Original Research Article

Determining frequency of genes of CTX-M and CTX-M-15 of producing *Enterobacteriaceae* of isolated extended-spectrum beta-lactamases from clinical samples of patients referred to training hospitals of Medical Sciences University, Khorramabad, Iran

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Abstract

Objective: The purpose of conducting this research was evaluation of the frequency of extended-spectrum beta-lactamases (ESBLs) in separated *Enterobacteriaceae* isolates from clinical samples in Khorramabad city and determination of their antimicrobial resistance pattern.

Materials and Methods: In this study, 240 isolates belonging to *Enterobacteriaceae* family were collected in time duration between March and June in 2014. The isolates were identified by standard biochemical tests. Producing isolates of enzymes of ESBLs were identified by combined disc method and based on the Clinical and Laboratory Standards Institute criterion, and then, frequency of genes of blaCTX-M and blaCTX-M-15 in positive phenotypic isolates was determined using polymerase chain reaction method.

Results: In the present research, the most frequency was, respectively, belonged to *Escherichia coli* with 76%, *Klebsiella pneumoniae* - 16.2%, *Citrobacter freundii* - 5.4%, *Proteus mirabilis* - 1.6%, and *Enterobacter* - 0.83%. The obtained results from determining the antibiotic sensitivity pattern in the separated isolates showed that the maximum resistance of different isolates was related to antibiotics of ampicillin 88% while the minimum antibiotic resistance of isolates was related to the amikacin antibiotic with resistance value of 2.5%. The obtained results from the combined disc phenotypic method in the present research showed that from 240 *Enterobacteriaceae* isolates, 59% was generators of ESBLs. In addition, 85% of positive phenotype *Enterobacteriacea* had genes of blaCTX-M-15 and blaCTX-M that totally formed 50.4% of all separated bacteria from the clinical samples.

Conclusion: The obtained results from the present research showed that the prevalence of ESBL enzymes and antibiotic resistance to ESBLs is high among the separated *Enterobacteriaceae* isolates from the clinical samples in Khorramabad city. Hence, policies of prescription of antibiotics and infection control in hospitals should be reviewed.

Keywords: CTX-M, CTX-M-15, *Enterobactericeae*, extended-spectrum beta-lactamase, Khorramabad, Iran, polymerase chain reaction

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INTRODUCTION

The appearance of antibiotic resistance among pathogens has become a big problem especially in health centers and hospitals in relation with public health. Various bacteria act by different mechanisms, hence, responds differently to antibacterial agents.[1-5] Different types of bacteria also may become resistant to antibacterial agents by various mechanisms.[6-10] Some bacteria can produce some enzymes that lead to change or destruction of chemical structure of antibiotics and finally cause inactivation of antibiotic and appearance of resistant phenotype by bacteria. The best example of this type of resistance is beta-lactamase enzymes that through beta-lactam ring hydrolysis cause inactivation of beta-lactam antibiotics.[11,12] Among drug resistances, resistance to beta-lactam is the main concern to treat bacterial infections.[13] The main mechanism of bacterial resistance against antibiotics of the beta-lactam class is production of beta-lactamase enzymes. These enzymes hydrolyze and inactivate antibiotics of beta-lactam before reaching penicillin-binding proteins in cytoplasmic membrane. The obtained results from other researches show that the maximum production of extended-spectrum beta-lactamase (ESBL) is respectively formed by *Klebsiella pneumoniae* and *Escherichia coli*.[4] ESBL can hydrolyze oximino-β-lactams completely such as third generation cephalosporins.[14,15] Beta-lactamases of CTX-M (cefotaxime hydrolyzing capabilities) are increasingly prevalent in *E. coli* and *K. pneumonia*. These enzymes based on changes of amino acid are divided into five main groups (CTX-M1, CTX-M2, CTX-M8, CTX-M9, and CTX-M25).[14]

Types of CTX-M2 and CTX-M3 have been spread in the whole world and are dominant types in different countries such as Argentina. More than 50 types of CTX-M have been identified until now.[16]

Approximately 5%–10% of hospitalized patients suffering from opportunistic infections are from organisms of this family. Types of *Salmonella* gender, *Shigella* gender, *Yersinia* gender, and some of *E. coli* strains are important factors of infections of digestive system so are known as enteric pathogen.[17] Some dispersed researches have been conducted in Iran about ESBL.[18]

According to importance of ESBLs, especially strains containing genes of bla-CTX-M in creation and development of resistance among *Enterobacteriaceae* isolates and according to this issue that such research has not been conducted in Khorramabad, so in the present research, we tried to help physicians in the treatment of patients and prevention of resistances due to ESBL by evaluating the value of abundance of these kinds of isolated ESBLs.

