

# Phenotypic and molecular characterization of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* isolates in Shiraz, Iran

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## ABSTRACT

**Introduction:** The aim of this study was to detect the prevalence of the extended-spectrum beta-lactamase (ESBL)-encoding CTX-M gene in *Escherichia coli* isolates. **Methods:** Phenotypic screening of 376 *E. coli* isolates for ESBL was conducted using disk diffusion. ESBL-producing isolates were tested using PCR and specific primers. The *bla*<sub>CTX-M</sub> cluster was identified using the RFLP method, and its genotype was sequenced. **Results:** From 202 ESBL-producing *E. coli*, 185 (91.5%) possessed CTX-M genes. CTX-M-1 subtypes were found in 98% of the isolates. The *bla*<sub>CTX-M</sub> gene was identical to CTX-M-15. **Conclusions:** A high prevalence of CTX-M-1-producing *E. coli* apparently exists in Shiraz, Iran.

**Keywords:** *Escherichia coli*. CTX-M gene. Iran.

*Escherichia coli*, one of the most common intestinal commensals, causes various hospital- and community-acquired infections<sup>(1)</sup>. Specifically, the pathogen can cause intestinal and extra intestinal infections such as gastroenteritis, urinary tract infection, and meningitis<sup>(1) (2)</sup>. *Escherichia coli* strains utilize several mechanisms of antibiotic resistance, one of which is beta-lactamase production<sup>(3)</sup>. The extended-spectrum beta-lactamases (ESBLs) are a group of beta-lactamases with the ability to hydrolyze the extended-spectrum cephalosporin and monobactam antibiotics<sup>(4)</sup>. The extended-spectrum beta-lactamases were first detected in *Klebsiella* spp., and then later identified in *E. coli* and other species of *Enterobacteriaceae*<sup>(3)</sup>. These enzymes are divided into several main groups [e.g., sulphhydryl variable (SHV), temoneira (TEM) and cefotaximases (CTX-M)]<sup>(5) (6)</sup>. Until the year 2000, SHV and TEM types were thought to be the most prominent ESBLs; however, since 2000, CTX-M types have emerged as new forms of ESBL that, unlike TEM and SHV, are more active against cefotaxime and ceftriaxone rather than ceftazidime<sup>(3)</sup>.

The CTX-M family includes more than 50 beta-lactamase enzymes that are divided into five main groups based on similarities in amino acid sequence, i.e., CTX-M-1, CTX-M-2,

CTX-M-8, CTX-M-9, and CTX-M-25<sup>(7)</sup>. All CTX-M enzymes contain a serine at position-238 that plays a significant role in increasing beta-lactamase activity<sup>(5)</sup>.

CTX-M-15-producing *E. coli* strains have now emerged in many countries and are a major cause of community-acquired infections, especially those that involve urinary tract infection (UTI)<sup>(4)</sup>. Furthermore, the *E. coli* ST131 and ST405 clones, which are part of virulence phylogenetic groups D and B2 respectively, are associated with the spread of CTX-M-15<sup>(4) (8)</sup>. Because CTX-M-producing *E. coli* is widespread among various communities, hospitals, and environments, the treatment of patients exposed to this strain should be considered carefully in order to prevent the spread of CTX-M-producing antibiotic-resistant strains<sup>(3) (5)</sup>. Furthermore, because these strains are also multidrug-resistant, it is essential that epidemiological studies be conducted to enable identification and treatment of the infections they cause<sup>(8) (9)</sup>. In the present study, our aim was to detect the prevalence of the CTX-M gene and its subtypes in clinical *E. coli* isolates collected from four hospitals in Shiraz, Southwest Iran.

This study was conducted from January to June, 2012, and involved analysis of 376 *E. coli* clinical samples from urine, stool, blood, wound, and sputum (Table 1), which were collected from Nemazee, Faghihi, Ali Asghar, and Dastgheib hospitals affiliated with Shiraz University of Medical Sciences, Shiraz, Iran. Bacteria were identified in samples by inoculating the clinical specimens into conventional culture media such as TSI, SIM, Simmons' Citrate Agar, MR/VP (Merck, Germany), and using the API 20E kit (bioMérieux, France). The bacterial isolates were then stored at -70°C in tryptic case soy broth containing 20% glycerol. Fresh subcultures were used for testing.

