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Assessment of *AmpC* Beta-Lactamase Genes among Clinical *Escherichia coli* Isolates

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ABSTRACT

***Background*:** *AmpC* beta lactamases play a significant role in creating resistance to third generation cephalosporins worldwide. They mostly express on chromosome of Enterobacteriaceae especially Escherichia coli and cause consequential problem in clinical treatment and lead to failure in diagnosis and phenotypic test recommended by Clinical and Laboratory Standards Institute.

***Methods*:** Totally 200 *E. coli* isolates from different hospitals of Tehran were collected. The isolates were screened by disk diffusion method according to the CLSI guidelines. The profiles and prevalence surveys of *AmpC* (*Dha*, *CITM*, *Mox* and *FOX*-type) β-lactamase genes in clinical isolates of *E. coli* by phenotypic and molecular methods.

***Results*:** Out of 200 *E. coli* isolated, 115 (89.8%) and 13 (10.2%) isolates were identified as ESBL- and AmpC- beta-lactamase producers, respectively. Among AmpC producers, 13 (100%) and 5 (38.5%) isolates was reported by PCR assay as *blaCITM* and *Dha*, respectively. *Mox* and *FOX* genes were not detected in any sample.

***Conclusion*:** Our results highlight the importance of using molecular detection methods to identify β-lactamase-producer that have resistance to antibiotics.

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Introduction

Following the using of antibiotics, resistance against them is appearing, growing and change to the main public health now days. Bacteria like a thinker organism find some way to change or destroy the chemical structure of antibiotics. So, they survive and start to reduces or ultimately inactive the antibiotics, it is cause of resistant phenotypes in bacteria (1, 2). In recent years, the production of *AmpC* β-lactamase via transferring on chromosome and plasmid among extended spectrum β-lactamases ESBLs (extended spectrum beta lactamases) has greatly increased. Their genes that have more activity compared with the initial generation of these resistance genes (TEM-1, TEM-2, and SHV-1). The new generation of genes encodes enzymes that are generally referred to ESBLs (3, 4). So, gram negative bacteria can acquire to newest resistance to beta lactam antibiotic. β-lactamases have beta lactamase enzymes that hydrolyze beta lactam rings and lead to the inactivation of beta lactam antibiotics (2). Currently, the increasing use of antimicrobial drugs, such as cephalosporin antibiotics, is resulting in the appearance of this group. Recent research has shown that one of the main mechanisms of antibiotic resistance among the Enterobacteriaceae family. It is the production of *AmpC* β-lactamase, which becomes a clinical concern (5). The ESBL phenomenon was initially reported in Europe in early 1983, but the phenotype was also quickly identified in America and Asia (6). Although it pass more than a decade since the discovery of beta-lactamases, but clinical significance of these enzymes have not understood. So, detection of them is needed to daily testing in clinical laboratories (7). According to recommendations from the Clinical and Laboratory Standards Institute (CLSI), isolates with reduced susceptibility to β-lactam antibiotics, especially third generation cephalosporin, are potentially ESBL-producers; this prediction is confirmed by using clavulanate in phenotypic tests (8). However, over the past decade, the appearance of *AmpC* β-lactamases among pathogens has led to a problem for ESBL diagnosis via the disk diffusion method because this enzyme is resistant to clavulanic acid (9, 10). The *AmpC* gene is chromosomally available in some bacteria, such as *Enterobacter cloacae*, *Citrobacter reundii*, *Morganella morganii* and *Hafnia alvei*. It express at the low levels in E. coli but can transfer by plasmid to this pathogens and induce resistance like other organism (11, 12). *AmpC* β-lactamases with this capability spread ESBL-producers, so make potent resistant against antimicrobial drug (9, 12). Therefore, this study focused on reporting the prevalence of *AmpC* beta-lactamases, such as *Dha*, *CITM*, *Fox* and *Mox*, and their detection by molecular methods (namely, PCR) in clinical isolates of *E. coli*.

