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Genotyping of *E. coli* Isolated from Urinary Tract Infection Patients Containing B-Lactamase Resistance Gene CTX-M Group 1 in Sanandaj Medical Health Centers

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Abstract

CTX-M-producing bacteria are known as a resistant source against *oxyimino-cephalosporin* such as cefotaxime and ceftazidime; although laboratory diagnosis of this gene has not been properly defined. The aims of this study are determining the rates of prevalence of CTX-M and CTX-M group 1 in the *Escherichia coli* (*E. coli*) obtained from urinary tract infections (UTI), and also determining their genetic relationship in the city of Sanandaj. In current study, 180 *E. coli* strains isolated from urinary tract infections were used. Sensitivity to common antibiotics was studied by the disc diffusion method. Phenotypic detection of isolated ESBL-producing starins was done by the combination disc test. CTX-M and CTX-M1 genes were detected using the PCR method and finally, the possible clonal relationship between isolates was determined using the REP-PCR method. 89 samples were ESBL-positive. The PCR assay used for detecting the CTX-M gene, showed that 48 samples out of 180 samples (26.66%) contained that gene; also among these 48 samples, 23 (12.77%) had CTX-M group 1. Based on the REP-PCR assay, 48 genotypes among 48 samples were CTX-M-positive. Results from the REP-PCR assay indicated that the clonal propagation theory of one epidemic strain of *Escherichia coli* is not apply, *i.e.* all CTX-M-producing species are not originated from one single strain and the gene is spread between different isolates. Therefore, hospitals and their employees must be more hygiene and, proper disposal of hospital waste can help to prevent the spread of different resistances.

Keywords

Escherichia coli, Urinary Tract Infection, ESBL, CTX-M, CTX-M Group 1, REP-PCR

1. Introduction

Extended-spectrum beta-lactamases (ESBLs) were reported for the first time in Germany 1983 [1]. The CTX-M family of ESBLs is a serious threat for global health [2] to the extent that in the previous decade, it was described pandemic [3]. CTX-M is the most prevalent ESBL in antrobactericeas that produce nosocomial and society-acquired infections [2] [4]. CTX-M genes are usually found on plasmids and derivative chromosomes of Beta-lactamase genes are from the *Kluyvera* spp. genus which are created by "multiple delivery mechanisms" [2] [5]. Normally, these plasmids are easily spread among microbial populations and they carry the resistant genes against other antibiotics such as aminoglycoside acetyltransferases and dihydropteroate synthases or other beta-lactamases [6]. This increase in resistance, to a large extend, is due to the spread of *E. coli* bacteria and *Klebsiella pneumonia* that carry CTX-M [7]. CTX-M group 1 contains six plasmid-dependent enzymes namely: CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15 and FEC-1 and, unprinted enzymes of CTX-M-22, CTX-M-23 and CTX-M-28 (corresponding gene bank numbers respectively are AY080894, AF488377 and AJ549244) [2]. For epidemiological study and determining the genetic relationships of resistant isolates, a rapid typing method could be a valuable tool. Repetitive Element Palindromic PCR (REP-PCR) is a suitable method for proliferation of repetitive elements of bacterial DNA, with the following characteristics: 1) low costs, 2) high discriminatory power, 3) high speed, and 4) reliable tool for typing and classification of a wide range of Gram-negative and some Gram-positive bacteria [8] [9].

A lot of research has been done on identifying CTX-M in *E. coli*. Woodford *et al*. (2004) conducted a study to identify the CTX-M in *E. coli* isolated from community and hospital in Britain. In this study, 291 CTX-M-producing samples were identified in Britain which 279 sample involved CTX-M 1and 12 samples involved CTX-M-9. The result of dendrogram indicated that 279 CTX-M-producing samples are related with each other's [10].

Leila Nasehi *et al*. (2010) studied the CTX-M, PER, SHV and TEM β-lactamase prevalence in *Lebsiella pneumoniae* isolated from clinical samples in Tehran. The results indicated that the prevalence of blaSHV, blaCTX-M, and blaTEM gens was 7.5%, 16%, 22.5 % and 23%, respectively [11].

