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Prevalence of CTX-M-Type β-Lactamases in Multi-Drug Resistant *Escherichia coli* Isolates from North of Iran, Rasht

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Abstract

Introduction: One of the most important resistance determinants in Enterobacteriaceae are extended spectrum β-lactamases (ESBLs). During the last decade, CTX-M types ESBLs have increased considerably and become the most common ESBLs worldwide which are the major causes of the urinary tract infections (UTIs). The aim of this study was to determine the antibiotic resistance patterns and the frequency of the CTX-M β-lactamases among multi-drug resistant (MDR) *Escherichia coli* (*E. coli*) isolates from northern Iranian patients with UTI.

Materials and methods: Thirty three *E. coli* isolates from urine samples were applied in this study. Double disk synergy test (DDST) was applied for identification of ESBL phenotypes in *E. coli* isolates. The ESBL related genes, CTX-M group (1, 2, 8 and 9), were amplified by polymerase chain reaction (PCR).

Results: All *E. coli* isolates showed sensitivity to piperacillin and 55% of the isolates were resistant to 3rd and 4th cephalosporins. The presence of the $bla_{\text{CTX-M}}$ gene in 88% of the ESBL producing isolates was approved based on molecular method. CTX-M (1, 2, 8 and 9) containing *E. coli* isolates showed resistance to more antibiotics than non-CTX-M isolates. CTX-M-1 was the most prevalent CTX-M determinant in ESBL producing *E. coli* isolates**.**

Discussion and conclusion: Based on the results of the present study, the preferred antibiotic against CTX-M type ESBL-producing *E. coli* strains in north of Iran, Rasht, should be piperacillin. Although, CTX-M type ESBLs prevalence was nearly low in the studied MDR *E. coli* isolates, but controlling these low prevalence isolates is important.

Key words: CTX-M, *E. coli*, ESBL, Multi-drug resistance, Urinary tract infection

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Introduction

β-lactamases are bacterial enzymes that have the ability to hydrolyze β-lactam antimicrobial drugs. Widespread use of antimicrobials has resulted in selection of bacteria with extended spectrum β-lactamases (ESBLs) production trait. ESBLs are able to hydrolyze early cephalosporins, peniclillins, oxyimino-thiazolyl cephalosporins (including third and fourth generation cephalosporins) and monobactams, but cannot hydrolyze carbapenems or cephamycins. They are prevented by inhibitors including tazobactam, sulbactam and clavulanic acid (1 and 2). A group of class A ESBLs is CTX-M enzymes that are quickly dispersing among Enterobacteriaceae in the world (3). These periplasmic enzymes which were encoded by plasmid give a high level of resistance to cefotaxime but show a low level of activity against ceftazidime (4). Earlier CTX-M-β-lactamases were cefotaximases, but more than 60% of CTX-M variants can hydrolyze both ceftazidime and cefotaxime nowadays (1 and 2).

Primary isolation of CTX-M-1 was done from a European patient in the late 1980s (5) and since that isolation >130 CTX-M allelic variants have been reported. These CTX-M variants have been categorized in 5 major phylogenetic groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, or CTX-M-25 based on their amino acid sequences (3 and 5).

Dissimilar to SHV and TEM enzymes, CTX-M enzymes have not originated from point mutations in the parent enzymes, but they have originated from the chromosomal *bla* genes of *Kluyvera* sp. followed by transmission to plasmids. Further mutations resulted in variation in the CTX-M family (1 and 2). Although many *Kluyvera* sp. include the bla_{klu} genes, they don't produce the β-lactamases and then are susceptible to cefotaxime because of weakly expression of these genes. The existence of a strong promoter upstream of the *bla* gene leads to cefotaxime resistance phenotype. In clinical isolates exhibiting CTX-M phenotype, insertion sequences (IS) including a strong promoter have been reported. These insertion sequences have not been observed in the natural isolates of *Kluyvera* (1 and 2).

In the past decade, CTX-M enzymes were the most common ESBL enzymes in clinical Enterobacteriaceae isolates, especially in ESBL-producing *Escherichia coli* in Europe, Asia, and South America (3 and 6), while SHV- and TEM-type ESBL enzymes have primarily been reported from ESBL-producing *K. pneumoniae* and *E. coli* clinical isolates in North America (6).

