To survey the frequency of ESBL-producing E. coli, a heterogeneous group of enzymes, are encoded by genes which have been described that confer extended-spectrum β-lactam (ESBL) resistance to third and fourth generation cephalosporins and monobactams but are inhibited by β-lactamase inhibitors[2]. ESBLs are grouped into four classes A, B, C and D enzymes. Cefotaximase (CTX-M), temoneira (TEM) and sulfhydryl variable (SHV) are class A ESBLs[3]. ESBLs, a heterogeneous group of enzymes, are encoded by genes which have been described that confer resistance to third and fourth generation cephalosporins and monobactams but are inhibited by β-lactamase inhibitors[2]. ESBLs are grouped into four classes A, B, C and D enzymes. Cefotaximase (CTX-M), temoneira (TEM) and sulfhydryl variable (SHV) are class A ESBLs[3]. Escherichia coli (E. coli) is a major agent of UTI and also the most frequent bacteria that produces different type of ESBLs[1,2]. Several investigators have reported different prevalence of ESBLs between 6% and 88% in various health care settings especially among Klebsiella pneumoniae (K. pneumoniae) and E. coli[4-5]. Although, until recently, TEM and SHV variants were the most ESBLs produced by Klebsiella spp., Enterobacter spp., and E. coli, the nature of ESBL dissemination has changed during the past few years and E. coli strains expressing CTX-M have presently replaced TEM and SHV as the most common

1. Introduction

Urinary tract infection (UTI) is one of the most frequent types of infections that probably affects one-half of all people during their lifetimes. The extensive consume of β-lactam antimicrobial agents in order to treat patients with UTI has recently led to the emergence of resistant strains all over the world. Beta-lactam resistance is mediated by extended-spectrum β-lactamase (ESBL)-producing Escherichia coli (E. coli) isolated from hospitalized patients with urinary tract infection and the determination of their antibiotic resistance patterns.

Methods: During 11-month study, 100 ESBL-producing E. coli were collected from 330 patients who met the definition of urinary tract infection. The phenotypic identification of ESBL was confirmed by double disk synergy test and combined disk diffusion test. In vitro, susceptibility to ESBL isolates than 14 antimicrobial agents was performed by Kirby-Bauer disk diffusion method. The frequency of blaTEM, blaSHV and blaCTX-M ESBL-producing E. coli was assessed by PCR method.

Results: The frequency of ESBL-producing E. coli was 40.8%. In vitro, susceptibility to ESBL-producing E. coli showed that the majority of isolates were highly susceptible to amikacin (74%) and imipenem (91%). The rates of resistance to other antibiotics varied from 33% to 96%. Through 100 tested isolates, the prevalence of blaTEM, blaSHV and blaCTX-M genes was determined to be 67%, 45% and 74% respectively. In comparison with other bla genes, the frequency of blaCTX-M was strikingly high.

Conclusions: Due to the increase of E. coli with multiple ESBL genes, continuous surveillance in order to appropriate antibiotics and the control of infections is necessary.
types of ESBLs\cite{6}, SHV $\beta$-lactamases confer high level resistance to ceftazidime, but not to cefotaxime and cefazolin, while CTX-M $\beta$-lactamases are more active against cefotaxime and ceftriaxone than ceftazidime\cite{6}. At first, the CTX-M family was described in 1992\cite{6}. It has only 40\% similarity to TEM and SHV\cite{6}. Based on amino acid similarities, five different groups of CTX-M types (1, 2, 8, 9, and 25) are known. The prevalence of specific CTX-M subgroups varies due to geographic region and studied country. In particular, CTX-M-15 is the most widely disseminated CTX-M genotypes currently\cite{6}. As mentioned, in recent years, according to the widespread of CTX-M-type enzyme, the epidemiology of ESBLs has dramatically increased since 1995 in the most parts of world\cite{7,8,9}. Given the increasing prevalence of TEM, SHV and CTX-M type of ESBLs, there is a serious threat to the clinical use of third generation cephalosporins for the treatment of severe infections and also limit the choice of effective antimicrobial drugs to carbapenems or colistin\cite{7}.

