

Original Article

Prevalence of *bla*_{CTX-M} Gene in Multi-Resistant *Escherichia coli* Isolated from Urinary Tract Infections, Tehran, Iran

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Abstract

Background: The emergence and increase in the incidence of Extended-spectrum beta lactamase (ESBL) producing *Escherichia coli* (*E. coli*) has become an emerging challenge especially in hospitalized patients with urinary tract infection (UTI). The aim of the present study was to survey the frequency of *bla* CTX-M genotype in ESBL producing *E. coli* isolated from hospitalized patients with urinary tract infection and determination of their antibiotic resistance pattern.

Materials and Methods: A total of 135 *E. coli* isolates were collected and isolated from patients with UTI. The isolates were subjected to confirmatory phenotype tests for the presence of ESBL. 75 *E. coli* isolates were confirmed as ESBL-positive by double disc synergy test. In vitro susceptibility of ESBL isolates to 15 antimicrobial agents amoxicillin, penicillin, ceftazidime, cefotaxime, ceftiofur, ceftriaxone, cefixime, cephalexin, co-trimoxazole, gentamicin, nalidixic acid, ciprofloxacin, nitrofurantoin, amikacin, and imipenem was performed by Kirby-Bauer's Disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2012) guideline. PCR method was used to identify *bla* CTX-M gene in 75 ESBL positive strains.

Results: PCR and sequence analysis showed that 75 (55.5%) isolates produced *bla* CTX-M genes. In vitro susceptibility of ESBL producing *E. coli* showed that all of them were resistant to amoxicillin and penicillin. The rates of resistance to the majority of tested antibiotics varied among 61% to 100 %, with the exception of amikacin (14.7%) and imipenem (2.7%). Our results showed that the frequency of *bla* CTX-M was strikingly high (93.3%) in patients with UTI.

Conclusion: These data confirmed that the frequency of *bla* CTX-M genes was high among *E. coli* isolated from patients with UTI. The trend of multidrug-resistant profile has been associated with *bla* CTX-M gene is alarming. Therefore, it is very important to establish a routine screening of ESBL in clinical isolates to prevent dissemination of resistant isolates in health care settings.

Keywords: ESBL, Beta lactamase, *Escherichia coli*, antimicrobial resistance

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Introduction

Extended-spectrum beta lactamase (ESBL) producing *Enterobacteriaceae* were reported in Europe and subsequently United States in the early 1980s. They have been detected in *Klebsiella* species and later in *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* and *Serratia marcescens* and other genera of the *Enterobacteriaceae* family¹. The emergence of ESBL, as an important cause of transferable multidrug resistance in gram-negative bacteria, particularly in *E. coli* is a global health problem since 1995^{2,3}. ESBLs are a heterogeneous group of enzymes that confer resistance to 3 and 4 generation of cephalosporins and monobactams⁴. The production of beta lactamase as a predominant cause of resistance to beta lactam antibiotics among bacteria is mostly mediated by acquisition of beta lactamase genes which is located on mobile genetic elements such as plasmids or transposons. According to the scheme of Ambler et al., ESBLs are grouped into four classes A, B, C and D on the basis of their amino acid sequences⁵. In comparison of other classes, classes A and C are the most common classes. Class A ESBLs hydrolyzes oxymino-cephalosporins and aztreonam and generally susceptible to clavulanate, sulbactam, tazobactam as beta lactamase inhibitors⁴.

At present, there are more than 400 ESBLs that have been clustered into nine different structural and evolutionary families based on amino acid sequence⁶. The most ESBLs can be divided into 3 genotypes: temoneira (TEM), sulfhydryl variable (SHV) and cefotaximase (CTX-M)^{6,7}. CTX-M, TEM and SHV are class A ESBLs^{1,4,6}.

The CTX-M beta lactamases are the most widespread enzymes. They were initially reported in the second half of 1980s. Rate of their disseminations in both over wide geographic areas and among a wide range of clinical bacteria has rapidly increased since 1995⁸. Several investigators believed that CTX-M ESBL was dominant type in east Asia since it has appeared or caused outbreaks in many countries^{4,9}.

Before 2000, the predominant ESBL genotypes were TEM and SHV variants which were produced by *Klebsiella spp.*, *Enterobacter spp.*, and *E. coli* but during the past few years, the nature of ESBL

dissemination has changed and *E. coli* strains expressing CTX-M have presently replaced TEM and SHV as the most common type of ESBLs¹⁰.

