

# Extended Spectrum $\beta$ -Lactamase-Producing Strains of *Escherichia coli* in Hospitalized Children in Isfahan, Iran

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**Background:** Beta-lactam antibiotics such as long-spectrum cephalosporins and carbapenems are the empirical treatment against urinary tract infections (UTIs). The common mechanism of resistance to  $\beta$ -lactamas in *Escherichia coli* is producing of ESBLs.

**Objectives:** The aims of this study was evaluation of the susceptibility of the ESBL-producing *E. coli* strains to ordinarily used antibiotics and to detect the presence of the four common ESBL genes: *blaTEM*, *blaOXA*, *blaCTX-M* and *blaSHV* using multiplex PCR method.

**Materials and Methods:** In a retrospective cross sectional study conducted between 2012 and 2013, out of 145 strains of *E. coli* were isolated from hospitalized children with UTIs. Susceptibility test was performed by disc diffusion method. All isolates were also tested for ESBL by the standard CLSI double disk diffusion method, using cefotaxime/ clavulanic acid and ceftazidime/clavulanic acid disks. Multiplex PCR was performed for detect of *blaCTX-MIV*, *blaOXA*, *blaTEM* and *blaSHV* genes.

**Results:** From 145 strains of *E. coli*, 51(35.2%) and 61(42.1%) isolates were resistance to ceftazidime and cefotaxime, respectively. ESBL production was noted in 59 (40.7%) strains. The amplification of  $\beta$ -lactamase genes showed the presence of *blaCTX-M* like genes in 22 (37.9%) strains, *blaTEM* in 42 (72.4%), *blaSHV* in 7 (11.9%) and *blaOXA* in 23 (39%) of the total 58 strains of *E. coli*.

**Conclusions:** Our data showed that, the high prevalence of beta-lactamase genes among isolates, and it. Our findings may provide useful insights in replace of the appropriate antibiotics and it may also prevents of ESBLs, mediated resistance problem.

**Keywords:** Beta-Lactamase; UTI; Children; *Escherichia coli*

## 1. Background

Enterobacteriaceae are a large and important family of Gram negative bacteria including many of same-more familiar pathogens such as *Escherichia coli* (1). *E. coli* is the most prevalent infecting organism in urinary tract infections (UTIs). UTI in Children is very important because If not diagnosed and treated properly and in time, a lot of different problems including kidney problems, chronic high blood pressure in adulthood etc. (2). One of the problems in the treatment of infectious diseases such as UTI around the world; is the growing antibiotic resistance. Beta-lactam antibiotics such as long-spectrum cephalosporins and carbapenems are the preferred treatments of UTIs. In recent years, the emergence of  $\beta$ -lactamas worldwide; increased the importance of infections caused by bacteria species carries these enzymes. The genes of extended-spectrum beta-lactamas (ESBLs) are encoded by elements such as plasmid, transposons, and integrons. ESBL producing organisms are among the fastest growing challenges in the area of infectious diseases. ESBLs are enzymes that confer resistance to most beta-lactam antibiotics, including penicillins, cephalosporins, and the monobactams aztreonam. Reliable identification of

ESBL-producing organisms in clinical laboratories can be challenging, so their prevalence is likely underestimated. However, the most common ESBL-producing organism is *E. coli* (3). In addition, ESBL-producing organisms are frequently resistant to many other classes of antibiotics, including cephalosporins. For this reason, a more appropriate name would be "multidrug resistant organisms". When producing these enzymes, these organisms become highly effective at inactivating various beta lactam antibiotics. In addition, ESBL-producing bacteria are frequently resistant to many classes of antibiotics, resulting in difficult to treat infections. Clinicians should be familiar with the clinical significance of these enzymes and potential strategies for dealing with this growing problem (4, 5).

## 2. Objectives

The aim of this study was to evaluate the susceptibility of ESBL producing *E. coli* strains to commonly used antibiotics and to detect the presence of the four common ESBL genes: *blaTEM*, *blaOXA*, *blaCTX-M* and *blaSHV* using multiplex PCR.