Materials and Methods

Bacterial strains

During this study, more than 500 clinical samples, including urine, stool, blood, wound and other samples were collected from hospitals in Tehran. Based on IMVIC standard biochemical tests, 200 isolates of *E. coli* were identified from these clinical samples. Following their identification, all isolates were stored in skim milk at -70 °C prior to testing.

Screening and phenotypic identification of ESBLs and AmpC

The isolates identified as *E. coli* were examined by the disk diffusion method according to the CLSI guidelines for initial screening of ESBL production. In this method, the following antibiotics were used: cefotaxime (30 µg), ceftazidime (30 µg), gentamicin (10 µg), amoxicillin (30 µg), imipenem (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), cotrimoxazole (1.25 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), and cefoxitin (30 µg) (Mast Diagnostics Ltd.) (7, 12). Isolates of *E.*

coli with reduced susceptibilities to cefotaxime (zone diameter ≥ 23 mm) and/or ceftazidime (zone diameter ≥ 18 mm) were selected for confirmatory tests with the combined disk method. For the combined disk method assay, the following antibiotic concentrations were used: ceftazidime, 30 µg; ceftazidime/ clavulanate, 30/10 µg; cefotaxime, 30 µg; and cefotaxime/clavulanate, 30/10 µg (Mast Diagnostics Ltd). Following incubation of plates at 37 °C for 24 hours, ESBL production was confirmed by an increase of at least five mm in the zone of inhibition in the presence of clavulanic acid compared with inhibition zones in the absence of clavulanic acid (14). Isolates which their effect β-lactamase were not inhibiting in confirmatory test by clavulanate were selected for *AmpC* producers, so did not display phenotype of ESBLs and also show resistance to cephamycin (15). Resistant isolates were evaluated by molecular assessment using PCR.

Polymerase chain reaction and sequencing of β-lactamase genes

The isolates tested as positive for *AmpC* were assessed for the presence of four subfamilies of *AmpC* beta lactamase genes (*Dha*, *CITM*, *Mox* and *FOX*). For this purpose, genomic DNA was extracted by Bioneer extraction kit according to the manufacturer's instructions.

PCR assays were performed to amplify *blaDha*, *CITM*, *Mox* and *FOX* by using universal primers (Table 1). Each PCR reaction contained the following reagents: 2.5 µl 10x Buffer, 2 µl MgCl2 (50 mM), 1 µl dNTP (10 mM), 1.5 µl of each primer (50 Pmol/µl), 1 µl Taq polymerase (5 U/µl), 2 µl template DNA (50 Pmol/µl) and 14.5 µl H2O in a final volume of 25 µl. The PCR program included the following steps: initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min and elongation at 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. Multiplex PCR for *blaDha* and *blaCITM* was also performed using these conditions. Subsequently, the amplicons were evaluated by electrophoresis on 0.8% agarose gels. Direct sequencing was performed on PCR products that corresponded to the expected amplification size of each cluster; these PCR products were extracted from gels using the Fermentas kit and then sent to Korea for sequencing. Sequences were compared with known sequences in the NCBI database with the MEGA 4 multiple-alignment program.

Primer Design

Submitted sequences is related to *Dha*, *CITM*, *Mox* and *FOX* genes of *E. coli* were 22, 24, 6 and 5 (respectively) in GenBank. The sequences of each cluster were aligned with the MEGA4 multiple-alignment program until analogous locals were identified. These locals were used for designing primers with Gene runner software. Subsequently, designed primers were tested on the submitted sequences by using BLAST. Then, four sets of universal primers were evaluated by using PCR.

