 g for 5 minutes. The supernatant, 1 µl of the total sample, was used as the source of template DNA for Multiplex PCR (8). A PCR amplification reaction was performed in 25 µl total volume containing, 12.5 µl ready to use 2x PCR master mix 1.5 µl Mix Forward primers (MOXMF, DHAMF, CITMF, ACCMF, EBCMF, FOXMF), 1.5 µl Mix Reverse primers (MOXMR, DHAMR, CITMR, ACCMR, EBCMR, FOXMR) and 1 µl template DNA and 8.5 ml distilled water. The sequence of the primers used for PCR amplification are listed in table 1 (6). The PCR program were as follows: initial denaturation at 95 ºC for 5 minutes, followed by 30 cycle of DNA denaturation at 95 ºC for 30s , primer annealing at 62 ºC for 30s, primer extension at 72 ºC for 1 minutes, and a final extension step at 72 ºC for 7 minutes. Six-microliter aliquots of PCR product were analyzed by agarose gel electrophoresis. Gels were stained with DNA safe stain and visualized by UV trans-illumination. A 100 bp DNA ladder from CinnaGen (Tehran, Iran) was used as a size marker. Negative controls (PCR mixtures containing water in place of template DNA) were also used in each run.

Sequence analysis of a CIT-like PCR amplicon

To identify the *AmpC* β-lactamase genes type, DNA sequence analyses of the amplicons were performed (Bioneer, Seoul, Korea). The primers used for sequencing were the same as those used to generate the amplicon. Each sequence was then compared with known β-lactamase gene sequences of the GenBank database using the Basic Local Alignment Search Tool (9).

Results

A total of 227 clinical isolates of *Escherichia coli* were isolated from four hospital and three private clinical laboratories of Karaj city. Isolates were identified as *E. coli* based on standard biochemical tests. Total isolates were subjected to antibiotic susceptibility test by Kirby-Bauer disc diffusion method. Up to 39% of the isolates exhibited a multidrug-resistance (MDR) phenotype.

Phenotypic Identification of ESBLs and AmpC

ESBLs and AmpC screening of strains by phenotypic combined disc test showed that out of 227 isolates, 12 (5.2 %) and 77 (34%) of *Escherichia coli* isolates were AmpC and ESBLs

Table 1. Primers used for Multiplex PCR.

Primer	Sequence (5´ to 3´)	Target (s)	Expected amplicon size (bp)
MOXMF	GCT GCT CAA GGA GCA CAG GAT	MOX-1 , MOX-2 , CMY-1 CMY-8 to CMY-11	520
MOXMR	CAC ATT GAC ATA GGT GTG GTG C	MOX-1 , MOX-2 ,CMY-1 CMY-8 To CMY-11	
CITMF	TGG CCA GAA CTG ACA GGC AAA	LAT-1 to LAT-4 CMY-2 to CMY-7- BIL-1	462
CITMR	TTT CTC CTG AAC GTG GCT GGC	LAT-1 to LAT-4 CMY-2 to CMY-7- BIL-1	
DHAMF	AAC TTT CAC AGG TGT GCT GGG T	DHA-1 , DHA-2	405
DHAMR	CCG TAC GCA TAC TGG CTT TGC	DHA-1 , DHA-2	
ACCMF	AAC AGC CTC AGC AGC CGG TTA	ACC	346
ACCMR	TTC GCC GCA ATC ATC CCT AGC	ACC	
EBCMF	TCG GTA AAG CCG ATG TTG CGG	MIR-1T , ACT-1	302
EBCMR	CTT CCA CTG CGG CTG CCA GTT	MIR-1T , ACT-1	
FOXMF	AAC ATG GGG TAT CAG GGA GAT G	FOX-1 to FOX-5b	190
FOXMR	CAA AGC GCG TAA CCG GAT TGG	FOX-1 to FOX-5b	

Table 2. Percentage of antimicrobial resistance of *AmpC* positive *Escherichia coli* isolates.

