*Incidence of Extended-Spectrum β-lactamases ESBLs producing *Escherichia coli* in patients with urinary tract infection

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Abstract

Two hundred seven isolates of *Escherichia coli* were isolated from urine samples collected from patient suffering from UTI obtained from Al-Diwaniya Teaching Hospital. During the period from March 2011 to May 2012, a total of 2000 urine samples were collected from patients with suspected UTI. Identification according to the traditional biochemical tests, then confirmed by VITEK 2 system. Detection of ESBLs by using initial screening and confirmatory test which carry out by disk approximation or double disk synergy test (DDS) and CHROMagar methods, showed that 186 (89.8%) / 207(100%) of *E. coli* were initially screened as positive for ESBL. CHROMagar CTX technique was the most 166 (80.2%) ambient method than the disk approximation or (DDS) 0(0.0%) tests regarding confirmation of ESBL production. The results revealed that Screening and confirmatory tests considered the most accurate method for detection of ESBL- producing isolates, while the disk approximation was not accurate method in detecting such enzymes. The results of PCR assay showed that, 50 (100%) and 49 (98.0%) of the $bla_{\text{CTX-M}}$ positive isolates harbored bla_{SHV} and bla_{OXA} genes, respectively. While, bla_{TEM}, bla_{PER}, bla_{VEB} and bla_{GES} genes were not detected in this investigation.

Introduction

Urinary tract infection (UTI) is one of the most common diseases among all age groups encountered in medical practice today (1). *E. coli* is the main caused of UTIs (2). Increased use of β-lactam antibiotics, particularly the third generation of cephalosporins, has been associated with the emergence of β-lactamases mediated bacterial resistance, which subsequently led to the development of ESBLs producing bacteria. ESBLs are enzymes that mediate resistance to extended spectrum e.g., third generation cephalosporins as well as monobactams (3), but not the cephamycins or carbapenems (4), produced by the Gram-negative bacteria more commonly in *E. coli* and *K. pneumoniae* (5). A shift in the distribution of different ESBLs has recently occurred in different part of the world, with a dramatic increase of CTX-M enzymes over TEM and SHV variants (6). CTXM- ESBL-producing *Escherichia coli* have

***The Research is apart of on Ph.D. Dissertation in the case of the First researcher**

emerged as a significant and developing problem in many parts of the world, occurring in patients in the community as well as in those with recent hospital contact (7). Because of inappropriate usage of antibiotic in treatment of infection caused by ESBL producing pathogens, it seems that studies about correct detection and antibiotic resistance pattern of these organisms are necessary. In recent years a few studies were done about the *E. coli* isolates producing ESBLs in our country (8, 9, 10, 11). Also despite of importance of CTX-M type β-lactamase, there is little information concerning *E. coli* isolates harboring $bla_{\text{CTX-M}}$ gene in Iraq, particular Al-Diwaniya province. Therefore, the present study was carried out to determine the prevalence of the genes encoding CTX-M, SHV, TEM, OXA, GES, VEB and PER responsible for ESBL production in the *E. coli* isolated from clinical specimens at Al-Diwaniya Teaching Hospital.

Materials and methods

Collection and Handling of Samples

During the period from March 2011 to May 2012, a total of 2000 urine samples were taken (by standard mid-stream "clean catch" method) from patients with clinical suspected urinary tract infection (UTI) according to (12).

Identification of Bacterial Isolates

Escherichia coli (207 isolates) *and* other bacterial isolates (248 isolates) were identified depending on the traditional morphological and biochemical tests according to the methods of (13) as mentioned in Table (1). Selected isolates were further confirmed as *E. coli* by the VITEK 2 identification system (BioMerieux, Marcy L'E toile, France). This system was prepared in accordance with the manufacturer's instructions fixed on their strips.

Extended-Spectrum β-lactamase (ESBL) Production

1. Initial Screening for ESBL Production

All *E. coli* isolates were tested for ESBL production by initial screen test. The isolate would be considered potential ESBL producer, if the inhibition zone of ceftazidime (30μg) disks was ≤ 22 mm, ceftriaxone (30μg) was ≤ 25 mm, and cefotaxime and aztreonam (30μg each) were ≤ 27 mm. (3).

2. Confirmatory Test

All bacterial isolates were tested also for confirmatory ESBL production by two methods as follows:

a. Disk Approximation Test

All *E. coli* isolates tested according to (14).

b. Detection of CTX-M ESBL by CHROMagar Technique

CTX-M β-lactamase CHROMagar plates were streaked with overnight growth of *E. coli*. The plates were incubated at 37°C for 24 hr according to manufacturer procedure. Blue colonies appearance indicated as CTX-M β-lactamase producer isolates. The growth of reference strain *E. coli* ATCC 25922 inhibited and used as negative control.