### 3. Materials and Methods

#### 3.1. Sampling and Bacterial Isolation

In a retrospective cross-sectional study conducted between 2012 and 2013, out of 145 strains of *E. coli* were collected from hospitalized children with UTIs in Esfahan, Iran. All of these strains of *E. coli* were isolated from urine samples of children (aged less than 15 years.), those were primary positive culture by urine culture. The strains were identified by biochemical and confirmed as *E. coli* by, Catalase, oxidase, MR (+), Indol production, VP (-) and citrate negative tests bacteriological tests if they had the following characteristics: Gram-negative bacilli, citrate-positive, fermentative, triple sugar iron (TSI) acid/acid, motile, H<sub>2</sub>S-negative, urease negative, oxidase-positive and catalase-positive. The confirmed isolates were kept preserved at -20°C. The standard strain of *E. coli* ATCC 27853 was used as control (6).

#### 3.2. Antibiotics Susceptibility

The susceptibility of the strains to antibiotics were performed by the standard strain of *E. coli* using the disc diffusion method on Muller-Hinton agar, as CLSI guideline (7).

The following antibiotic discs were used: piperacillin (PP, 100 µg), cefoxitin (FOX), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), imipenem (IMI, 10 µg), trimethoprim-sulfamethoxazole (STX, 25 µg), amikacin (AN, 30 µg), Tetracyclin (TE, 30 µg), ciprofloxacin (CP, 5 µg).

#### 3.3. Phenotypic Detection of ESBL producing isolates Strain

Each *E. coli* isolate should be considered a potential ESBL producer if the test results are as follows: ceftazidime < 22 mm, cefotaxime < 27 mm.

The ESBL presence was screened using the disc diffusion method on Muller-Hinton agar. The synergy between clavulanic acid (a beta lactamase inhibitor) and a third generation cephalosporin (cefotaxime, ceftazidime) was detected following the recommendations of CLSI. The clavulanic acid inhibits the production of ESBL by *E. coli*. In brief, pairs of discs containing cefotaxime (30 µg) and ceftazidime (30 µg) with and without clavulanic acid (10 µg) were placed on opposite sides (at a distance of 20 - 30 mm) of the same inoculated plate containing Muller-Hinton agar (BBL-Becton Dickinson). A positive test result was defined as a ≥ 5 mm increase in the zone diameter compared to a disk without clavulanic acid. An expansion of > 5 mm or 50% (according to the particular product as the manufacturer's guideline) indicated ESBL production. Pairs of discs containing an extended-spectrum cephalosporin (cefotaxime, ceftazidime or cefpodoxime) with and without clavulanic acid were placed on opposite sides of the same inoculated plate. The inhibition zone was measured after following overnight incubation in aerobic condition at 37°C. *Klebsiella pneumonia* ATCC 700603 (positive control) and *E. coli* ATCC 25922 (negative control) were used for quality control of the ESBL tests (6).

#### 3.4. The Extended Spectrum β-Lactamase-Producing Genes Detection

The detection of the gene sequences coding for the *bla*TEM, *bla*OXA, *bla*SHV and *bla*CTX-M-IV enzymes was performed using multiplex PCR and the primers used to amplify the abovementioned genes are listed in Table 1. Lack of homology of the primer sets with other genes for L-lactamases and other genomic regions of the enterobacteriaceae was tested by comparison with the nucleotide sequence data in Gene Bank (7).

**Table 1.** The primer sequencing for detection of *bla*TEM, *bla*CTX-M, *bla*OXA and *bla*SHV genes <sup>a</sup>

Gene	Primer Sequences, 5' -3'	Product Size, pb <sup>b</sup>	References
<b><i>bla</i>CTX-M-IV</b>		501	10
F	GACAAAGAGAGTGCAACGGATG		
R	TCAGTGGGATCCAGACGAAA		
<b><i>bla</i>TEM</b>		431	10
F	AGTGCTGCCATAACCATGAGTG		
R	CTGACTCCCC GTCGTGTAGATA		
<b><i>bla</i>OXA-1</b>		296	10
F	ATTATCTACAGCAGCGCCAGTG		
R	TGCATCCACGCTCTTGGTG		
<b><i>bla</i>SHV</b>		214	10
F	GATGAACGCTTCCCATGATG		
R	CGCTGTATCGCTCATGGTAA		

<sup>a</sup> Abbreviations: F, forward; R, reverse.

<sup>b</sup> Nucleotide numbering begins at the initiation codon of genes.