E. coli is an important pathogen and is the causal agent of urinary tract infections (UTIs) (7). Extended-spectrum cephalosporins are usually applied for treating upper UTIs (8). Resistance to these antibiotics has been reported increasingly among *E. coli* strains causing UTIs, as the result of distribution of strains producing extended-spectrum-beta-lactamases (ESBLs), particularly from the CTX-M type (9 and 10). Intestinal colonization which occurs more frequently than actual infections has fueled this increase (11).

The aim of this study was to determine the antibiotic resistance patterns and the frequency of the CTX-M β-lactamase between multi-drug resistant (MDR) *E. coli* isolates from northern Iranian patients with UTI.

Materials and methods

Samples and strains

Urine samples were collected from appropriate patients in early morning mid-stream using sterile, wide mouthed glass bottles with screw cap tops between May and July of 2012. Samples were maintained in an ice-box until laboratory analysis. It didn't last more than one hour between sample collection and sample analysis.

Urine samples were cultured on nutrient, blood and MacConkey agar plates and incubated at 37°C for 18 to 24 h. The usual bacteriological methods were applied for cultivation, isolation and identification of isolates from urine samples. The isolates were stored at -70 °C in tryptic soy broth containing 15% glycerol until processing. The isolates entitled as E1 to E33.

Antibiotic susceptibility test

Disc diffusion test was applied to identify the susceptibility of isolates to the bellow antimicrobials: piperacillin (100 μg), streptomycin (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), chloramphenicol (30), amoxicillin-clavulanic acid (20/10 μg), cefepim (30 μg), ceftriaxone (30), ceftazidime (30), imipenem (10), cephalothin (30 μg), cefotaxime (30 μg) and cefuroxime (30 μg). *E. coli* ATCC 25922 and ATCC 35218 were used as the reference strains to control the quality of the applied antimicrobial agents. MDR were described as resistance to three or more antimicrobials. Thirty three MDR *E. coli* strains were recognized (12).

Phenotypic detection of ESBLs

DDSTs were performed by placing disks of ceftazidime, cefotaxime, and cefepime (30 μg each) at 20 or 30 mm distance (center to center) from a disk containing AMC (amoxicillin, 20 μg and clavulanic acid (CLA), 10 μg). When the cephalosporin zone was expanded by the clavulanate, ESBL production was supposed. It means the zones produced by the disks with clavulante were ≥5 mm larger than those without inhibitor (12).

PCR detection of bla_{CTX-M}

Genomic DNA was extracted by boiling a dense suspension in sterile distilled water (no. 1 McFarland standard) and then 2–3 μL of the boiled cell suspension was applied as the DNA template in the PCR assays. PCR of bla_{CTX-M} genes in *E. coli* isolates was performed using 4 primer pairs specific to each type of CTX-M including CTX-M-1, 2, 8 and 9 for detecting 516, 779, 569 and 393 bp bands, respectively (13).

Initial denaturation was done at 94ºC for 10 min followed by 30 cycles of 1 min at 94ºC, 30 sec for annealing (according to primer type) (Table 1), 1 minute at 72ºC for elongation and final extension was conducted at 72°C for 10 min. A reagent blank was included in every PCR assay which contained all components of the reaction mixture except for the bacteria. PCR products were subjected to electrophoresis in 1% agarose gel (type II, Sigma, USA) in TBE buffer at room temperature at 100 volt (50 mA) for 1 h. DNA bands were visualized by gel staining with ethidiumbromide (0.5 mg/ml) for 30 min and then photographed (14).

Results

Antibiotic susceptibility test

The bacteria isolated from urine. The resistance patterns of the thirty-three isolates were shown in Table 2. All *E. coli* isolates (33 strains) identified as multi-drug resistant (MDR) and also 55% of the isolates were resistant to 3rd and 4th cephalosporins. All isolates displayed 100% sensitivity to piperacillin.

ESBL-producing *E. coli* prevalence

Screening of ESBLs by DDST indicated that eight isolates (24%) were ESBL producers. Phenotypic confirmatory disc diffusion test confirmed that 8 of the 33 isolates were ESBL producers. All the ESBL isolates were completely resistant to tetracyclines, cephalothin, cefotaxime, cefuroxime and amoxicillin-clavulanic acid, but rates of resistance was as bellow: streptomycin 25%, cefepime 62%, ceftriaxone 87%, chloramphenicol 75%, ceftazidime 50%, ciprofloxacin 50% and imipenem 62%.