Antibiotics	No. of sensitive strain (%)	No. of Intermediate strain (%)	No. of resistant strain (%)
Cefepime	3 (25)	2 (16.67)	7 (58.33)
Ceftazidime	0	0	12 (100)
Ceftriaxone	0	0	12 (100)
Cefotaxime	0	0	12 (100)

Table 3. Percentage of antimicrobial resistance of ESBL positive *Escherichia coli* isolates.

Antibiotic	No. of sensitive strain (%)	No. of Intermediate strain (%)	No. of resistant strain (%)
Cefepime	7 (9)	18 (23)	52 (68)
Ceftazidime	12 (15)	5 (6)	60 (79)
Ceftriaxone	0	0	77 (100)
Cefotaxime	0	0	77 (100)

Table 4. Most common antibiotic resistance patterns of MDR, ESBL and *AmpC* positive *Escherichia coli* isolates.

Most common antibiotic resistance patterns of MDR *E. coli* isolates	
Pattern of resistance	No. of resistant strains (%)
FEP-CTX-CRO-CAZ	57 (64%)
FEP-CTX-CRO	2 (2%)
CTX-CRO	15 (17%)
CTX-CRO-CAZ	15 (17%)
Total	89 (100%)
Most common antibiotic resistance patterns of ESBL positive *E. coli* isolates	
FEP-CTX-CRO-CAZ	50 (65%)
FEP-CTX-CRO	2 (3%)
CTX-CRO	15 (19%)
CTX-CRO-CAZ	10 (13%)
Total	77 (100%)
Most common antibiotic resistance patterns of AmpC positive *E. coli* isolates	
FEP-CTX-CRO-CAZ	7 (58%)
FEP-CTX-CRO	0 (0%)
CTX-CRO	0 (0%)
CTX-CRO-CAZ	5 (42%)
Total	12 (100%)

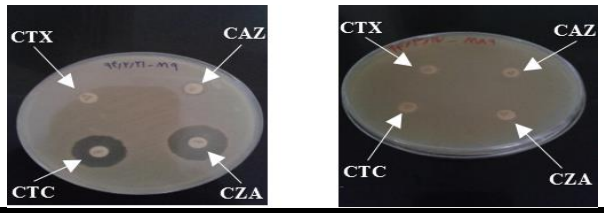

Figure 1. Screening and phenotypic identification of ESBLs and AmpC Right figure: Positive AmpC phenotypic test Left figure: Positive ESBL phenotypic test CTX: cefotaxime, CAZ: ceftazidime, CTC: (clavulanic acid + cefotaxime), CZA: (clavulanic acid + ceftazidime).

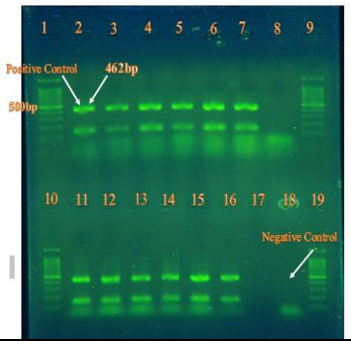

Figure 2. PCR Amplification of *bla*$_{CITM}$ Lanes 1, 9, 10 and 19: 100 bp DNA marker. Lane 2: CITM from the positive control. Lanes 3-7 and 11-16: Clinical isolates harboring *bla*$_{CITM}$. Lane 8: Clinical isolate without *bla*$_{CITM}$. Lane 18: Negative control.

producers, respectively (Figure 1). Antibiotic resistance pattern were observed as follows: cefepime (30%), ceftazidime (36%), ceftriaxon (42%), cefotaxime (47%). Cefepime and ceftazidime were the most effective drugs in vitro. Tables 2 and 3 indicate percentage of antimicrobial resistance of *AmpC* positive and ESBL positive *E. coli* isolates, respectively. Table 4 shows the most commonly identified combinations of antimicrobial agents in multidrug-resistant, ESBL positive and *AmpC* positive isolates of *E. coli*.

