Detection of *CTX-M*β-lactamase in *Escherichia coli* using Disk diffusion, combined disk, MIC and Multiplex PCR methods

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Abstract: Introduction: β-lactamase enzymes are *CTX-M*enzymes that hydrolyze cephalotine and cefaloridine better than penicillin, and also preferentially hydrolyze cefotaxime over ceftazidime. The aim of the study was to investigate the prevalenceof ESLB-producing *E. coli*, and molecular evaluation of CTX-Mβ-lactamases using Multiplex in *E. coli*strains. **Materials and methods:** A total of 200 *E.coli*isolates werecollected from clinical specimens and the antibiotic susceptibility of strains was determined using Disk Diffusion method. The production of ESBL enzymes was determined using Combined Disk,Cefotaxime and Ceftazidime with and without Clavulanic Acid. Also, MIC of Ceftazidime and Cefotaxime was determined with and without Clavulanic Acid using Agar Dilution method. Finally, the presence of *CTX-M*genes was detected by specific primers using Multiplex PCR method. **Findings:** The drug resistance of separated isolates was determined in relation to 14 anti- Antibiotics. In the Combined Disk method,increase in inhibition zone diameter by ≥ 5mm against ceftazidime and cefotaxime with and without Clavulanic Acid as ESBL producers were identified. There were 155 isolates (77.5%) in Combined Disk method as ESBL-producing*E. coli.*Amongthese ESLB-producing isolates, 66 ESBL samples contained MIC_{CAZ}= 16-32 (µg/ml), and 24 samples contained 64 to 512. Also, 48 samples contained MIC_{CTX}=4-8 and 97 samples contained 16 to 512 for CTX. 4/57% of these isolates in Multiplex PCR method were *CTX-M*-producers. **Conclusion**: The results show that the production of β-lactamase ESBL enzymes in the strains is high (77.5%), which is confirmed by Agar Dilution and Combined Disk methods. PCR method shows higher prevalence of *CTX-M*enzyme. Therefore, appropriate treatment is recommended in the country to prevent the spread of ESBL-producing *Escherichia coli* strains.

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Introduction

E. coliis one of the bacteria capable of producing ESBL enzymes. This bacterium is the key member of Enterobacteriaceae family and it is the main reason for many hospital-acquired infections such as gastroenteritis, enteritis, neonatal meningitis, sepsis and urinary tract infections. E. coli is the most prevalent form of gram-negative bacilli isolated from clinical specimens. Also, it is the reason behind more than 80% of community-acquired urinary tract infections as well as hospital-acquired ones [1-2]. ESBLs were reported for the first time from Japan in 1986, and from then on, they have spread in different parts of the world. This enzyme has been reported inE.coli, K. pneumoniae and *Kluyvera ascorbata,* but it is also found in other Enterobacteriaceae[1-2]. These β-lactamase enzymes are divided into 5 clusters: CTX-M-1,which the main ones are CTX-M 1, 3, 10, 12, 15 and UOE-1. They emerge from *Kluyvera ascorbata* and they are encoded by Plasmid. CTX-M-2 Includes CTX-M 2, 4, 5, 6, 7, 20; CTX-M-44 also belongs to this cluster which is previously referred to as TOHO-1.FEC-1also belongs to this family. They also emerge from Kluyvera ascorbata and are encoded by Plasmid. CTX-M-8is the most important member of the cluster CTX-M-40, and they emerge from *Kluyvera georgiana*. CTX-M-9include 9, 13, 14, 16, 17, 18, 19 - 21, 24, 27, 46, 47, 48, 49 and 50 CTX-M. CTX-M-45belongs to these families which arepreviously referred as TOHO-2. They, like the previous group, emerge from *Kluyvera georgiana*is one. The origin of CTX-M-25which Includes 25, 26, 39 and 41CTX-Ms, unlike previous clusters, is still known. These clusters are identified based on their amino acid sequences [2].

These β-lactamases have little relation with the members of the *TEM* and *SHV*β-lactamases, but they are more similarities between chromosomal enzymes of a*mp-C*except *KLU-1*, and between *KLU-2*with*CTX-M*enzymes and they seem to be the derivatives of one kind of species [3]. Clinical studies show that *CTX-M*enzymes hydrolyze cephalotine and cefaloridine better than penicillin, and also these enzymes hydrolyze cefotaximepreferentially over ceftazidime and hydrolysis of ceftazidime is not that strong to cause clinical resistance to this bacterium. The presence of iodine amino acid in 237thposition, which exists in all *CTX-M*enzymes, plays an important role in the Extended-spectrum of β-lactamase activity of these enzymes [4].

