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BETA LACTAMASE SUSCEPTIBILITY PATTERNS AND PREVALENCE OF ESBL GENES IN UROPATHOGENIC *Escherichia coli*

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ABSTRACT

Escherichia coli is one of the main causes of urinary tract infections (UTIs). The pathogenic isolates are becoming increasingly resistant to antibiotics especially beta-lactam by their ability to produce extended-spectrum beta-lactamases (ESBLs). The aim of this study was to isolate *E. coli* from UTIs, and detection the antimicrobial resistance and its association with ESBL genes. CHROM agar Orientation medium was used for rapid detection of *E. coli* from urine specimens. According to phenotype of ESBL detection by Double Disk Synergy Test (DDST), eighty-four of *E. coli* isolates were ESBL producing. Polymerase chain reaction (PCR) was used for detection beta-lactamase genes (*bla*CTX-M, *bla*TEM and *bla*SHV). The percentage of ESBL-producing *E. coli* isolates was 57.5 %. The most frequent gene was *bla*TEM (82.1 %) followed by *bla*SHV (67.9 %) and *bla*CTX-M (34.5 %) genes. The most common ESBL genotype among our isolates was *bla*SHV and *bla*TEM (33.3 %). *E. coli* isolates that able to produce ESBL were susceptible to Meropenem and Imipenem, 94.1 % and 90.5 % respectively, and showed 100 % resistance to Amoxicillin, Cefotaxime and Cephalexin. The negative ESBL isolates exhibited high resistance to Amoxicillin and Cephalexin, and high sensitive to other antibiotic in comparison with the positive ESBL isolates. In conclusion, the most effective antibiotics against ESBL-producing uropathognic *E. coli* were carbapenems. The cephalosporins resistance is mainly due to ESBL production, where TEM-type gene was the most frequent among our local isolates. Study on ESBL producers among *E. coli* strains helps to control drug resistance cases and choose the right antibiotics for therapy of UTI.

Key words: *E. coli*, ESBL, UTI, Antibiotic Resistance.

INTRODUCTION

One of the most frequent diseases that are encountered in clinical practice is urinary tract infections (Baral *et al*., 2012). The developing countries suffer from the high frequency of UTIs and this due to the bad environmental condition, very poor hygiene and malnutrition. The main identified causative agents for community acquired UTIs were *E. coli*, *Klebsiella* spp. and *Enterococcus* (Foxman, 2002). The patients with UTIs does not responded to antibiotics therapy and this a representative example of the increasing complications of antimicrobial resistance (Mukherjee *et al*., 2013). Antibiotic resistance in Gram negative bacteria due to various mechanisms, such as enzymetic inactivation of antibiotics, altered target sites, efflux pump, and decreased permeability by the porins (Rao *et al*., 2014). Extended Spectrum beta-Lactamase enzymes are consider the main important mechanism for beta lactam resistance, these enzymes hydrolyze all penicillins, most of cephalosporins and monobactames, but they cannot hydrolyze carbapenemes (Bonnet, 2004; Gupta, 2007). The most ESBLs can be divided into 3 genotypes: sulfhydryl variable (SHV), temoneira (TEM) and cefotaximase (CTX-M). CTX-M, SHV and TEM are class A ESBLs (Schaumburg *et al*., 2013). The ESBL producer's bacteria can acquire different resistance genes and this give raise their multidrug resistance, further limiting the drug of choice (Crémet *et al*., 2009). ESBL enzymes have the ability to hydrolyze aztreonam and third-generation cephalosporin, but the common characteristic is the inhibition by clavulanic acid, also, ESBL-producing bacteria reveal co-resistance to other classes of antibiotics, and this lead to the therapeutic option limitation (Rawat and Nair, 2010). The multidrug resistance in gram-negative bacteria, particularly *Escherichia coli*, is mainly due to the emergence of ESBL and this resistance mechanism consider as a serious global problem of public health. Therefore, the purpose of the present study is determining of uropathogenic antimicrobial resistance pattern to beta- lactam antibiotics had commonly been used and investigate the prevalence of ESBL type of *E. coli* isolated from patients with UTI and detection the genes correlated with these enzymes.

MATERIALS AND METHODS

Isolation and identification of *E. coli*: This study was performed at Al-Kindy Teaching Hospitals in Baghdad, Iraq, between January and July 2016. Out of 450 Urine sample, a total of 146 isolates

were collected from UTIs. Blood agar and McConkey agar were used for isolation uropathogenic *E. coli* and CHROM agar Orientation medium for rapid detection of these bacteria. The isolates were identified according to the biochemical tests, with an API 20E system (bioMerieux, France).