### 3.5. DNA extraction

The total DNA content of each ESBL-positive isolate was extracted by boiling method. Briefly a, single colony from a 16-hour culture in nutrient agar (Hi-Media, India) was inoculated in 50  $\mu$ L of TES (50 mM Tris hydrochloride (pH = 8.0), 5 mM EDTA, 50 mM NaCl), and the suspension was heated at 95°C for 10 minutes and centrifuged at 15,000 rpm for 2 minutes. The supernatant containing was transferred to new sterile DNase free-RNase free micro tubes (8).

### 3.6. Rapid Detection of *bla*OXA, *bla*SHV, *bla*TEM and *bla*CTX-M IV Genes by Multiplex Polymerase Chain Reaction Technique

Multiplex PCR was performed for amplification of *E. coli* *bla*OXA, *bla*SHV, *bla*TEM and *bla*CTX-M IV genes, using the primers are listed in Table 1. According to previous protocols (9).

Two microliters of total DNA was included in multiplex PCR in a 50  $\mu$ L reaction mixture. The mixture for the detection of *bla*OXA, *bla*SHV, *bla*TEM and *bla*CTX-M IV genes contained 1x PCR buffer (10 mmol/L Tris-HCl [pH 8.3]), 1.5 mmol/L MgCl<sub>2</sub>, 0.125 mmol/L of each deoxynucleotide triphosphate, 10  $\mu$ mol/L of each primer, and 2 U of AmpliTaq polymerase (Fermentas R, Korea).

Amplification was carried out with the following thermal cycling conditions: initial DNA denaturation at 94°C for 10 minutes, 36 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 40 seconds and extension at 72°C for 50 seconds, followed by a final extension at 72°C for five minutes. Agarose gel electrophoresis of the amplified DNA with 100 bp size marker (Fermentas R, Korea) has done for one hour, at 80 V in a 2% agarose gel in TAE buffer (40 mmol/L Tris-HCl (pH = 8.3), 2 mmol/L acetate, 1 mmol/L EDTA).

DNA was stained using DNA safe stain to detect the specific band and the gels were visualized using UV strains illumination, and photographed (9, 10) (Figure 4).

### 3.7. Statistical Analysis

For the statistical analyses, the statistical software SPSS version 18 for Windows (SPSS Inc., Chicago, IL) was utilized. Continuous variables were described as mean  $\pm$  standard deviation (SD) and compared using compared with standard student t test, or and calculates median range. All the tests were two-tailed and  $P < 0.05$  was considered statistically significant.

## 4. Results

The medical records of the 145 index patients were reviewed. About 50% of patients were below one year old, 89.7% (n = 130) were female and 1.3% (n = 15) were male.

The antibiotic resistance rates of our studied *E. coli* strains is listed in Figure 1. In the disk diffusion method, the highest resistant rate was observed to trimethoprim-sulfamethoxazole (70.3%) and tetracycline (62.8%) the most and the lowest resistant was observed to amikacin (5%), piperacillin (4%) and cefoxitin (13%) the least resistant antimicrobial agents against *E. coli* strains. In fact, of 145 *E. coli* isolates, 62 isolates didn't show any antibiotic sensitivity to cefotaxime and ceftazidime. In addition, 59 isolates were identified as ESBL producing bacteria by combined disk test. Our result did not show any significant relationship between antibiotic resistance and gender of patients using chi-squared analysis ( $P = 0.79$ ).

The results of multiplex PCR revealed a predominance of the *bla*TEM gene among the strains of *E. coli* (42 strains). A large number of *E. coli* strains carried two or more ESBL genes, and three strains carried all four gene types. Eleven strains showed none of these four genes. The rate of strains carrying the *bla*CTX-M-IV, *bla*OXA-1 and *bla*SHV genes were 22, 24 and 6 isolates respectively. Presence of these genes analyzed using chi-square test and no difference was observed (Figures 2 and 3).