Among *CTX-M* enzymes, *TOHO-1*group is effective on a wide spectrum of β-lactams, compared to other groups and the reason is the lack of hydrogen bond and flexibility in chain of Ω reactant and the area of this circle. This enzyme can easily hydrolyze cefotaxime and it is inhibited by Tazobactam better than Clavulanic acid [4]. The first *CTX-M* endemics were reported in Latin America and Eastern Europe, but from 2000, numerous cases of spread of these enzymes were report in the Western Europe countries such as Greece, France, England and even Mongolia[4-5].

CTX-M enzymes, except the TOHO-*2*enzyme, contain 291 amino acids with the molecular mass of about 28KDa with isoelectric points (PIs) rangingfrom of 7.4 to 9, and they have the highest Hydrolytic activity against narrow-spectrum Cephalosporins. Their activity against cefotaxime is often 35-fold greater than that against ceftazidime. For example, the*SFO-1*enzyme which is encoded by plasmid has more hydrolytic activityagainst Cefepime and Cefpirome andexhibits no hydrolytic activity against the 7-α-substituted β-lactams (Cefoxitin), and they have Imipenem[40]. The aim of this study was to determine the prevalence of ESBL- producing *E. coli*and molecular evaluation *CTX-M*β-lactamases using Multiplex PCR method in *E.coli*strains

Materials and methods

Collection of clinical samples

In this Descriptive-analytical and Cross-Sectional study, a total of200 E. coli isolates were collected from 5 hospitals in Tehran city (Children's Medical Center, Tehran Heart Center, Bagiatallah, Milad and Mehr) in the first 6 months of 1390. These bacteria were separated from various clinical samples such as wound, blood, tissue, secretion, urine and faces, and they were confirmed by biochemical tests.

Disk Diffusion test

This test is the most common test used to test for antibiotic resistance on the agar. Antibiotic Susceptibility of Isolated strains was determined using Disk diffusion method (Kirby-Bauer) as recommended by CLSI against 14 against antibiotics: Cefoxitin 30 µg (FOX), Ceftazidime 30 µg(CAZ), Cefotaxime 30 µg (CTX), Cefepime 50 µg (CPM), Aztreonam 30 µg (ATM), Erythromycin 15 µg (ERY), Gentamicin 10 µg (GM), Tetracycline 30 µg (TE), Co-trimoxazole 25 µg (SXT), Co-amoxiclav 30 µg (AX), Ampicillin 25 µg (AM), Imipenem 10 µg (IPM), Amikacin 30 µg (AN), Ciprofloxacin 30 µg (CP).

Combined Disk test for the detection of ESBLs

Producing ESBL enzymes using Combined Disk method and using cefotaxime and ceftazidime against antibiotics with and without phenotypic acid was determined. In this test, ceftazidime 30µg + 10µg Clavulanic avidand Cefotaxime 30µg+ 10µg Clavulanic avidwere used with Ceftazidime and with Cefotaxime alone. After 24 hours of incubation at 37°C, production of ESBLs by increasing the size of the inhibition zone diameter by about ≥ 5 mm against ceftazidime and cefotaxime in combination of each of them with Clavulanic Acid was the sign of ESBL production.

Determination of the antibiotic minimum inhibitory concentration (MIC) using agar dilution method

In this study, antibiotic minimum inhibitory concentration of Cefotaxime and Ceftazidime was determined using antibiotic powders with and without Clavulanic acid.

Test method for the determination of the minimum inhibitory concentration (MIC)

MIC Method was also used for ceftazidime and cefotaxime and it was used on sensitive samples as well. The test was carried out forresistant samples in 6 dilutionsof 16, 32, 64, 128, 256 and 512µg / ml, and on sensitive samples with dilutions of<5/0, 5/0, 1, 2, 4 and 8µg / ml. MIC in the presence of an inhibitor was also performed in the same way in which 4 mg/ml in constant concentration was added to all Muller Hinton agar plates containing sequential concentrations of antibiotics. Rates of the inoculated bacteria was 10^4 cfu / spot. Finally, plates were kept incubated at 37 ° C for 24-18 hours. (Desired powders were obtained from Himedia Co, India). For quality control of MIC, Antibiogram methods, and Combined Disk *E.coli ATCC 25922*were employed.