Moreover, CTX-M-type ESBLs have emerged within the community, particularly among *E. coli* and *K. pneumoniae* isolated from urinary tract infections (UTIs), with a widespread prevalence and multidrug resistance in many worldwide countries⁴.

The emergence of ESBL as an important cause of transferable multidrug resistance in gram-negative bacteria, particularly *Escherichia coli* is now a serious problem of public health worldwide. However, there are still few reports on the prevalence of ESBL producing *E. coli* in the hospitals of Iran. With this background, we investigated the prevalent ESBL type of *E. coli* isolated from patients with UTI and then determination of their pattern of antimicrobial resistance to antimicrobial agents had commonly been used.

Methods

Bacterial isolates: A total of 75 ESBL-producing *E. coli* were recovered from 135 patients with UTI during August 2013 to April 2014. Urine samples were obtained from a midstream into standardized, sterile a period of 9 containers and delivered to the laboratory within 2 hours after had been collected. Identification was done based on culture characteristics, gram stain and routine standard biochemical tests. Colony count method was done according to surface streak procedure by using calibrated loops. The cultured plates were incubated in aerobic conditions at 37°C for 24-48 hours. The result of equal or more than 10⁵ CFU/mL was considered as positive UTI and a less than 10² CFU/ml was interpreted as negative UTI. The result of 10²-10⁴ CFU/ml was repeated¹¹.

Screening ESBL producing isolates by phenotypic method: ESBL production was confirmed phenotypically by double disc synergy test (DDST) according to the Clinical and Laboratory Standards Institute (CLSI) criteria for ESBL screening¹². According to the CLSI protocol, DDST was done by using cefotaxime (30 µg) and ceftazidime (30 µg) with and without clavulanic acid (10 µg). Discs were placed 25 mm apart from each other on Muller-Hinton agar (MHA, Oxoid, UK) plate inoculated with 0.5

McFarland suspension of the tested isolates. 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 ESBL¹³. *E. coli* ATCC 25922 was used as control strain.

Antimicrobial susceptibility testing: The susceptibility testing of the ESBL-producing *E. coli* isolations to antibiotics were examined by Kirby-Bauer's disk diffusion method according interpretive criteria recommended by CLSI guidelines. Antibiotic susceptibility test was carried out on Mueller Hinton agar (MHA, Oxoid, UK) to the following agents: Gentamicin (GEN 10 µg), ciprofloxacin (CIP 5 µg), amikacin (AK 30 µg), ceftazidime (CZX 30 µg), imipenem (IMP 10 µg), cefotaxime (CTX 30µg), cefoxitin (CX 30 µg), cephalixin (CN 30 µg), co-trimoxazole (COT 25 µg), amoxicillin (AMX 30 µg), penicillin (P 10 µg), nalidixic acid (NA 30 µg), nitrofurantoin (NIT 300 µg), ceftriaxone (CTR 30 µg) and cefixime (CFM 5 µg). Antibiotic disks used in this research were supplied by HiMedia Laboratories Pvt, Ltd., Mumbai, India.

Briefly, the bacterial suspension equivalent to a no. 0.5 McFarland standard was inoculated on Mueller-Hinton agar (Oxoid, UK) and then antibiotics were placed at distances of 22 mm apart. After 24h incubation at 37°C, diameter of inhibition zones was read and their results were interpreted as susceptible, intermediate, and resistant. *E. coli* ATCC 25922 was used as control strain. Samples confirmed as *E. coli* were stored in

Tryptic Soy Broth (TSB; Merck, Germany) containing 20% glycerol at -70°C and were subjected to further molecular identification.

DNA extraction and identification of *bla* CTX-M gene by PCR: Confirmed ESBL producing isolates with the combination disk diffusion test were characterized for the presence of CTX-M beta lactamase. Hence, DNA was extracted from bacterial cells by using QIAamp DNA isolation columns (Qiagen, Hilden, Germany) according to the manufacturer's procedure. The concentration of extracted DNA was assessed by spectrophotometer. The PCR reactions for detection *bla* CTX-M genes were done within a total volume of 25 µL. The mixture of reaction contained 1x buffer (10 mM Tris-HCl, 50 mM KCl), 0.2µM of each deoxynucleoside triphosphate, 1 mM MgCl₂, 0.5 µM of forward and reverse primers of CTX-M genes, and 1 Unit of Takara Taq (Takara Shuzo Co., Ltd., Shiga, Japan). The Primer sequences, which were used for detection of *bla* CTX-M genes in this study, were as follows: CTX-M-F (5'-ACGCTGTTGTTAGGAAGTG-3') and CTX-M-R (5'-TTGAGGCTGGGTGAAGT-3'). PCR conditions for amplification of 857 bp fragment of the CTX-M gene was carried out by the thermocycler (AG 22331; Eppendorf, Hamburg, Germany) as follows: initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 58°C for 30 second, and extension at 72°C for 1 min, was repeated for 36 cycles; a final extension at 72°C for 10 min.