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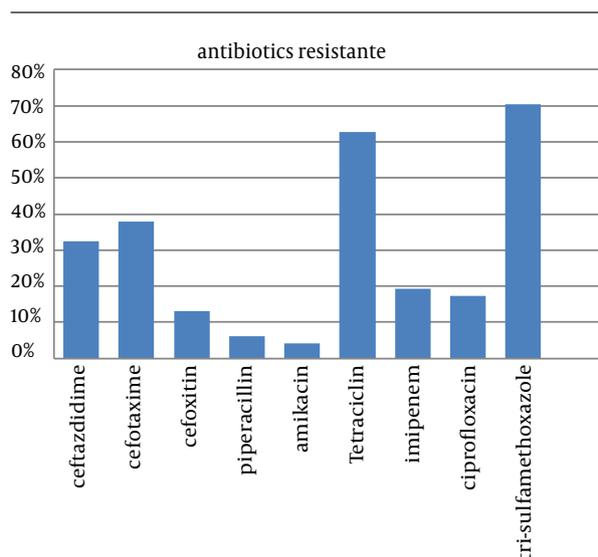


Figure 1. Antibiotic Susceptibility of the 145 Tested Strains

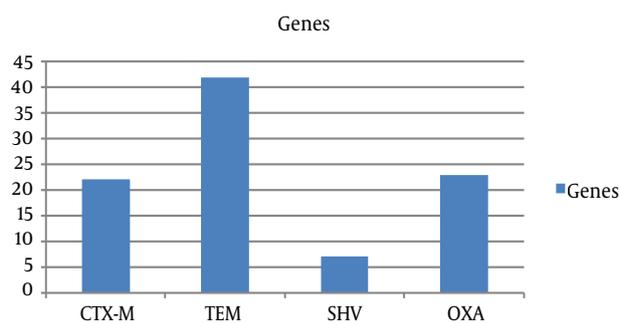
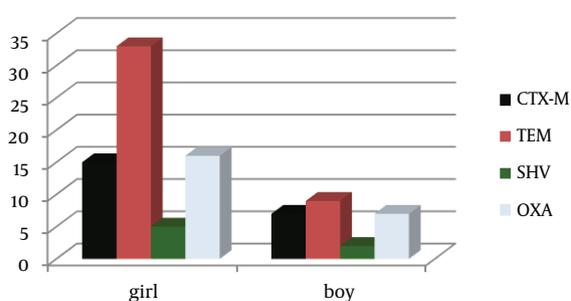
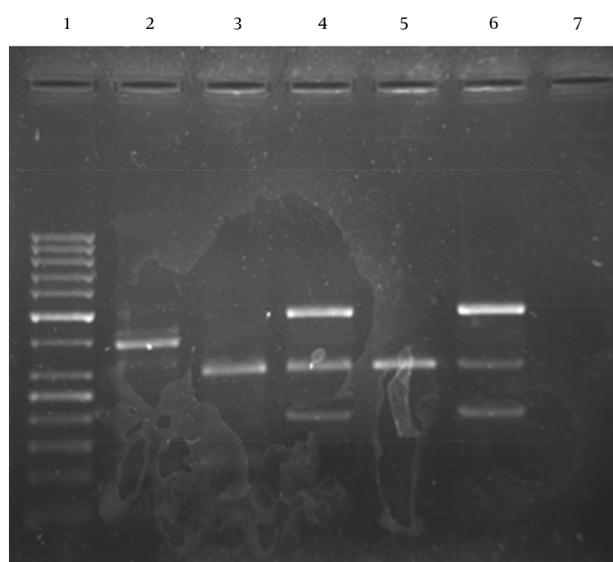


Figure 2. Types of Extended Spectrum beta-Lactamase Genes



**Figure 3.** Types of the ESBL genes in patients

**Figure 4.** Gel Electrophoresis of Multiplex Polymerase Chain Reaction Products Following Amplification with Specific Primers



From left to right: lane 1, marker; lane 2, *blaTEM*; lane 3, *blaOXA*; lane 4, *blaCTX* *blaOXA* *blaSHV*; lane 5, positive control (*blaOXA*); lane 6, *blaCTX* *blaOXA* *blaSHV*; lane 7, negative control.

## 5. Discussion

The ESBL distribution in Europe has shown a dramatic increase of the *blaCTX-M* gene instead of *TEM* and *SHV* genes (6). The results of our study confirmed a high prevalence of *blaTEM* and *blaCTX-M-IV* genes, while the prevalence of *blaSHV* genes was low (Figure 3).

