Multiplex PCR Detection of *blaCTX* and *blaSHV* Resistance Genes in *Escherichia coli* Isolated from Milad Hospital in Tehran

Shima Mirzaie Parsa 1, Mohammad Javad Soltani Banavandi 1, 2, *, and Mohammad Hassan Shahhosseiny 3, 4

1Department of Microbiology, Kerman Branch, Islamic Azad University, Kerman, Iran
2Department of Microbiology, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran
3Iranian Gene Fanavar Institute, Tehran, Iran

*Corresponding author: Department of Microbiology, Kerman Branch, Islamic Azad University, Kerman, Iran. Email: mj.soltani@yahoo.com*

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**Abstract**

**Background:** Nosocomial infection caused by antibiotic resistance bacteria is increasing in the world. Resistance to extended-spectrum beta-lactamase (ESBLs) is one of the most important antibiotic resistance mechanisms.

**Objectives:** According to prevalence of *E. coli* producing ESBL, the aim of this study was to identify the *blaCTX* and *blaSHV* genes, which are the causing genes of the resistance to beta-lactam antibiotics by Multiplex-PCR method.

**Methods:** A total of 100 isolates of *E. coli* were collected from the Milad Hospital in Tehran. Disk diffusion method was done and ESBL positive isolates were determined by combination disc test. DNA extraction of them was carried out and the presence of *blaCTX* and *blaSHV* genes was evaluated by using the M-PCR method.

**Results:** By using the molecular method on all isolates, 46 of them were positive for the *CTX* gene, three isolates were positive for the *SHV* gene, and none of the isolates have both *blaCTX* and *blaSHV* genes. Antibiogram test was done on all samples in which 17 of them were resistant to antibiotics.

**Conclusions:** Based on the goals of this study for investigation of frequency of *SHV* and *CTX* antibiotic resistance genes in collected isolates in order to improve PCR detection method, achieved results emphasized that the M-PCR method, with high accuracy and sensitivity, should be used as the substitution of phenotype tests in clinical laboratories. In addition, avoiding the indiscriminate prescribing of antibiotics is an important necessity.

**Keywords:** *Escherichia coli*, ESBL, *blaCTX*, *blaSHV*, PCR

1. **Background**

With the discovery of antibiotics (Fleming, 1928), the emergence of new antibiotics and their spread use in the treatment of bacterial infectious diseases, bacterial resistance against the anti-bacterial materials happened (1). One of the different strategies employed by the bacteria in order to be safe from the bad and destructive effects of antibiotics, which are being used in the gram negative bacteria against the beta-lactamase antibiotics, is the production of the beta-lactamase enzymes, which inactivate them through the hydrolysis of the central core in the beta-lactamase antibiotics (2, 3). The continuity in the use of these groups of antibiotics leads into the emergence of ESBLs, and the first identification of worldwide detection of ESBL have been done in the early 1980s in Germany (4-6). To date, approximately more than 200 different types of ESBLs have been discovered in the world as most of them have been seen in the family of Enterobacteriaceae (7). Transmission of ESBL-producing organisms caused an increase in hospital-acquired infections worldwide (8). One of the methods to classify Beta-lactamase, have been divided into four groups, A, B, C, and D based on the type of substrate, ability of prevention, and physical characteristics; regarding this classification, the widespread enzymes were located in group A and they include the derivatives of mutated enzymes such as *TEM* and *SHV* (9-11). Beta-lactamase of *CTX-M* types are widely distributed through the plasmids carrying ESBL, which are lacking *TEM* and *SHV*; for the first time, they were reported in Europe in the late 1980s (12, 13). *Escherichia coli* is one of the most common bacterial agents that has been isolated from human infections (14). This bacterium is the original member of the Enterobacteriaceae family and it is the main factor of many hospital infections such as sepsis, neonatal meningitis, gastroenteritis, and especially urinary tract infections (15).
2. Objectives

Therefore, studying the antibiotic sensitivity patterns as well as the presence of beta-lactam genes in the clinical samples of *E. coli* were considered as the aims of this research.