Agarose gel electrophoresis was done a 1.2% agarose

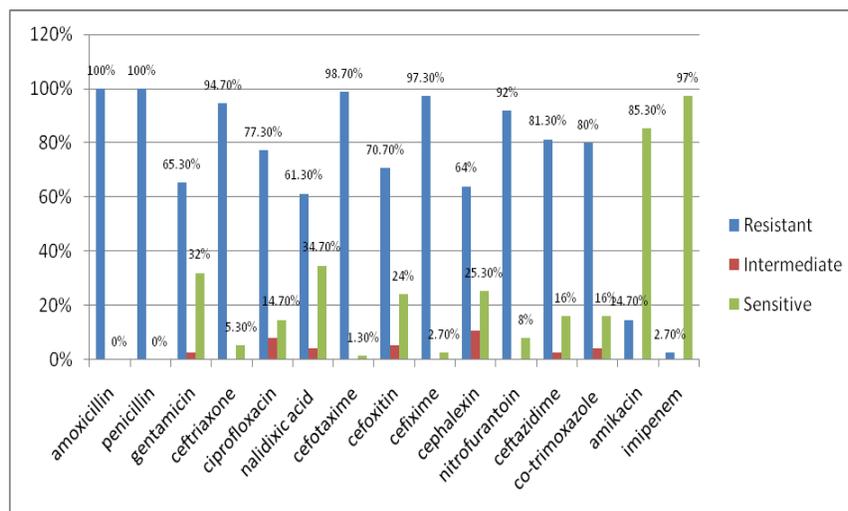


Figure 1. The susceptibility pattern of 75 ESBL producing *E. coli* isolates to 15 antimicrobial agents

gel at 80V for 2 hours. After electrophoresis fragments were stained by Ethidium Bromide, and then visualized with ultraviolet light. PCR products were sequenced and nucleotide sequences were compared with sequences in the GenBank and EMBL databases using the BLASTN local alignment search tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical analysis: The results were analyzed using with SPSS software for Windows, version 17.0 (SPSS Inc., Chicago, IL).

Results

In this study, from the 135 *E. coli* isolates, 75 (55.5%) were ESBL producer. Eighty percent (n=60) of the positive patients were female and 20% (n=15) were male. The average age was 49.6 years old and patient's age ranged from 2 to 73 years old. Patients were classified in 4 different age groups. The age distribution was 16% for ≤20 year, 14.6% for 20-35 years, 33.3% for 36 to 60 years, and 36% for ≥60 years. The results of disk-diffusion antimicrobial susceptibility testing revealed the full resistance to amoxicillin and penicillin and high level of resistance to other tested antimicrobial agents. The majority of isolates were resistance to cefotaxime 98.7%, cefixime 97.3%, ceftriaxone 94.7%, nitrofurantoin

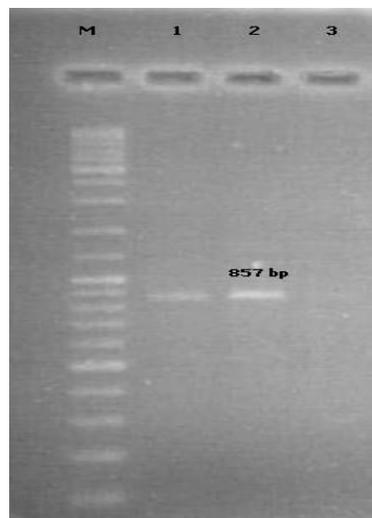


Figure 2. Lane M, ladder (Fermentas, UK); lane 1, the 857 bp PCR product of *bla* CTX-M; lan 2 positive control (*K. pneumonia* ATCC 700603); lan 3 negative control (*E. coli* ATCC 25922)

