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CTX-M extended-spectrum β -lactamase-producing *Klebsiella* spp, *Salmonella* spp, *Shigella* spp and *Escherichia coli* isolates in Iranian hospitals



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ABSTRACT

This study was conducted in Iran in order to assess the distribution of CTX-M type ESBLs producing Enterobacteriaceae. From January 2012 to December 2013, totally 198 *E. coli*, 139 *Klebsiella* spp, 54 *Salmonella* spp and 52 *Shigella* spp from seven hospitals of six provinces in Iran were screened for resistance to extended-spectrum cephalosporins. After identification and susceptibility testing, isolates presenting multiple-drug resistance (MDR) were evaluated for ESBL production by the disk combination method and by Etest using (cefotaxime and cefotaxime plus clavulanic acid). All isolates were also screened for *bla*_{CTX-M} using conventional PCR. A total of 42.92%, 33.81%, 14.81% and 7.69% of the *E. coli*, *Klebsiella* spp, *Salmonella* spp and *Shigella* spp isolates were MDR, respectively. The presence of CTX-M enzyme among ESBL-producing isolates was 85.18%, 77.7%, 50%, and 66.7%, in *E. coli*, *Klebsiella* spp, *Salmonella* spp and *Shigella* spp respectively. The overall presence of CTX-M genes in Enterobacteriaceae was 15.4% and among the resistant isolates was 47.6%. This study indicated that resistance to β -lactams mediated by CTX-M enzymes in Iran had similar pattern as in other parts of the world. In order to control the spread of resistance, comprehensive studies and programs are needed.

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Introduction

The emergence of new enzyme groups that have a typical extended spectrum β -lactamase (ESBL) resistance phenotype,

but are non-TEM and non-SHV derivatives, have been reported in Enterobacteriaceae.¹ CTX-M-type β -lactamases constitute a novel group of molecular class A ESBLs encoded by transferable plasmids. These enzymes exhibit overall preference for cefotaxime (CTX; hence the name CTX-M) and ceftriaxone

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along with higher susceptibility to tazobactam than clavulanate.^{1,2}

The explosive dissemination of CTX-Ms around the world has been referred to as “CTX-M pandemic” due to their increasing description worldwide.³ These enzymes have different prevalence rates in various members of the Enterobacteriaceae family. They mostly exist in enterobacterial species such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella enterica* serovar Typhimurium. In contrast, production of ESBLs is uncommon among the isolates of *Shigella sonnei*.⁴

Firstly, two CTX-M-type enzymes were reported in Europe in 1989.⁵ But, they did not become predominant over the other ESBL enzymes until the first decade of the XXI century, in which an extraordinary spread of these enzymes was observed both in hospital and community settings.⁶ Nowadays, a variety of different types of this enzyme has been identified.³ At the time of writing this article, more than 160 CTX-M-type β -lactamases have been identified in various clinical isolates (www.lahey.org/studies), classified in five families according to the amino acid sequence: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25; two additional groups have been reported recently: CTX-M-74 and CTX-M-75,⁶ two of which (CTX-M-2 and CTX-M-8 types) are shown to be similar (95–100%) to the chromosomally encoded β -lactamase of *Kluyvera ascorbata* and *Kluyvera georgiana*, respectively,^{7,8} while the original sources of the other enzymes of this group remain unknown. The *bla*_{CTX-M} genes have been found on a number of plasmids

and are commonly associated with IS mobile elements, such as ISEcp1, found flanking the open reading frame region of the *bla*_{CTX-M} genes.⁹

The CTX-M group has become the most common type of ESBL in Latin America, but is also becoming more common in Europe and has been recently reported in the UK.³ Also, in Asia, previous reports have identified CTX-M producing isolates in China, Korea, Japan, and India.¹⁰ Resistance to broad-spectrum β -lactams is becoming an ever-increasing problem in Iran.¹¹ In this work, we investigate the distribution of CTX-M-type enzymes in the clinical isolates of *Enterobacteriaceae*, including *Escherichia coli*, *Klebsiella* spp, *Salmonella* spp, and *Shigella* spp in seven hospitals from six provinces of Iran.

