Available online at **www.ijmrhs.com**

ISSN No: 2319-5886

International Journal of Medical Research & Health Sciences, 2018, 7(9): 81-89

Detection of bla CTX-M, bla TEM-01 and bla SHV Genes in Multidrug Resistant Uropathogenic *E. coli* Isolated from Patients with Recurrent Urinary Tract Infections

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ABSTRACT

***Introduction**: Extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae that give antibacterial resistance to 3rd generations cephalosporin and broad-spectrum penicillin, using ESBL genes CTX-M, TEM, SHV, which are encoded in bacterial plasmid genome. This study was concerned with detection of bla CTX-M, bla TEM-01 and bla SHV genes in multidrug resistant uropathogenic E. coli isolates in adult patients with recurrent Urinary Tract Infections (UTIs). **Material and Methods:** Total 47 of uropathogenic E. coli (UPEC) was isolated from patients with urinary tract infection who attended Azadi Teaching Hospital in Duhok city. The isolates were collected from June 2016 to December 2016 and investigated by using antimicrobial susceptibility assay according to Clinical and Laboratory Standards Institute (CLSI) guidelines and by using Phoenix (BD). All these UPEC isolates were investigated for subgroups gene of bla CTX-M such as CTX-M-14, 15, 24, 27, bla SHV subgroups gene such as SHV-11, 12 and bla TEM-1 by using uniplex and multiplex PCR. All UPEC samples were screened by Double Disc Approximation (Synergy) test (DDAT) for detection of the positivity to ESBLs production. **Results:** Out of 47 UPEC isolates, bla CTX-M15 subgroup gene was detected in 72.3%, CTX-M14 was detected in 42.6%, bla subgroup gene CTX-M24 was detected in 27.7% and the last subgroup gene bla CTX-M27 was detected in 12.8%. **Conclusion:** The current study found that an acute uncomplicated UTI affects a large proportion of the population. The study confirmed that the E. coli to be a major uropathogen. ESBL encoding genes, the resistance among males were higher than females particularly the distribution of bla TEM genes.*

Keywords: Extended-spectrum beta-lactamase, CTX-M, TEM, SHV, *Escherichia coli*, UTIs

INTRODUCTION

β-lactamase is a group that belongs to Extended Spectrum β-lactamase (ESBLs) that can be able to hydrolyze third generation cephalosporins and aztreonam sharing the ability of clavulanic acid to inhibit this group. The fundamental changes in the substrate spectra of the enzymes make β-lactamase-mediated resistance to βlactam antibiotics [1].

Since the 1980s, Enterobacteriaceae-producing ESBLs such as SHV and TEM types have been established as a major cause of hospital acquired infections. As well as, in 1990s, Urinary Tract Infections (UTIs) and diarrhea have also been found to be ESBL producers caused by several community acquired pathogens, these include *E. coli*, *Salmonella, Shigella, and Vibrio cholera* [2-4]. The genes location responsible for production of ESBL is located on the plasmid that also carries genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol [5].

Bacterial resistance can happen by several mechanisms: alterations of the cell permeability, the site of action may be changed leading to lose the antibiotic susceptibility mediated by the increment of the efflux pump activity and by degradation of antibiotic enzymes [6-8]. ESBLs can be divided into 3 major types which are TEM, SHV and CTX-M, these enzymes have point mutation in the active site of native β-lactamases, particularly TEM-1, TEM-2,

and SHV-1 [9,10]. The CTX-M types have different 50 types divided into 5 groups according to its amino acids into CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 [11]. The CTX-M-15 b-lactamase an enzyme associated with mosaic plasmids, belongs to the CTX-M-1 group, firstly recorded in India during 2001, but is now becoming worldwide [12].

In the last decade, *E. coli* producing CTX-M (especially CTX-M-15) has worldwide distribution as a cause of community acquired UTIs [10]. The ESBL producing microorganisms required can be exactly detected by phenotypic tests, but there are also many applications of genotypic tests for all genes detection responsible for beta-lactamase production [13].