92%, ceftazidime 81.3%, co-trimoxazole 80%, ciprofloxacin 77.3%, cefoxitin 70.7%, gentamicin 65.3%, cephalixin 64%; nalidixic acid 61.3%. The rates of resistance to the majority of tested antibiotics varied from 61% to 100 %, with the exception of amikacin (14.7%) and imipenem (2.7%). None of isolates were sensitive to all antibiotics. Only one isolate was resistant to all of tested antibiotic. Antimicrobial susceptibility pattern of the ESBL-

Table 1: Multi- drug resistance pattern of 75 ESBL producing *E. coli* isolates

Resistant patterns	Antibiotics No.	Total number of resistant isolates (%)
AMX, P, CTX	3	10 (13.3)
AMX, P, CFM		
AMX, P, CTR		
AMX, P, NIT	4	8 (10.7)
AMX, P, CTX, CFM		
AMX, P, CTX, AK		
AMX, P, CTX, NIT		
AMX, P, CTX, CX	5	12 (16)
AMX, P, CTX, CZX		
AMX, P, CTX, CFM, GEN		
AMX, P, CTX, CFM, AK		
AMX, P, CTX, CFM, CTR, NIT		
AMX, P, CTX, CFM, CTR, COT	6	11(14.7)
AMX, P, CTX, CFM, CTR, AK		
AMX, P, CTX, CFM, CTR, CN, COT	7	
AMX, P, CTX, CFM, CTR, CN, AK		
AMX, P, CTX, CFM, CTR, CX, NIT, COT, CZX		
AMX, P, CTX, CFM, CTR, CX, NIT, COT, CZX, IMP	9	34 (45.3)
AMX, P, CTX, CFM, CTR, CX, NIT, COT, CZX, IMP		
AMX, P, CTX, CFM, CTR, CX, CIP, NIT, COT, NA, GEN	10	
AMX, P, CTX, CFM, CTR, CX, CN, CZX, CIP, NA, COT, GEN, NIT, AK, IMP	11	
	15	

GEN: Gentamicin, CIP: ciprofloxacin, AK: amikacin, CZX: ceftazidime, IMP: imipenem, CTX: cefotaxime, CX: cefoxitin, CN: cephalixin, COT: co-trimoxazole, AMX: amoxicillin, P: penicillin, NA: nalidixic acid, NIT: nitrofurantoin, CTR: ceftriaxone and CFM: cefixime

producing strains to 15 tested antibiotics are shown in figure 1.

Multi drug resistance (MDR) was defined as resistance to three or more classes of antibiotics¹⁴. MDR was observed in all of isolates. Frequencies of MDR to three, four, five, six and more antibiotics were 10 (13.3%), 8 (10.7%), 12 (16%), 11 (14.7%) and 34 (45.3%). Of the 75 MDR isolates, the most prevalent patterns were resistance to more than 6 (n=34; 45.3%), followed by 5 (n=12; 16%) and 6 (n=11; 14.7%) of antimicrobial agents. The predominant resistance profile among our isolates was included resistance to 9 antibiotics amoxicillin, penicillin, cefotaxime, ceftriaxone, cefexime, nitrofurantoin, co-trimoxazole, ceftazidime and cefoxitin (20%), followed by resistance to 5 antibiotics amoxicillin, penicillin, cefotaxime, gentamicin and cefexime (13.3%).

PCR detection of *bla* CTX-M gene in ESBL producing *E. coli* showed that 70 out of 75 (93.3%) isolates were positive for *bla* CTX-M gene.

Discussion

The spread of ESBL-producing bacteria is a significant public health threat due to the consuming limitation of therapeutic options for infections. Dissemination of ESBL-producing bacteria could be attributed to the presence of multiple risk factors such as inappropriate use of broad-spectrum antimicrobials, inappropriate prescription, long duration of hospital stay and transfer of ESBL genes by transposable elements such as plasmid and integron in health care settings¹⁵.

Our findings showed Females had a higher rate of isolation of ESBL producing *E. coli* (80%) that is in agreement with findings of other investigators¹⁶. In the present study, the most infected age group with ESBL-producing *E. coli* was ≥ 60 years (36%). Our results, regarding the age of the patients, is in agreement with recent data that states aging is a risk factor for beta lactamase-mediated resistance in patients infected with *enterobacteriaceae*¹⁷.