MATERIALS AND METHODS

Collection of samples

This descriptively and cross-sectional study were done between March 2014 and June 2014. A total of 240 isolates of bacteria were tested which were related to *Enterobacteriaceae* family from different clinical samples such as blood, cerebrospinal fluid, urine, respiratory tract secretions, and wound (except stool) of patients referred to training hospitals of Shohadaye Ashayer, Shahid Rahimi and Shahid Madani in Khorramabad city (Lorestan province, Iran). By culturing the collected samples on the environment of special Gram-negative such as Mac Cancan agar (Merck Company, Germany), we tried to purify bacteria.

Identification of isolates

Using the standard biochemical tests such as Gram-stain, oxidation test oxidation, triple sugar iron agar, sulfide indole motility, to evaluate mobility, indole, and gas production, methyl red-Voges Proskauer, citrate, and lysine decarboxylase were identified.[9] The identified strains were stored using environment of trypticase soy broth and glycerol (20%) in −70°C to be not destructed to conduct next stages.

Antibiotic test and identification of phenotypic strains of generating extended-spectrum beta-lactamase

The antibiotic sensitivity pattern in strains was determined using disc diffusion method (Kirby-Bauer) according to criteria of Clinical and Laboratory Standards Institute (CLSI-2011).[20] Discs were products of Haymdya Company (India) that included ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), aztreonam (30 μg), ampicillin (30 μg), ciprofloxacin (5 μg), and amikacin (30 μg).

From the passage of the stored strains on blood agar medium (Merck Company, Germany), a single colony of them was inoculated to 5 ml of Mueller-Hinton broth medium (Merck, Germany). This test was conducted based on the method of Watts *et al.* (2008). Finally, results of inhibition zone of each antibiotic and its diameter were measured by a ruler also were compared with CLSI guidelines.[19,21]

Combination disc method

All isolates were tested by combination disc to the phenotypic evaluation of the production of ESBLs enzymes. This was conducted based on the method of Woodford (2004).[21]

Extraction of bacterial genome

The extracted DNA related to isolates was required to conduct polymerase chain reaction (PCR) which was used as a pattern in the reaction.

Polymerase chain reaction

The purpose of conducting PCRs was amplification of genes of blaCTX-M and blaCTX-M-15 from DNA of the extracted DNA from clinical isolates under effect of specific primers. For this purpose, first the used primers were selected from published papers in valid scientific journals.[20] Thermodynamic evaluation and testing specificity of these primers were conducted by primer-blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Specifications of the primers and length of their products have been provided in Table 1.

Preparation of master mix

For preparation of the reaction, the Master PCR Kit (Sina Klon Company, Iran) was used which contained magnesium, buffer, dNTPs, and Taq polymerase enzyme. PCR was conducted in the volume of 25 μl for the both genes of blaCTX-M and blaCTX-M separately. In this order that 1 μl of forward primer, 1 μl of revers primer with a final concentration of 10 pmol, 1 μl of DNA as the pattern, 12.5 μl of master mix containing 2XTaq (Sina Klon Company, Iran), and 9.5 μl sterile twice distilled water were used.

Polymerase chain reaction test

Condition of the reaction for the both genes of blaCTX-M and blaCTX-M-15 was in this way that proliferation of the intended part was conducted using thermocycler instruments of Eppendorf (made in Eppendorf Company, Germany) with an initial denaturation temperature of 94°C for 5 min, then 36 cycles with denaturation temperature of 94°C for 30 s, connection of 52°C in 40 s, and development stage of 72°C in 50 s and final development stage of 72°C for 5 min.

PCR was conducted in the volume of 25 μl for the both genes of blaCTX-M and blaCTX-M separately. In this order that 1 μl of forward primer, 1 μl of revers primer with a final concentration of 10 pmol, 1 μl of DNA as the pattern, 12.5 μl of master mix containing 2XTaq (Sina Klon Company, Iran), and 9.5 μl sterile twice distilled water were used. Meanwhile, the clinical isolates of *K. pneumonia* which had genes of blaCTX-M and blaCTX-M-15 and had been prepared from Iran's Pasteur Institute to positive control. The negative control included all required materials to conduct PCR and only instead of the pattern DNA, 1 μl of deionized water was added to vial and was used as the negative control.