CTX-M in ESBL-producing *E. coli*

As shown in Fig. 1 and Table 3, except for one ESBL-producing isolate that could not produce any type of the CTX-M, the other isolates had one or three $bla_{\text{CTX-M}}$ genes minimally. Among the $bla_{\text{CTX-M}}$ genes, CTX-M-1 and CTX-M-9 showed the highest (62.5%) and the lowest (25%) abundance, respectively.

Table 1- Primers used in this study for PCR detection of $bla_{\text{CTX-M}}$

Primers	Sequence (5́-3́)	Annealing temperature (°C)	Size of PCR Product (bp)
CTX-M-1-F CTX-M-1-R	GCT GTT GTT AGG AAG TGT GC CCA TTG CCC GAG GTG AAG	56.5	516
CTX-M-2-F CTX-M-2-R	ACG CTA CCC CTG CTA TTT CCT TTC CGC CTT CTG CTC	53.5	779
CTX-M-8-F CTX-M-8-R	CGG ATG ATG CTA ATG ACA ACGTC AGA TTG CGA AGC GTC	55.0	569
CTX-M-9-F CTX-M-9-R	GCA GAT AAT ACG CAG GTG CGG CGT GGT GGT GTC TCT	53.5	393

Table 2- Percentage of susceptible (S), intermediate (I) and resistant (R) *E. coli* strains from UTIs

Antimicrobial agent (mg)	Diffusion zone	Breakpoint (mm)		*E. coli* isolates (n=33)
		S (%)	**I (%)**	**R (%)**
Streptomycin (10 mg)	≤11	5 (15.15)	17 (51.5)	11 (33.33)
Cefepime (30 mg)	≤14	14 (42.42)	5 (15.15)	14 (42.42)
Ceftriaxone (30 mg)	≤13	9 (27.27)	4 (12.12)	20 (60.60)
Amoxicillin-clavulanic acid (20/10 mg)	≤13	1 (3.03)	2 (6.06)	30 (90.90)
Chloramphenicol (30 mg)	≤12	17 (51.51)	1 (3.03)	15 (45.45)
Ceftazidime (30 mg)	≤14	12 (36.36)	3 (9.09)	18 (54.54)
Ciprofloxacin (5 mg)	≤15	14 (42.42)	7 (21.21)	12 (36.36)
Imipenem (10 mg)	≤15	18 (54.54)	3 (9.09)	12 (36.36)
Cephalothin (30 mg)	≤14	4 (12.12)	0 (0)	29 (87.87)
Cefotaxime (30 mg)	≤14	8 (24.24)	4 (12.12)	21 (63.63)
Tetracyclines (10 mg)	≤11	5 (15.15)	2 (6.06)	26 (78.78)
Cefuroxime (10 mg)	≤14	6 (18.18)	4 (12.12)	23 (69.69)