DNA Extraction

For DNA extraction Cinna Gen Kit was used in this method. For this purpose, was taken from overnight bacteria culture and it was solved in 500 Landa TE Buffer (10 min centrifugation at 7500 g), Then we removed the surface liquid and added100 ml protease buffer and kept it at 95 ° C for 10 minutes. Then, 400 ml lysis solution was added and

homogenized thoroughly using vortex. Subsequently, 300 ml Precipitation solution was added and it was vortexed for 3 to 5 seconds at - 20 ° C for 10 min for 10 min. Then, we kept in 12,000 g Centrifugation for 10 min, drained and dried the micro tubes.We added 1 ml of wash buffer on the bacteria pellet and centrifuged at 12,000 rpm for 5 minutes, and we drained the washed the buffer and kept it at 65 ° C for 5 minutes. We shook the pellet in 50 ml solvent buffer and kept it at 65 ° C for 5 minutes. Finally, we centrifuged for 30 seconds, and poured the supernatant in sterile micro tube for PCR and kept it in refrigerator at - 20 ° C.

Multiplex PCR

To evaluate the prevalence of β-lactamase CTX-M genes, primers listed in Table 1 were used. 5 µl of extracted DNA Extracted PCR Master mix with final volume of 25µl (each vial contains 1 µl Mgcl2 (From 50 mM stock), 2 µl10X buffer, 2 ml dNTP (from 10 mmol stock) And 1 ml from each primer (from mix primer 10 pM) and 1 ml of Taq Polymerase enzyme) was added and (CinnaGen) marker bp 100 was used to confirm the molecular weight of the amplified products in PCR Used and the results were analyzed using electrophoresis in 2% Agarose gel.

The primers which were used to identify CTX-M gene are as follows(6):

Table 1. Gen names, Nucleotide sequence and Product size used in current study

Gene Name	Nucleotide sequence	Product Size
CTX-M1 (F)	GGT TAA AAA ATC ACT GCG TC	850
CTX-M1 (R)	TTG GTG ACG ATT TTA GCC GC	
CTX-M2 (F)	ATG ATG ACT CAG AGC ATT CG	850
CTX-M2 (R)	TGG GTT ACG ATT TTC GCC GC	
CTX-M9 (F)	ATG GTG ACA AAG AGA GTG CA	850
CTX-M9 (R)	CCC TTC GGC GAT GAT TCT C	

Thermo-cycler programming was carried out for CTX-M gene as follows: 3 subtypes *(CTX-M1-CTX-M2AndCTX-M9)* was considered for this gene using Multiplex PCR method. The initial 4-min denaturation at 94 ° C, then 35 cycles of denaturation at 94 ° C for 45 s, the pairing level of primers 55 ° C for 45 seconds, and the primer elongation at 72 ° C for 1 min. Finally, final elongation was done at 72 Celsius for 10 min.

Results

Among200 *E. coli* strains Isolated from clinical specimens, 63 samples (5/31%) were related to urine samples, 38 samples to (19%) to faces, 35 samples (17.5%) to wound, 28 samples to tissue, 21 samples to secretions and 15 samples (5/7%) were related to blood. Resistance of the isolated strains against various antibiotics is as follows:

The highest percentage of antibiotic resistance belongs to Erythromycin and Ampicillin by about 5/93% and 91% respectively, and the lowest percentage of resistance belongs to Imipenem with 5/0% and Amikacin with 5/15%. Disc diffusion results in resistant, average resistant and susceptible *E. coli* strains are given in table 2. (The analysis was carried out using SPSS 20).

Table 2.Antibiotic resistance inclinical strains of*E.coli*

Row	Name	Number and percent		
		Resistant	Average resistant	Susceptible
1	Ceftazidime	(45%) 90	(5/4%) 9	(5/50%) 101
2	Cefotaxime	(72%) 144	(5/6%) 13	(5/21%) 43
3	Cefepime	(36%) 72	(7%) 14	(57%) 114
4	Cefoxitin	(54%) 108	(5/15%) 31	(5/30%) 61
5	Aztreonam	(5/39%) 79	(15%) 30	(5/45%) 91
6	Gentamicin	(5/36%) 73	(5/12%) 25	(51%) 102
7	Erythromycin	(5/93%) 187	(6%) 12	(5/0%) 1
8	Tetracycline	(75%) 150	(5/9%) 19	(5/15%) 31
9	Co-amoxiclav	(85%) 170	(5/6%) 13	(5/8%) 17
10	Cotrimoxazole	(57%) 114	(4%) 8	(39%) 78
11	Ampicillin	(91%) 182	(5/3%) 7	(5/5%) 11
12	Imipenem	(5/0%) 1	(5/1%) 3	(98%) 196
13	Amikacin	(5/15%) 31	(17%) 34	(5/67%) 135
14	Ciprofloxacin	(39%) 78	(3%) 6	(58%) 116