Table 1: Specifications of the primers and length of their products

Size (bp)	Primer sequence	PCR target
590	F: 5-TTTGCGATGTGCAGTACCAGTAA-3 R: 5-CGATATCGTTGGTGGTGCCATA-3	blaCTX-M
885	F: AGAATAAGGAATCCCATGGTT R: ACCGTCGGTGACGATTTTAG	blaCTX-M15

PCR: Polymerase chain reaction

Electrophoresis

Buffer of ×10 was prepared, but ×1 buffer was used to conduct electrophoresis.[12] Electrophoresis product of PCR was conducted on agarose gel of 1% in the method of Mirsalehian *et al.*[22] In this evaluation, the *K. pneumoniae* strains with ATCC 700603 was used as the positive control, and distilled water was used as the negative control.

The method of analyzing data

The information was analyzed through the statistical software of SPSS (version 16.0). The descriptive statistic methods were used to analyze the obtained results statistically. Because with PCR method in average, approximately 20% of *Enterobacteriaceae* have gene of CTX-M and according to the frequency of *Enterobacteriaceae* of positive phenotypic in the clinical samples, by considering the reliability of 0.95 and with accuracy assumption of 0.05, 240 *Enterobacteriaceae* were evaluated in this research.

RESULTS

Results of separation of isolates

Members of *Enterobacteriaceae* family make wide range of infections such as infections of the gastrointestinal tract, urinary tract, and septicemia.[20] Nowadays, the producing *Enterobacteriaceae* of ESBLs have been propounded as a major problem in medical bacteriology because their treatment is very difficult and also controlling the made infections by them face some difficulties in hospital.

From 240 isolates, 86 male and 154 female subjects, and from each clinical sample, only one isolate was evaluated [Table 1]. The patients were in the age range between 18 and 72 years. The clinical isolates were separated from samples such as blood, cerebrospinal fluid, urine, respiratory system, and wound that value of relative frequency of each one has been summarized in Table 2. The clinical samples of the hospitalized patients (58%) and outpatients (42%) were related to three hospitals of Shohadaye Ashayer, Shahid Rahimi, and Shahid Madani that the frequency of each is shown in Table 2.

To purify and accurate determination of the identity of isolates, again inoculation was formed to blood agar, and MacCancan agar mediums and the separated colonies were identified by using standard biochemical tests. The relative frequencies of the separated isolates were provided in Table 3. According to this issue that the outbreak of infections due to generating *Enterobacteriaceae* of beta-lactamase is increasing in whole world and Iran.[23,24] Hence, in the present research that was conducted in time interval of March to July in 2014 in Khorramabad city, on 240 isolates belonging to *Enterobacteriaceae* family, the relative frequency of types of the separated bacteria belonging to *Enterobacteriaceae* family, the antibiotic resistance pattern in them, frequency of generating isolates of ESBLs enzymes in the phenotypic method of combination disc, and finally the frequency of presence of blaCTX-M and blaCTX-M-15 in positive phenotypic isolates using PCR method were evaluated.

Table 2: Number of the studied patients in separation of gender

Gender	*n* (%)
Male	86 (36)
Female	154 (64)
Total number	240 (100)

Table 3: The relative frequency of the different clinical samples

Type of sample	Number of sample (%)
Blood	63 (26)
Spinal fluid	22 (9)
Urine	113 (47)
Respiratory tract secretions	26 (11)
Wound exudates	16 (7)
Total	240 (100)

Table 4: The relative frequency of types of the separated bacteria belonging to *Enterobacteriaceae* family

Name of bacteria	*n* (%)
Escherichia coli	182 (76)
Klebsiella pneumoniae	39 (16.2)
Citrobacter frondii	13 (5.4)
Proteus mirabilis	4 (1.66)
Enterobacter	2 (0.83)
Total	240 (100)

Based on the results of this research, the maximum and minimum relative frequencies of the separated bacteria belonged to *Enterobacteriaceae* family were related to *E. coli* (76%) and *Enterobacter* (0.83%). Further information related to types of the separated bacteria belonging to *Enterobacteriaceae* family are specified in Table 4. Based on the obtained results in the present research, from 240 isolates belonging to *Enterobacteriaceae* family, the maximum frequency, respectively, belonged to *E. coli* with 76% (182 isolates), *K. pneumoniae* 16.2% (39 isolates), *Citrobacter* 5.4% (13 isolates), *Proteus mirabilis* 1.6% (4 isolates), and *Enterobacter* 0.83% (2 isolates).

The results related to the antibiotic sensitivity pattern for 240 isolates belonging to *Enterobacteriaceae* family in accordance with the guideline of CLSI were provided in Table 4.