According to the above description, the aims of this study are determining the rate of prevalence of CTX-M and CTX-M group 1 genes in the *Escherichia coli* responsible for urinary tract infection and, determining the genetic relationship between isolated starin using the typing method of REP-PCR.

2. Material and Methods

2.1. Sampling

In 2015, 325 urine samples were collected from Sanandaj laboratories. *E. coli* isolated in 180 samples that their presence were confirmed by biochemical test.

2.2. Antibiotic Sensitivity and Phenotypic Identification of ESBLs

The antibiotic sensitivity of the samples was conducted using the disc diffusion method and based on the Clinical and Laboratory Standards Institute (CLSI standards); also, the antibiotics that were used are listed in Table 2. ESBL-producing isolates were detected by the CLSI combination disc test [12]. At first, bacterial suspensions equivalent to Mc-Farland half of resistant isolates were cultured on Molar-Hinton agar media; then, two Ceftazidime and Cefotaxime discs and also, two discs of these materials combined with clavulanic acid were placed 25 mm apart on the media. After incubation, if the difference in diameters of the halos around the combined discs and the halos of the initial discs is ≥5 mm, the isolate is considered as a positive ESBLs phenotype.

2.3. Determining MIC by the E-Test

The E-test was performed with the antibiotics of Ceftazidime and Cefotaxime for all the samples that were detected as ESBLs. In this method, after making a bacterial suspension by the Mc-Farland half method, it was placed on the Molar Hinton agar plate; then, E-test strips, each representing one specific antibiotic, were placed on the Molar Hinton agar and after 24 h of incubation in 37˚C, a triangular growth zones of inhibition was formed. Then by referring to the table provided by the company that had created the E-test strips, the susceptibility of *E. coli* bacteria to the mentioned antibiotics was determined.

2.4. DNA Extraction

DNA of the bacterium was extracted using gram-negative DNA extraction kit (Sina Gene, Iran). The extracted DNA was checked by the agar gel electrophoresis.

2.5. Detection of Resistant Genes

In this study,primers from previous studies were used which their characteristics are listed in Table 1 [13] [14]. The PCR reaction was done with the final volume of 25 µl that contained 12.5 µl of PCR Master Mix (containing DNA polymerase, salts, magnesium, dNTPs and optimized reaction buffer), 1 µl of each primer, 2 µl of the sample's DNA and 8.5 µl distilled water. The conditions of reaction for each primer are shown in Table 1. The product of PCR was analyzed by electrophoresis in agar gel 1.5% and finally it was visible under UV.

2.6. REP-PCR

In order to determine the genetic relationships between ESBL-producing samples, the

Table 1. Primers and the conditions of their reaction.

Product size	PCR condition	Primer (5′→3′)	Target
759 bp	94˚C, 5 min; 35 cycles of 94˚C, 45 s; 58˚C, 45 s; 72˚C, 60 s	Forward ACGCTGTTGTTAGGAAGTG Reverse TTGAGGCTGGGTGAAGT	CTX-M
864 bp	94˚C, 2 min; 30 cycles of 95˚C, 45 s; 58˚C, 30 s; 72˚C, 45 s	Forward GGTTAAAAAATCACTGCGTC Reverse TTGGTGACGATTTTAGCCGC	CTX-M-1 group

typing method of REP-PCR was used. The REP-PCR reaction was done with the final volume of 25 µl containing: 12.5 µl of PCR Master Mix, 1 µl of the sample's DNA, 1 µl of each primer and 9.5 µl distilled water. REP1 (5'-IIIGCGCCGICATCAGGC-3') and REP2 (ACGTCTTATCAGGCCTAC-3') primers were used for amplification of repetitive sequences in the bacterial genome [15]. The reaction took place in XP Thermal Cycler with the following circumstances: Initial denaturation (2 min at 95˚C), then, 35 cycles of denaturation (1 min at 92˚C), annealing (1 min at 40˚C), extension (8 min at 65˚C) and at last, final extension (8 min at 65˚C). The products of REP-PCR were electrophoresed in Agar gel 1.5%. In the end, the bonds became visible by UV ray and then the image was recorded. The dendrogram relating to the analysis of fingerprinting was drawn by the algorithm of the Unweighted Pair-Group Method (UPGMA) using the software NTSYS v2.02e.