Antibiotic Susceptibility Test: This test was conducted using Kirby Bauer method by agar diffusion test. *E. coli* colonies were taking from overnight growth on blood agar and re-suspended in Mueller-Hinton broth (HiMedia). This suspension was adjusted to an equivalent 0.5 McFarland and used for testing the susceptibility of bacterial isolates on Mueller-Hinton agar (HiMedia). Meropenem (MRP 10µg), Imipenem (IMP 10µg), Ceftazidime (CZX 30µg), Cefotaxime (CTX 30µg), Cephalexin (CN 30µg), Ceftriaxone (CTR 30µg), Cefixime (CFM 5µg), Cefepime (FEP 30µg), Amoxicillin (AMX 30µg), Amoxycillin clavulanate (AMC20/10µg) and Piperacillin tazobactam (PIT 100/10µg), (MAST, UK) were placed on the Mueller-Hinton agar plates, the incubation was at 35 °C for 18 h. and then the inhibition zone was measured and the data were compared according to the test cultures by CLSI. The results were interpreted by CLSI breakpoint interpretative Criteria (CLSI, 2012).

Screening ESBL producing isolates by phenotypic method: Double Disk Synergy Test (DDST) was conducted for phenotypically ESBL production. In this test we can use one of the two discs (Ceftrixon (30µg) and Ceftazidime (30µg)); everyone was placed 16 to 20 mm apart from the Amoxycillin clavulanate (20/10µg). The incubation was at 37°C for 24 h. The ESBL producers exhibit the zone of cephalosporin disc towards the clavulanic acid disc (Peter-Getzlaff, 2011). For confirmation the results, Phenotypic Disc Confirmatory Test (PDCT) was used as recommended by CLSI. *E. coli* ATCC 25922 was used as control strain.

DNA extraction and identification of ESBL genes by PCR: Bacterial DNA was extracted from cells by using DNA extraction Kit (Promega, USA) according to the procedure of the manufacture. The DNA concentration was estimated by spectrophotometer. The Primer sequences, which were used for detection of ESBL genes in this study, were as in Table 1.

Table 1. Primer sequences for PCR detection of extended-spectrum beta-lactamases genes in *E. coli.*

Target gene	Oligonucleotide primer sequence 5' to 3'	Amplicon size (bp)	Reference
*bla*SHV-1F *bla*SHV-1R	GGCCGCGTAGGCATGATAGA CCCGGCGATTTGCTGATTTC	714	Abujnah *et al*., 2015
*bla*CTX-M-1F *bla*CTX-M-1R	GAAGGTCATCAAGAAGGTGCG GCATTGCCACGCTTTTCATAG	560	Abujnah *et al*., 2015
*bla*TEM-1F *bla*TEM-1R	CAGCGGTAAGATCCTTGAGA ACTCCCCGTCGTGTAGATAA	643	Abujnah *et al*., 2015

PCR conditions for amplification of ESBL gene was carried out by the thermocycler (Applied Biosystems) as follows: initial denaturation at 94°C for 5 min, denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min, was repeated for 30 cycles; a final extension at 72°C for 10 min (Abujnah *et al*., 2015).

Agarose gel electrophoresis was done a 1.2% agarose gel at 80V for 2 hours. After electrophoresis fragments were stained by Ethidium Bromide, and then visualized with ultraviolet light.

RESULTS AND DISCUSSION

In this study, it was found a significant bacterial growth among the most common uropathogens. Out of the 450 urine samples, 210 of the patient's urine samples had bacterial growth. Due to the specificity of CHROMagar Orientation medium with respect to color and colony morphology, this medium made the differentiation of bacterial colonies of uropathogenes from urine samples. The colonies of *E. coli* on this medium were obtained dark pink to reddish as showed in figure 1.

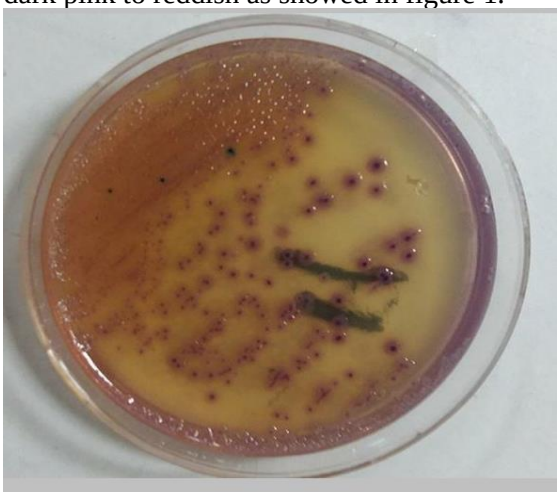

Figure 1: Colonies of *E. coli* on CHROMagar Orientation medium (dark pink to reddish).