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**TABLE 1 - Distribution of *bla*<sub>CTX-M</sub> genes in ESBL-positive *Escherichia coli* isolated from hospital patients in Shiraz, Iran.**

Sample	Total number	ESBL (+)	<i>bla</i> <sub>CTX-M</sub>
Urine	303	156	145
Blood	17	9	8
Sputum	11	8	7
Wound	5	2	1
Stool	7	3	2
Fluid	7	6	5
Abdominal fluid	6	4	4
Other samples	20	14	13
<b>Total</b>	<b>376</b>	<b>202</b>	<b>185</b>

ESBL: extended-spectrum beta-lactamase. CTX-M: cefotaximases.

Antimicrobial susceptibility of the identified isolates was determined by using the disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI)<sup>(10)</sup>. Eight antimicrobial agents were tested: ceftazidime, ceftriaxone, cefotaxime, cefepime, ceftizoxime, aztreonam, amikacin (30µg of each agent), and ciprofloxacin (5µg) (Rosco, Denmark). Production of ESBL in the isolates was determined by using the combination disk method with antibiotic disks containing ceftazidime, cefotaxime, and cefepime (30µg per antibiotic) either alone or in combination with clavulanic acid (10µg) (Rosco, Denmark). The results were interpreted as previously described<sup>(10)</sup>. Quality control for the production of ESBL was assessed using *E. coli* ATCC 25922 (negative control) and *Klebsiella pneumoniae* ATCC 7881 (positive control).

Deoxyribonucleic acid was extracted from ESBL-positive isolates using an AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions, and extracted DNA was stored at -20°C.

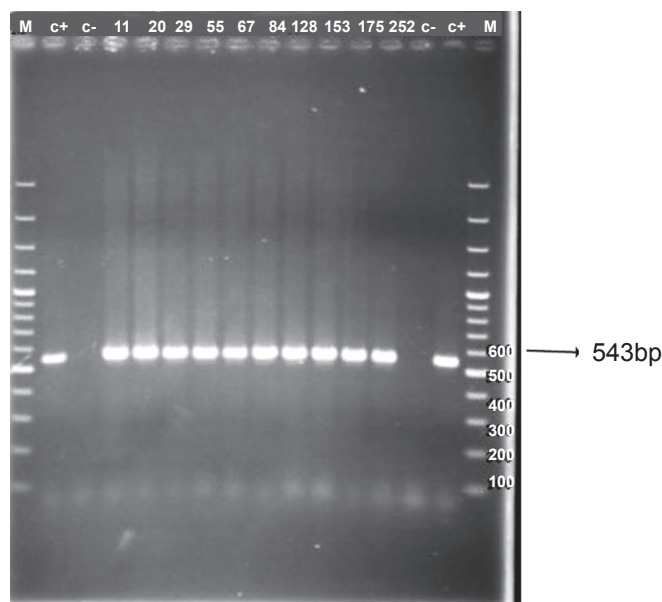
All 202 ESBL-positive isolates were screened for the presence of *bla*<sub>CTX-M</sub> by using polymerase chain reaction (PCR) with previously published primers and methods<sup>(11)</sup>. For PCR amplifications, 2µL of DNA was added to a 50-µL solution containing 1µL of deoxynucleotide triphosphates (dNTPs), 1.5µL MgCl<sub>2</sub>, 2µL of each primer, and 0.3µL of Taq polymerase (CinnaGen, Iran) in 1x PCR buffer. An Eppendorf Thermocycler (Germany) was used for amplification, with cycling parameters comprising an initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 30s, annealing at 51°C for 40s, amplification elongation(extension) at 72°C for 30s, and a final extension at 72°C for 3 min.

The amplified CTX-M product (10µL) was directly subjected to digestion with 9U PST-I and 4U Pvu-II enzymes (Fermentas, Lithuania) in One-Phor-All Plus Buffer (10mMTris acetate; pH 7.5), 10mM magnesium acetate, and 50mM potassium acetate for 18h at 37°C. The restriction fragments of the PCR