3. Results

3.1. Bacterial Isolated

All the samples were separated from different patients that had referred to sanandaj diagnostic laboratories. The rates of prevalence of urinary tract infection based on sex and age are shown in Figure 1 and it is logical that UTIs are more prevalent in women.

3.2. Sample Antibiotic Sensitivity

CLSI standard was used to determine the sensitivity. Samples sensitivity to antibiotics was measured and presented in Table 2 as sensitive, semi sensitive, and resistant.

3.3. Phenotypic Detection of ESBL Producers

89 *E. coli* samples (49.44%) were detected as ESBL producers by the combination disc test (Figure 2). Antibiotic susceptibility of the positive- and negative-ESBL samples are compared in Figure 3.

3.4. Results of E-Test

The results of this E-test for the ESBL-producing samples were in the range of 2 - 4 µg/ml for Cefotaxime and 1 - 16 µg/ml for Ceftazidime (Figure 4).

3.5. Detection of Resistant Genes

Among SBL-producing samples, 48 out of 89 samples contained the CTX-M gene; also 23 out of 48 samples, were detected as CTX-M group 1.

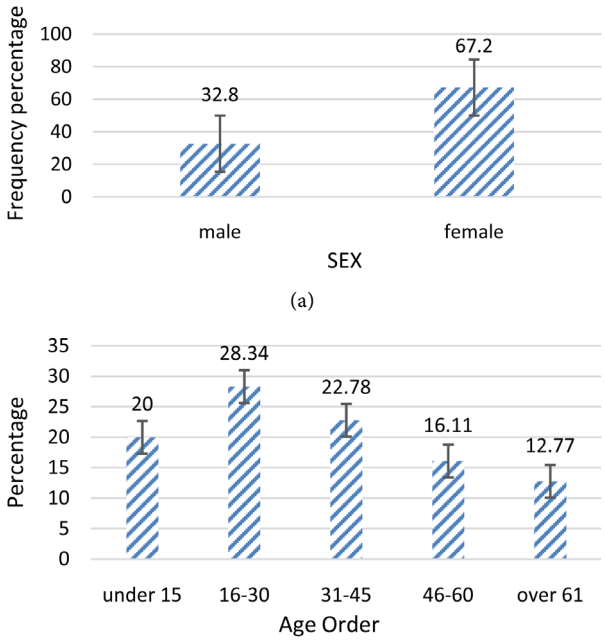

Figure 1. (a) The rate of prevalence of UTI based on sex; (b) The rate of prevalence of UTI based on age.

Figure 2. Phenotypic detection of ESBL producers by the combination disc test.

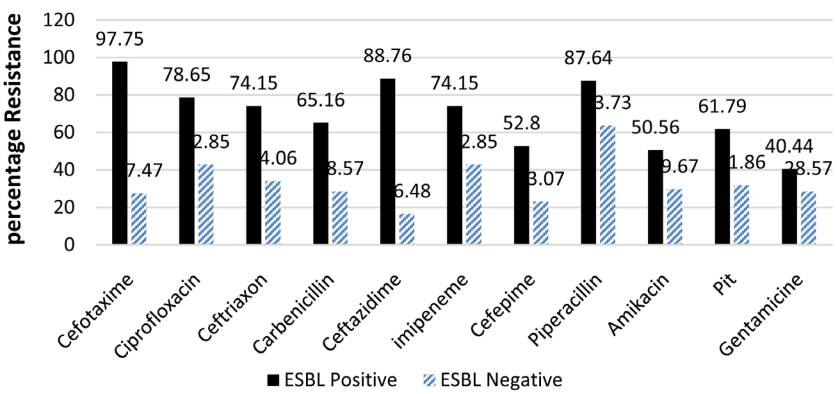

Figure 3. Comparison of the susceptibility profiles of positive and negative ESBL-producing *E. coli.*

Figure 4. Non-growth triangle due to increased antibiotic density around T-Test strip.

