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Dissemination of CTX-M-Type Beta-lactamase Among Clinical Isolates of *Enterobacteriaceae* in Markazi Province, Iran

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Background: Organisms producing CTX-M-lactamases are known as the source of resistance to Oxyiminocephalosporins such as Eeftriaxone and Ceftazidime. However, the laboratory detection of these strains is not well defined.
Objectives: The aim of this study was to determine the presence and prevalence of known CTX-M-beta- beta-lactamase genes in clinical isolates of *Enterobacteriaceae* from Arak educational hospitals, Iran.
Materials and Methods: During a 10-month period (May to February 2010), 350 randomly *Enterobacteriaceae* isolates were obtained from the clinical laboratories of different hospitals of Arak University of Medical Sciences, Iran. Antibiotic susceptibility was tested by CLSI disk diffusion and extended spectrum beta-lactamase (ESBL) confirmatory tests. Minimum Inhibitory Concentration (MICs) was determined by broth micro dilution. All of the ESBL-producing isolates were examined by PCR to detect the presence of *bla* CTX-M genes.
Results: In phenotypic confirmatory test, 154 (44%) out of 350 clinical isolates were ESBL positive. Using molecular assay, 154 strains potentially producing extended-spectrum-beta -lactamases were examined for the presence of CTX-M enzymes. 92.2% isolates CTX-M - 1, 28.5% isolates CTX-M-2, 17.5% isolates CTX-M-8, and 38.3% isolates CTX-M-9 genes detected by PCR.
Conclusions: The levels of resistance to Ceftazidime were remarkably variable among CTX-Mproducers. This study provides further evidences of the global dissemination of CTX-M type ESBLs and emphasized on the need for their epidemiological monitoring.

Keywords: CTX-M beta-lactamese; Enterobacteriaceae; extended-spectrum ß-lactamases

1. Background

The recent global spread of *Escherichia coli* producing CTX-M extended-spectrum β-lactamases (ESBL) is a major concern because of new microbiological and epidemiological features.

Plasmid-mediated extended-spectrum β-lactamases extended spectrum beta-lactamase (ESBLs) are increasingly frequent among clinical isolates of the family *Enterobacteriaceae* throughout the world. The development of new enzyme groups that have a typical ESBL resistance phenotype but are non-TEM and non-SHV derivatives have recently been reported (1). CTX-M-type-lactamases constitute a novel group of enzymes encoded by Transferable plasmids (1). The CTX-M-type enzymes are a group of molecular class A extended-spectrum β- lactamases (ESBLs)that exhibit a general preference Tocefotaxime (CTX; hence the CTX-M name) and Ceftriaxoneand are capable to hydrolyze broad-spectrum Cephalosporins and are inhibited by Clavulanic acid, Sulbactam and Tazobactam. These enzymes are emerging in members of the family *Enterobacteriaceae*, that can cause resistance to CTX and other expanded-spectrum-lactams (2).

The first two CTX-M-type enzymes were reported in European countries in 1989 (1, 3). To this point, more than 40CTX-M-type-lactamases have been identified in various clinical isolates, mostly in Enterobacterial species such as *Escherichia coli*, *Klebsiella pneumonia* and *Salmonella enterica* serovar *Typhimurium*. These enzymes have been classified in to five major phylogenetic branches based on their amino acid sequence homologies (4).

The CTX-M group I including CTX-M-1,-3,-10,-12, -15,-28,-30 (5): and FEC-1; the CTX-M group II including CTX-M-2,-4,-5,-6,-7,-20 and Toho-1;the CTX-M group III including CTX-M-8; the CTX-M group IV including CTX-M-9,-13,-14 (also named CTX-M-18),-16,-17,-19,-21,-24,-27 and Toho-2;and the CTX-M group V with CTX-M-25 and CTX-M-26. The CTX-M group consist of five groups, each group contains different types. Beta-Lactamases of the CTX-M group II are

