Concurrent of blaCTX-M and blaNDM-1 genes in clinical isolates of Escherichia coli from northern Iran

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Abstract. The emergence of E. coli producing extended-spectrum β-lactamases and metallo β-lactamases has been reported as an important cause of treatment failure. The present study aimed to evaluate the existence of blaCTX-M, blaTEM, blaSHV, blaNDM-1 and blaIMP-1 genes among E. coli isolated from patients in Babol, Northern Iran. The pattern of antibiotic resistance and the prevalence of multidrug-resistant (MDR) E. coli isolates were determined. E. coli isolates were separated from clinical specimens and antimicrobial susceptibility test (AST) was performed using the disk diffusion method. These isolates were further evaluated for the production of ESBLs and MBLs enzymes using cefotaxime (CTX), ceftazidime (CAZ) disks with and without clavulanic acid, and two CAZ with 2 mercaptopropionic acid disks, respectively. The ESBLs and MBLs positive isolates were analysed for the existence of blaCTX-M, blaTEM, blaSHV, blaNDM-1 and blaIMP-1 genes with the conventional PCR method. Of 259 isolates, 117 β-lactamase producing E. coli were detected. MDR isolates were observed in 110/117 (94.9%) E. coli. Among 117 isolates, ESBLs, MBLs and coproduction of ESBL and MBL enzymes were observed in 45, 7 and 65 isolates, respectively. PCR analysis showed that the predominant genes were blaCTX-M-15 (95.5%) and blaNDM-1 (31.9%) among ESBL and MBL producing E. coli, respectively. Also, the concurrent occurance of the blaCTX-M with blaTEM, blaSHV, blaNDM-1 and blaIMP-1 genes were demonstrated. In conclusion, high prevalence rate of MDR isolates, particularly ESBL and MBL producing E. coli, observed in the current study shows the necessity of control and management strategies for the aforementioned isolates. Also, the early detection of concurrent ESBLs and MBLs producing E. coli is necessary to avoid treatment failure and prevent the distribution of such bacteria.

INTRODUCTION

Escherichia coli is the most prevalent species in clinical laboratories and is the main cause of enteric infections in humans. This species also cause extraintestinal infections such as urinary tract infections, sepsis and neonatal meningitis (Allocati et al., 2013). Therefore, it is considered as an important pathogen in health care settings. The main transmission route of enteric E. coli strains is fecal-oral, through the ingestion of contaminated food or water, and extraintestinal infections are mainly related to nosocomial and community-associated infections (Allocati et al., 2013).

Over the past few decades, antibiotic resistance development has been frequently reported among various microorganisms. Several reports indicate that E. coli strains resistant to the first line of antibiotics are prevalent throughout the world (Nepal et al., 2017). The resistance of E. coli against other antibiotics has become widespread which causes major challenges in the management of infectious diseases (Fair & Tor, 2014). Many investigations have been carried out to find the mechanisms of drug
resistance in bacteria, which show that the most common antimicrobial resistance mechanism is the production of hydrolytic enzymes, particularly β-lactamases (Singh-Moodley & Perovic, 2016; Moxon & Paulus, 2016; Shaikh et al., 2015). Extended-spectrum β-lactamases (ESBLs) are frequently reported in Enterobacteriaceae such as E. coli (Nepal et al., 2017) and other life-threatening bacteria like Pseudomonas spp. and Acinetobacter baumannii (Shaikh et al., 2015). Several types of ESBLs genes have been identified and the most prevalent gene types around the world are blaCTX-M type, blaTEM-type, and blaSHV-type (Dallenne et al., 2010; Shaikh et al., 2015).

Carbapenems are the last option for the treatment of infections caused by multidrug-resistant gram negative bacteria. These antibiotics are suggested for treatment of infectious diseases caused by ESBL producing bacteria (Ibadin et al., 2017; Wan Nor Amilah et al., 2012). Carbapenemases are another group of β-lactamases which are less frequent than ESBLs, however they have been reported in different regions throughout the world. Carbapenemase producing bacteria cause serious problems for the treatment of infectious diseases since they are usually highly resistant to the most β-lactams and other antibiotics considered for the treatment of multidrug-resistant (MDR) isolates. Metallo β-lactamases (MBLs) including the New Delhi metallo β-lactamase-1 (NDM-1) and IMP-1 are class B carbapenemases which are very important as they cause often resistance to all β-lactam antibiotics (Kost et al., 2017). The rate of MBL producing Enterobacteriaceae, particularly in E. coli, has rapidly increased around the world, which is considered a serious global public health problem (Nepal et al., 2017).