* According to 2011 CLSI guidelines

S= Susceptible, I= Intermediate, R= Resistant

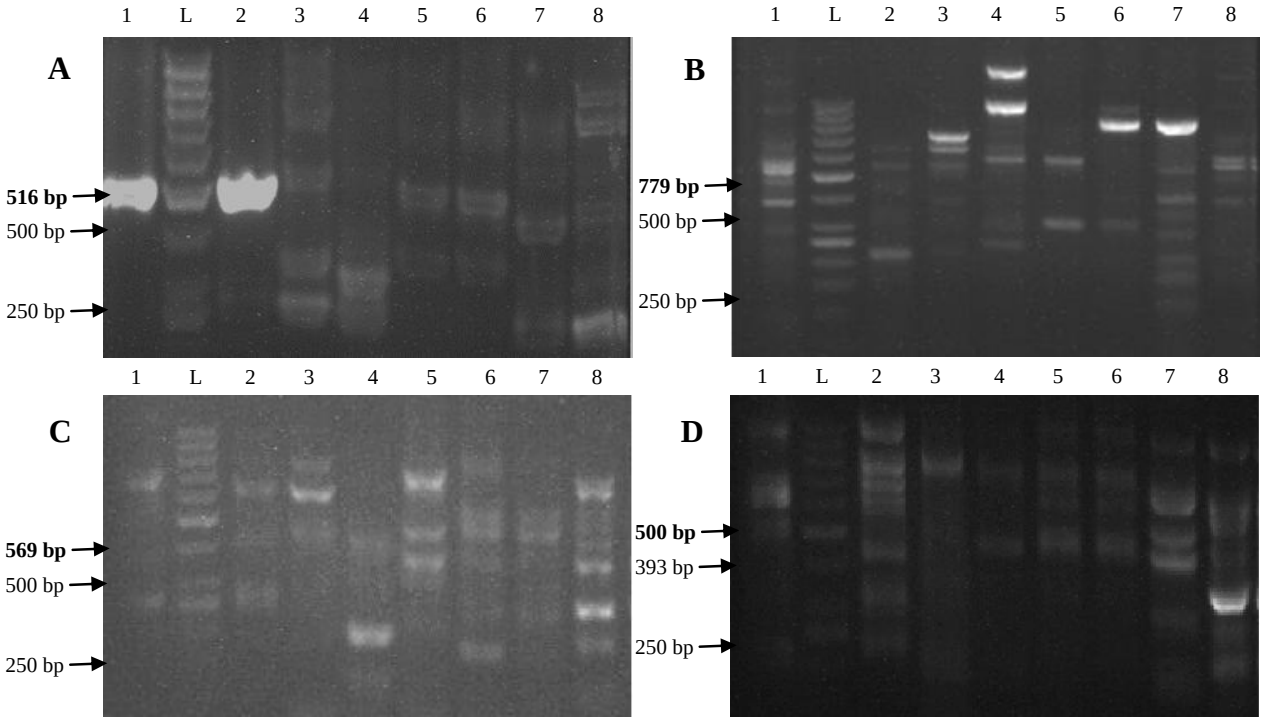

Fig. 1- PCR amplification of *bla*$_{CTX-M-1}$ (A), *bla*$_{CTX-M-2}$ (B), *bla*$_{CTX-M-8}$ (C) and *bla*$_{CTX-M-9}$ (D) genes. Lanes: L, DNA ladder; 1-8, ESBL *E. coli* isolates

Table 3- The *bla*$_{CTX-M}$ genes in ESBL-producing isolates

Isolates	CTX-M-1	CTX-M-2	CTX-M-8	CTX-M-9
1	+	-	-	-
2	+	-	-	-
3	-	+	-	-
4	-	-	-	-
5	+	-	-	-
6	+	+	+	-
7	-	+	+	+
8	+	-	+	+

+: Producing CTX-M, -: Non-producing CTX-M

CTX-M and antibiotic resistance

For considering the relation between the existence of *bla*$_{CTX-M}$ genes and the pattern of antibiotic resistance, the isolates with the higher and lower number of *bla*$_{CTX-M}$ genes and also isolates with and without ability to produce CTX-M, were compared according to the profiles of antibiotic resistance (Fig. 2 and Table 4). The two variables didn't have significant correlation statistically (correlation coefficient or r = 0.245).

The results showed that isolate E1 with one type of CTX-M in contrast to isolates E6, E7 and E8 with three types of CTX-M was sensitive to coamoxyclave, but resistance pattern of isolate E2 was nearly similar to the three isolates mentioned above. Isolate E3 versus isolates E6 and E8, was sensitive to imipenem and chloramphenicol and compared with isolate E7, was sensitive to imipenem only.

Table 4- The resistance pattern of eight ESBL-producing isolates to different antibiotics

Antibiotics	ESBL isolates							
	E1	E2	E3	E4	E5	E6	E7	E8
Streptomycin	R	R	R	R	I	R	R	I
Cefepim	R	R	R	R	S	I	I	R
Ceftriaxone	R	R	R	R	R	R	R	R
Amoxicillin clavulanic acid	S	R	R	I	R	R	R	R
Chloramphenicol	R	R	S	R	R	R	R	R
Ceftazidime	I	R	R	S	I	R	R	S
Ciprofloxacin	R	R	I	R	I	I	I	R
Imipenem	R	R	S	S	I	R	R	R
Cephalothin	R	R	R	R	R	R	R	R
Cefotaxime	R	R	R	R	R	R	R	R
Tetracyline	R	R	R	R	R	R	R	R
Cefuroxime sodium	R	R	R	R	R	R	R	R
Piperacillin	S	S	S	S	S	S	S	S