Implication for health policy/practice/research/medical education:
Today, an increased bacterial resistance against antibiotics has become a major worldwide concern. During the past years, increasing rates of infections by extended spectrum beta-lactamase (ESBL) - producing isolates has greatly limited the use of noncarbapenem beta-lactam antibiotics, thus the therapeutic importance of carbapenem and non-beta lactam antibiotics has risen incrementally. The cotransmission of antibiotic resistance genes via plasmids may also compromise the effectiveness of many antibiotics. Therising antibiotic resistance rates among clinical isolates have resulted in increased morbidity and mortality and extended periods of hospitalization, consequently, increased economic costs.

structurally related to the naturally produced beta-lactamase of *Kluyvera ascorbata* (6), and in CTX-M group III, is related to beta-lactamase of *K. georgiana* (7), and CTX-M group I enzymes are related to the beta-lactamases of *K. cryocrescens* (8). Although an identical enzyme to CTX-M-3 was isolated from *K.ascorbata* (9). The CTX-M group IV is related to enzymes from *Kluyvera* spp. isolated in Guyana, which was identical to CTX-M-14 (10).

CTX-M-producing strains were initially found in west Europe, they have now been observed over a wide geographical area, including Latin America(4), Africa, Asia (11), some parts of eastern Europe (12) and recently, North America (13).

2. Objectives

In this work we report the dissemination of various CTX-M-Type beta-lactamases (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9) in clinical isolates of *Enterobacteriaceae* from Arak educational hospital of University of Medical Sciences, Iran.

This study provides evidences about the global dissemination of CTX-M type ESBLs and emphasizes on the need of its epidemiological monitoring.

3. Materials and Methods

3.1. Bacterial Strains

350 randomly selected *Enterobacteriaceae* were isolated, during a 10-month period (May to February 2010), from clinical specimens Arak educational hospitals of Medical University, Iran. These organisms were screened for the presence of ESBL and then investigated for the presence of CTX-M-Type beta-lactamese. The isolated bacteria were kept at -70 ˚C before tested.

3.2. Phenotypic Detection of ESBL

ESBL producers were detected by combination disk methods (CLSI) (14). CAZ/CA (10 μg of clavulanic acid and 30 μg of CAZ) and CTX/CA (10 μg of CA and 30μg of CTX) (provided by Mast-German) disks were placed on the inoculated plates containing Muller Hinton agar. A positive test result was defined as a ≥ 5 mm increase in diameter of inhibition zone compared to a disk without Clavulanic acid (15).

Antibiotic susceptibility tests and Minimum Inhibitory Concentration (MIC) of antimicrobial agents that are usually active against the *Enterobactericaeae* were determined by an antibiotic disk diffusion method on Mueller-Hinton (MH) agar (Figure 1). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as controls.

MICs of Ceftazidime and Cefotaxime were determined by a micro dilution test using Cation-Adjusted Mueller-Hinton (MH) broth, in accordance with the Criteria of the Clinical and Laboratory Standards Institute (15). The MIC panels were prepared in house. Plates were incubated at 35°C for 18 hours before reading the results. *E.coli* ATCC25922 was used as a reference for quality control of in vitro susceptibility testing.

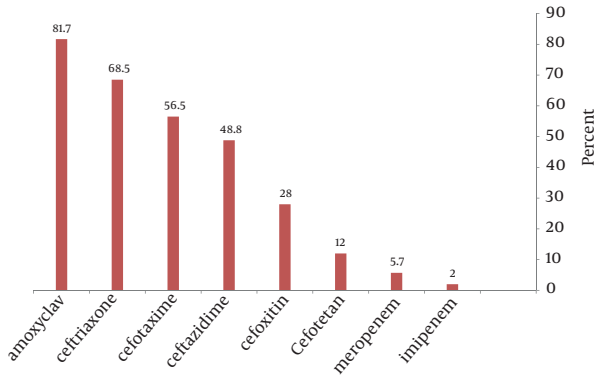


Figure 1. Resistance Rate to Antibiotics Among Clinical Isolates of *Enterobacteriaceae* Members