Material and methods

This cross-sectional study was performed on the isolates collected from seven hospitals of six provinces in Iran. They included the provinces of Tehran, Kurdistan, Sistan and Baluchestan, Mazandaran, Khuzestan, and Fars. Tehran isolates were collected from two hospitals, namely Milad and Mehr. The geographic distribution of the surveyed hospitals and isolates collected from different provinces are shown in Fig. 1 and Table 1, respectively. During the studied period, totally, 198 *Escherichia coli*, 139 *Klebsiella* spp, 54 *Salmonella* spp, and 52 *Shigella* spp were collected and used for further investigations. The studied samples included urine (mostly), blood,

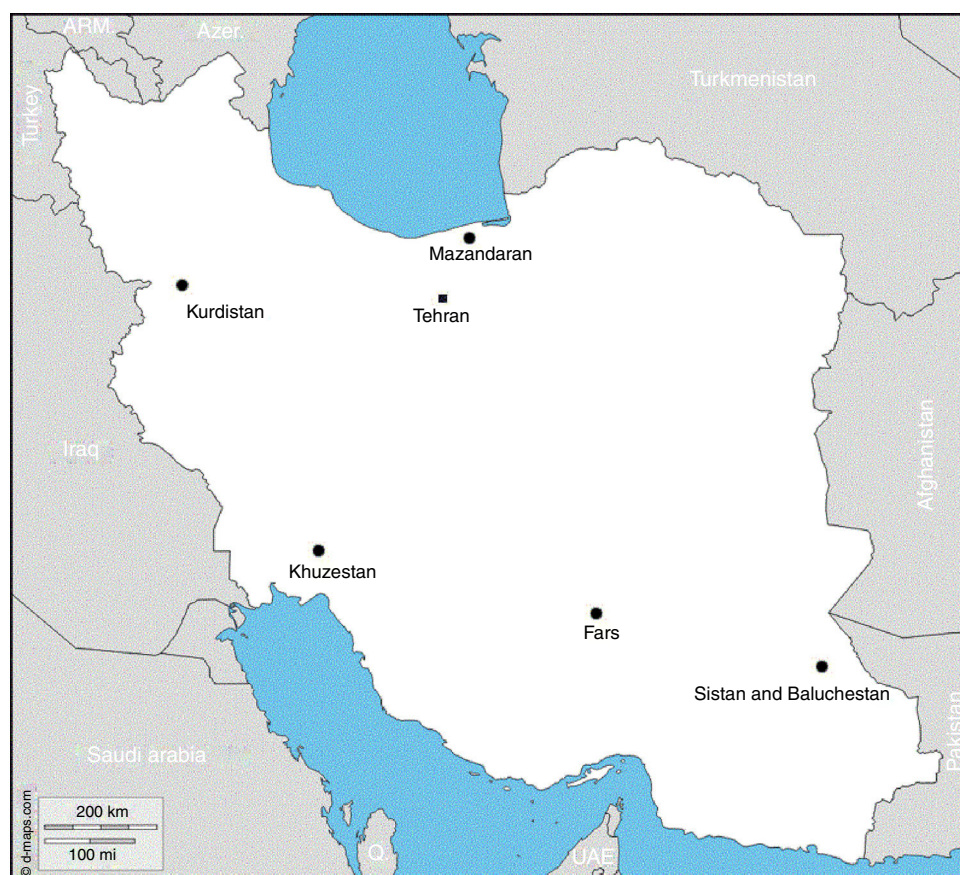


Fig. 1 – Geographic locations of hospitals surveyed in this study.

Table 1 – Total isolates collected from different provinces in Iran.