The concurrent presence of ESBLs and MBLs in bacteria limits the choices of antibiotic therapy and often leads to failure in treatment (Nepal et al., 2017). Moreover, the lack of a standard phenotypic test for the detection of MBLs in clinical laboratories increases the difficulty of diagnosis (Ibadin et al., 2017).

The prevalence of ESBLs and MBLs encoding genes in E. coli strains are different based on geographical location, therefore the current study was carried out to investigate distribution of blaCTX-M, blaTEM, blaSHV, blaNDM-1 and blaIMP-1 genes among E. coli, isolated from patients at Babol University of Medical Sciences affiliated Hospitals, in the North of Iran. Furthermore, antibiotic susceptibility of ESBL and MBL strains were determined.

**MATERIALS AND METHODS**

**Phenotypic methods**

In total, 259 E. coli isolates were obtained from clinical samples. Antimicrobial susceptibility test (AST) for cefotaxime (CTX), ceftazidime (CAZ), cefpodoxime (CPD), cefepime (CPM), ertapenem (ETP) and aztreonam (ATM), (Mast disks, UK) were performed using the disk diffusion method according to CLSI standard protocol (Shahandeh et al., 2015; Wanyne, 2014). Then, all isolates were examined for ESBLs and MBLs production using screening and confirmatory phenotypic methods as described below. For ESBL, all isolates which were resistant to CAZ and CTX (CAZ < 22 mm, CTX < 27 mm), were examined by CAZ and CTX disks with and without Clavulanic acid (CA). These isolates were also tested using CAZ disks alongside 2-mercaptopyropionic acid (2-MPA) disk to evaluate MBLs production (Shahandeh et al., 2015). The interpretation of these confirmatory methods was performed based on Wins et al., 2006 and Arakawa et al., 2000, respectively. In brief, for ESBLs producing E. coli, a considerable increase of growth inhibitory zone presents around the disks containing Clavulanic acid and CAZ or CTX, and for MBLs producer, a distinct growth inhibitory zone appears between the disk containing CAZ and the filter disk containing 2-MPA. No change is observed for non-ESBLs and non-MBLs
isolates (Figure 1). *E. coli*, ATCC25922, was used as a control strain.

An AST using cefoxitin (FOX), ciprofloxacin (CIP), cotrimoxazole (TS), and gentamicin (GM) was carried out for the bacteria which were confirmed positive for ESBLs and MBLs production by confirmatory phenotypic methods. Bacterial isolates which were resistant to at least three antibiotics of different families, were considered multi-drug resistance (MDR).

**DNA extraction, PCR and Sequencing**

Total DNA was extracted from isolates which were identified as ESBLs and MBLs producing bacteria using a commercial DNA extraction kit according to manufacturer’s instructions (Signage, Iran). Then, PCR analysis of *blaCTX-M*, *blaTEM*, *blaSHV*, *blaIMP-1* and *blaNDM-1* genes was performed. Details of the genes encoding β-lactamases and the primers pairs are listed in Table 1. The PCR conditions for *blaCTX-M*, *blaTEM*, *blaSHV*, *blaIMP-1* and *blaNDM-1* were adjusted according to previous publication (Dallenne et al., 2010; Shahcheraghi et al., 2013). *Klebsiella pneumoniae* ATCC 700603, *Acinetobacter baumannii* (Azizi et al., 2016) and *E. coli* K12 (Solgi et al., 2017b) were used as positive control strains for *blaCTX-M*, *blaTEM*, *blaSHV*, *blaIMP-1* and *blaNDM-1* genes, respectively.

PCR products were electrophoresed on a 2% agarose gel and the gels were evaluated under UV light and photographed by gel documentation system (Vilber, Lourmat, France). Furthermore, the PCR product of selected amplicons for each gene was subjected to sequencing with forward primers (Bioneer Company, South Korea). The sequencing results were evaluated with Chromas (v.2.6.4) software and BLAST analysis (www.ncbi.nlm.nih.gov/blast).

**Ethical consent**

This work was approved by the ethical committee of Babol University of Medical Sciences, Babol, Iran (Ethical number: MUBABOL.REC.1394.223).