CHROMagar Orientation has a specificity of 99.3 % for *E. coli*, rending the species confirmatory test largely unnecessary. This fact, together with the ability of the medium to limit the spread of bacteria, allowed the presumptive identification of several microorganisms directly from the primary plates (Samra *et al*., 1998). The results of previous studies revealed that the use of CHROMagar Orientation medium streamlined the urine culture process and increased bench throughput by reducing both workload and turnaround time in our laboratories (Manickam *et al*., 2013). Biochemical tests and API 20E system (bioMerieux, France) were used for confirmation the identification of *E. coli* isolates.

Among the isolates, the most common organisms identified were *Escherichia coli* (146). This study demonstrated that *E. coli* remain the leading uropathogen being responsible for 32.4 % of UTI in our local hospital. This is in consistence with findings of other studies in which *E. coli* was the most frequently reported isolate from patients with UTIs (Wei Tan and Chlebicki, 2016).

Among *E. coli*, 84 (57.5%) isolates be being found to ESBL positive while 62 were ESBL negative. The detection of ESBL enzymes by *E. coli* isolates was conducted by DDT method as sown in figure 2.

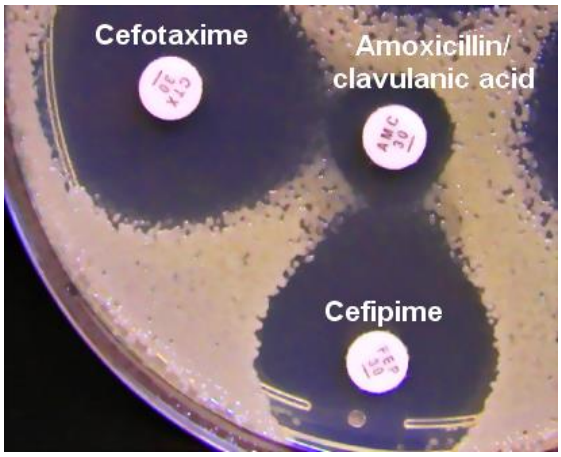

Figure 2: Double-disk synergy test for extended spectrum beta-lactamases in *E coli*, positive result. AMC-Amoxycillin + Clavulanate, CTX- Cefotaxime, FEP–Cefepime (A clear extension of the edge (Syne-rgy) of the FEP inhibition zone towards the disc containing clavulanate).

Our study revealed that 57.5 % of *E. coli* isolates to be ESBL producers. Aggarwal *et al.* reported 40% of *E. coli* to be ESBL producers from Rohtak, Haryana.

In another study in Nepal, among 97 MDR Gram negative isolates, 63 (64.9%) showed the ESBL screening positive result, in which *E. coli* accounts to be 15.32 % (Shrestha *et al*., 2016). The rates of ESBL-producing *E. coli* were higher in comparison with other countries such as turkey 17%, Lebanon 13.3% and Korea 9.2% (Ananthan and Subha, 2005; Shahcheraghi *et al.*, 2009).

The differences in results may be due to different patterns of antibiotic usage. Our study confirmed the global trend towards increased resistance to beta lactum antibiotics. The routine disk diffusion susceptibility test is not appropriate for ESBL detection and this may be lead to inappropriate use of antibiotics and treatment failure. It is emphasized that hospitals should employ appropriate tests for their detection and avoids indiscriminate use of third generation cephalosporins. The ESBL mutant which are derived from broad spectrum beta lactamase (eg. TEM-1, TEM-2, SHV-1) mediate resistance to extended spectrum third generation antibiotic cephalosporins such as cefotaxime. These mutants are specific to third and fourth generation cephalosporins but not to cephamycins or carbapenams (Hakenbeck and Coyette, 1998).