The most precise molecular techniques are Polymerase Chain Reaction (PCR) used for identification and confirmation of the presence of ESBLs [14]. These techniques give a significant importance in clinical microbiology laboratories [15].

The aim of the present study was to recover the uropathogenic *E. coli* isolates from UTI patients, and detection of *bla* SHV, *bla* TEM, *bla* CTX-14, CTX-15, CTX-24, and CTX-27 beta-lactamase genes using conventional PCR.

PATIENTS AND METHODS

Bacterial Isolation

Total 47 urine samples were collected from adult patients suffering from UTIs that attended Azadi Teaching Hospital during June 2016 to December 2016. All samples were cultured on MacConkey agar and Kligler agar and incubated for 24 hours at 37°C.

By using standard biochemical tests such as the oxidase test, lactose fermentation in Mac Conkey and triple sugar iron agar, in addition to the MRVP test were used for identification of *E. coli* isolates [16].

All isolates were stored at -18°C in nutrient broth containing 40% glycerol [17]. All isolated samples were recognized as *E. coli* ESBLs producing enzymes using BDTM Phoenix system (Becton-Dickinson Diagnostic System, Sparks, MD), that has been conducted in bacteriology department of the laboratory in Azadi Teaching Hospital, Duhok.

Screening of ESBL-Producing Isolates

The disk synergy test was used for detection and screening of ESBL-producing bacteria by using Muller-Hinton agar. All isolates were inoculated on the surface of the plates with the concentration of the bacterial suspension [OD] adjusted to 0.08-0.13 at 650 nm corresponding to the 0.5 McFarland standard.

Then, an Augmentin (AUG) disk (20 µg amoxicillin+10 µg clavulanic acid) was placed at the center of the plate while Ceftazidime (CAZ) disk (30 µg), Cefotaxime (CTX) disk (30 µg), Ceftriaxone (CRO) disk (10 µg), Aztreonam (AZT) disk (30 µg) were placed 25-30 mm apart along an axis passing through the center of the AUG disk. After inoculating plates, overnight incubation at 37°C in ambient air. If a hallow zone of inhibition was seen around each disks then it was expanded to AUG [18].

DNA Extraction and Amplification of the *bla* Genes

All molecular steps were done in Duhok Veterinary Research Center (DVRC) in the College of Veterinary Medicine, University of Duhok. Extraction of genomic DNA was fulfilled according to the boiling method of Shakibaie, et al. [19]. The concentration and the purity of the extracted DNA were determined by nano drop system. The detection of β-lactamase genes *bla* TEM, *bla* CTX and *bla* SHV in the positive ESBL isolates by PCR using the specific oligonucleotide primers are listed in Table 1.

Table 1 Nucleotide sequences of primers used in this study

bla genes	Primer sequences	Reference
CTX-M-14	5'-TTATGCGCAGACGAGTGCGGTG-3'	Ponnusamy, et al., [20]
	5'-TCACCGCGATAAAGCACCTGCG-3	
CTX-M-15	5' GAGCCGACGTTAAACACCGCCA-3	
	5'-GCTGCACCGGTGGTATTGCCTT-3'	
CTX-M-24	5'-TTATGCGCAGACGAGTGCGGTG-3'	
	5'-GCGTCATTGTGCCGTTGACGTG-3'	
CTX-M-27	5'-TTATGCGCAGACGAGTGCGGTG-3'	
	5'-GCCACCGAGCTGGGCAATCAAT-3'	
TEM-M-01	5'- CCAAACGACGAGCGTGACACCA-3'	
	5'-AGCGCAGAAGTGGTCCTGCAAC-3'	
SHV-11	5'-CGCCGCCATTACCATGAGCGAT-3	
	5'-CCGGGAAGCGCCTCATTCAGTT-3'	
SHV-12	5'-CGCCGCCATTACCATGAGCGAT-3'	
	5'-ACCCGATCGTCCACCATCCACT-3'	

By thermal cycler, amplification of extracted DNA was carried out according to Shakibaie, et al. (Table 2) [19].