Bacteria	Province							Total
	Tehran (Milad)	Tehran (Mehr)	Kurdistan	Sistan and Baluchestan	Mazandaran	Khuzestan	Fars	
<i>E. coli</i>	78	14	37	30	6	25	38	198
<i>Klebsiella</i> spp	80	6	11	2	1	9	30	139
<i>Salmonella</i> spp	46	1	2	0	0	4	1	54
<i>Shigella</i> spp	50	0	0	0	0	2	0	52

sputum, wound, respiratory and vaginal secretions, ascites, biopsies, and body fluids. Identification of the isolates was done using conventional standard biochemical tests, including plating in blood agar, chocolate agar, MacConkey agar, *Salmonella-Shigella* agar (Merck, Germany), triple sugar iron agar, urea agar, SIM medium, and IMVic (indole, methyl red, Voges-Proskauer, and citrate).¹²

ESBL confirmatory test

To confirm ESBL production, those isolates that were identified as resistant to ceftriaxone using the disk diffusion susceptibility test were further subjected to the disk combination method.^{13,14} The combination disk method utilized discs containing only cefotaxime (CTX) (30 mg) and other disk containing cefotaxime (CTX) (30 mg) combined with clavulanic acid (CLA) (10 mg) (MAST Diagnostics, Merseyside, UK). ESBL producing isolates were identified as those with a greater than 5 mm increase in the zone with the single antimicrobial compared with the combined antimicrobial, i.e. demonstrating ESBL inhibition.¹⁵

Antimicrobial susceptibility testing

The antimicrobial susceptibility test was done by Kirby-Bauer disk diffusion methodology according to the guidelines of Clinical and Laboratory Standards Institute.¹⁶ Antibiogram was done for tetracycline (30 µg), ampicillin (10 µg), amoxicillin (20 µg), cephalothin (30 µg), gentamicin (10 µg), amikacin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefixime (30 µg), imipenem (10 µg), ciprofloxacin (5 µg), and antibiotics (MAST Diagnostics, Merseyside, UK).

To assess the effectiveness of the antibiotic concentration gradient, E-test (AB Biodisk, Dalvagen, Sweden) using the cefotaxime concentration gradient strips was applied. For this purpose, E-test was studied in a medium containing clavulanic acid (4 µg/ml) and a simple medium simultaneously. The expected reduction in MIC in the presence of the β -lactamase inhibitor was $\geq 3 \log_2$ dilution steps.¹⁷

Polymerase chain reaction (PCR)

Isolates with an ESBL phenotype were further investigated for *bla*_{CTX-M} alleles. Bacterial DNA was extracted from the isolates by boiling the bacterial suspensions. DNA isolates with the concentration of 0.1 ng/µl were used as the templates for PCR analysis. PCR was done by CINNAGEN MsterMix (Cinnacolon, Iran), which was performed with the universal primers

CTX-M-U1 (5'-ATG TGC AGY ACC AGT AAR GTK ATG GC-3') and CTX-M-U2 (5'-TGG GTR AAR TAR GTS ACC AGA AYC AGC GG-3'), as described previously.¹⁸ Amplification was carried out in a Techne TC-512 thermocycler (Eppendorf, Hamburg, Germany) as follows: 30 cycles of 50 s for denaturation at 94 °C, 50 s for annealing at 58 °C, and 50 s for primer extension at 72 °C, followed by terminal extension at 72 °C for 10 min. Initial denaturation at 95 °C for 5 min was used for both amplifications. Electrophoresis of PCR products was performed on 1% agarose gel. The gel staining was performed in ethidium bromide for 20 min and visualized in gel documentation system (Bio-rad, Hercules, USA).

Sequencing

To confirm PCR products, sequencing was randomly done for 114 isolates by Takapozist Company, Iran (on behalf of Bioneer Company, Korea). All the sequencing results were interpreted by LaserGene software (DNASTAR, MADISON) including SeqMan, MegAlign, and EditSeq software.