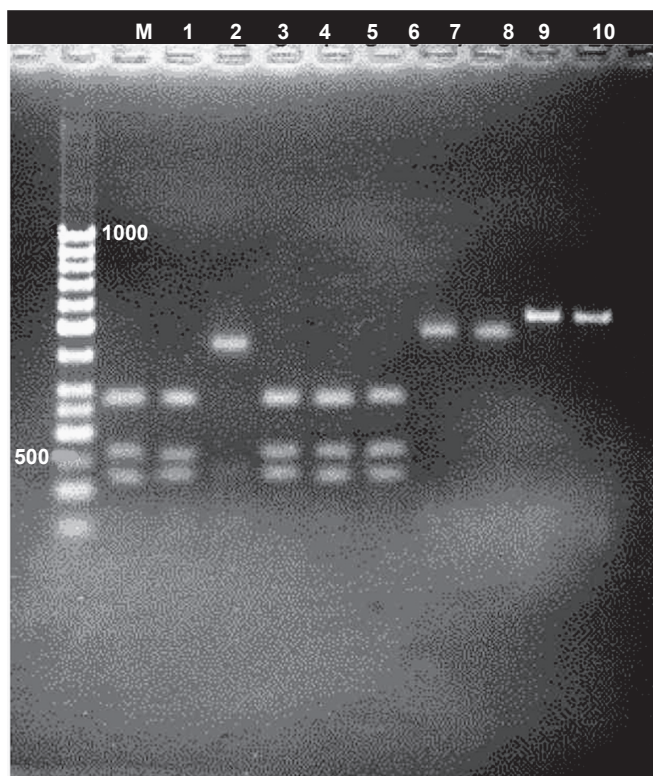
products were analyzed by electrophoresis in 3% agarose containing ethidium bromide (Imogene, Iran). Six PCR products were randomly selected and purified using the AccuPrep® Gel Purification Kit (Bioneer, Korea). They were subsequently sent to Bioneer Corp., Korea, for sequencing (GenBank accession numbers: KC465234/1, KC465233/1, and KC465232/1). The relationship between the presence of the *bla*<sub>CTX-M</sub> gene and antibiotic susceptibility was analyzed using non-parametric tests (chi-squared test), with analysis conducted in Statistical Package for the Social Sciences (SPSS) v18 software.

In total, 376 *E. coli* specimens were confirmed. These included 303 (80.6%) urine, 17 (4.5%) blood, 11 (2.9%) sputum, 7 (1.9%) stool, 7 (1.9%) fluid, 6 (1.6%) abdominal fluid, 5 (1.3%) wound, and 20 (5.3%) other specimens (**Table 1**). Of these, 211 and 165 isolates were recovered from inpatient and outpatient settings, respectively. Following isolation and confirmation by biochemical tests using the API-20E kit, the antimicrobial susceptibility tests of the 376 *E. coli* specimens showed that the highest and lowest resistance was due to ceftriaxone (57.4%) and amikacin (13%), respectively. Resistance to the following antibiotics was also detected: cefotaxime (57.4%), aztreonam (56.1%), ceftazidime (50%), ciprofloxacin (48.1%), ceftizoxime (42%), and cefepime (37.5%).

Of the 202 (53.7%) *E. coli* isolates with an ESBL phenotype, 185 (91.5%) strains carried *bla*<sub>CTX-M</sub> genes (**Figure 1**). The frequency of CTX-M genes in inpatients and outpatients was 122 (65.9%) and 63 (34.1%), respectively. In total, 182 (98.4%) CTX-M-producing isolates belonged to the CTX-M-1 group (**Figure 2**). A randomly selected *bla*<sub>CTX-M</sub> gene was sequenced and was shown to be identical to CTX-M-15.



**FIGURE 1 - DNA genomic patterns of PCR products measured by electrophoresis. Lane 1: DNA size marker; Lanes 2 and 3: positive (*Klebsiella pneumoniae* ATCC 7881) and negative (*Escherichia coli* ATCC25922) controls. Lanes 11 to 252 represent *bla*<sub>CTX-M</sub> genes (543bp). DNA: deoxyribonucleic acid; PCR: polymerase chain reaction.**



**FIGURE 2** - RFLP patterns obtained using *Pst*I and *Pvu*II restriction endonucleases (Lanes 1-8). Lanes 9 and 10: undigested 543-bp PCR products. RFLP: restriction fragment length polymorphism; PCR: polymerase chain reaction.