Table 2. Outlines patterns of susceptibility and resistance among ESBL and non-ESBL pathogens to 11 commonly used beta-lactam antibiotics. It was obvious that the most of *E. coli* isolates were susceptible to carbapenemes (Imipenem and Meropenem) with sensitivity rang 90.5 % to 96.7 %. Also, the results revealed that the most isolates were resist to Amoxicillin and Cephalexin with the resistance range 91.9 % to 100 %. In 7 out of 11 antibiotics, ESBLs had a greater percentage of resistance when compared to non-ESBL resistance (figure 3). Ceftazidime, Cefotaxime, Ceftriaxone, Cefixime and Amoxycillin clavulanate has about the non-ESBLs resistance representing 24.2 %, 48.4 %, 19.4 %, 27.4 % and 12.9 % versus the 84.5 %, 100 %, 80.9 %, 73.8 % and 66.6 % of ESBLs respectively. The highest percentages of susceptibility among ESBLs were in response to Imipenem 90.5%, Meropenem 94.1%, Piperacillin tazobactam 65.5 % and Cefepime 42.8 %.

Table 2. Percentages of antimicrobial susceptibility rate of 84 ESBL *E. coli* isolates against 11 antimicrobial agents.

Antibiotic*	Positive ESBL isolates (N= 84)			Negative ESBL isolates (N= 62)
	Resistant	Intermediate	Sensitive	Resistant
IPM	7 (8.3	1(1.2 %)	76 (90.5 %)	3 (4.8 %)

MEM	5 (5.9 %)	0 (0 %)	79 (94.1 %)	2 (3.2 %)
CAZ	71 (84.5	2 (2.3 %)	11 (13.2 %)	15 (24.2 %)
CTX	84 (100	0 (0 %)	0 (0 %)	30 (48.4 %)
CN	84 (100	0 (0 %)	0 (0 %)	57 (91.9 %)
CTR	68 (80.9	3 (3.6 %)	13 (15.5 %)	12 (19.4 %)
CFM	62 (73.8	1 (1.2 %)	21 (25 %)	17 (27.4 %)
FEP	44 (52.4	4 (4.8 %)	36 (42.8 %)	9 (14.5 %)
AMX	84 (100	0 (0 %)	0 (0 %)	60 (96.8 %)
AMC	56 (66.6	0 (0 %)	28 (33.3 %)	8 (12.9 %)
PIT	27 (32.2	2 (2.3 %)	55 (65.5 %)	7 (11.3 %)

Meropenem: MEM, Imipenem: IMP, Ceftazidime: CAZ, Cefotaxime: CTX, Cephalexin: CN, Ceftriaxone: CTR, Cefixime CFM, Cefepime: FEP, Amoxicillin: AMX, Amoxycillin clavulanate: AMC, Piperacillin tazobactam: PIT.

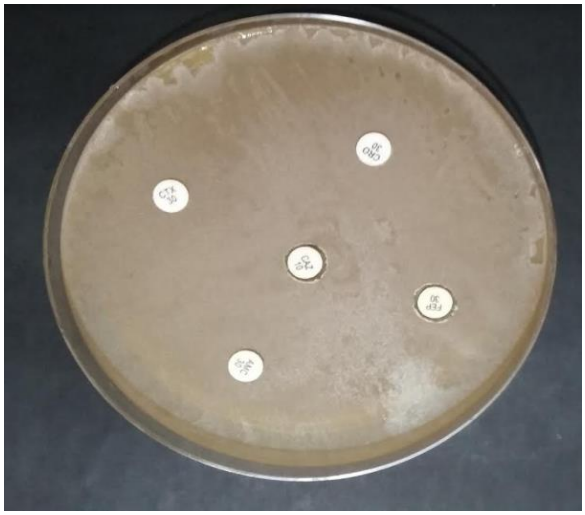

Figure 3: Disc diffusion susceptibility test of ESBL *E. coli* on Mueller Hinton agar medium showed the high resistance to Cephalosporins.

The current study indicated to the significant correlation between production of ESBL enzymes by *E. coli* isolated from UTIs and the resistance of cephalosporins especially third generation such as Ceftazidime, Cefotaxime and Ceftriaxone. Antibiotic resistant in uropathogenic strains of *E. coli* are increasingly found and are a serious problem in many areas (Ma and Wang, 2013). The local study of Mahmood, 2011 (22) indicated to the high prevalence of *E. coli* among UTI patients in Baghdad city and these isolates were resist to Ampicillin, Trimethoprim and Cefoxitin but the antibiotic Imipenem revealed inhibitory activity against them.