Statistical analysis

Statistical analysis was performed in SPSS 12.0 (SPSS, Chicago, IL, USA). The variables were analyzed by univariate analysis using χ^2 or Fisher's exact test, as appropriate. Statistical significance was set at $p < 0.05$.

Results

During the studied period, 198 *E. coli*, 139 *Klebsiella* spp, 54 *Salmonella* spp, and 52 *Shigella* spp were totally investigated. In the present study, MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. A total of 42.92%, 33.81%, 14.81% and 7.69% of the *E. coli*, *Klebsiella* spp, *Salmonella* spp and *Shigella* spp isolates were MDR, respectively. Increasing the diameter of the disk containing β -lactamase inhibitor (over 5 mm) represents the production of ESBL enzymes, whose data are shown in Table 2. The CTX-M was detected in 15.4% of the isolates and the presence of this gene in all of the resistant isolates was 47.6%.

Among *E. coli* isolates from the provinces of Fars, Sistan and Baluchestan, Kurdistan, Mazandaran, and Tehran's Mehr hospital, 100% of ESBL-producing isolates were CTX-M positive. Also, 50% of Kurdistan and 75% of Tehran's Milad hospital were CTX-M positive. Totally, 88.37% of the MDR and ESBL positive *E. coli* isolates (cephalosporinase) contained CTX-M and

Table 2 – Test result of E-test and double-disk diffusion for diagnosis of ESBL-producing and Cephalosporinase-producing isolates.

Bacteria:	Total isolates	Resistant (%)	ESBL producing in resistant isolates	Total ESBL producing isolates	Cephalosporinase producers in resistance isolates
<i>E. coli</i>	198	85 (42.92%)	54 (63.52%)	54 (27.27%)	45 (52.94%)
<i>Klebsiella</i> spp	139	47 (33.81%)	36 (66.59%)	36 (25.9%)	32 (68.08%)
<i>Salmonella</i> spp	54	8 (14.81%)	2 (25%)	2 (25%)	3 (75%)
<i>Shigella</i> spp	52	4 (7.69%)	3 (75%)	3 (5.77%)	2 (25%)

consisted of 46.91% of the total MDR isolates detected by PCR technique. In the case of *Klebsiella* spp, 87.09% of the MDR isolates were ESBL-producing and contained CTX-M genes, which consisted of 56.25% of the total MDR isolates. Among ESBL-producing *Salmonella* spp isolates, 50% contained CTX-M gene that consisted of 14.28% of the total MDR isolates.

Totally, out of 52 *Shigella* spp isolates, 7.69% were MDR, 5.77% of which was ESBL-producing. All the patients with drug resistance were under 10 years old and all resistant isolates were related to Tehran's Milad Hospital. Seventy-five percent of the resistant isolates belonged to male and 25% were female, and all the isolated bacteria were from stool. In the resistant isolates, 100% were resistant to ampicillin, amoxicillin, cephalothin, gentamicin, and ceftazidime. Also, 75% and 50% were resistant to tetracycline and ceftizoxime, respectively. All the isolates were sensitive to ciprofloxacin and imipenem. Seventy-five percent of the resistant isolates had ESBL-producing enzymes that were identified by the double disk diffusion method. All ESBL-producing isolates were determined by cefotaxime E-test for cephalosporinase production and the results of all the isolates were positive. In the genomic analysis of resistant genes, 75% of ESBL-producing *Shigella* spp isolates or nearly 50% of the MDR isolates contained CTX-M gene. Also, the sequencing results indicated that CTX-M15 present in all the isolates was examined.

Discussion

In the present study, we examined the presence of ESBL-producing isolates and identification of CTX-M enzymes involved in resistance to third-generation cephalosporins as possible. This study showed that resistance to β -lactams mediated by CTX-M enzymes in Iran had similar pattern as in other parts of the world.