Multiplex PCR

Multiplex PCR was performed on all 12 resistant isolates. Presence of bla_{CIT} gene was confirmed in 11 isolates and other *AmpC* gene family have not found in any isolate. CITM sequence analyses also showed high similarity to the submitted sequences related to these genes (bla_{CYM-42}) in GenBank (Figure 2). CITM sequence obtained in this study was submitted to GenBank under accession number KU211646.

Discussion

Due to the appearance of bacterial strains resistant to third generation cephlosporins, since 1993 cephalosporins of 4th generation have been developed for therapy among them. Cefepime is the most active 4th generation cephalosporin and is active against bacteria producing chromosomally and plasmid-mediated extended broad-spectrum β-lactamase enzymes (10). Resistance to third and fourth generation cephalosporins is increasing in human-infecting bacteria very quickly and one of the major causes of this resistance is antibiotic misuse (11). Resistance mechanisms to cefepime involve the production of extended-spectrum β-lactamases and metalloenzymes, which hydrolyze cefepime (10). These enzymes can spread within the community and in hospitals (10). Therefore, cefepime resistant pathogens are a major threat to infection treatment and control programs.

In this study more than 35% of the screened *E. coli* isolates were resistant to third generation-cephalosporine, the rate of resistance to third generation- cephalosporine in the study of Wassef et al. was 74.9% which is considerably higher than our results. In our study resistance to cefepime, fourth generation, was 30%. This finding is comparable to those of Mansouri et al. who have reported 28.6% resistant to cefepime, 73 resistant isolate out of the 255 (10). Resistance to third and fourth generation- cephalosprins among AmpC producer *E. coli* isolates were 100% and 58.33% respectively. Eleven (4.8 %) of *E. coli* isolates produced AmpC as detected by the phenotypic methods and/or the PCR (after excluding one *E. coli* isolate for being positive for AmpC by phenotypic method and negative by the PCR) and 77 (34%) of them were ESBL positive that is almost the same as in our previous study (12). ESBL and AmpC producing organisms are clinically relevant and remain an important cause for failure of therapy with caphalosporins.

This is the first time, to our knowledge, that AmpC-producing *E. coli* isolates have been detected in the immigration friendly city of Karaj. As mentioned above, in this study, among 227 screened *E. coli* isolates 12 isolates (5.2%) were found to be *AmpC* positive by phenotypic test. This finding is comparable to those of Wassef et al. who have reported 5.8% AmpC producing isolates among all identified Gram negative bacilli by screening disc diffusion methods (2).

Currently, there are no CLSI-recommended tests that actually detect AmpC –β-lactamases. Reducing the spread of plasmid-mediated AmpC resistance in hospitals and control the movement of this resistance mechanism require the identification of genes involved (2). In this study, among 12 positive screens for AmpC in confirmatory test, the result of multiplex PCR was 91% positive.

Extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases have been extended worldwide, failure to detect these enzymes has contributed to their uncontrolled spread and

sometimes to therapeutic failures (13). In many clinical centers, there is no attention to the identification of AmpC β-lactamases based on phenotypic or genetic screening tests. Based on data of this study, we suggest that laboratories are required to improve their reorganization methods for complete detection of AmpC and ESBLs producer organisms.

Conclusion

As ESBL-producing organisms exhibit co-resistance to many classes of antibiotics, especially the fluoroquinolones (12), and AmpC producing organisms are generally resistant to broad-spectrum penicillins, oxyimino- and 7-α-methoxy-cephalosporins, and aztreonam (14), then antibiotic options in the treatment of ESBL and ampC producing organisms are extremely limited (15) and occurrence of isolates co-expressing AmpC-β-lactamases and ESBLs can create serious problems in future. Failure to treatment of ESBL and AmpC producing organisms is largely due to the lack of clinical awareness; thus, development of diagnosis methods for complete detection of β-lactamase enzymes is important for resistance control and the treatment with high achievement.

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Conflict of interest

None declared conflicts of interest.

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