Table1. Primers Used for Amplification

Gene	Primer Sequence	Product Size, bp	Annealing Temp, °C	MgCl2 Concentration, mM
blaCTX-M1	F: 5' AAGACTGGGTGTGGCATTGA 3'	670	52	2
	R: 5' AGGCTGGGTGAAGTAAGTGA 3			
blaCTX-M2	F: 5'CGACGCTACCCCTGCTATT 3'	552	60	3
	R: 5' CCAGCGTCAGATTTTTCAGG3'			
blaCTX-M8	F: 5' CGC TTT GCC ATG TGC AGC ACC3'	307	58	2
	R: 5' GCT CAG TAC GAT CGA GCC 3'			
blaCTX-M9	F: 5' GCTTTATGCGCAGACGAGTG 3'	666	50	2
	R: 5' GCCAGATCACCGCAATATCA 3'			

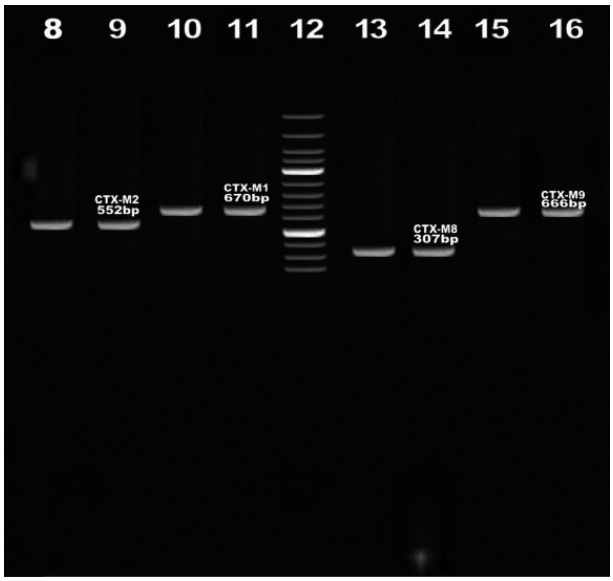
3.3. DNA Extraction and PCR Experiments

Genomic DNA template preparation using boiling methods and PCR amplification for CTX-M beta-lactamase genes were as follow: denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1minute, annealing for 1minute (according to Table 1), extension at

72°C for 1 minute and final extension at 72°C for 5 minutes. The primers, and fragment size are listed in Table 1.

PCR products were subjected to electrophoresis on horizontal 1% agarose gels in TBE 1X buffer, loaded with 5 μL of reaction mix and stained with ethidium bromide after electrophoresis (Figure 2).

Figure 2. Gel Electrophoresis of the PCR Products (blaCTX-M1 , blaCTX-M2, blaCTX-M8 and blaCTX-M9 Genes)



lane12: molecular weight marker (100 bp ladder), lane 9,11,14 and 16:positive control ; lane 8,10,13,15:isolated strains which produced blaCTX-M1 , blaCTX-M2, blaCTX-M8 and blaCTX-M9

3.4. Statistics

Statistical analysis was performed using SPSS for windows version 11.5. Resistances were compared by Chi-square test. P value < 0.05 was considered to be statistically significant.

4. Results

4.1. Bacterial Strains and Antibiotic Susceptibility Patterns

From May to February 2010, a total of 350 positive samples for *Enterobacteriaceae* were processed. 60% of the specimens were collected from hospitalized patients and 40% from outpatients urine (n = 251), wound (n = 42), blood (n = 29), sputum (n = 28) samples. The resistance rate to the antimicrobial agents was as follows: Amoxyclav (81.7%), Ceftriaxone (68.5%), Cefotaxime (56.5%), Ceftazidime (48.8%), Cefoxitin (28%), Cefotetan (12%), Meropenem (5.7%), Imipenem (2%).

Among the tested third-generation Cephalosporins, the highest resistance was found against Ceftriaxone and Cefotaxime (68.5% and 56.5%, respectively).

Resistance phenotypes of ESBL- producing isolates were identified as follow: 80.5% (n = 108) of isolates *E. coli* , 71.9% (n = 41) of isolates *Klebsiella pneumoniae*, and in 50% (n = 5) of isolates *Enterobacter cloacae,* the ESBL producers were identified using the combined disk methods. The majority of ESBL producer that were mainly resistant to Cefotaxime and Ceftazidime, were obtained from urine (n = 251) followed by wound (n = 42), blood (n = 29), sputum (n = 28) specimens (Table 2). Also the most common ESBL positive phenotype was observed among hospitalized patients (48.5%).