The maximum antibiotic resistance of different isolates of *Enterobacteriaceae* was to ampicillin antibiotics (88%) and to cefotaxime (43%) while the minimum antibiotic resistance of these isolates was related to amikacin antibiotic with resistance value of 2.5%.

The obtained results from determination of antibiotic sensitivity pattern in the separated isolates showed that the maximum antibiotic resistance of different isolates of *Enterobacteriaceae* was related to ampicillin (88%) and cefotaxime (43%) while the minimum antibiotic resistance of isolates was related to amikacin with resistance value of 2.5%. In the present research, values of resistance to cefotaxime and ceftazidime were, respectively, equal to 43% and 39% that are higher compared with research of Nijssen

Table 5: Frequency of the presence of blaCTX-M and blaCTX-M-15 in 141 positive phenotypic isolates

Number of positive phenotypic isolates	Number of positive isolates of blaCTX-M	Percent	Number of positive isolates of blaCTX-M 15	Percent
141	111	78.72	75	53.19

Table 6: The relative frequency of presence of the evaluated genes in the 141 evaluated positive phenotypic isolates

Bacteria name (number of positive ESBL isolates)	blaCTX-M 15 (number of isolates)	blaCTX-M (number of isolates)	Ctx-m + ctxm-15
Escherichia coli (108)	59	85	50
Klebsiella (22)	10	16	10
Proteus (2)	1	2	1
Enterobacter (2)	1	2	1
Citrobacter (7)	4	6	3
Total (141)	75	111	65

ESBL: Extended-spectrum b-lactamase

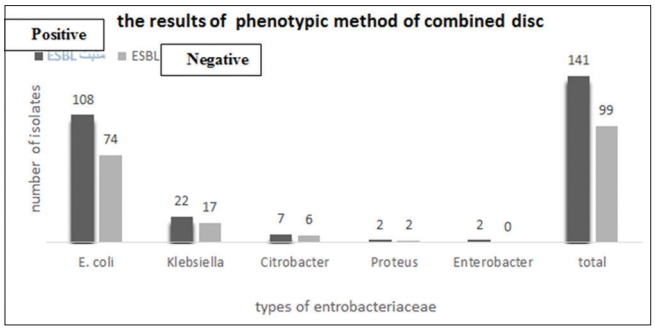

Figure 1: The comparative graph of number of positive and negative extended-spectrum beta-lactamases for bacterial isolates of *Enterobacteriaceae* family

et al.[25] and Jones *et al.*[26] with value of 18.7% and 10%. The resistance values to cefotaxime and ceftazidime were 44% and 42% in the conducted research by Mousavian *et al.* on the isolates of *Enterobacteriaceae* which are consistent with the present research.[27]

Results of phenotypic method of combination disc to identify producing isolates of ESBL enzymes are provided in Figure 1. In this test, after preparation of Mueller-Hinton agar medium, microbial suspension with the concentration of half McFarland was completely inoculated in the mentioned medium and then ceftazidime discs (30 μg), ceftazidime-clavulanic acid (30-10 μg), cefotaxime (30 mg), and cefotaxime-clavulanic acid (30–10 mg), (prepared by the MAST Company, England) were put on the medium in the least distance of 2.5 from each other. After 24 h, the incubation at 37°, inhibition zone around the disc containing clavulanic acid as an inhibitor of ESBLs enzymes was tested to discs without clavulanic acid. If inhibition zone around the disc containing clavulanic acid was higher than or equal to 5 mm to without clavulanic acid, the intended strain according to CLSI criterion was considered as generator of ESBLs [Figure 1].

The obtained results from phenotypic method of combination disc in the present research showed that from 240 isolates of *Enterobacteriaceae,* 59% were generator of ESBLs which their maximum was *E. coli* 74% and the minimum case was *Enterobacter* (2%). In fact, among 94 resistant isolates to ceftazidime 70 isolates and among 103 resistant isolates to cefotaxime the 84 isolates were phenotypically generator of ESBLs. This issue shows that existence of ESBL enzymes has higher importance in the creation of bacterial resistance to cephalosporins compared with other resistance mechanisms such as loss of purines of the outer membrane in Gram-negative bacteria.[28]