Combined disk test for the detection of ESBLs

There were 155*E.coli*isolates with ESBL Phenotype. Boronic acid was also used in this experiment which only helped to identify 3 ESBL isolates. Number and the percentage of antibiotic resistance in isolates with ESBL phenotype are shown in table 3.

Table 3. Percentage of antibiotic resistance in isolates with ESBL phenotype

antibiotic	Total	Blood N=12	Tissue N=22	stool N=26	Secretion N=19	Urine N=46	Wound N=30
Ampicillin	143(92.2%)	10(6.9%)	20(13.9%)	23(16%)	17(11.8%)	43(30%)	30(20.9%)
Amikacin	26(16.7%)	0	4(15.3%)	2(7.6%)	3(11.5%)	11(42.3%)	6(23%)
Imipenem	0	0	0	0	0	0	0
Ciprofloxacin	66(42.55)	6(9%)	12(18.15)	6(9%)	7(10.6%)	18(27.2%)	17(25.7%)
Ceftazidime	82(52.9%)	7(8.55)	15(18.2%)	13(15.8%)	10(12.1%)	22(26.8%)	15(18.2%)
Tetracycline	118(76.1%)	10(8.45)	18(15.2)	20(16.9%)	13(11%)	37(31.35)	20(16.9%)
Cefotaxime	84(54.1%)	3(3.5)	13(15.4%)	15(17.8%)	7(8.3%)	27(32.1%)	19(22.6%)
Erythromycin	143(92.2%)	9(6.2%)	22(15.3%)	22(15.3%)	18(12.5%)	43(30%)	29(20.2%)
Aztreonam	78(50.3%)	4(5.1%)	15(19.2%)	9(11.5%)	9(11.5%)	23(29.4%)	18(23%)
Cotrimoxazole	98(63.2%)	4(4%)	14(14.3%)	18(18.3%)	11(11.2%)	32(32.6%)	19(19.3%)
Gentamicin	65(41.9%)	6(9.2%)	15(23%)	3(4.6%)	13(20%)	15(23%)	13(20%)
Cefepime	71(45.8%)	3(4.2%)	9(12.6%)	12(16.9%)	6(8.4%)	22(30.9%)	19(26.7%)
Coamoxiclav	137(88.3%)	9(6.5%)	20(14.5%)	24(17.5%)	18(13.1%)	38|(27.7%)	28(20.4%)
Cefoxitin	86(55.4%)	3(3.4%)	19(22%)	16(18.6%)	7(8.1%)	24(27.9%)	17(19.7%)

In this table, the percentage of antibiotic resistance in isolateswhich were identified in ESBL Combined Discwas investigated. From the total of 155 isolates with ESBL phenotype, the highest percentage of antibiotic resistancebelongs to Ampicillin, erythromycin andcoamoxiclavwith 143, 143 and 137 isolates, respectively;and the lowest resistance belongs to Amikacinwith 26 isolates. Also, no resistanceantibiotic was observed against Imipenem in ESBL isolates. Also, the highest percentage of resistance belongs to urine and wound and the lowest percentage of resistance belongs to blood samples. No resistance was observed against Amikacin antibiotic in blood samples. However, resistance to Amikacin in urine samples was the highest in all samples by 42.3 %.

Results fromthe Determination of MIC using Agar dilution method

Using agar dilution method, minimal inhibitory concentration of growth was carried out for ceftazidime and cefotaxime antibiotics. According to CLSI Standard table, $MIC_{CAZ} \geq 16$ and $MIC_{CTX} \geq 4$ µg / mlare considered as resistant. Results for MIC Ceftazidime for isolates of Escherichia coli are shown in Table 4.