Table 2. Frequency of *Enterobacteriaceae* Isolates Based on ESBL Phenotype and Sample Type

	Sputum, %	Blood, %	Wound, %	Urine, %
ESBL positive phenotype	68.5	83.4	76	75.8
ESBL negative phenotype	31.5	16.6	24	24.2

4.2. MIC Determination

The MICs of the resistant isolates for Ceftazidime ranged between 16 and ≥ 512 μg/mL, whereas for Cefotaxime this amount varied between 64 and ≥ 512 μg/mL. According to our result more than 85% of the putative ESBL producers had $MIC_{CAZ} \geq 16$ and 98% of ESBL producer had $MIC_{CTX} \geq 64$. Nevertheless, a high diversity of the resistancelevel to Cefotaxime was observed for CTX-M-positive strains, as illustrated by the broad range of MICs (64 to ≥ 512 μg/mL) (Table 3).

Table 3. MIC Result to Micro Broth Dilution Method

Isolates, μg/mL	≤ 4	8	16	32	64	128	256	≥ 512
Ceftazidime	11	11	39	33	27	20	10	3
Cefotaxime	1	1	1	0	11	36	43	16

4.3. Detection of CTX-M-Producing Isolates

4.3.1. PCR Amplification

The resistant phenotypes of the 154 (44%) isolates were suggestive of CTX-M-type ESBL production that was obtained by Screening bla_{CTX-M} determinants by PCR using four sets of Specific primers for the CTX-M family of ESBLs. Out of 154 ESBL positive, 92.2% isolates were CTX-M - 1, 28.5% isolates were CTX-M-2, 17.5% isolates were CTX-M-8,

and 38.3% isolates were CTX-M-9 genes detected by PCR (Figure 2) (Table 4).

Also Table 5 shows the frequency of the beta-lactamase genesbased in isolated microorganisms from patients in different status.

Table 4. Frequency of ESBLs Producing Isolates by PCR[a]

Isolates, %			CTX-M-Typebeta lactamase
Enterobacter cloacae	*E.coli*	*K.Pneumonia*	
60	95.3	87.8	CTX-M-1
0	35.1	14.6	CTX-M-2
20	16.6	19.5	CTX-M-8
20	45.3	21.9	CTX-M-9

[a] $P < 0.001$

Table 5. Frequency of the beta-lactamase Genes Based on Patients Status

Frequency	Hospitalization, %	Outpatients, %
CTX-M-1	91.1	49.2
CTX-M-2	24.5	36.5
CTX-M-8	8.8	34.6
CTX-M-9	38.2	38.4

5. Discussion

There has been a dramatic increase in the number of CTX-M beta-lactamase producing organisms that were reported in the literature (5). This class of beta-lactamases has been recognized worldwide as an important mechanism of resistance to Oxyimino-cephalosporins used by Gram-negative pathogens. In most of the cases, organisms producing these enzymes display higher levels of resistance to Cefotaxime and Ceftriaxone than Ceftazidime (5). However, phenotypic differentiation of organisms produce CTX-M beta-lactamases from organisms that produce other types of ESBLs can be difficult (12). Therefore, susceptibility testing which relies on identifying organisms that are resistant to Cefotaxime and/or Ceftriaxone but susceptible to Ceftazidime is not a reliable approach (5).

In our study antimicrobial susceptibility testing showed that the majority of isolates were resistant to at least one of the third-generation Cephalosporines. In a Study by Jeong et al. in 2004 in North Korea showed (16), Ceftazidime and Cefotaxime resistance was respectively 11% and 14%. Retrospective studies about resistance to antibiotics showed an increasing trend (17, 18).