Table 2 Components of PCR

No.	Components	Volume (µl)
1	PCR Master mix 2X KAPA2G Fast ready mix	12.5
2	Distilled water DNA-free, RNA-free	8.5
3	Forward primer (10 pmol/µl)	1.0
4	Reverse primer (10pmol/µl)	1.0
5	DNA template	2.0
Total		25.0

The cycling conditions were an initial denaturation at 94°C for 3 minutes, template denaturation at 94°C for 30 seconds, annealing at 48°C (for CTX), 60°C (for SHV), 58°C (for TEM) for 30 second and extension 72°C for 1 minute for a total of 30 cycles, with a final extension at 72°C for 10 minutes.

A positive control used in this study was provided by DVRC. The amplicons were electrophoresed in 1.5% agarose gel and visualized after staining with red safe under transilluminator equipped with a digital camera (Biometra, Goettingen, Germany). A 100bp ladder (KAPA, Germany) was used as molecular weight marker.

Statistical Analysis

Statistical analysis was performed using the SPSS (version 22.0) statistics software. In this cross-sectional study, SPSS was used to determine the frequency and spread of antibiotics resistance and resistance-related genes.

RESULTS

Tables 3 and 4, shows the frequency of gene encoding ESBLs among age grouping ranging from 25-65 year giving more gene resistance than other groups including CTX-M, TEM-01and SHV.

Table 3 Frequency distribution of genes (ctxm14, 15, 24 and 27) encoding ESBLs among age groups

Age of patients (Year)	CTXM14		Total	Sig	CTXM15		Total	Sig	CTXM24		Total	sig	CTXM27		Total	sig
	Positive	Negative			Positive	Negative			Positive	Negative			Positive	Negative		
15-24	4 (36.4%)	7 (63.6%)	11 (100%)	0.901**	9 (81.8%)%	2 (18.2%)	11 (100%)	0.519**	3 (27.3%)	8 (72.7%)	11 (100%)	0.519**	2 (18.2%)	9 (81.8%)	11 (100%)	0.794**
25-65	14 (43.8%)	18 (56.2%)	32 (100%)		23 (71.9%)	9 (28.1%)	32 (100%)		9 (28.1%)	23 (71.9%)	32 (100%)		4 (12.5%)	28 (87.5%)	32 (100%)	
66 and Above	2 (50%)	2 (50%)	4 (100%)		2 (50%)	2 (50%)	4 (100%)		1 (25.0%)	3 (75.0%)	4 (100%)		0 (0.0%)	4 (100%)	4 (100%)	
Total	20 (42.6%)	27 (57.4%)	47 (100%)		34 (72.3%)	13 (27.7%)	47 (100%)		13 (27.7%)	34 (72.3%)	47 (100%)		6 (12.8%)	41 (87.2%)	47 (100%)	

Table 4 Frequency distribution of genes (TEM-M 01and SHV-11) encoding ESBLS among age groups

Age of patients (Year)	TEM-M 01		Total	Sig	SHV-11		Total	Sig
	Positive	Negative			Positive	Negative		
15-24	9 (81.8%)	2 (18.2%)	11 (100%)	0.854**	1 (9.1%)	10 (90.9%)	11 (100%)	0.046**
25-65	25 (78.1%)	7 (21.9%)	32 (100%)		0 (0.0%)	32 (100%)	32 (100%)	
66 and Above	4 (100%)	0 (0.0%)	4 (100%)		1 (25.0%)	3 (75.0%)	4 (100%)	
Total	38 (80.9%)	9 (19.1%)	47 (100%)		2 (4.3%)	45 (95.7%)	47 (100%)	

The most prevalent infection with *E. coli* among age group 25-65 year which represented 65%, while age groups 15-24 year and 66 year and above with 30.5% and 4.5 % respectively. On the other hand, the occurrence of *bla* gene TEM-M01 is higher than other genes 78.1% followed by 71.9% for CTX-M15. It is obvious from this table, the positivity of other *bla* genes CTX-M14, CTX-M24 and CTX-M27 among the same age group were 43.8%, 28.1% and 12.5% respectively. As regard, the SHV-11 gives less percentage in positivity (9.1%).