Table 2. Results of antibiotic sensitivity determination based on disc diffusion.

	Resistant N. (%)	Intermediate N. (%)	Susceptible N. (%)
Cefotaxime	144 (57.8)	8 (4.4)	68 (37.8)
Ciprofloxacin	87 (48.3)	22 (12.2)	71 (39.4)
Ceftriaxon	72 (40)	25 (13.9)	83 (46.1)
Carbenicillin	67 (37.3)	17 (9.4)	96 (53.3)
Ceftazidime	59 (32.8)	35 (19.4)	86 (41.8)
Imipeneme	81 (45)	24 (13.3)	75 (41.7)
Cefepime	52 (28.9)	16 (8.9)	112 (62.2)
Piperacillin	111 (61.7)	25 (13.9)	44 (24.4)
Amikacin	39 (21.7)	33 (18.3)	108 (60)
Piperacillin-tazobactam	38 (21.1)	46 (25.6)	96 (53.3)
Gentamicine	54 (30)	8 (4.4)	118 (65.6)

3.6. Results of REP-PCR

The next step was determining the genetic relationship between the samples. After drawing the dendrogram for the obtained results from REP-PCR (Figure 5), ESBL-producing samples which were patterned as the samples that had 100% genetic similarity, were considered as one pattern and, other samples each were considered as a separate pattern. Based on this, 89 patterns exist among 89 ESBL-producing samples; so 89 positive-ESBL samples, had 89 different genotypes (Figure 6).

In this dendrogram, 10 clusters which labeled by letters A-J can be observed. Each cluster A-C-D-F-G-H is divided by two sub-clusters.

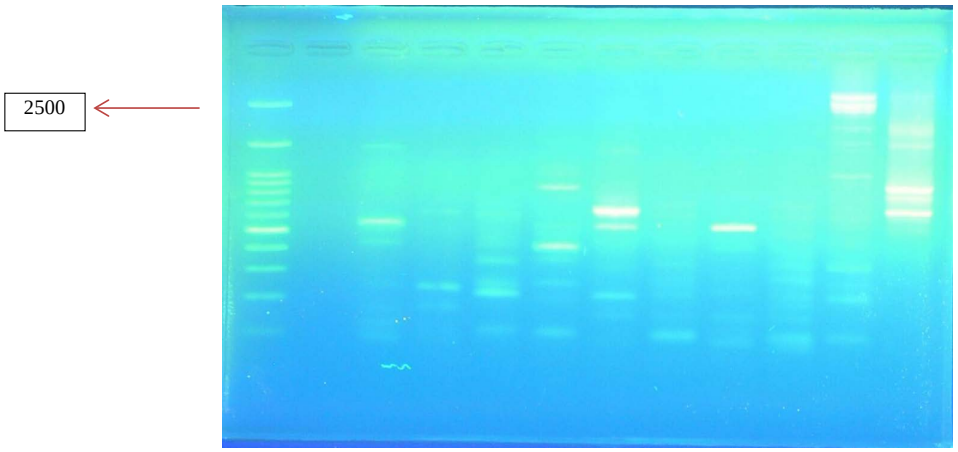

Figure 5. Bands created by rep-PCR for sample type.