In the present research, the maximum isolates that were identified as positive phenotype according to the results of phenotypic method were *E. coli* types that from 182 isolates; 108 isolates (60%) were positive phenotype. The outbreak of generating isolates of ESBLs is different in different cities of Iran so that in the conducted research by Ghafourian *et al.*[29] from 288 separated *K. pneumoniae* isolates from the clinical samples of hospitals in the cities of Tehran, Ilam and Tabriz were, respectively, 50.7%, 39.4%, and 45.8% were generator of ESBLs.[30] In another conducted research by Mousavian *et al.* in Dezful city, frequency of generating *Enterobacteriaceae* of ESBLs was obtained 30.5% that its outbreak in isolates of *K. pneumoniae* and *E. coli* has been, respectively, reported 45.4% and 28.8%. In another conducted research by Zaniani *et al.*[31] in Mashhad city, 43.9% of *E. coli* isolate, and 56.1% of *K. pneumonia* isolates were producers of beta-lactamase that also in this research 74% of *E. coli,* and 17% of *Klebsiella* were producers of beta-lactamase which are consistent with this research. Also in the research of Feizabadi *et al.*[32] among the 104 separated *K. pneumonia* from four hospitals in Tehran city, 72.1% of isolates were producers of enzymes of ESBLs. In the conducted research by Gholipour *et al.*[33] in Isfahan city, from 245 isolates of *E. coli* 107 isolates (43.6%) and from 55 isolates of *Klebsiella* 21 isolates (38%) became positive phenotype. In a conducted research by Mousavian *et al.*[27] in Ahwaz city from 240 separated isolates from different clinical samples, value of outbreak of generating strains of ESBLs using the phenotypic method of combination disc was obtained 45%. So that isolates of *Klebsiella* with 66.6% had the maximum frequency and after that were, respectively, *E. coli* with 46% and *Enterobacter* with 41.5%.

Values of outbreak of types of generating *Enterobacteriaceae* of ESBLs in other countries such as European countries are different from country to another country so that the value of isolates of ESBLs in Germany in two bacteria of *E. coli* and *K. pneumonia* has been reported 1.5% while these enzymes in the separated isolates from Russia, Poland, and Turkey has been reported 39%–47%.[34]

The difference in outbreak of ESBLs in *Enterobacteriaceae* in various parts of Iran and the world may be due to different causes such as the consumption pattern of antibiotics, especially extended-spectrum cephalosporins, amount of consumption, and difference in time of collecting isolates.[17]

Results of phenotypic method of combination disc became positive in 141 different isolates of *Enterobacteriaceae*. Results of the frequency of the presences of blaCTX-M and blaCTX-M-15 in 141 positive phenotypic isolates using PCR method were provided in Table 5.

Results of frequency of presence of blaCTX-M and blaCTX-M15 in positive phenotypic isolates by using polymerase chain reaction method

After extraction of genome of isolates and conducting test PCR, the obtained products from PCR were evaluated using horizontal electrophoresis and gel docking device, based on comparing their length with marker and the obtained results from positive control and negative control [Figures 2 and 3].

Frequencies of blaCTX-M and blaCTX-M15 in 141 evaluated positive phenotypic isolates in this research were, respectively, obtained 79% (111 isolates) and 53% (75 isolates). Totally, from 141 positive phenotypic isolates, 121 isolates were considered positive using PCR method as ESBL. Frequencies of the presence of the evaluated genes were provided in Table 6. In the present research, among 141 positive phenotype isolates, 75 isolates had blaCTXM-15, and 65 isolates had both genes of blaCTX-M and blaCTX-M15. In the conducted research by Safari *et al.* in Arak city from the 350 isolates belonging to the evaluated *Enterobacteriaceae* family 154 phenotype isolates became positive that the frequencies of encoder genes of beta-lactamase enzyme of CTXM-1, CTXM-2, CTXM-8, and CTX-M-9 were, respectively, obtained 92%, 25%, 17.5%, and 38.3%.[35]

Evaluation of the significant relationship between results of antibiogram and genotypic results

The obtained results from antibiogram in the phenotypic method of combination disc and obtained results from PCR in genotypic result were evaluated using SPSS software. Based on this, the genotypic and phenotypic relationship was evaluated between each strain that its final result for all isolates for antibiotics of cefotaxime and ceftazidime were mentioned in Tables 7 and 8. It shows that in isolates that do not have the studied genes, number of sensitive isolates is more and in isolates that have the intended genes number of the resistant isolates is more that show relationship of existence of gene to antibiotic resistance.