Results

Two hundred clinical isolates of *E. coli* were collected during a period of six months. Isolates included 125 (62.5%) urine and/or urinary catheters, 48 (24%) stool, 18 (9%) blood, 5 (2.5%) wound sites and 4 (2%) were from other clinical samples. The resistance pattern of these 200 *E. coli* isolates to 11 antimicrobial agents are shown in Figure 1. Most of the isolates were highly resistant to oxyimino cephalosporins but remained they were susceptible to imipenem. Up to 70% of the isolates exhibited an MDR (Multi Drug Resistance) phenotype. Based on the disk diffusion method, 128 (64%) of these *E.coli* isolates were resistant to ceftazidime and cefotaxime; these isolates were selected as positive for possible ESBL production and were therefore assessed by the combined disk assay. In the combined disk assay, of the 128 screened isolates, 115 (89.8%) and 13 (10.2%) isolates

Table 1. Primers used for amplification.

Target(s)	Primer Sequence (5`to 3 ` as synthesized)		Expected Amplicon size (bp)
LAT-1 to LAT-4, CMY-2 to CMY-7 CMY-21,-22,-24,-28, -32,-33,-43,-44, BIL-1	CITMF	CAGCCTCTTTCTCCACATTTGC	575
	CITM R	CAGTTTTAATGGTTGCAGGACG	
FOX-1 to FOX-5b	FOXMF	TGAGCTTCCAGGCCAATCC	167
FOXMR		GATGGGATAGTTGCGATTGGC	
DHA-1, DHA-2	DhaMF	ATCGGCCTGTTTGGTGC	469
DhaMR		AGGAAGCACGGTTATACGG	
MOX-1, MOX-2, CMY-1,CMY-8 to CMY-11	MOXMF	CTATTCGAAAGAGGACAAGCC	218
MOXMR		AGGCGTAACTCTCCCAACC	

were identified as ESBL- and *AmpC*-producers, respectively.

β-lactamase producing *E. coli* isolates were more common in urinary samples (80%). PCR was performed on 13 resistant *AmpC*-producing isolates. Results indicated that of these isolates, 13 (100%) and 5 (38.5%) isolates had *blaCITM* and *Dha*, respectively. *Mox* and *FOX* genes were not detected in any sample (Figure 2). *CITM* and *Dha* sequences that were analyzed from our submitted samples showed up to 90% similarity to these genes in GenBank.

Discussion

Beta-lactamase producer are recognized as a resistance source to cephalosporins that is increasing in *Escherichia coli* in Iran. *AmpC* beta lactamases with inducible ability increase their genes in the vast numbered and spread quickly. We evaluated clinical *E.coli* isolates with reduced susceptibility to third-generation cephalosporins for their *AmpC* phenotype (16).

In this study, four groups of *AmpC* genes, namely, *DHA*, *MOX*, *CITM* and *FOX*-type, were analyzed. Thus the two hundred clinical *E. coli* isolates, 128 were resistant to ceftazidime and cefotaxime based on the disk diffusion assay. However, only 115 isolates from 128 were identified as ESBL-producers based on the results of the combined disk assay, which is the CLSI-recommended confirmatory method. Currently, reported some methods to detection of *AmpC* β-lactamases by researcher, but it is not establish guildline in CLSI .Thus there are challenges for diagnostic *AmpC* β-lactamase enzymes phenotypically (17). This lack of guidelines leads to a problem for ESBL screen via phenotypic methods and make false negative results for ESBL screenings (18). Resistance to oxyiminocephalosporins (ceftazidime and cefotaxime) and cephamicins could be indicative of the presence of *AmpC* enzyme, but these results could also be due to decrease permeability of the outer membrane (19). However, according to the CLSI recommendations, isolates that yield negative confirmatory test results are potentially producers of *AmpC* and should be further evaluated by molecular methods (14, 20). Due to the variation present in the subfamilies of *AmpC* genes, four sets of universal primers that covered the member of each family were designed, and these primers were confirmed by BLAST. Thirteen isolates were screened by PCR, and 100% and 38.5% of strains were