The emergence of ESBLs, as an important cause of transferable multidrug resistance in Gram-negative bacteria particularly E. coli, is now a serious problem in the public health world. However, there are still few reports on the prevalence of nosocomial ESBL-producing E. coli in hospitals of Iran. With this background, we investigated the ESBL type prevalent of E. coli isolated from hospitalized patients with UTI and then determined their pattern of antimicrobial resistance as commonly antimicrobial agents consume.

2. Materials and methods

2.1. Study setting and bacterial isolates

The present descriptive study was performed on hospitalized patients with UTI. A total of 245 E. coli isolates were collected from the 330 urine specimens of UTI hospitalized patients during a period of 11-month from August 2014 to June 2015. Urine samples were obtained by a wide mouth sterile container from the study subjects. UTI was defined as > 10^5 leukocytes per milliliter urine and the growth of a single pathogen of > 10^5 CFU/mL from urine specimens. All of patients were instructed for proper and correct urine specimen collection. All of included cases had history of nosocomial UTI. Hospital acquired infection was confirmed by clinical examination of physicians. All the urine samples were transported to the laboratory and processed immediately. Identification was done based on culture characteristics, Gram stain and routine standard biochemical tests. Colony count semi-quantitative method was done according to surface streak procedure using calibrated loops. The cultured plates were incubated in aerobic conditions at 37 °C for 24–48 h. The result of equal or more than 10^5 CFU/mL was considered as a positive UTI and a less than 10^5 CFU/mL was interpreted as a negative UTI. The result of 10^2–10^4 CFU/mL was repeated\cite{7}. Repeated E. coli isolates were excluded from the study.

2.2. Phenotypic test for ESBL detection

ESBL production was confirmed phenotypically by double disk synergy test (DDST) and combined disk diffusion test according to the Clinical and Laboratory Standards Institute (CLSI) criteria for ESBL screening\cite{7}.

2.3. DDST

DDST was done by using cefotaxime (30 μg) and ceftazi-
dime (30 μg) with and without clavulanic acid (10 μg) disks on Mueller-Hinton agar (Oxoid, Basingstock, UK) with 25 mm apart from each other. An increase of equal or more than 5 mm in zone diameter for either antimicrobial agent tested with clavulanic acid versus its zone when tested without clavulanic acid indicated the presence of ESBL\cite{7}.

2.4. Combined disk diffusion method

According to the CLSI protocol, test was done by using both ceftazidime (30 μg) disk alone and the combination of clav-
ulanic acid (30 μg/10 μg). Disks were placed 25 mm apart from each other on Mueller-Hinton agar inoculated with 0.5 McFar-
land suspension of the test isolate. More than or equal to 5 mm increase in zone diameter for either antimicrobial agent tested with the combination of clavulanic acid versus its zone when tested alone was interpreted as ESBL producer\cite{7}. E. coli ATCC 25922 was used as quality control.

2.5. Antimicrobial susceptibility testing

Isolates were tested to evaluate the pattern of antimicrobial susceptibilities by Kirby-Bauer disk diffusion method according to interpretive criteria that have been recommended by CLSI guidelines. The following antimicrobial agents were used in this study: aztreonam (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), amikacin (30 μg), ceftazidime (30 μg), imipenem (10 μg), cefotaxime (30 μg), piperacillin (100 μg), cefoxitin (30 μg), cephalaxin (30 μg), co-trimoxazole (25 μg), amoxicillin (30 μg), penicillin (10 μg), nalidixic acid (30 μg). In this research, antibiotic disks were supplied by HiMedia Labora-
tories Pvt. Ltd., Mumbai, India. Briefly, after preparation of bacterial suspension, the turbidity of each of them was adjusted equivalent to a No. 0.5 McFarland standard and then inoculated on Mueller-Hinton agar. After overnight incubation at 37 °C, diameters of inhibition zones were read and the results were interpreted as susceptible, intermediate, and resistant. The confirmed samples as E. coli were stored in tryptic soy broth (Merck, Germany) containing 20% glycerol at −70 °C and were subjected to further molecular identification.