The present study was conducted on 84 ESBL-producing *E. coli* isolates. The results exhibited that all of isolates were resistant to amoxicillin, Cephalexin and Cefotaxime. According to the reports in over 800 laboratories from 31 countries by the European Antibiotic Resistance Surveillance System, the resistance to third generation cephalosporins was increased (Gagliotti *et al*., 2011). Also, due to low standard hygiene and indiscriminate use of antibiotics, the percentage of *E. coli* resistance to Ampicillin, aminoglycosides, tetracycline and sulphonamides in developing countries are higher than industrialized countries (Sana *et al*., 2011).

The phenotypically identified ESBL-producing *E. coli* were subjected to PCR using *bla*CTX-M, *bla*-TEM and *bla*SHV specific primers. Of the 84 phenotypically ESBL-producing *E. coli* isolates, all the isolates (100 %) were positive for ESBL genes. The detected *bla*CTX-M, *bla*TEM and *bla*-SHV were present alone or in combination with each other. The TEM type was the most prevalent among *E. coli* isolates 82.1%, followed by the SHV 67.9% and CTX-M 34.5% types, respectively (Table 3). The results of PCR-products electrophoresis showed genomic patterns related to *bla*SHV (714 bp), *bla*TEM (643 bp) and *bla*-CTX-M (560 bp), (Figures 4, 5 and 6).

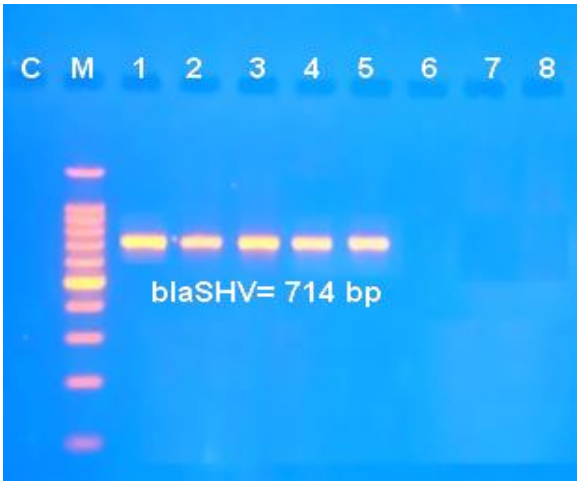

Figure 4: Electrophoresis of the amplified products of *bla*SHV (714 bp) gene by a PCR in a 2 % agarose gel. Lane 1 to 5; positive result of gene detection in *E. coli* isolates. Lane C, Negative control (PCR product without the DNA template). Lane M, 100 bp DNA ladder.

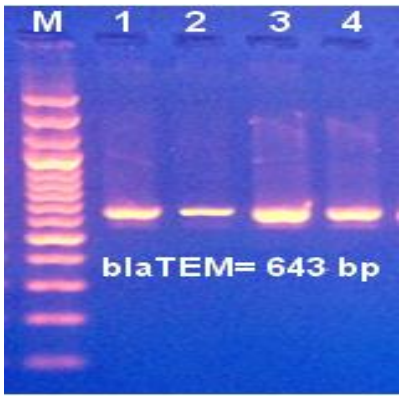

Figure 5: Electrophoresis of the amplified products of *bla*TEM (643 bp) gene by a PCR in a 2 % agarose gel. Lane 1 to 5; positive result of gene detection in *E. coli* isolates. Lane M, 100 bp DNA ladder.

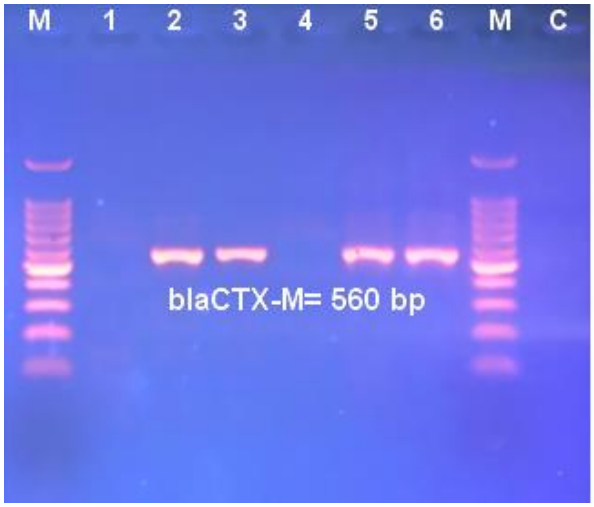

Figure 6: Electrophoresis of the amplified products of *bla*CTX-M (560 bp) genes by a PCR in a 2 % agarose gel. Lanes 2,3,5 and 6; positive result of gene detection in *E. coli* isolates. Lane C, Negative control (PCR product without the DNA template). Lane M, 100 bp DNA ladder.