3. Methods

3.1. Sample Collection and Antibiotics Sensitivity and Resistance Pattern

In this research study, a total of 100 clinical isolates of *E. coli* related to urine isolates, scars, blood, respiratory secretions, and chips of patients in the microbiology clinical laboratory of Milad Hospital in Tehran, in 2015, were collected. All isolates, after the accurate identification and biochemical tests performed at -80°C, were kept. The sensitivity pattern and antibiotic resistance were defined by the disc diffusion method in accordance with the clinical and laboratory standards (CLSI, 2015) under the culture conditions of Mueller Hinton agar. The discs (Mast) were: Ampicillin, cephalexin, ofloxacin, nitrofurantoin, cefuroxime, nalidixic acid, imipenem, ceftriaxone, ofloxacin, cefixime, cotrimoxazole, amikacin, ceftizoxime, tetracycline, gentamicin, norfloxacin, ciprofloxacin, chloramphenicol, cefotaxime, meropenem, imipenem, ceftriaxone, fluoxetine, cefuroxime, tobramycin, chloramphenicol, cefotaxime, ceftriaxone, and tetracycline, gentamicin, norfloxacin, ciprofloxacin, cefuroxime, tobramycin, chloramphenicol, cefotaxime, meropenem.

After placing the antibiotic discs regarding the standard distance on the surface of the related environment, the plate was put in the incubator (37°C) for 18 to 24 hours. To identify the isolates producing ESBLs, the confirmation test was used. The testing discs, based on the combination discs, were ceftazidime and cefotaxime plus clavulanic acid, as well as cefotaxime and cefotaxime plus clavulanic acid under the conditions of Mueller Hinton Agar in a distance of 20 mm (from each other). Finally, after 24 hours of incubation at 37 degrees, *E. coli* producing ESBLs were determined through increasing the diameter size of halo (about 5 mm or more than it) around the ceftazidime disk/ clavulanic acid or cefotaxime clavulanic acid.

3.2. DNA Extraction from Bacteria

DNA of all samples was extracted by using the DNG-Plus kit (SinaClone, Iran).

3.3. Primers

Nucleotide sequence of the primers used for tracking *blaSHV* and *blaCTX* genes for PCR test is shown in Table 1 (16).

3.4. PCR Method Optimization

Optimization of Multiplex PCR test in order to detect the *blaCTX* and *blaSHV* genes in *E. coli* was done through the optimization of the concentration of components, which were used in the polymerase chain reaction process. Materials and the required M-PCR factors were: 10 × PCR buffer 2.5 µL, forward primer (CTX) (0.2 µM ) 0.5 µL, reverse primer (CTX) (0.2 µM ) 0.5 µL, forward primer (SHV) (0.2 µM) 0.5 µL, reverse primer (SHV) (0.2 µM) 0.5 µL, MgCl₂ (50 mm) 0.75 µL, dNTP mix (10 mm) (thermo scientific) 0.5 µL, Taq DNA polymerase (thermo scientific) 0.3 (1.5 unit) µL, DNA template or POS control 5 µL, D.D.W 14 µL, and total volume 25 µL. PCR was performed using an initial denaturation condition for five minutes at 94°C, 30 amplification cycles (denaturing 94°C for one minute, annealing at 60°C for one minute, and extension at 72°C for 1 minute) and a final elongation step for seven minutes at 72°C in the PCR machine (My Gene™ (Model MG96G)).

Then, PCR product of each isolate was mixed with 2 µL of loading buffer (guide buffer) (IGF) and electrophoresis was done with the use of agarose gel (CinnaGen, Iran), 1.5%, along the size marker of Ladder mix (Thermo Scientific). At the end, it was determined that the PCR products of CTX gene and SHV gene were fragments of 593 bp and 747 bp, respectively.

3.5. Determination of Limit of Detection and Specificity of PCR Test

Determination of limit of detection of PCR test was done by the serial dilution method with the dilutions of 10⁴ to 10⁻⁷ separately for SHV and CTX genes. In addition, the DNA of eight strains consists of different bacteria such as *Salmonella*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, *Candida albicans*, and *Brucella*, which were used for M-PCR of CTX and SHV genes, respectively, for determination of the specificity test.

4. Results

4.1. Results of Multiplex PCR Test on the Clinical Isolates

M-PCR test was done on extracted DNAs from the clinical isolates of *E. coli*. Among 100 isolates from the patients, 46 of them were positive for CTX gene and three of them were positive for the SHV gene. Based on these results, 49% of them were positive and none of them had these two genes together (Figures 1 and 2).
Table 1. Nucleotide Sequence of the Primers for PCR Test

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequences</th>
<th>Amplicon Size in bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF:CTX-M-U1</td>
<td>5’-ATG TGC AGY ACC AGT AAR GTK ATG GC-3’</td>
<td>593</td>
</tr>
<tr>
<td>PR:CTX-M-U2</td>
<td>5’-TGG GTR AAR TAR GTS ACC AGA AYC AGC G-3’</td>
<td></td>
</tr>
<tr>
<td>Bla-SHV SE</td>
<td>5’-ATG CGT TAT ATT CGC CTG TG-3’</td>
<td>747</td>
</tr>
<tr>
<td>Bla-SHV AS</td>
<td>5’-TGC TTT GTT CGG GCC AA-3’</td>
<td></td>
</tr>
</tbody>
</table>

4.2. Determination of Limit of Detection and Specificity of PCR Test

The limit of detection of PCR test for CTX gene was 100 CFU and the SHV gene was 1000 CFU. In addition, by doing the PCR test on eight different strains of various bacteria for determining the specificity of test, no product was observed.