Our finding is in accordance with a similar study performed in Lebanon in 2011. According to other previous studies, the *blaSHV* gene was more prevalent among *K. pneumoniae* than *E. coli* strains (11). In two recent similar studies in Iran in 2007 and 2010, the frequency of *blaTEM* gene was estimated 46.4% and 68.8%, respectively (12, 13). The rate of *blaTEM* in our study was estimated about 73% that showed an increasing rate in these years.

In the past several years, the emergence of new variants of ESBL producers, especially *blaCTX-M* suggested the

involvement of co-resistance to other drug classes during endemic conditions. This co-resistance is due to the transmission of different types of resistance genes within the same clone (9).

Several studies showed that *blaCTX-M* genes were commonly found in large plasmids those which often carry other genes, conferring resistance to other antimicrobial agents including aminoglycosides, fluoroquinolones, chloramphenicols, tetracyclines and others (particularly *blaOXA-1*, *blaTEM-1*, *tetA*, *aac*) This may explain the high rate of transmission of the *blaCTX-M* gene among the *E. coli* strains by acquiring R-plasmid; often, the high prevalence of the *blaCTX-M* resistance gene is combined with other resistance genes in these strains (9-11).

The *blaCTX-M* gene predominates in Europe, while in other countries ESBL genes are more diverse (2, 9). In the United Kingdom, a recent dramatic increase of the ESBL producing strains was observed both in hospitals and in the community, and this increase was attributed to *blaCTX-M-15* (7). In Italy, the prevalence of *E. coli* producers of ESBL also increased with a predominance of *blaTEM* (45.4%), *blaSHV-12* and the emergence of *blaCTX-M* and *blaPER* (14).

For rapid screening of ESBL-producing isolates, multiplex PCR is a suitable tool. Recently, multiplex PCR for detection of *blaTEM*, *blaSHV* and *blaOXA-1* genes, and *blaCTX* was described (10).

In the Europe predominate gene is *blaCTX-M*, while in other countries the ESBL genes are more diverse. In Turkey, a study by Ozgumus et al. showed that 15% of the Enterobacteriaceae isolates were producers of ESBLs and commonly *blaTEM* and *blaSHV* genes were detected. Of these strains, 5 isolates were carried *blaTEM* gene, 12 carried *blaTEM* and *blaSHV* and 3 isolates carried none of the two genes (15). In another similar study in Thailand, 235 ESBL-producing *E. coli* strains were studied; 87.3% of the strains were positive for *blaCTX-M*, 77% for *blaTEM*, and only 3.8% had the *blaSHV* gene. In addition, a few strains produced *blaVEB-1* and *blaOXA-10* (16).

Every organization should usually check the resistant rate of pathogens and the antibiotics consumption and report the information to infection control programs (17).

The present study showed high resistance patterns to the most effective antibiotics used against *E. coli* producers of ESBLs such as third generation cephalosporins. On the other hand, this study revealed an alarming decrease in the susceptibility of our isolates to carbapenems and fluoroquinolones (Figure 2).

Similar results were found in a study in Lebanon, but other studies in the Middle East areas showed higher susceptibility rates to fluoroquinolones (11).

Similar to the results of another studies performed in Iran by Mehrgan et al. the sensitivity of the same isolates as our study to cefoxitin was 81.4% (18).

Our results about aminoglycosides showed that amikacin remains the most active drug (82%), which is in accordance with antibiotic susceptibility results of another similar study conducted in Saudi Arabia (19).

The high frequency of ESBLs was found first to *K. pneumonia* strains which produce *blaTEM* and *blaSHV* and then were detected in *E. coli* strains producing and *blaCTX-M*, which became the major type (20, 21).

Our study has had limitation. This was a retrospective cross-sectional study and we could not investigate any independent factors. Further studies should be performed to clarify whether the presence of ESBL genes affects the clinical outcome.

In conclusion, the multiplex PCR investigated in this study showed excellent performance and could supplement phenotypic tests in the ESBLs detection. The ESBL genes prevalence (*blaSHV*, *blaTEM*, *blaCTX-M* and *blaOXA* genes) in the present study was relatively high and these genes could emerge among clinical isolates of *E. coli*. Since patients with infections caused by ESBL-producing bacteria are at an intensified risk of treatment failure, fast determination of these organisms is necessary.

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