TABLE 4. MIC Ceftazidime for *E.coli*isolates

µg / ml	≤ 4	8	32-16	128-64	512-256	> 512MB
CAZ	83,	27,	66,	13,	1	10th
CAZ + CA	149,	33,	8	4	2	4

Concentration serial was obtained from 5/0 to ≥ 512 micrograms per ml of ceftazidime and was it tested. Also, MIC ceftazidime with clauvanic Acid was carried out for resistant strains. In this experiment, 90 *E.coli* isolateshad $MIC_{CAZ} \geq 16$ and they were considered resistant. In using Clavulanic Acid inhibitor, this number was reduced to18 isolates. In Figure 1, the determination of MIC in the subsequent concentration of CAZ in the serial concentration is shown. Cefotaxime MIC was done similar to ceftazidime concentrations. Also, MIC cefotaxime with clauvanic acid was performed for resistant strains. In this test, 145 isolates had $MIC_{CTX} \geq 4$ in which they were reduced to 65 isolates in using Clavulanic Acid, which are shown in table 5 of MIC Cefotaxime for Escherichia coli isolates.

TABLE 5. MIC Cefotaxime for *E.coli*isolates

µg / ml	1 ≥	2	8-4	32-16	128-64	512-256	≥ 512MB
CTX	49,	6	48,	21,	34,	21,	21,
CTX + CA	129,	6	58,	0	1	0	6

PCR test for *CTX-M*gene

Among 155 samples, 89 samples (4/57%) contained enzymatic gene of *CTX-M-1*. The highest percentage of samples (29 samples) belongs to urine sample and the lowest percentage of samples belongs to clinical blood sample (2 samples). Maximum resistance belongs to ampicillin Penicillin, coamoxiclav and erythromycin, each with 77 samples

and minimum resistance to Amikacin with 16 samples. Meanwhile, no resistance was observed for Imipenem. Other family of *CTX-M* enzymes was not identified.Thereaction of PCR to B-lactamase gene is shown in Figure 2.

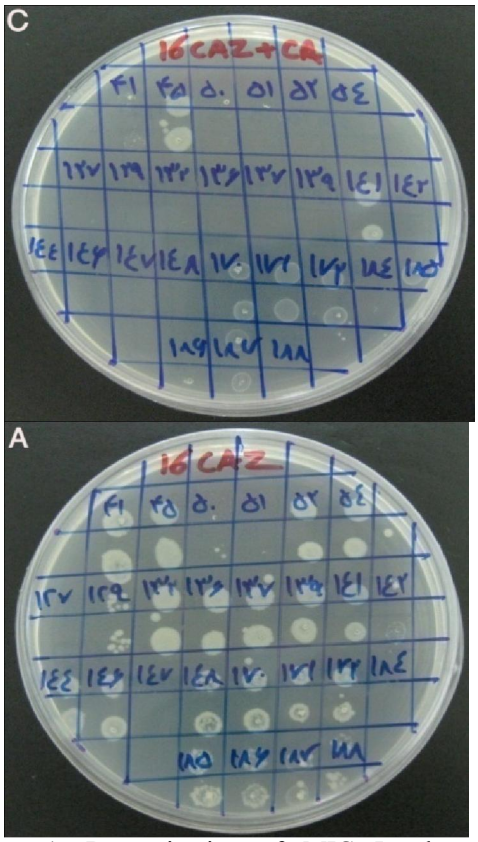

Figure 1. Determination of MIC In the CAZ subsequent concentration with(C) and without ClavulanicAcid (A)

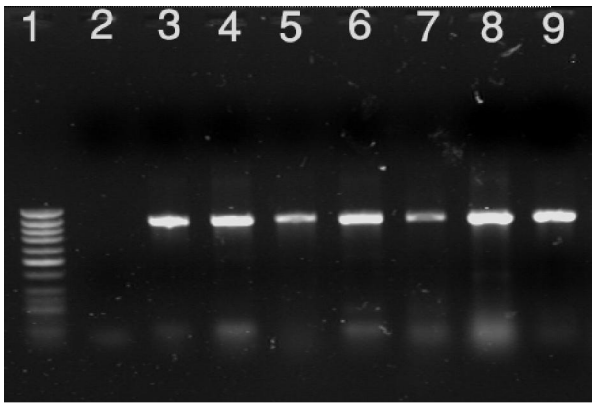

Fig 2. PCR For *-1CTX-M-1*gene: Well -1: Ladder 100bp, Well 2: negative control, wells 3: positive control,and Wells from 4 to 9 show positive clinical samples.