E: isolates, R= resistant, I= intermediate and S= sensitive

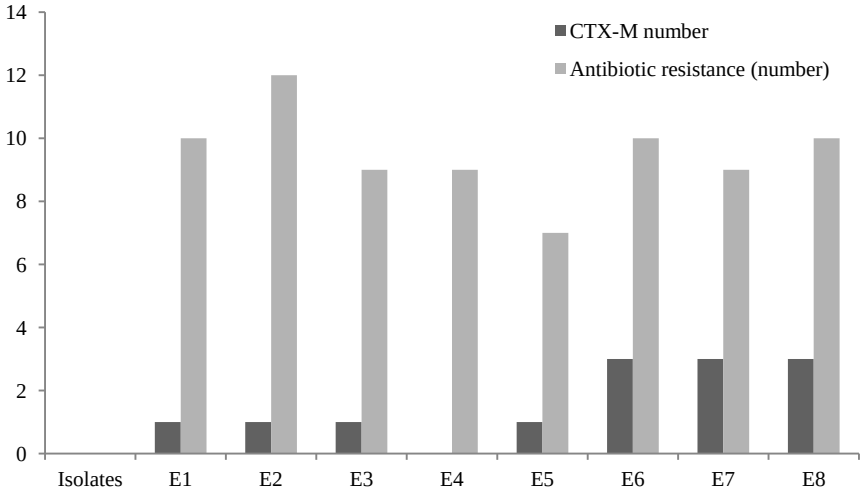

Fig. 2- The relation between the number of *bla*$_{CTX-M}$ genes and the pattern of resistance to antibiotics (number of antibiotics that each isolate was resistant to it)

Isolate E4 without ability to produce CTX-M, in comparison with the isolates E1, E2, E3 and E5 with ability to produce one type of CTX-M, was sensitive to imipenem versus isolate E1, sensitive to imipenem and ceftazidime versus E2 and finally sensitive to ceftazidime versus E3, but was resistant to cefepim compared to E5. Isolate E4 in comparison with isolates E6 and E7, was sensitive to imipenem and ceftazidime and versus E8, was sensitive to imipenem.

Discussion and conclusion

Resistance to β-lactam antimicrobial drugs among gram-negative bacteria is mostly related to ESBLs (1 and 2). Isolates producing CTX-M type ESBLs are often resistant to fluoroquinolones and aminoglycosides. Plasmids containing *bla*$_{CTX-M}$ gene are completely associated with resistance phenotype to macrolides, tetracyclines, sulphonamides, trimethoprim, and chloramphenicol. It seems that fluoroquinolone or multi-drug resistance

lead to CTX-M maintenance due to co-selection process (1 and 2).

Until the early 2000, most reports of ESBLs were associated with SHV or TEM derived enzymes worldwide. An extensive spread and high degree of diversification of CTX-M β-lactamases has observed in the first decade of 2000. These enzymes have been increasingly reported from *E. coli* isolates from community acquired UTIs and also infections in the hospitals worldwide and nominated as "CTX-M pandemic". Currently the most broadly distributed CTX-Ms are CTX-M-1, CTX-M-3, and CTX-M-15 from group 1 and CTX-M-9 and CTX-M-14 from group 9 (1 and 2).

A number of studies have emphasized on synchronized origins of certain CTX-M types in different geographic locations at the same time period, but plasmid studies proposed that they originated independently (1 and 2).

Yazdi *et al*. studied 246 *E. coli* isolates from UTI specimens in Tehran and concluded that 47.1% of the isolates were resistant to the third generation cephalosporins (cefotaxime and ceftazidime). In these isolates, 44.3% were ESBLs producing. By molecular study on these ESBLs producing isolates, it was demonstrated that 68.8% of the isolates included *bla*$_{\text{CTX-M}}$ gene (15). In the study conducted in Tabriz on 32 *E. coli* isolates from the patients suffering from a hospital infection, 93.7% isolates were ESBLs producing. All the isolates including the ESBLs producing isolates were susceptible to imipenem and 83.7% of the isolates were susceptible to amikacin. 31.2% of the isolates included *bla*$_{\text{CTX-M}}$ gene (16).