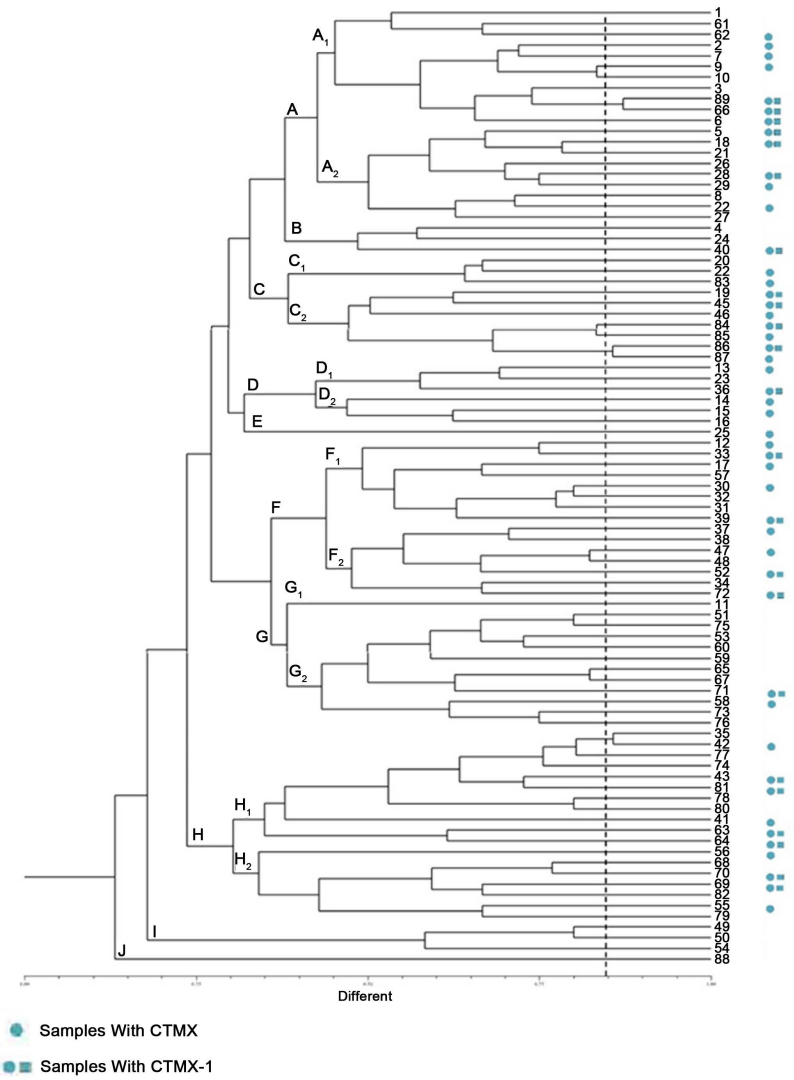

Figure 6. Dendrogram related to rep-PCR analysis shows 89 genetic patterns in 89 ESBL producing samples.

4. Discussion

In 1992, a new type of ESBL that gave high level of resistance against Cefotaxime to bacteria was detected in members of *Antrobactericeas* [16] [17]. This new family of ESBLs are from the class A in the Ambler's classification. As mentioned before, CTX-M is described pandemic. According to several reports, the number of CTX-M B. lactamases is rapidly increasing [18]. In some reports from France [19], Sweden [20], and India [21] as well, the prevalence of this B. In a study performed in Tabriz on 188 *E. coli* separated from urine samples of outpatient and hospitalized patients, it was revealed that 84.1% of the isolates were contained the CTX-M type 1 B. lactamase gene [22]. In order to find a suitable strategy for stopping further spread of this gene, worldwide studies are required. According to **Figure 1**, UTI is most prevalent in ages between 16 - 30 and 31 - 45. This result could be attributed to this fact that most sexual intercourses occur in these periods. Also, UTI was more prevalent in women, which seem logical because of anatomical reasons.