There are different reports from different geographical regions on the specific focus of multi-drug resistance.¹⁹ So, to determine the prevalence of resistance in Iran, we decided to evaluate the occurrence of such resistance from different parts of the country. The presence of CTX-M in all of the resistant isolates was 47.6%, a rate that was similar to the prevalence in the studies of other parts of the world. In a similar study from South Korea on 603 Enterobacteriaceae, 6.8% of the total isolates and 44.1% of resistant isolates were carrying CTX-M genes.²⁰

There are different reports on the prevalence of these enzymes in Enterobacteriaceae family from Iran and other parts of the world. In a study from Tehran hospitals, ESBL

production rate in *Klebsiella pneumoniae* was 72.1%, 45.2% of which had *bla*_{CTX-M}.²¹ Another study from Tehran on the clinical isolates of *E. coli* revealed 56.69% production of ESBL, 35.78% of which was positive for *bla*_{CTX-M} genes from the CTX-M-I group and 2.1% was positive for *bla*_{CTX-M} genes from the CTX-M-III group.²² A study in Kurdistan Province reported that 34.85% of Enterobacteriaceae family was ESBL producing.²³

The low number of ESBL-producing isolates in the present study might be because of the diversity of clones examined in this study. In the studies that have been done in other parts of the world, different prevalence rates have been reported. The prevalence of these enzymes in European countries for *E. coli* and *Klebsiella* spp has been 5.4% and 23–25%, respectively.²⁴ This rate is very low in Japan; i.e. in *E. coli* and *Klebsiella* spp, it was less than 0.1% and 0.3%, respectively.¹³ In other parts of Asia, 4.8% Korea,¹³ 8.5% Taiwan,²⁵ 12% Hong Kong,²⁶ and 28% in Malaysia²⁷ have been reported.

E. coli is the predominant pathogen that carries CTX-M enzymes and the isolates carrying this gene are often isolated in UTI, bacteremia, and gastroenteritis.²⁸ In this study, the most ESBL-producing *E. coli* carrying CTX-M genes was from urine, bacteremia, respiratory infections, and gastroenteritis isolates, respectively.

In the case of *Klebsiella* spp, CTX-M enzymes are important after SHV and TEM. ESBL producing isolates can be isolated from respiratory infections, intra-abdominal infections, and blood flow.^{28,29} But, in the present study, most of the ESBL-producing *Klebsiella* carrying CTX-M genes were isolated from the samples of urinary tract infections and respiratory tract, wound, and blood. Sex distribution of the patients complied with the kind of infections. Most isolates of *E. coli* were in women with urinary tract infections. Conversely, most of the patients with *Klebsiella* spp with respiratory infections were men.

The presence of CTX-M enzyme among ESBL producing isolates of *E. coli*, *Klebsiella* spp, *Salmonella* spp, and *Shigella* spp was 85.18%, 77.7%, 50%, and 66.7%, respectively. In a similar study in the United Kingdom and Ireland, similar results to those stated in this study, i.e. 83% of ESBL-producing *E. coli* and 74% of ESBL-producing *Klebsiella*, were found which were due to CTX-M genes.³⁰ It is obvious that β -lactam resistance due to CTX-M enzymes has similarly grown in Iran and other parts of the world, which shows the need for comprehensive studies and programs to control the spread of resistance.

The frequency and distribution of CTX-M genes in different geographic regions examined in this study showed that, due to the indiscriminate use of new generation antibiotics in the more developed regions, a higher level of resistant genes

was identified. As a statistical comparison of the rates of resistant isolates of Tehran's Milad Hospital and Medical Center in Sanandaj (Kurdistan Province), significant differences were observed (p -value: 0.0007).

Certainly, as shown in the present study, comprehensive control of antibiotics is extremely important. The most important way to control the spread of resistance is control of risk factors. The present study suggested the necessity for identifying patterns of transmission and spread of antibiotic resistance in wider geographical areas and developing a comprehensive program to manage and control the use of antibiotics.

Conflicts of interest

The authors declare no conflicts of interest.

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