Of totally 350 isolates, 203 isolates (58%) showed ESBL phenotype detected by combination disk method which is different from the reported rates of ESBLs in other countries in our region such as India (97.1%), Turkey (57%) and Korea (68.7%) (19, 20). As 154 (44%) of 203 Ceftazidime resistance or Cefotaxime resistance isolates were ESBL positive in this study, it appears that ESBL production has a significant role in resistance to Cephalosporines rather than other mechanisms of resistance such as the loss of porins and efflux pumps in our research (21).

In this study, the best coverage against ESBL-producing isolates was obtained with Imipenem. Although, the Carbapenem resistance has been rarely reported in past, *but its* resistance rates have recently increased. Nonetheless , Carbapenem remains as the first choice for treatment of infections caused by ESBL-producing *Entrobacteriaceae*. It has been estimated that the worldwide Carbapenem resistance is near 2% (22). When national data are taken into account, this estimation are 0 to 8% (23) Most ESBLs caused resistance to one or more of the Oxyminobeta-lactams, the beta-lactamase does not always increase the MICs (24). The MICs of Ceftazidime in majority of ESBL positive isolates (n = 132) were > 16 μg/mL.

Several studies revealed that distribution of ESBLs is widespread in our country. So the prevalence of *E. coli* and *K. pneumoniae* in 2009, 2010 in Iran were reported 52.5%, 59.2% (25, 26). In this study, the prevalence of ESBL producers among *E. coli* strains was 80.5%, the this amount among *K. pneumonia* strains was 71.9% and the among *E. cloacae* strains was 50%.

In this study, the frequency of CTX-M-1,-2,-8,-9 among isolated bacteria were 92.2%, 28.5%, 17.5%, 38.3% respectively. It has been reported that the presence of CTX-M-type beta-lactamases in *Enterobacteriaceae* isolates is up to 80% globally (27). In Turkey, recent studies reported that CTX-M-type beta-lactamases are very common in *E. coli* Isolated. It was reported that CTX-M-type beta-lactamase was obtained from 86.8% of community and hospital originated ESBL-producing strains (n = 61) (28). In another study, CTX-M-type beta-lactamase was detected in 13 (76.5%) of 17 ESBL-producing *Enterobacteriaceae* strains collecting from community (29). In a more recent study from Turkey, a high prevalence (98%) of CTX-Mtypebeta-lactamases was found in ESBL positive *Enterobacteriaceae* strains (n = 51) isolated from urinary tract infections (30).

Similar studies in Sudan, Korea, Thailand showed that the frequency of CTX-M-1 were respectively 45.9%, 16.4%, 14% compared with our findings is less abundant (31-33). Also, other studies showed the dissemination of CTX-M8 gene in Iran are higher than other parts of the world (34) On the other hand, in this study, the frequency of CTX-M-9 in isolates was less than Germany, America, Canada (ranged 38.5%-58.5%) (35, 36).

Today, an increase in bacterial resistance against antibiotics has become a major worldwide problem. During the past years, increasing rates of infections by extended spectrum beta-lactamase (ESBL)- producing isolates has greatly limited the use of non-carbapenem beta-lactam antibiotics, and thus the importance of Carbapenemand non-beta lactam antibiotics with therapeutic purposes

has incrementally increased. The co-transmission of antibiotic resistance genes via plasmids may also affect the effectiveness of many antibiotics activities. Increased amount of antibiotic resistance rates among clinical isolates have resulted to higher morbidity and mortality and extended periods of hospitalization, consequently, has increased the economic costs (37).

From the results of this study, can be concluded that the number of ESBL – producing *Enterobacteriaceae* in patients enrolled in this study is higher compared with other parts of the world. *Klebsiella* species and *E. coli* are compromising the major microbial population. In order to resolve this problem, it is important to emphasize on the balanced and cyclic use of extended spectrum β-lactam drugs and imply an appropriate infection control strategy in hospitals. In addition, regular surveillance of resistance to antimicrobial agents is necessary.

Our findings were supported by the fact that many CTX-M cases were observed during hospitalization. These significant public health implications mean that the spread of bacteria producing ESBLs (particularly CTX-M enzymes) requires precise monitoring, enhanced surveillance and modifications have to be made in the antibiotic utilization policies with careful consideration, before the resistance predicaments worsen.

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Authors' Contribution

All authors had equal contribution.

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