Discussion

In recent years *CTX-M* enzymes as the most common extended-spectrum B-lactamase have been reported, especially in Europe and North America. Different types of this enzyme have been identified and reported. Therefore, in this study we evacuated the prevalence of this type of β-lactamase enzyme. This enzyme derives from an environmental bacterium called*Kluyvera* and shows higher activity against Cefotaximethan Ceftazidime.So in the present study ESBL and MIC disks were used to identify the phenotype of enzyme-producing isolates using Agar Dilution and Combined Disk methods for Ceftazidime and Cefotaximeantibiotics. The gene involved in the production of this enzyme was placed on Mobile elements called ISEcp1, which can exist in different regions of the genome of the bacterium. *E. coli*is the most dominant pathogen-carrying CTX-M enzyme gene. The strainswhich carry this gene are often isolated in UTI, Bacteremia, and Gastroenteritis infections [8].

Considering the results, 89 positive CTC-M samples were identified in our isolates, showing the total of 57.4 % of CTX-M gene prevalence in collected *E.coli* isolates during this period. The prevalence of this gene in isolated samples was similar to the prevalence reported in studies in other parts of the world. In a similar study which was conducted on 603 *Enterobacteriaceae* samples, 6.8% of samples and 44.1% of resistant samples were CTX-M gene carrier[9]. In a study by Eisner et al, which was conducted in Austria on CTX-M-producing *Enterobacteriaceae*, it was reported that 58% of studied *E.coli* were CTX-M-producer [10].

In the studies carried out in Iran the prevalence of this enzyme in the studies by Soltan Dalal et al carried out in Tehran, it was reported that out of 161 E.coli isolates, 79.5% were ESBL producer, in good agreement with our results which showed 77.5%. The similarity can be attributed to the source of this bacterium, because in both of these studies the collected samples were from hospitals in Tehran. Another study conducted in the country was the one by Mobayen in 1386 and the obtained result was 25.1% [11]. Samples in the mentioned study were collected from hospitals in Tabriz. The conflicting results may be because of the method of bacterial isolation. In a similar study by Pak-Leung Ho at Pokfulam University, the wide-spectrum antibiotic resistance in 46 samples of E. coli with E. coli Double disk Synergism method was investigated and they could detect ESBL-producing bacteria, in which 8 samples were detected to be ESLB producer. Also, from these resistant bacteria, 7contained CTX-M plasmid, which could detect various types ofCTX-

M9, CTX-M38, CTX-M24 and CTX-M14 [12]. In another study by Jonas Bonnedahl on Wide-spectrum resistance *Enterobacteriaceae* in the southern France, by investigating E.coli and *K. pneumoniae*in clinical samples using MIC and PCR methods on $bla_{\text{CTX-M,}}$ they found that 47.1 % were resistant to tetracycline and ampicillin, and 4.9 % were ESBL-producing enzymes and 6% were CTX-M plasmid carriers [13]. The different results can be attributed to the completely different sources of collected samples and more importantly to the high control measures in France and other European countries in preventing such resistance. Also, in a study by Shahcherani,200 strains of *E.coli* were studied with different clinical samples using Disk Agar Diffusion (DAD) and PCR methods and it was found that 52.5% contained ESBL gene, in good agreement with our results [14]. More extensive studies can be fruitful to identify the various species and sub- species of the Extended-spectrum B-lactamase enzymes in the identification of effective antibiotics for the treatment of resistant infections and prevention of the spread of resistance. Also, the identification of prevalent bacterial clones can be effective in health unit strategies and hospital management. Also, in recent studies in the other parts of the world, 71.42 % of ESBL-producing E. coli isolates in Greece [15], 63.1 % of ESBL -producing E. coli isolates in India were CTX-M genecarriers [3]. Also, in a comprehensive study in the hospitals of Sweden, 92% were CTX-M genes producer [15]. What is obvious is the simultaneous and parallel development of resistance to β-lactamases through CTX-M enzymes worldwide and Iran, highlighting the importance of extensive studies and controlling the increase of resistance.

Conclusion

The results show that the prevalence of ESBL-producing E. coli is high (5/77 %). Disk Diffusion, Combined Disk, MIC and PCR methods show higher prevalence of CTX-M among ESBL-producing *E.coli* strains. ESBL production is considered as a major threat to the widespread use of extended-spectrum Cephalosporins. Therefore, we should be careful in choosing the appropriate antibiotic for the treatment of infections suspected of Beta-lactamase-producing organisms. Also, strains with decreased susceptibility against ceftazidime and cefotaxime should be tested for ESBL genes and also under-treatment strains should be tested regularly.

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