**RESULTS**

One-hundred and seventeen out of 259 (45.2%) isolates were recognised as ESBL and MBL producing bacteria by confirmatory phenotypic methods (Figure 1). Of 117 isolates, 45(38.4%) produced only ESBLs and 7(6%) produced only MBLs. The concurrence of ESBL and MBL production

![Figure 1](image-url)
was observed in 65 (55.6%) isolates. AST results showed that 109 out of 117 (93.2%) 
*E. coli* isolates were resistant to CPD and CTX. Ninety-three out of 117 (79.5%) were 
susceptible to ETP. In total, 111 out of 117 (94.9%) isolates were detected as MDR 
bacteria (Table 2).

PCR analysis revealed that the predominant ESBL gene was *bla*CTX-M-15 [105/110(95.5%)] followed by *bla*TEM-1 [48/110 (43.6%)] and *bla*SHV-33 [8/110 (7.3%)] in ESBL producing *E. coli* isolates. Forty-one and eight isolates carried two and three genes simultaneously, in that order. Figure 2 shows the PCR analysis of CTX-M, SHV, TEM, and NDM-1 genes from *E. coli* isolates.

The *bla*CTX-M-15 gene was exclusively detected in 51.8% (57/110) isolates and a 
combination of this gene with the other studied genes was observed in 58/110 (52.7%) isolates (Table 3). All isolates which were positive for *bla*CTX-M-15 gene were 
resistant to at least one of the following antibiotics in addition to the *β*-lactam 
group: GM, CIP, and TS. Fifty-three (50.5%, 53/105) isolates were considered MDR as 
a result of resistance to the aforementioned antibiotics.

PCR analysis of 72 MBLs producing *E. coli* showed the existence of *bla*NDM-1 and *bla*IMP-1 genes among 21 (29.2%) and 6 (8.3%) isolates, respectively. Also, 2/72 (2.8%) isolates were positive for *bla*NDM-1
Table 3. The frequency of blaCTX-M-15, blaTEM-1 and blaSHV-33 genes in ESBL-producing E. coli (110 isolates)

<table>
<thead>
<tr>
<th>Genes</th>
<th>blaCTX-M-15</th>
<th>blaTEM-1</th>
<th>blaSHV-33</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaCTX-M</td>
<td>57 (51.8%)</td>
<td>40 (36.4%)</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>blaTEM</td>
<td>40 (36.4%)</td>
<td>1 (0.9%)</td>
<td>7 (6.3%)</td>
</tr>
<tr>
<td>blaSHV</td>
<td>1 (0.9%)</td>
<td>7 (6.3%)</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>98 (89.1%)</td>
<td>48 (43.6%)</td>
<td>8 (7.3%)</td>
</tr>
</tbody>
</table>

Figure 2. Gel electrophoresis of PCR products of blaCTX-M, blaSHV, blaTEM, and blaNDM-1 genes from E. coli isolates. Lanes: M, 50 bp DNA size marker; 1&5, CTX-M(500 & 550 bp); 2, blaCTX-M negative isolate; 3&4, blaSHV positive (713 bp) and negative isolates; 6&7, blaTEM negative and positive isolates (800 bp); 8, blaNDM-1 positive isolate.

and blaIMP-1 genes concurrently. Twenty-one out of 29 (72.4%) isolates which carried blaNDM-1 or blaIMP-1 genes, were susceptible to ETP.

The nucleotide sequences of blaCTX-M-15, blaTEM-1, blaSHV-33 and blaNDM-1 genes were deposited in the Gene Bank database under accession numbers. MG770114, MG745167, MG745166, MG770113 and MG995853.

DISCUSSION

Recent studies on drug resistance mechanisms against β-lactam antibiotics in bacteria have demonstrated that the production of β-lactamase is the most important strategy involved in this phenomenon (Ibadin et al., 2017; Sadeghi et al., 2016). In the current study, high resistance rates (88.1%) to third and fourth generation cephalosporins were observed while resistance to ertapenem was low (11.1%) (Table 2). These results are supported by other studies, which found that the resistance rate to cephalosporins was 76.8% and 94.8%, whereas resistance to ertapenem was 13.3% and 1.9% in Azarbaijan, Iran (Sadeghi et al., 2016) and Lebanon (Dandachi et al., 2016), respectively.