Figure 1, illustrates distribution and percentage of different genes among total 47 of *E. coli* isolates producing ESBLs. In general it has been found that among CTX-M *bla* genes, (34/47) isolates carried CTX-M15 enzymes encountered higher percentage (72.3%) which is more predominant while (20/47) isolates (42.6%) carrying CTX-M14 (13/47) 27.7%, (6/47) 12.8% isolates produced CTX-M24 and CTX-M27 respectively. On the other side, TEM *bla* genes (38/47) accounting (80.9%) and (2/47) isolates harbored SHV-11 registered only (4.3%).

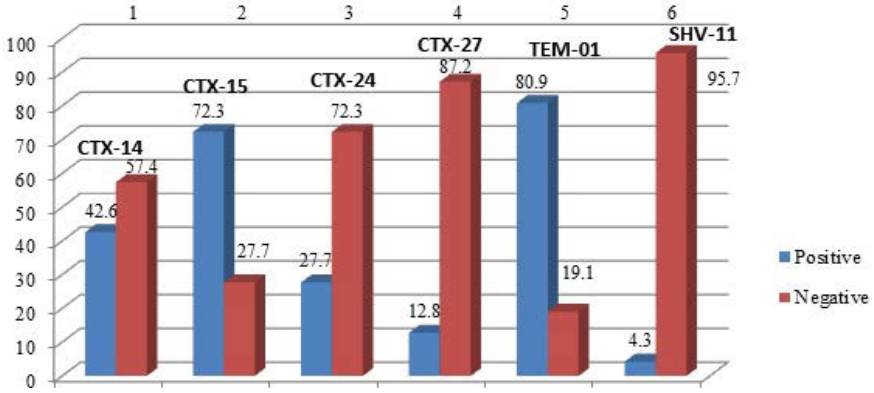

Figure 1 Distribution of CTX-M, TEM and SHV genes with their percentages among *E. coli* isolate producing ESBLs enzymes

The distribution of ESBL producing genes is summarized in Figure 2. This pattern observed detection of single production of genes encountered (21%) and some combination of genes ESBLs belonging to different groups composed from 2 genes, 3 genes, 4 genes, 5 genes are (36.2%), (27.7%), (10.6%) and (4.3%) respectively.

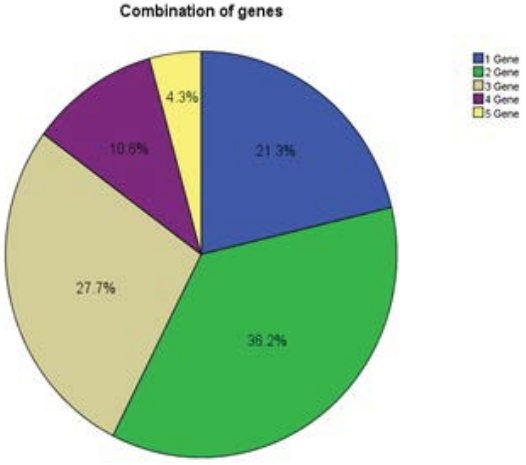

Figure 2 Distribution of genes encoding ESBLs among 47 isolates *E. coli*

A total of 47 of *E. coli* were isolated from patients with UTIs, 27(57.4%) male and 20(42.6%) female, the major source for ESBL-producing bacteria is urine (Table 5).

Table 5 Frequency of ESBL producing *E. coli* according to gender

Gender	No. of Examined Patients	%
Male	27	57.4%
Female	20	42.6%
Total	47	100.0%

Regarding ESBLs encoding genes, the occurrence of resistance among male was high comparing with female patient as well as the distribution of *bla* genes among males is higher than females (Figure 3).