2.6. DNA extraction and PCR of blaTEM, blaSHV and blaCTX-M genes

DNA was extracted from pure colony of an overnight growth of E. coli isolates on Luria-Bertani agar (Oxoid, UK) by using QIAamp DNA isolation columns (Qiagen, Hilden, Germany) according to the manufacturer’s procedure. The concentration of extracted DNA was assessed by spectrophotometer. E. coli isolates that included in the study were screened by PCR method and using specific oligonucleotide primers to determine blaTEM, blaSHV and blaCTX-M genes. Primer sequences and their size were used for the detection of blaTEM, blaSHV and blaCTX-M genes in this study, which are listed in Table 1.

The PCR reactions for detection of blaTEM, blaSHV and blaCTX-M genes were done in a total volume of 25 μL by using Master Mix Red, Taq DNA polymerase with MgCl₂, amplicon.
PCR program used in this study for amplification of blaTEM, blaSHV and blaCTX-M genes are presented in Table 2.

Table 2
PAC programs used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Program</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaTEM</td>
<td>5 min 94 °C initial denaturation 45 s 94 °C denaturation 30 s 54 °C annealing 1 min 72 °C extension 5 min 72 °C final extension</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaSHV</td>
<td>5 min 94 °C initial denaturation 45 s 94 °C denaturation 1 min 56 °C annealing 1 min 72 °C extension 5 min 72 °C final extension</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaCTX-M</td>
<td>3 min 94 °C initial denaturation 1 min 94 °C denaturation 30 s 58 °C annealing 1 min 72 °C extension 10 min 72 °C final extension</td>
<td>36</td>
</tr>
</tbody>
</table>

The amplicons were separated by 1.2% agarose gel electrophoresis at 80 V for 2 h. After electrophoresis, fragments were stained by ethidium bromide and visualized by using ultraviolet light. PCR products were sequenced. Then, nucleotide sequences were compared with sequences in the GenBank and European Molecular Biology Laboratory databases by using the BLAST.

2.7. Statistical analysis

The results were analyzed by using SPSS version 17.0 (SPSS Inc., Chicago, USA).

3. Results

In this study, from 330 urine specimens of hospitalized patients with UTI, 245 (74.2%) E. coli isolates were collected and 100 (40.8%) isolates of them were ESBL producer and recruited for the study. The mean age was 45 years old and the range of patient's age was from 11 months to 75 years old. There were 71 (71%) females and 29 (29%) males and the male: female ratio was 0.41. The occurrence of UTI was high in the age's group 36–60 years (59%) and also, between the age of 20 and 35 years (4%) was low and equal. A total of 25 (25%) patients had a history of UTI, and 32 (32%) patients had taken antimicrobial therapy. Over 63% of them had been treated with the β-lactam antibiotics. In this research, the same number of ESBL positive isolates were detected by both phenotypic methods of DDST and disk diffusion test.

The result of disk diffusion susceptibility testing of clinical samples revealed that all of the E. coli producing ESBLs were resistant to amoxicillin and penicillin. The susceptibility rates of isolates to other antibiotics were: co-trimoxazole 96%; ceftazidime 82%; cefotaxime 78%; cefoxitin 69%; gentamicin 65%; cephalexin 63%; nalidixic acid 61%; piperacillin 39%; aztreonam 33%. Most of isolates were highly susceptible to amikacin 74% and imipenem 91%. The results also showed that none of isolates were sensitive to all antibiotics. In vitro, the susceptibility of ESBL-producing strains to 14 antibiotics are shown in Figure 1.

Isolates resistant to at least three or more unrelated antibiotics were determined via multidrug-resistant[13]. All of isolates

![Figure 1](image1.png)

Figure 1. The suscpetibility pattern of 100 ESBL-producing E. coli isolates to 14 antimicrobial agents.
A: Amoxicillin; B: Penicillin; C: Co-trimoxazole; D: Cefazidime; E: Cefotaxime; F: Cefoxitin; G: Ciprofloxacain; H: Gentamicin; I: Cephalexin; J: Nalidixic acid; K: Piperacillin; L: Aztreonam; M: Amikacin; N: Imipenem.

![Figure 2](image2.png)

Figure 2. Detection of gene encoding blaCTX,M in ESBL-producing E. coli by PCR.
Lane M: Ladder (Fermentas, UK); Lanes 1–4: The 857 bp PCR product of blaCTX,M; Lane 5: Positive control (K. pneumoniae ATCC 700603); Lane 6: Negative control (E. coli ATCC 25922).
showed resistance to more than two antibiotics. Frequencies of multidrug-resistant to 3, 4, 5, 6 and more antibiotics were 7 (7%), 10 (10%), 8 (8%), 11 (11%) and 64 (64%).