This replacement not only is due to excessive transfer and release of the mentioned genes because of mobile genetic factors but also another cause of that is placement of these genetic platforms on the clones that the simultaneous

Table 7: Evaluation of statistical relationship between the results of polymerase chain reaction method and the resistance pattern of ceftazidime antibiotic number of sensitive isolate

Gene	Ceftazidime		*P*
	Number of sensitive isolate (%)	Number of resistant isolate (%)	
CTX-M			
Have	57 (51)	51 (46)	0.537
Do not have	41 (36.9)	92 (32.6)	
CTX-M-15			
Have	47 (62.6)	31 (41.3)	0.041*
Do not have	51 (77.2)	113 (6603)	

*Positive significant relationship

Table 8: Evaluation of statistical relationship between the results of polymerase chain reaction method and the resistance pattern of cefotaxime antibiotic

Gene	Cefotaxime		*P*
	Number of sensitive isolate (%)	Number of resistant isolate (%)	
CTX-M			0.048*
Have	64 (57.6)	44 (39.6)	
Do not have	40 (36)	90 (81)	
CTX-M-15			0.038*
Have	51 (68)	27 (36)	
Do not have	54 (81)	111 (59.4)	

*Positive significant relationship

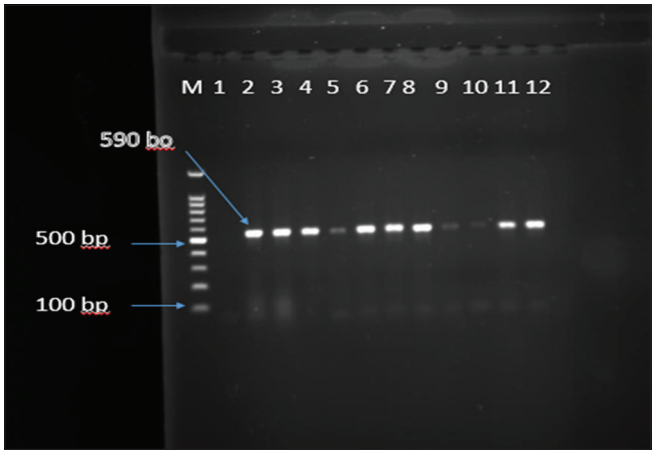

Figure 2: Results of polymerase chain reaction for blaCTX-M with 590 bp band compared with marker 100 bp. Product of polymerase chain reaction, M: Marker, sink 1: Negative control, sink 2: Positive control (*Klebsiella pneumoniae* ATCC 700603), sinks 4, 6, 7, 8, and 3: Positive samples sink 9 and 10 negative samples in terms of blaCTX-M

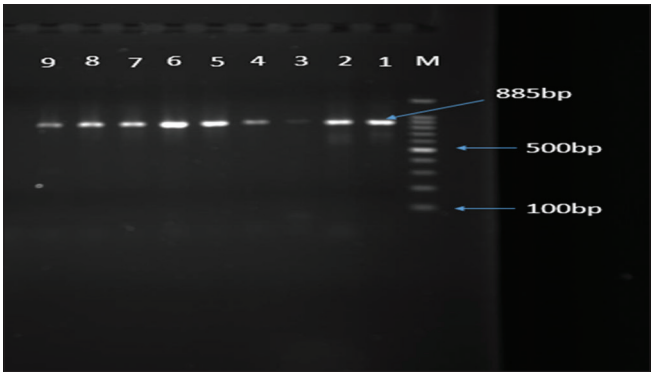

Figure 3: Results of polymerase chain reaction gene of blaCTX-M 885 bp bond compared with marker 100 bp product of polymerase chain reaction, M: Marker, sink 1: Positive control (*Klebsiella pneumoniae* ATCC 700603), sinks 2–9: Positive samples and sink 3: Negative samples in terms of gene 15 blaCTX-M with size of 885 bp

Table 9: Antibiotic sensitivity pattern of 240 different isolates of *Enterobacteriaceae* to used antibiotics

Antibiotics (μg)	Total, *n* (%)	*Escherichia coli* (*n*=182), *n* (%)	*Klebsiella* (*n*=39), *n* (%)	*Enterobacter* (*n* =2), *n* (%)	*Proteus* (*n*=4), *n* (%)	*Citrobacter* (*n*=13), *n* (%)
Ceftazidime (30)	94 (39)	71 (39)	18 (46)	1 (50)	0	4 (31)
Cefotaxime (30)	103 (43)	77 (43)	19 (49)	1 (50)	1 (25)	5 (38)
Ceftriaxone (30)	100 (42)	74 (40.3)	20 (51)	1 (50)	1 (25)	5 (38)
Aztreonam (30)	91 (38)	66 (36)	19 (49)	1 (50)	0	5 (38)
Ampicillin (30)	210 (88)	157 (86)	37 (95)	2 (100)	3 (75)	11 (85)
Ciprofloxacin (5)	90 (37.5)	67 (36.6)	17 (44)	1 (50)	1 (25)	4 (31)
Amikacin (30)		2 (0.54)	3 (8)	0	0	1 (7.6)

resistance to other antibiotics such as fluoroquinolones and aminoglycosides is their specifications. This issue causes more resistance of them by antibiotics. Among enzymes of CRX-M, producing isolates of CTX-M15 enzymes because of their ability to create infection among humans and animals and have been reported from the entire world have special importance.[36]