Peerayeh *et al*. studied 200 non-duplicate clinical isolates of *E. coli* which were collected from Tehran hospitals. 70% of these non-duplicate *E. coli* isolates were ESBL positive. 61.8% of CTX resistant *E. coli* isolates included the CTX-M-1 group alleles. None of the isolates were containing CTX-M groups 2 and 9. The resistance of CTX-M-1 containing *E. coli* isolates to antibiotics were 93.2% to amoxicillin and amoxicillin-clavulanic acid, 70.7% to ceftazidime, 68.5% cefoxitin, 65.1% to cefepime, 86.5% to aztreonam, 94.3% to erythromycin, 38.2% to gentamicin, 75.2% to tetracycline, 67.4% to co-trimoxazole, 14.6% to amikacin, and 43.8% to ciprofloxacin. No imipenem resistant isolate was identified (17).

The prevalence of ESBLs producing isolates was 43.8% in the study that was done by Mansouri *et al*. on 338 *E. coli* and 75 *K. pneumoniae* clinical isolates which were collected from Kerman hospitals. The abundance of *bla*$_{\text{CTX-M}}$ gene among ESBLs and AmpC producing isolates of *K. pneumoniae* and *E. coli* were 21.3% and 13.3%, respectively. The results of their study revealed that imipenem was more active than other antibiotics (18). Goudarzi *et al*. mentioned that CTX-M-15 was detected in 74% of the *E. coli* isolates were collected from urine specimens of patients with UTI who had referred to the Tehran Children Medical Center, between November 2012 and July 2013. 51% and 24% of the isolates were resistant to cefotaxime and ceftazidime, respectively.

Based on their study, imipenem was more active than other antibiotics (19).

In another study, CTX-M type ESBLs production was investigated in 160 *E. coli* isolates collected from three university hospitals of Tehran and based on the results, 35.78% and 2.1% were positive for CTX-M-1 and CTX-M-3, respectively (20). In the study conducted by Soltan Dallal *et al*. on 188 *E. coli* isolates were collected from Tabriz hospitals, 43.6% were ESBLs producers which in these isolates, CTX-M-1 group prevalence was very high (84.1%). All isolates were sensitive to imipenem (21).

In Al-Agamy *et al*. investigation on 152 *E. coli* strains, 31 ESBL-positive *E. coli* strains identified and $bla_{\text{CTX-M}}$-15 gene was highly prevalent in these ESBL-positive strains (96.77%). The $bla_{\text{CTX-M -27}}$ gene was only detected in one strain. All of the CTX-M producers were sensitive to imipenem but were insensitive to cefotaxime and ceftazidime (22). In the study was done by Hayakawa *et al.*, 84.6% of *E. coli* isolates included a $bla_{\text{CTX-M}}$ gene which mostly were as CTX-M-15 type (74.8%). CTX-M *E. coli* strains showed more resistant to multiple antibiotics than non-CTX-M ones (23).

In Pallecchi *et al*. survey, 88% of the *E. coli* isolates from a healthy population of children living in Bolivia and Peru, harbored a CTX-M-type. An increased diversification of CTX-M-type ESBLs was also reported compared to 2002 (24).

Eckert *et al*. analyzed 19 clinical isolates of the family *Enterobacteriaceae* (16 *E. coli* and 3 *Klebsiella pneumoniae* isolates) collected from Paris hospitals, France, from 2000 to 2002. These strains showed a particular extended-spectrum cephalosporin resistance profile characterized by a higher level of resistance to cefotaxime and aztreonam than to ceftazidime. The $bla_{\text{CTX-M}}$ genes were responsible for this resistance, with a predominance of CTX-M-15 (25). Shibata *et al*. identified resistance to either oxyimino-cephalosporin in 21.7% of 1456 strains isolated in 57 of 132 clinical facilities from 2001 to 2003 and these strains were found to harbor $bla_{\text{CTX-M}}$ genes by PCR. $bla_{\text{CTX-M}}$ genes belonging to the CTX-M-1, CTX-M-2, and CTX-M-9 clusters were determined in 17.9%, 50.7% and 31.2% of the strains, respectively (26).