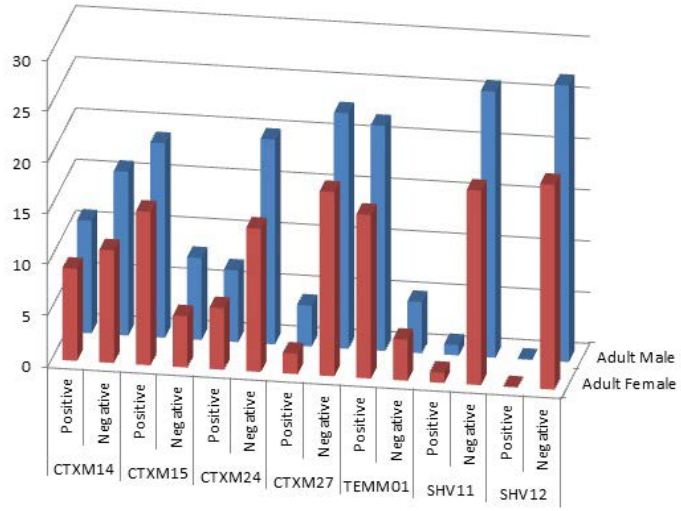

Figure 3 Occurrence of ESBLs encoding genes producing according to gender

Figures 4-6 illustrates PCR positive band products of ESBLs uropathogenic *E. coli* genes *bla* SHV-11, *bla* TEM-01 and *bla* CTX-M14 respectively.

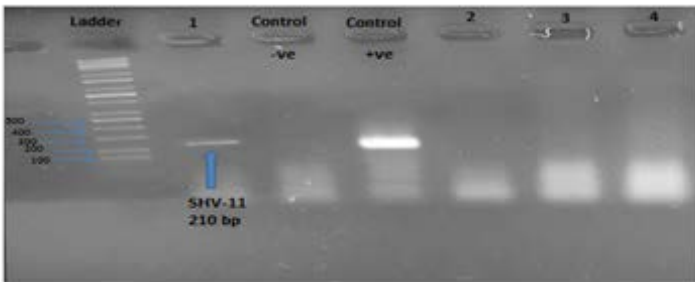

Figure 4 PCR products of UTI samples (1,2-4) on 1.5% agarose gel/red safe electrophoresis, Ladder 100 bp molecular weight marker, lane 1 positive SHV-11 gene (210bp) sample, lanes 2,3 and 4 negative samples

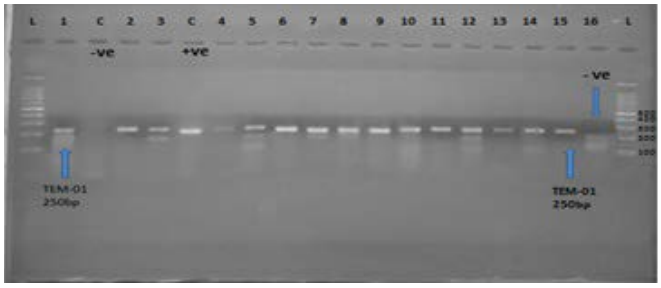

Figure 5 PCR products of UTI samples (1-16) on 1.5% agarose gel/red safe electrophoresis, ladder 100bp molecular weight marker, lanes (1, 2, and 3, 4-15) positive samples TEM-01 gene 250bp, lane 16 negative samples

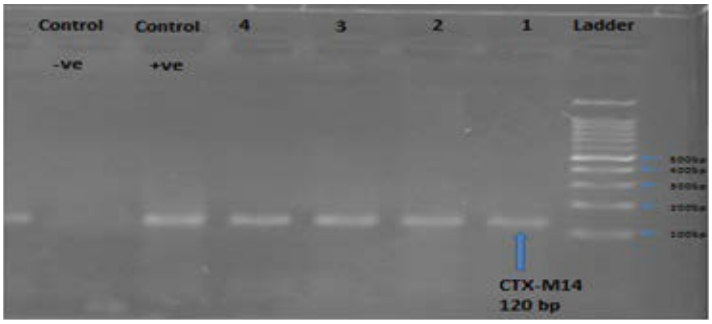

Figure 6 PCR products of UTI samples (1-4) on 1.5% agarose gel/red safe electrophoresis, ladder 100bp molecular weight marker, lanes (1, 2, 3 and 4) positive samples CTX-M14 gene 120bp

DISCUSSION

Molecular characterization and identification of beta-lactamase would be important for the reliable of antimicrobial resistance and epidemiological investigations. In different parts of the world until now, ESBL genes have been studied in order to give reports about the diversity of ESBL genes among uropathogenic *E. coli* which is isolated from all over the world. In the present study, three types of ESBL genes and subgroups were detected: CTX-M14, CTX-M15, CTX-M24, CTX-M27, TEM-01, SHV-11and SHV-12 which are the most prevalent ESBLs genes type produced by uropathogenic *E. coli*.