In recent years, the spread of ESBL producing *E. coli* isolates have been reported threateningly in many regions of the world^{18,19}. Several studies exhibited that the prevalence of ESBL-producing bacteria is a serious problem of public worldwide health and their distribution can be vary according to geographic

region, country and studied institution²⁰. The present study showed that out of 135 tested samples 75 (55.5%) were ESBL positive. Our results were lower than in comparison to other study carried out in Turkey (84%)¹², Portugal (67.9%)¹⁷, Sudan (92.2%)²¹, Egypt (87%)²² and higher than those reported in Colombia (11.7%)¹⁹, Japan (20.4%)²³, China (36.7%)²⁴, Thailand (13.2%)²⁵, Saudi Arabia (30.6%)²⁶. These differences could be attributed to type and volume of samples, duration of study and drug regimens in different geographical regions.

The susceptibility pattern of ESBL producer's isolates to antibiotics has decreased recent years in many countries¹⁰. The results obtained by this study revealed that all of ESBL producer's isolates were resistant to amoxicillin and penicillin. The lowest rates of resistance in ESBL-producing isolates were observed for imipenem (2.7%) and amikacin (14.7%). These resistance profiles were in accordance with other studies²⁷. In present study, an alarming rate of decrease in the susceptibility not only to beta lactam but also to different antibiotic families including trimethoprim/sulfamethoxazole, sulfamethoxazole - ones, quinolones and aminoglycosides were seen. A possible reason of high resistance in our study might be contributed to the presence of ESBL in these strains. Overall, this is an alarm for clinicians that consumption and prescription of these antibiotics must be changed.

Due to carrying of multi resistant genes by plasmid and integron and also their readily transfer between and within microbial pathogens, bacteria with multiple resistances to antibiotics are widely distributed in hospitals and increasingly being isolated from community¹². Multiple resistances to antibiotics are frequently observed among ESBL producers hence the presence of an ESBL is a good marker of the MDR phenotype and resistance to newer beta lactam antibiotics²⁸. It is important to note that all of isolates showed MDR phenotype. The prevalence of MDR is increasing throughout the world^{1,4}. In this respect, several studies expressed that the existence of MDR phenotype among *E. coli* isolates have lower frequencies in Europe and United States than in Asia and Africa. In a study done in Sudan in 2012, Ibrahim et al. showed that from 232 *E. coli* isolates, 214 (92.2%) isolates were MDR²¹. In compare to studies

were performed in Egypt²², Ethiopia²⁹, Nigeria³⁰, Saudi Arabia²⁶ a high prevalence of MDR were seen in our study. This high prevalence of MDR in our study could be due to differences in the type of samples, Population investigated, poor health condition in hospitals and the social and geological factors.

The incidence of CTX-M among plasmid-mediated ESBL strains is continuously increasing and *bla* CTX-M has already become the most common ESBL gene²⁰. As expected, the frequency of CTX-M enzymes in our study was very high that is in accordance with recent data. Vaida et al., studied 175 non-duplicate clinical isolates (62 *E. coli* and 113 *K. pneumoniae*) showed that 47 (76%) *E. coli* and 98 (87%) *K. pneumoniae* isolates were identified as ESBL producers and CTX-M-encoding genes were found in the majority of *E. coli* (96%) and *K. pneumoniae* (71%) isolates showing the ESBL phenotype³¹.

In Portugal, Mendonca et al. (2007) have studied 180 of *E. coli* recovered in various Portuguese hospitals from cases of community-acquired and nosocomial infections, found that CTX-M producer's isolates were prevalent among community-acquired infections (56%), urinary tract infections (76%), and patients ≥ 60 years old (76%)³².

In a nationwide study of ESBL-producing *E. coli* from 10 different centers in Turkey between 2011 and 2012, the presence of the three common ESBL genes: TEM, CTX-M and SHV using PCR method, were evaluated. Then, they found that CTX-M1 group is the most common type of class A beta lactamases among ESBL-producing *E. coli* strains in Turkey (83.18%)²⁰.

These higher rates of CTX-M among our isolates may be associated with high mobilization of the encoding genes so that Barlow et al. (2008) reported increased tenfold in movement of *bla* CTX-M genes via plasmid in compare to other class A beta lactamases³³.

Conclusion

Currently, CTX-M-producing *E. coli* strains are serious problem of public health in Iran which may be related to free access, misuse and abuse of third generation cephalosporins, especially cefotaxime.

Our study suggests that *E. coli* strains carrying *bla* CTX-M genes are widespread in Iran. With regards ESBL mediated resistance to wide range of antimicrobial classes; it is worthwhile that routine screening of ESBL in clinical isolates carried out in order to prevent dissemination of resistant isolates in our hospitals.

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