The predominant resistant profile among our isolates consisted of 10 antibiotics amoxicillin, penicillin, co-trimoxazole, ceftazidime, cefotaxime, cefoxitin, ciprofloxacin, cephalixin, nalidixic acid and aztreonam (13%), followed by resistance to 8 antibiotics amoxicillin, penicillin, co-trimoxazole, ceftazidime, cefotaxime, cefoxitin, cephalixin and aztreonam (11%).

The PCR data of ESBL-producing strains revealed that bla<sub>CTX-M</sub> genes were the most frequent ESBL types (74%), followed by bla<sub>TEM</sub> (67%) and finally bla<sub>SHV</sub> (45%) respectively (Figures 2–4). Forty-five (45%) out of 100 isolates carried more than one type of β-lactamase genes. Coexistence of the bla<sub>CTX-M</sub> and bla<sub>TEM</sub> was detected in 22 isolates (22%), bla<sub>CTX-M</sub> and bla<sub>SHV</sub> in 12 isolates (12%), bla<sub>TEM</sub> and bla<sub>SHV</sub> in 8 isolates (8%) and bla<sub>CTX-M</sub>, bla<sub>SHV</sub> and bla<sub>TEM</sub> in 3 isolates (3%). Fifty-five isolates (55%) carried only one of ESBL genes.

4. Discussion

The increase of resistance to antibiotics and the existence of multidrug-resistant in ESBL producers have become an emerging public health problem due to the clinical failure of empirical treatment protocols<sup>[14]</sup>. Although the mechanism of resistance among ESBL-producing <i>E. coli</i> may be varied, it has been documented that the most common mechanism of resistance to β-lactam antibiotics in <i>E. coli</i> isolates is β-lactamase production.

The present study was conducted on 100 ESBL-producing <i>E. coli</i> isolates. The results exhibited that all of isolates were resistant to amoxicillin and penicillin and none of them were sensitive to all tested antibiotics. The lowest rates of resistance in ESBL-producing isolates were observed for imipenem 8% and amikacin 18%. This is an alarm for clinicians that the consumption and prescription of these antibiotics must be changed. Our finding is in accordance with recent data<sup>[15]</sup>.

In a study, there were 100 specimens of <i>E. coli</i> isolated from the urine samples and their resistance to antibiotics penicillin, amoxicillin, cephalixin, ceftriaxone and imipenem was 100%, 100%, 43%, 46% and 2% respectively<sup>[13]</sup>. These results exhibited a high frequency of resistance among <i>E. coli</i> isolates to the common antibiotics especially penicillin, amoxicillin and cephalosporins such as cefotaxime and ceftazidime which are used routinely in the treatment of UTIs. According to another study that was conducted by the European Antibiotic Resistance Surveillance System in 2006, continuous increase of resistant to third generation cephalosporins has been reported in over 800 laboratories from 31 countries<sup>[16]</sup>. Overall, due to indiscriminate use of antibiotics and low standard of personal and community hygiene, the percentage of <i>E. coli</i> resistance to chloramphenicol, ampicillin, tetracycline, aminoglycosides and sulphonamides in developing countries is higher than industrialized countries<sup>[17]</sup>.

In our study, the most age group infected with ESBL-producing <i>E. coli</i> was 36–60 years (59%). Our finding, regarding the age of the patients, is in accordance with recent data that state aging is a risk factor for β-lactamase-mediated resistance in patients infected with enterobacteria.

In recent years, ESBL-producing <i>E. coli</i> isolates have been increased frequently from different parts of the world particularly Asia<sup>[18]</sup>. In our study, the ESBL-producing <i>E. coli</i> isolates were 40.8% of all <i>E. coli</i> collected over a period of 11 months. The prevalence of ESBL-producing strains varies due to geographic region, studied country and institution. In this respect, several studies expressed that the ESBL production is much less frequent in Europe than in Latin America and Asia, and they are even less frequent in the Pacific than in North America<sup>[19]</sup>.