The result shown that CTX-M15 higher accounting than other subgroup (72.3%) of the CTX-M ESBLs genes compared with (80.9%) of TEM-01 and SHV-11 (4.3%). The situation on 1990, there was a dramatic turn around when there is a dominant in TEM and SHV and rarely recognized CTX-M types.

The ESBLs producing genes distributions in the current study, in a large predominance of CTX-M is similar to those who reported in many countries like Argentina, the UK, Spain, France and India [21-25], among some others countries where have endemic CTX-M-producing Enterobacteriaceae been described such as [26] have designed phylogenetic analysis of ESBL producing gene CTX-M of uropathogenic *E. coli* isolates, derived from commensal phylogenetic type of human. Similarly, the study of [27] have reported that among all ESBL producing *E. coli*, 95% of strains, carried genes from family *bla* CTX-M, as well as [28] were also reported that 88% of ESBL producing *E. coli* isolates carried CTX-M *bla* genes subgroups. The present study found that ESBL producing genes *bla* CTX-M subgroups *bla* CTX-M-14, blaCTX-M-15, *bla* CTX-M-24 and CTX-27 are found in all the UPEC strains (100%). Depending on this result, it was argued that the epidemiology of the current ESBLs is consistent to our neighboring countries and Europe [29,30]. The higher rate of CTX-M-15 which is subgroup of CTX-M among isolates of the present study is associated with increase of encoding gene mobilization. In case of TEM-01, as mentioned, the TEM-01 was encountered 80.9%.

The predominant ESBLs genes in other several studies were diverse. In Italia , Portugal, Turkey were performed 45.4%, 40.9% and 72.7% respectively, TEM-01was the most predominant genotypes [31-33]. The variations of results in prevalence rate of these studies of ESBLs may be as a result of different reasons such as difference in volume and type of antibiotic consumption and differences in time by which isolates were collected [34].

In the current study, a high prevalence of ESBL producing *E. coli* was in males 27 (57.4%) than in females 20 (42.6%) despite low number of infection. This result was despite the results that registered earlier by [35]. At the same time, one of the studies was analyzed that there is some differences between male and female patients in producing ESBL infections, indicating that male patients with *E. coli* are more likely and prone for having the CTX-M plasmid gene around 68.2 age group [36]. Prior to this, plasmid gene combination association with demographic factors had not been described in the literature.

The most frequent UTIs are in females, and most of the UTIs caused by bacteria from the intestinal microbiota. This give explanation for the highest frequency of infection by UTIs that occurred in women comparing with men, the reason might be due to a shortening in urethra that making colonization easier [37,38].

At the same time another mechanism for the lower frequency of UTIs in men would be explained that the prostatic fluid which has antibacterial substance [39]. Multiple ESBLs-producing *E. coli* had been occurs worldwide, therefore,

CTX-M, TEM, and SHV plasmid genes, and their combinations which detect resistance of antibiotics and severity of clinical course variations depending on the geographical location, ward admitted, the hospital area, groups of patients and even type of infection [40-43]. Therefore, it is important to determine the localization of ESBLs producing bacterial strains and Extended-spectrum beta-lactamase plasmid genes frequency for establishing the local resistance pattern, in order to begin with effective empiric antibacterial therapy [44].

CONCLUSION

The frequency of gene encoding ESBLs among age grouping ranging from 25-65 year give more gene resistance than other groups including CTX-M, TEM-01 and SHV. ESBL encoding genes, the resistance among males were higher than females particularly the distribution of *bla* TEM genes.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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