positive for *bla-CITM* and *bla-Dha*, respectively. Subsequently, multiplex PCR was performed on all of the strains with an *AmpC*-positive phenotype (10.2%). Results showed that isolates were exhibiting false-negative results harbored *AmpC* enzymes. Research shows that Infection isolate related to *AmpC* producers belong to CITM subfamily frequently. Two isolates that were identified as *AmpC*-producers based on results from the combined disk method encoded both CITM and Dha genes. This survey improved upon CLSI recommendations. Because, in a confirmatory test among 13 positive screens for *AmpC*, CITM PCR results were 100% positive. Therefore, we argue that it is prudent to investigate bacteria that are resistant to third-generation cephalosporin but are not inhibited by clavulanate. So, they are examined for their resistance genotype by molecular methods (such as PCR). These practices could be useful to prevent of spread of *AmpC* β-lactamases that are transported by plasmid to other sensitive bacteria. It would be lead to a reduction in public health risks ultimately (14, 21). Based on the results of our study, we believe that all laboratories should attempt to investigate the presence of *AmpC* β-lactamases. That would be complicated, and cause false negative in antibiotic resistance screening results. Therefore, it is better that all of the laboratories attend to effect of *AmpC* β-lactamases in phenotypic tests to prevent from false negative results .In addition use of molecular methods to confirm and achieve ideal solutions.

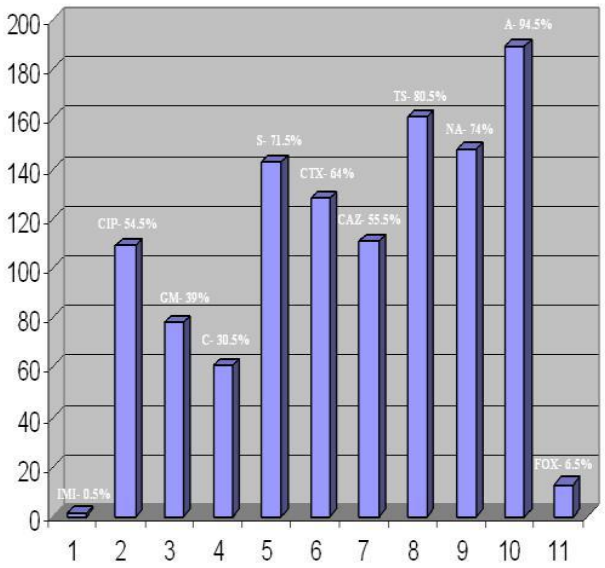

Figure 1. Pattern of resistance to 11 antimicrobia agents among 200 E. coli isolates.

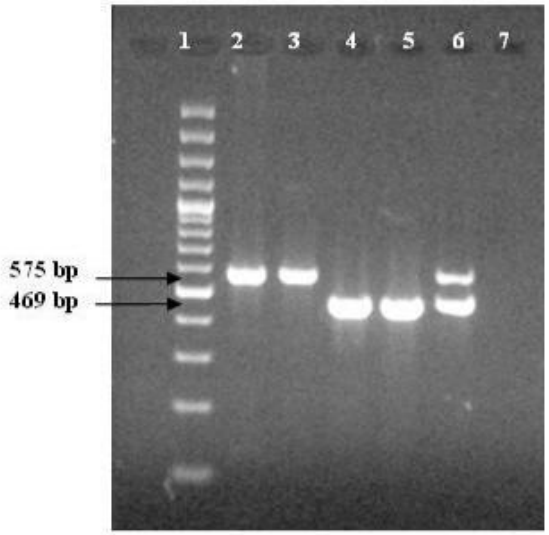

Figure 2. Lane 1: 100 bp DNA marker; Lanes 2 and 3: clinical isolates expressing the bla (CITM) gene; Lanes 4 and 5: clinical isolates expressing the bla (DHA) gene; Lane 6: clinical isolates expressing the bla (CITM, DHA) genes; and Lane 7; negative control.

Conclusion

Evaluating of existence and prevalence of *AmpC* producers among clinical isolates is highly recommended. Thus, improving of detection methods even innovation of new assay to recognize of these genes could be best move in this context in near future.

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Conflict of interest

The authors declare no conflicts of interest.

Financial disclosure

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