R Iroha *et al*. surveyed the presence of ESBLs in 44 clinical isolates of *E. coli* from out-patients in two university teaching hospitals in South-Eastern Nigeria. A high prevalence of CTX-M-1 cluster - ESBLs was observed in South-Eastern Nigeria and further confirmed the worldwide distribution of CTX-M ESBL in clinical isolates (27). Rani Varkey *et al*. screened ESBL producing isolates of 361 blood samples. PCR analysis showed that 71% of the *E. coli* isolates were carrying $bla_{\text{CTX-M}}$ genes (28).

In the present study, the CTX-M ESBLs frequency was approximately low in the studied MDR *E. coli* isolates comparing to other mentioned studies, but the results further emphasize that CTX-M-1 is now one of the most common CTX-M β-lactamases worldwide. Nevertheless, controlling of these low prevalence isolates is important. Unlike most of the mentioned

studies, all CTX-M producing isolates in the present study were resistant to cefotaxime but susceptible to piperacillin, while in other investigations it has been shown that CTX-M producing isolates were susceptible to imipenem. Therefore, the preferred antibiotic against CTX-M type ESBL-producing *E. coli* strains in north of Iran, Rasht, should be piperacillin. Further study is needed to clarify the presence of the types of ESBL genes among the MDR *E. coli* isolates in this geographic location.

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شیوع بتالاکتامازهای نوع CTX-M در جدایه‌های *E. coli* با مقاومت چندگانه دارویی از شمال ایران، رشت

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چکیده

مقدمه: بتالاکتامازهای با طیف گسترده (ESBLs) به‌عنوان مهم‌ترین شاخص مقاومت به آنتی بیوتیک‌ها در بین انتروباکتریاسه‌ها در سراسر جهان هستند. در طول دهه گذشته، انواع CTX-M از ESBL شیوع بالایی داشته و بیشتر در ارتباط با مسببین مهم عفونت ناحیه ادراری (UTI) هستند. هدف از این مطالعه بررسی الگوی مقاومت به آنتی بیوتیک و فراوانی بتالاکتامازهای CTX-M در بین جدایه‌های *E. coli* با مقاومت چندگانه دارویی از بیماران مبتلا به UTI در شمال ایران است.

مواد و روش‌ها: ۳۳ جدایه *E. coli* از نمونه‌های ادرار استفاده شد. فنوتیپ‌های ESBL جدایه‌ها با استفاده از آزمون سینرژی دیسک دوتایی (DDST) تعیین شدند. ژن‌های مرتبط با ESBL، شامل گروه‌های (۱، ۲، ۸ و ۹) با استفاده از واکنش زنجیره‌ای پلیمراز (PCR) تکثیر شدند.

نتایج: تمام جدایه‌های *E. coli* حساس به پیپراسیلین بوده و ۵۵ درصد آن‌ها مقاوم به سفالوسپورین‌های نسل سوم و چهارم بودند. تحلیل مولکولی حضور ژن CTX-M در ۸۸ درصد جدایه‌های تولید کننده ESBL نشان داده شد. جدایه‌های *E. coli* حاوی CTX-M (۱، ۲، ۸ و ۹) بسیار مقاوم به چند آنتی بیوتیک نسبت به جدایه‌های فاقد CTX-M بودند. CTX-M-1 به‌عنوان شایع‌ترین نوع CTX-M در جدایه‌های *E. coli* تولید کننده ESBL بود.

بحث و نتیجه‌گیری: بر اساس نتایج مطالعه حاضر، آنتی بیوتیک ترجیحی برای کنترل سویه‌های *E. coli* تولید کننده ESBL نوع CTX-M در شمال ایران، رشت، باید پیپراسیلین باشد. اگرچه، شیوع ESBL نوع CTX-M در بین جدایه‌های *E. coli* با مقاومت چندگانه دارویی کم بود، اما کنترل همین فراوانی کم نیز دارای اهمیت است.

واژه‌های کلیدی: CTX-M، اشیرشیا کلی، بتالاکتاماز وسیع الطیف، مقاومت چندگانه دارویی، عفونت ناحیه ادراری

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