The obtained results from the present research showed that the outbreak of ESBL enzymes and antibiotic resistance to antibiotics of ESBL among the separated *Enterobacteriaceae* isolates from the clinical samples of Khorramabad city is high that this issue has made the antibiotic resistance a big concern. Therefore, policies of prescription of antibiotics and infection control in hospitals should be reviewed. It should be noted that the mechanism action and the resistant pattern are different in various bacteria.[7]

DISCUSSION

Members of *Enterobacteriaceae* family make wide range of infections such as infections of the gastrointestinal tract, urinary tract, and septicemia.[20] Nowadays, the producing *Enterobacteriaceae* of ESBLs have been propounded as a major problem in medical bacteriology because their treatment is very difficult and also controlling the made infections by them face some difficulties in hospital. According to this issue that the outbreak of infections due to generating *Enterobacteriaceae* of beta-lactamase is increasing in whole world and Iran.[23,24] Hence, in the present research that was conducted in time interval of March to July in 2014 in Khorramabad city, on 240 isolates belonging to *Enterobacteriaceae* family, the relative frequency of types of the separated bacteria belonging to *Enterobacteriaceae* family, the antibiotic resistance pattern in them, frequency of generating isolates of ESBLs enzymes in the phenotypic method of combination disc, and finally frequency of the presence of blaCTX-M and blaCTX-M-15 in positive phenotypic isolates by using PCR method were evaluated.

Based on the obtained results in the present research, from 240 isolates belonging to *Enterobacteriaceae* family, the maximum frequency, respectively, belonged to *E. coli* with 76% (182 isolates), *K. pneumoniae* 16.2% (39 isolates), *Citrobacter* 5.4% (13 isolates), *P. mirabilis* 1.6% (4 isolates), and *Enterobacter* 0.83% (2 isolates).

The obtained results from the determination of antibiotic sensitivity pattern in the separated isolates showed that the maximum antibiotic resistance of different isolates of *Enterobacteriaceae* was related to ampicillin (88%) and cefotaxime (43%) while the minimum antibiotic resistance of isolates was related to amikacin with the resistance value of 2.5%. In the present research, values of resistance to cefotaxime and ceftazidime were, respectively, equal to 43% and 39% that are higher compared with research of Nijssen *et al.*[25] and Jones *et al.*[16] with value of 18.7% and 10%. The resistance values to cefotaxime and ceftazidime were 44% and 42% in the conducted research by Mousavian *et al.* on the isolates of *Enterobacteriaceae* which are consistent with the present research. Antibiotic sensitivity pattern of 240 different isolates of Enterobacteriaceae to used antibiotics was indicates in Table 9.[27]

The obtained results from the phenotypic method of combination disc in the present research showed that from 240 isolates of *Enterobacteriaceae,* 59% were generator of ESBLs which their maximum was *E. coli* 74% and the minimum case was *Enterobacter* (2%). In fact, among 94 resistant isolates to ceftazidime 70 isolates and among 103 resistant isolates to cefotaxime the 84 isolates were phenotypically generator of ESBLs. This issue shows that existence of ESBL enzymes has higher importance in the creation of bacterial resistance to cephalosporins compared with other resistance mechanisms such as loss of purines of the outer membrane in Gram-negative bacteria.[28] In the present research, the maximum isolates that were identified as positive phenotype according to the results of phenotypic method were *E. coli* types that from 182 isolates, 108 isolates (60%) were positive phenotype. The outbreak of generating isolates of ESBLs is different in different cities of Iran so that in the conducted research by Ghafourian *et al.*[29] from 288 separated *K. pneumoniae*