The frequency of ESBL-producing <i>E. coli</i> was 40.8% in our study, which was lower than that in Turkey (84%)<sup>[11]</sup>, Portugal (67.9%)<sup>[20]</sup>, India (66.7%)<sup>[20]</sup>, United Arab Emirates (41%)<sup>[21]</sup>, but was higher than those in Japan (20.4%)<sup>[22]</sup>, Kuwait (31.7%)<sup>[23]</sup>, Saudi Arabia (30.6%)<sup>[24]</sup>, Thailand (13.2%)<sup>[25]</sup>, and Colombia (11.7%)<sup>[26]</sup>. This may be due to the differences in the type of samples, in the time of sample’s collection, consumption of antibiotics and differences.
The rate of resistance to multiple antibiotics among ESBL-producing isolates is usually common due to carrying multi-resistant genes and plasmids. In the present study, our isolates were resistant not only to β-lactam but also to different antibiotic families including trimethoprim/sulfamethoxazole, fluoroquinolones and aminoglycosides. This obtained resistance pattern was similar to those commonly described in other studies.

As previously noted, the most prevalent types of ESBLs are SHV-, TEM- and CTX-M-type that arise mainly due to mutations in β-lactamase genes. We detected a variety of β-lactamases among the *E. coli* isolates namely, SHV, TEM and CTX-M. Although TEM and SHV variants were the most common ESBLs during the past decade, newly CTX-M β-lactamases have emerged as prevalent ESBL worldwide type compared to the TEM and SHV genotypes. Regarding the different types of ESBLs, as expected, CTX-M enzymes were the most common ESBL types (74%) followed by TEM (67%) and SHV (45%). The high prevalence of CTX-M gene in our study, we are in agreement with some earlier reports. In 2007, Seputiene et al. showed the high prevalence of CTX-M among *E. coli* (96%) and *K. pneumoniae* (71%) isolates, showing the ESBL phenotype. In a study which was done on 181 unduplicated *E. coli* strains isolated in nine different hospitals in three Portuguese regions in 2007, it showed that CTX-M producer strains were prevalent among UTIs (76%). Another study that was conducted in Turkey (2013) showed that CTX-M was the most prevalent β-lactamase among ESBL-producing *E. coli* isolates (83.18%). On the basis of our results, it can be argued that current ESBL epidemiology in our study is consistent with some neighboring countries and Europe. These higher rates of CTX-M among our isolates may be associated with high mobilization of the encoding genes. Barlow et al. reported increased tenfold in movement of β-lactamases from the community in Morocco. Our isolates were molecularly confirmed to have two or more ESBL enzymes as the prevalent ESBL type.

In several studies, the predominant genotypes of ESBLs were diverse. In studies performed in Italia (45.4%), Portugal (40.9%), and Turkey (72.7%), the most predominant ESBL genotype was TEM.

Our survey demonstrated a relatively high prevalence of SHV gene (45%), which was lower than Sweden (63%) and India (57.5%), but was higher than those in Thailand (3.8%), Saudi Arabia (23.1%) and Turkey (9.09%). The co-existence of different β-lactamases genes within the same isolates has been reported by several investigators. Our findings showed that about one-half of the ESBL-producing *E. coli* isolates were molecularly confirmed to have two or more ESBL genes. The most common ESBL genotype among our isolates was CTX-M and TEM (22%). This result is in agreement with findings of Harada et al. in Japan, Sharma et al. in India, and Hassan et al. in Saudi Arabian who reported TEM + CTX-M as the most common type.

Our study suggests that *E. coli* strains carrying multiple ESBL genes are widespread in Iran. CTX-M-producing *E. coli* strains are currently a problem in Iran which may be related to the misuse of third generation cephalosporins, especially cefotaxime. Therefore, isolation and detection of ESBL-producing strains are essential for the selection of the most effective antibiotic for treatment and also infection control. Overall, continuous monitoring and effective infection control measures, rational use of drugs and also the use of carbapenems instead of extended-spectrum cephalosporins, quinolones, and aminoglycosides for treating infections in which ESBL-producing strains are likely to emerge could be effective.

Conflict of interest statement

The authors report no conflict of interest.

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References