4.3. Antibiogram Test Result

This test was done on 100 isolates of E. coli based on CLSI standards and 17 isolates of them were considered as positive ones by disc diffusion test. By using the PCR method on these 17 positive isolates, 16 of them were found positive for CTX, and no positive SHV isolates were found (Table 2).

5. Discussion

The beta-lactamase genes in bacteria, particularly ESBLs gene, are one of the effective factors in increasing the resistance to the related antibiotics such as cephalosporin. Organisms that carry these genes increase pathogenicity and mortality among the individuals, as well, the continuance of increasing in antibiotic resistance may endanger the society with serious problems (17, 18). ESBLs are the beta-lactamase molecules of class A or D, which are able to hydrolyze the oxi-imuno-cephalosporin in an equal size or about 10% more than benzyl penicillin (9, 19). Different types of these enzymes are increasing (20). One of the most successful strategies for overcoming the resistance of beta-lactamase is the use of its inhibitors. This mechanism is based on the preventive activity by covalent joints.
or links to the active site of beta-lactamase; class A, tazobactam, sulbactam, and clavulanic acid are the preventive materials of beta-lactamase inhibitors, which are commonly used in clinical studies (21). In addition, enlargement of the active site, which increases the activity level against cephalosporin may increase the sensitivity of ESBLs to the inhibitors of beta-lactamase (22). Comparison of the results of other studies shows that the frequency and prevalence of ESBLs is not the same in different parts of the world and, also their dispersion is not similar through the countries or even in different cities (23). The long period of hospitalization, overuse of antibiotics (especially cefazidime), hospitalization in the ICU ward, and the use of urinary catheters are the factors for producing ESBLs (24). ESBLs-producing strains are usually the ones with multiple resistance (25). Until 1999, most ESBLs isolated from the patients were TEM and SHV, however, in recent studies CTX-M beta-lactamase was isolated from patients and around the world, Enterobacteriaceae members containing blaCTX-M gene will be isolated (26). It should be noted that there is extended-spectrum activity of the SHV-enzyme (27). The difference of the actions may be caused by the replacement of the different amino acids in the active site of enzymes (28-30). Since, phenotypic methods can only show the presence or absence of ESBL as well, the detection of this type of resistance in laboratory conditions was so difficult, and some of the resistant strains show their resistance under the in vivo conditions in spite of showing sensitive phenotype under the in vitro conditions. Therefore, for detecting a wide variety of beta-lactamase, extending the molecular methods such as PCR, which leads to a rapid and effective treatment and prevents the spread of resistant isolate of bacteria can be felt (31). In the present study, among 100 isolates of *E. coli*, 49% of them consisted of ESBLs, which were obtained by Multiplex-PCR as well, 46% of *E. coli* isolates contained CTX genes and 3% of them consisted of SHV genes. Shahcheraghi et al. in 2007, in Iran, showed that among 200 samples of *E. coli*, 105 (n = 105 (52.5%)) of them consisted of ESBL genes and 12 of them (n = 12 (6%)) consisted of blaSHV genes (15). In 2008, Meyer et al. showed that the frequency of *E. coli* producing ESBL in the ICU wards of Germany in 2001 was increased from 14% to 52.1% in 2007. The researchers believed that considering resistant *E. coli* against ESBL is important (32). During this year, Ho et al. studied the resistance of antibiotic in *E. coli*. By studying 46 samples by the DDST method, they could determine the bacteria producing ESBL, and among 46 samples, eight of them produced ESBL (33). In 2008, the studies by Mobasher Kare Jeddi et al. in Iran, showed that among 41 isolates of *E. coli*, 40 or 97.56% of them were positive ones and seven or 17.07% of them consisted of blaSHV genes (22). In 2010, Yazdi et al. in their studies in Iran declared that among 246 isolates of *E. coli*, 109 (44.3%) were the ones producing ESBL. blaSHV and blaCTX-M genes were founded in the isolates of ESBL (77 or 70.6% and 75 or 68.8%), respectively. In addition, 54 or 49.5% of them consisted of two genes; blaSHV and blaCTX-M ones (34). In the other study by Bali et al. in Turkey, among 44 samples of *E. coli*, 10 or 22.72% of them consisted of CTX-M genes and eight or 18.18% consisted of SHV genes (35). In the study by Soltan Dallal, in 2011 in Iran, it was shown that among 200 isolates of *E. coli*, 128 or 64% of them produced beta-lactamase, and after passing PCR process for determining CTX-M gene, it was defined that among 128 strains consisted of beta-lactamase, 99 of the related isolates (77.34%) consisted of the considered gene (36). In 2013, Altinkum et al. in Turkey, showed that among 62 samples of *Klebsiella pneumonia* and *E. coli*, 33% and 17.4% of them were produced beta-lactamase. The results were gained by using two methods such as disk dispersion and polymerase chain reactions and they were shown that 81% of *Klebsiella pneumonia* and 94.8% of