Our findings showed that 94.9% of the studied isolates were MDR. Different prevalence rates of MDR are reported
among various bacteria throughout the world (Ibadin et al., 2017; Nepal et al., 2017). 

blaCTX-M-15 and blaCTX-M-14, are among the most important blaCTX-M genes in bacterial species affecting humans and animals, which have worldwide distribution (Allocati et al., 2013). In the present study, PCR analysis showed that the most prevalent gene in this region was blaCTX-M-15 (95.5%). Several studies indicate that blaCTX-M-15 is the most prevalent gene among ESBL producing bacteria (Araque & Labrador, 2018; Khaleque et al., 2017; Sadeghi et al., 2016) but this is in contrast with other studies indicating that the blaCTX-M gene has low prevalence in Europe (Dallenne et al., 2010; Karanika et al., 2016; Valenza G, 2014). This dissimilarity may result from the differences in geographical regions and health care systems. According to Allocati et al. (2013), possible explanations for the high prevalence of CTX-M are as follows: (1) the insertion of blaCTX-M gene in plasmids and transposons results in rapid transmission between several kinds of bacteria; (2) the concomitance of this gene alongside other genes which are responsible for resistance to aminoglycosides and fluoroquinolones (Allocati et al., 2013). In line with previous studies, our study showed that more than 50% of the E. coli isolates, which were resistant to ammoglycosides and fluoroquinolones, carry the CTX-M-15 gene. In addition, the frequency of concurrent blaCTX-M and blaTEM genes obtained in the present study was higher than the surveys conducted by Haghhighatpanah et al., in Iran and Singh et al., in India. However the frequency of coexistence between blaCTX-M, blaTEM and blaSHV genes was lower than those studies (Singh et al., 2012).

The last option of β-lactam antibiotics for the treatment of infections caused by resistant bacteria are carbapenems (Ibadin et al., 2017). Recently, the emergence of MBLs producing bacteria isolated from clinical specimens and their distribution throughout the world has raised a major public health concern (Nepal et al., 2017). One important MBLs is the New Delhi metallo-β-lactamase (NDM-1) and its variants (Khan et al., 2017). The present study showed that the prevalence of E. coli encoding the blaNDM-1 gene was high in our region (23 out of 72, 31.9%). In Iran, the first report of NDM-1 producing K. pneumoniae isolate was reported in 2013 (Shahcheraghi et al., 2013). Subsequently, the blaNDM-1 gene was detected in 6 of 40 (15%) (Shahcheraghi et al., 2017), 17 of 54 (31.5%) of Enterobacteriaceae isolates (Solgi et al., 2017a), and 4 of 170 (2.4%) of K. pneumoniae isolates (Shoja et al., 2017) in Iran.

Our findings demonstrated that the studied isolates were resistant to a broad range of antimicrobial agents. The coexistence of blaCTX-M-15 with blaNDM-1 and blaIMP-1 genes was observed in 22 out of 65 (33.9%) and 6 out of 65 (9.2%) of the E. coli isolates, respectively. The coexpression of ESBLs and MBLs in Enterobacteriaceae, particularly in E. coli, and K. pneumoniae isolates, has been reported by several studies (Shoja et al., 2017; Solgi et al., 2017a). However, ESBLs producing isolates like E. coli are resistant to other families of drugs such as aminoglycosides, cotrimoxazole, tetracycline and fluoroquinolones in addition to β-lactam antibiotics (Tewari et al., 2018). Furthermore, MBLs producing bacteria are usually resistant to aminoglycosides and fluoroquinolones (Ibadin et al., 2017). On the other hand, E. coli is the most common gram negative bacilli isolated from clinical specimens (Dumaru et al., 2019). Therefore, the cohabiting of different β-lactamase encoding genes causes therapeutic problems to clinicians as there are limited treatment choices (Ibadin et al., 2017). In addition, the production of these enzymes are largely undetected by routine tests (Ibadin et al., 2017). Our results showed that 72.4% of isolates which carried MBLs genes, were susceptible to ertapenem using the AST method which is an important result obtained from the present study. This is supported by results obtained by Singh-Moodley and Perovic, 2016 which found that the blaNDM gene in some Enterobacteriaceae...
bacteriaceae isolates were susceptible to ertapenem by the AST method (Singh-Moodley & Perovic, 2016).

CONCLUSION

The current study demonstrated that the prevalence of ESBLs and MBLs producing MDR E. coli isolates are high in the studied region. Also, the high co-occurrence of blaCTX-M-15 with blaTEM-1 and blaNDM-1 genes are alarming and suggest that these genes may rapidly distribute throughout our country. Furthermore, isolates carrying the NDM-1 and IMP-1 genes were susceptible to ertapenem, which demonstrates the necessity of a new phenotypic method for the accurate detection of these bacteria. These findings indicate the need for the establishment of adequate strategies to control the increase of ESBLs and MBLs producing bacteria.

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Conflict of interest
There are no conflict of interest.

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