Extended-spectrum beta-lactamases produced in *E. coli* account for the most common forms of antibiotic resistance against extended cephalosporins and monobactams<sup>(6)</sup>. Cefotaximases is now identified as a predominant ESBL that is increasingly disseminated among clones of *E. coli*, and it creates numerous problems for treating infections caused by this bacterium<sup>(5)</sup>. Although strains that produce the CTX-M enzyme are often detected in hospital infections, the acquisition of these strains from community settings has increased substantially over recent years<sup>(2)</sup><sup>(7)</sup>. The increased prevalence of such infections may be due to the indiscriminate use of extended-spectrum cephalosporins and other beta-lactams. Moreover, transmission and prevalence of the *bla*<sub>CTX-M</sub> gene could be attributed to several causes, which include the use of contaminated medical equipment, prolonged hospitalization of patients, low quality hospital hygiene and infection control systems, transmission through animal products, contact with repositories of CTX-M-producing bacteria, dissemination via the hands of medical personnel, recombination of the *bla*<sub>CTX-M</sub> gene with insertion sequences and transposons, and the spread of other bacteria such as *Kluyvera* spp. and *Klebsiella* spp.<sup>(3)</sup><sup>(8)</sup>.

Our results suggest that CTX-M type beta-lactamases are widespread in Shiraz, Iran. In previous studies, CTX-M production was significantly associated with resistance to fluoroquinolones and amino glycosides, and decreased susceptibility to cefotaxime and ceftriaxone led to a high

prevalence of multi-drug resistant strains<sup>(3)</sup><sup>(4)</sup>. In our study, 125 (67.5%) of the 185 identified CTX-M-producing isolates were resistant to ciprofloxacin ( $p < 0.001$ ).

Our results indicated that *E. coli* isolates were more susceptible to ceftazidime, ceftizoxime, and cefepime than they were to cefotaxime and ceftriaxone: a finding that is consistent with other reports<sup>(7)</sup>. Cefotaxime and ceftriaxone are the third generation cephalosporins most commonly used for treating nosocomial and community acquired infections in Shiraz hospitals. Furthermore, 13% and 48.1% of the isolates from this study were resistant to amikacin and ciprofloxacin, respectively. Therefore, our results suggest that ESBL-producing strains in Shiraz have become strongly resistant to antibiotics such as ciprofloxacin. Nevertheless, amikacin remains an effective drug that could still be prescribed to treat infections caused by *E. coli*. The prevalence of ESBL-producing bacteria, according to the reports from other countries, differs in various geographical regions<sup>(12)</sup>.

In our study, the prevalence of ESBLs was 53.7%, which is consistent with the results of other reports in Iran (e.g., Mirzaee et al.)<sup>(1)</sup>. indicated that 135 of 250 strains were ESBL-producing<sup>(1)</sup>. However, 91.5% of ESBL-producing strains in our study contained the *bla*<sub>CTX-M</sub> gene, which is higher than the rates reported by other investigators in Iran<sup>(1)</sup><sup>(13)</sup><sup>(14)</sup>. Our analysis of samples using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method indicated that more than 98% of isolates contained CTX-M-1 subtypes from the CTX-M group; this is in agreement with reports of the predominant subgroup in most other countries and other areas of Iran<sup>(1)</sup><sup>(14)</sup><sup>(15)</sup>. The restriction patterns of the CTX-M-1 subtypes were similar, with restriction bands observed at 267bp, 156bp, and 120bp. According to Shahcheraghi et al.<sup>(15)</sup> and Sultan Dallal et al.<sup>(14)</sup>, CTX-M-1 is the predominant gene among Iranian isolates; after sequencing, 98% of randomly selected samples were found to be similar to CTX-M-15 reported from most countries<sup>(4)</sup>. CTX-M-15 was found in three sequenced isolates and it may be the most common group 1 type in the Shiraz geographic region. This CTX-M type is also relatively common among European isolates and has been responsible for community outbreaks of multi-drug resistant *E. coli* infections in the United Kingdom and elsewhere<sup>(5)</sup><sup>(9)</sup>.

In conclusion, this study demonstrated significant differences in susceptibility to various antibiotics among CTX-M-producing *E. coli* isolated in Shiraz, Iran, and a high prevalence of *bla*<sub>CTX-M-1</sub> beta-lactamase was observed in these isolates. Therefore, similar studies are necessary to fully determine the frequency of the *bla*<sub>CTX-M</sub> gene and to identify the role of broad-spectrum cephalosporins in the treatment of infections caused by multi-drug resistant CTX-M-producing *E. coli*.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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