The current result is in agreement with findings of Bajbai *et al*. (2017) which indicated that the most prevalent gene was *bla*TEM (48.7%) of the urinary isolates of enterobacteriaceae in a tertiary care hospital. ESBL are the result of point mutations of TEM-1, TEM-2 and SHV-I genes, more than 130 TEM type and more than 50 SHV type have been reported from various places of the world (Paterson *et al*., 2005).

The results of genotypes pattern demonstrated that 53 tested isolates 63.1 % of *E. coli* had at least two ESBL genes and the most prevalent genotype was the present of two genes, *bla*SHV and *bla*-TEM, 33.3% (Table 3).

Table 3: Genotypes of ESBL Genes Detected in Extended Spectrum β-lactamases-producing *E. coli* Isolates.

Genotypes	*E.coli* (n=84)
One genotype	
*bla*CTX-M	3 (3.6 %)
*bla*TEM	19 (22.6 %)
*bla*SHV	9 (10.7 %)
Total	31 (36.9 %)
Tow or thee combination genes	
*bla*CTX-M + *bla*TEM + *bla*SHV	9 (10.7 %)
*bla*CTX-M + *bla*TEM	5 (6 %)
*bla*CTX-M + *bla*SHV	11 (13.1 %)
*bla*SHV + *bla*TEM	28 (33.3 %)
Total	53 (63.1 %)

In our study, the frequency of ESBL-producing *E. coli* was 57.5 %, which was lower than that in Turkey 84% (Bali *et al.*, 2010) and India 66.7% (Hawkey, 2008), but was higher than those in Kuwait 31.7% (Mokaddas *et al.*, 2008), Saudi Arabia 30.6% (Hassan *et al.*, 2014), this may be due to the differences in the time of sample's collection and the type of antibiotics consumption. The current study revealed that TEM enzymes were the most common ESBL types followed by SHV and CTX-M. The high prevalence of TEM gene in our study, we are in agreement with some earlier reports. In studies conducted in Turkey 72.7% (Bali *et al.*, 2010), Italia 45.4% (Carattoli *et al*., 2008) and Portugal 40.9% (Fernandes *et al*., 2014), the most predominant ESBL genotype was TEM. The findings of Akya et al. (2013) revealed that 53% of ESBL producing isolates contained *bla*TEM gene, when testing their susceptibility to antibiotics, 81.43% of the isolates were resistant to ampicillin. Several investigators indicated to the co-existence of different ESBL beta-lactamases genes within the same isolates (Oteo *et al*., 2010). Our results showed that about 63.1% of the ESBL-producing *E. coli* isolates were molecularly confirmed to have two or more ESBL genes. The most common ESBL genotype among our isolates was *bla*SHV and *bla*TEM (33.3 %). One of the studies of the enerobacteriaceae isolates from health care centers revealed to the most prevalent of *bla*SHV and *bla*TEM among ESBL strains and the spread of ESBL-producing isolates to the community seems to be related to previous nosocomial acquisition (Arpin *et al*., 2003). The mutants of TEM β-lactamases are being recovered that maintain the ability to hydrolyze third-generation cephalosporins and are referred to as complex mutants of TEM (CMT-1 to -4) (Paterson *et al*., 2005). Our results don't agree with the findings of local study in Zakho, Iraq which referred that CTX-M type ESBL was the most dominant ESBL (87.2%) among ESBL-producing uropathogenic *E. coli*, while those for TEM-type and SHV-type

were 54.5% and 21.8% respectively (Polse et al., 2016).

CONCLUSION

Our results showed that ESBL positive uropathogenic *E. coli* are beta-lactams resistant and pose challenges to clinicians in determining the appropriate drugs which effectively treat UTI. The emergence of ESBL-producing uropathogens revealed the obvious ability of bacteria to evolve and survive against antibiotics. Also, there is correlation between the activity of ESBL enzymes and the cephalospornis resistance and the molecular basis of this mechanism may due to the ESBL genes especially the TEM type. The present study emphasizes the need for implementation of strict hospital infection control policies, such as control of the use of non-prescribed antibiotics and continuous monitoring of antibiotic susceptibility profiles of uropathogenic *E. coli* isolates.

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