isolates from the clinical samples of hospitals in cities of Tehran, Ilam, and Tabriz, were, respectively, 50.7%, 39.4%, and 45.8% were generator of ESBLs.[30] In another conducted research by Mousavian *et al.* in Dezful city, the frequency of generating *Enterobacteriaceae* of ESBLs was obtained 30.5% that its outbreak in isolates of *K. pneumoniae* and *E. coli* has been, respectively, reported 45.4% and 28.8%. In another conducted research by Zaniani *et al.*[31] in Mashhad city, 43.9% of *E. coli* isolate, and 56.1% of *K. pneumonia* isolates were producers of beta-lactamase that also in this research, 74% of *E. coli* and 17% of *Klebsiella* were producers of beta-lactamase which are consistent with this research. Also in the research of Feyzabadi *et al.*[32] among the 104 separated *K. pneumonia* from four hospitals in Tehran city, 72.1% of isolates were producers of enzymes of ESBLs. In the conducted research by Gholipour *et al.*[33] in Isfahan city from 245 isolates of *E. coli* 107 isolates (43.6%) and from 55 isolates of *Klebsiella* 21 isolates (38%) became positive phenotype. In a conducted research by Mousavian *et al.*[27] in Ahwaz city from 240 separated isolates from different clinical samples, value of outbreak of generating strains of ESBLs using phenotypic method of combination disc was obtained 45%. So that, the isolates of *Klebsiella* with 66.6% had the maximum frequency, and after that were respectively *E. coli* with 46% and *Enterobacter* with 41.5%.

Values of outbreak of types of generating *Enterobacteriaceae* of ESBLs in other countries such as European countries are different from country to another country so that the value of isolates of ESBLs in Germany in two bacteria of *E. coli* and *K. pneumonia* has been reported 1.5% while these enzymes in the separated isolates from Russia, Poland, and Turkey has been reported 39%– 47%.[34]

The difference in outbreak of ESBLs in *Enterobacteriaceae* in various parts of Iran and the world may be due to different causes such as the consumption pattern of antibiotics, especially extended-spectrum cephalosporins, amount of consumption, and difference in time of collecting isolates.[17] In the present research from 141 isolates of positive phenotype, *Enterobacteriaceae* 121 isolates (85%) had genes of blaCTX-15 and blaCTX-M that totally forms 50.4% of whole isolated bacteria from clinical samples.

The most isolates that had this genotype were strains of *E. coli* (94 isolates), *K. pneumonia* (16 isolates), *Citrobacter* (7 isolates), *Proteus* (2 isolates), and *Enterobacter* (2 isolates). The 20 positive phenotype isolates did not have these two genes that probably have other ESBL enzymes such as TEM or SHV or other enzymes.[37]

The encoder genes of beta-lactamase enzymes of blaCTX-M are transferred among bacteria by mobile genetic factors such as plasmids and transposons.[38] Studies in the last recent years apart from a few exceptions have shown that encoder genes of blaCTX-M enzymes have been approximately replaced to the other enzymes of ESBL in *Enterobacteriaceae*.

This replacement not only is due to excessive transfer and release of the mentioned genes because of mobile genetic factors but also another cause of that is the placement of these genetic platforms on the clones that the simultaneous resistance to other antibiotics such as fluoroquinolones and aminoglycosides is their specifications. This issue causes more resistance of them by antibiotics. Among enzymes of CRX-M, producing isolates of CTX-M15 enzymes because of their ability to create infection among humans and animals and have been reported from the entire world have special importance.[36]

In the present research, among 141 positive phenotype isolates, 75 isolates had blaCTXM-15, and 65 isolates had both genes of blaCTX-M and blaCTX-M15. In the conducted research by Safari *et al.* in Arak city from the 350 isolates belonging to the evaluated *Enterobacteriaceae* family, 154 phenotype isolates became positive that the frequencies of encoder genes of beta-lactamase enzyme of CTXM-1, CTXM-2, CTXM-8, and CTX-M-9 were respectively obtained 92%, 25%, 17.5%m and 38.3%.[35]

The obtained results from the present research showed that the outbreak of ESBL enzymes and antibiotic resistance to antibiotics of ESBL among the separated *Enterobacteriaceae* isolates from the clinical samples of Khorramabad city is high that this issue has made the antibiotic resistance a big concern. Therefore, policies of prescription of antibiotics and infection control in hospitals should be reviewed. It should be noted that the mechanism action and the resistant pattern are different in various bacteria.[7]

CONCLUSION

According to high capability of isolates belonging to *Enterobacteriaceae* family to achieve resistance to various antibiotics, the continuous monitoring should be conducted to evaluate changes related to antibiotic sensitivity pattern in these bacteria. In addition, it is suggested that in the future researches typing methods be used to find genetic relation between resistant isolates and to find the source of infection in hospitals.

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Conflicts of interest

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