In this study, CTX-M gene was detected in the *E. coli* isolated separated from UTIs in a specified period (2015). 48 out of 89 ESBL-producing samples (53.93%) contained the CTX-M gene. In addition, the results indicated that 23 samples (47.91%) of these 48, contained CTX-M group 1. Our findings showed the high prevalence of CTX-M enzyme in the ESBL-producing *E. coli* in the Sanandaj. Furthermore, it was observed that almost half of these enzymes were from the CTX-M group 1. CTX-M-15, which is in CTX-M group 1, has the most rate of prevalence worldwide [2] [23] [24]. As it can be seen in **Figure 2**, ESBL-producing samples had high resistance against Cefotaxime (97.75%), Ciprofloxacin (78.65%), Ceftriaxone (74.15%), Carbenicillin (65.16%) Ceftazidime (88.76%), Imipenem (74.15%), Piperacillin (87.64%) and Piperacillin-tazobactam (61.79%) and, a moderate resistance against gentamicin (40.44%), Amikacin (50.56%) and Cefepime (52.8%).

In a study in India, resistance of ESBL-producing isolates against non-beta lactam antibiotics were as follows: 93.8% to Ciprofloxacin, 79.1% to Sulfamethoxazole and 14.7% to Amikacin [21]. It is possible that genes that code resistance against these antibiotics are transferred alongside the ESBL genes. In a study conducted in the USA, among 20 isolated bacteria resistant to antibiotics that were separated from patients from hospitals and nursing homes, 17 bacteria contained 54-kb plasmid that endoced the resistance to Ceftazidime by TEM-10.This plasmid was the mediator of resistance against Co-trimoxazole, Gentamicin and Tobramycin [25].

According to reports, CTX-M B. lactamases hydrolyze Cefotaxime more than Ceftazidime [26]. In the present study, 95.83% of the CTX-M B. lactamase-producing isolates that were detected in the study, were resistant to or an intermediary for Cefotaxime, while lack of sensitivity to Ceftazidime was equal to 87.5%.

In order to differentiate between the two following hypotheses, the REP-PCR method was necessary. 1) An epidemic *E. coli* strain had been spread among all the patients, so one ancestral strain is possibly the cause of spread of resistance. 2) CTX-M gene had been spread among different *E. coli* isolated.

This study recognized 48 different genotypes as positive among 48 CTX-M samples; therefore, the results of this experiment indicated that the clonal propagation theory of one epidemic *E. coli* strain is not applicaple. This means that not all the types of CTX-M producers were originated from one single strain and, the gene had been spread among different isolates. Therefore, it can be concluded that one plasmid or mobile genetic element (MGE) containing the CTX-M gene, is responsible for the spread of the gene among different isolated of *E. coli*.

In the drawn dendrogram, it was observed that the samples in Clusters B, E, I and J do not contain the CTX-M-resistant gene and the number of samples in these clusters is very low. For instance, in clusters E and J there are only one sample and in clusters B and I, there are 3 samples and based on that, it can be concluded that the samples without the CTX-M gene, have a lower survival rate and their spread among the patients are lower.

5. Conclusions

In this study genotyping of *E. coli* from urinary tract of infection patients containing B-lactamase resistance gene CTX-M group 1 was assessed. 48 out of 89 ESBL-producing samples (53.93%) contained the CTX-M gene. In addition, the results indicated that 23 out of (47.91%) of these 48 samples, contained CTX-M group 1. The samples without the CTX-M gene, have a lower survival rate and the spread among the patients is lower.

Our findings showed the high prevalence of CTX-M enzyme in the ESBL-producing *E. coli* in the Sanandaj. Also, UTI was more prevalent in women, which seemed logical because of anatomical reasons. According to the obtained results from REP-PCR, it was concluded that the resistant genes were spread among different isolates. So hospitals and their staff must be more hygiene and, proper disposal of hospital waste and using antibiotics only by the doctors' order can help to prevent the spread of resistances.

6. Limitations and Recommendations for Future Research

Limitations of the current study were lack of proper access to some of the reagents and instruments in the tests and due to financial constraints, the study was under-powered, and because of small sample size, it is impossible to generalize the study results, certainly. For future studies, the results of study recommended that in order to generalize the results, study be repeated with big enough population of patients. Also, it is suggested that other ESBL gens prevalence and risk factors related to the spread of ESBL genes can be studied in future.

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