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Antibiogram Test Results</th>
</tr>
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<tbody>
<tr>
<td>Ofl oxacin</td>
<td>33</td>
</tr>
<tr>
<td>Flu oxacil</td>
<td>0</td>
</tr>
<tr>
<td>Gent amicin</td>
<td>41</td>
</tr>
<tr>
<td>Cef to xime</td>
<td>19</td>
</tr>
<tr>
<td>Cef roxime</td>
<td>14</td>
</tr>
<tr>
<td>Tob ramycin</td>
<td>16</td>
</tr>
<tr>
<td>Cip ro floxacin</td>
<td>40</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>8</td>
</tr>
<tr>
<td>Imipenem</td>
<td>1</td>
</tr>
<tr>
<td>Cef to xime</td>
<td>20</td>
</tr>
<tr>
<td>Amikacin</td>
<td>2</td>
</tr>
<tr>
<td>Cef xime</td>
<td>1</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>41</td>
</tr>
<tr>
<td>Chlor amphenicol</td>
<td>2</td>
</tr>
<tr>
<td>Cefoxolin</td>
<td>25</td>
</tr>
<tr>
<td>Cephal oxin</td>
<td>19</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>41</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>58</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0</td>
</tr>
<tr>
<td>Nitrofuranto in</td>
<td>3</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>23</td>
</tr>
<tr>
<td>Nor floxacin</td>
<td>1</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>58</td>
</tr>
<tr>
<td>Cefazidime + clavulanic acid/cefotaxime + clavulanic acid</td>
<td>0</td>
</tr>
</tbody>
</table>
E. coli were produced beta-lactamase, type of CTX-M (37). In 2015, Miraalami et al. determined the frequency of E. coli strains producing beta-lactamase genes such as SHV and CTX-M by Multiplex PCR, and in this study, among 55 samples, 41 of them consisted CTX-M genes (about 74.54%), furthermore, SHV gene was not observed in these samples. The results of these tests show that the production of beta-lactamase enzymes was observed in 70% of the considered samples (38). In a study by Mohammed et al. in 2016, of 108 various clinical samples obtained from hospitals in Egypt (such as urine, blood, pus, and mucus) where 41 (78.8%) were isolated from urine, 12 (66.6%) from blood, 14 (48.2%) from respiratory secretions and two (22.2%) from pus, the tests found that 65 examples of them are Gram-negative bacilli. Escherichia coli (46.1%) was the most commonly isolated organism among Gram-negative bacilli, followed by Klebsiella pneumoniae (26.2%), Pseudomonas aeruginosa (10.7%), Enterobacter cloaceae (6.1%), Proteus mirabilis (3.07%), and Acinetobacter baumanii (4.6%), while Citrobacter freundii and Proteus vulgaris gave 1.5%. Antibiotic susceptibility testing was determined by Kirby-Bauer technique and interpreted according to CLSI. Production of blaTEM, blaSHV, and blaCTX-M genes was done by polymerase chain reaction (PCR). A total of 24 isolates (72.7%) had blaTEM, and five (15%) showed blaSHV, while 12 (36%), six (18.2%), and zero (0.00%) harbored blaCTX-M-1, blaCTX-M-9, and blaCTX-M-8/25, respectively (39). Therefore, the rapid diagnosis of drug resistance contributing to community health can prevent further antibiotic resistance.

5.1. Conclusions

The production of ESBLs is significantly increasing. Therefore, in order to treat the infections with a factor producing beta-lactamase, antibiotics must be chosen with accuracy (40). Rapid diagnostic techniques such as M-PCR have to be used to eradicate the spread and transmission of these ESBL-producing strains and the correct treatment of these strains in hospitals (16, 41).

According to our results, the prevalence of blaSHV gene in samples collected from Milad Hospital is less than other studies mentioned in this article; in addition, the prevalence of blaCTX gene was almost similar to some other researches.

Supplementary Material

Supplementary material(s) is available [here](#). To read supplementary materials, please refer to the journal website and open PDF/HTML.

Footnotes

**Conflict of Interests:** The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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**References**