ECC CHROMagar

The ECC CHROMagar was prepared according to manufacturer recommendations by dissolving 33 g / L of sterile distilled water, homogenized with motility in heating 100˚C, autoclaving at 121°C for15-20 minutes.

CHROMagar Supplemented Solution

The supplements were prepared according to manufacturer recommendations by dissolving 125 mg/ml of CTX-M supplement in sterile distilled water and ethanol (equal mixture, vol/vol), homogenized and added in the proportion of 2 ml/l of final melted ECC CHROMagar after cooled at 45˚C. The medium were poured into plates and used in the same day of preparation.

DNA Extraction

Extraction of DNA from bacterial cells performed by salting out method according to (15).

Polymerase Chain Reaction (PCR) Assay

Preparing the Primers Suspension and PCR Materials

The DNA primers were resuspended by dissolving the lyophilized product after spinning down briefly with TE buffer molecular grad depending on manufacturer instruction as stock suspension. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer , Master Mix type Go Tag Green Master mix(2X Green Taq Reaction buffer pH 8.5, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3mM $MgCl_2$) (Promega, USA). Molecular Weight DNA Marker, 100 bp Ladder with Loading dye (100-1500 base pairs (bp). The ladder consists of 11double strand DNA fragment ladder with size of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500 bp. The 500 bp present at triple the intensity of other fragments and serve as a reference. All other fragments appear with equal intensity on gel (Promega, USA).

PCR Protocols

The concentration of extracted DNA measured and controlled by using biophotometer based on Promega manufacturer's instruction recommendations. The DNA extract of uropathogenic *E. coli* isolates subjected to *bla*-genes in Table (1) by monoplex PCR. All PCR components assembled in PCR tube and mixed on ice bag under sterile condition , Protocols of PCR reaction mixture volumes (Promega protocol,final volume 25 µl) : Master mix 2X (12.5 µl), Primer forward (10µM) 2.5 µl, Primer reverse (10µM) 2.5 µl, DNA template 5 µl and PCR grade water 2.5 µl .

Table-1: Primers of Monoplex PCR ESBL (Bioneer, Korea)

Type	Primer name	Gene name	Oligo sequence (3'-5')		Product size (bp)	Reference
ESBL	CTX-M	bla_{CTX-M}	F	CGCTTTGCGATGTGCAG	550	**16**
			R	ACCGCGATATCGTTGGT		
	TEM	bla_{TEM}	F	AAACGCTGGTGAAAGTA	822	**17**
			R	AGCGATCTGTCTAT		
	SHV	bla_{SHV}	F	ATGCGTTATATTCGCCTGTG	753	**18**
			R	TGCTTTGTTATTCGGGCCAA		
	OXA	bla_{OXA}	F	ATATCTCTACTGTTGCATCTC	619	**19**
			R	AAACCCTTCAAACCATCC		
	GES	bla_{GES}	F	ATGCGCTTCATTCACGCAC	846	**20**
			R	CTATTTGTCCGTGCTCAGG		
	PER	bla_{PER}	F	AGTCAGCGGCTTAGATA	978	**20**
			R	CGTATGAAAAGGACAATC		
	VEP	bla_{VEB}	F	GCGGTAATTTAACCAGA	961	**20**
			R	GCCTATGAGCCAGTGTT		

PCR Thermocycling Conditions

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in Table (2) for monoplex PCR.

Table-2: Programs of monoplex PCR thermocycling conditions

Gene name	Temperature (°C)/ Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	extension		
bla_{CTX-M}	94/30 sec	94/30 sec	60/1 min	72/1 min	72/10 min	35
bla_{TEM}	94/30 sec	94/30 sec	45/1 min	72/1 min	72/10 min	35
bla_{SHV}	94/30 sec	94/30 sec	60/1 min	72/1 min	72/10 min	35
bla_{OXA}	94/5 min	94/50 sec	55/50 sec	72/1 min	72/5 min	30
bla_{VEB}	93/3 min	93/1min	55/1 min	72/1 min	72/7 min	40
bla_{GES}	93/3 min	93/1min	55/1 min	72/1 min	72/7 min	40
bla_{PER}	93/3 min	93/1min	55/1 min	72/1 min	72/7 min	40

Results

Identification of Bacterial Isolates

In this investigation, all urine samples were routinely cultured on MacConkey and blood agar plates. The bacterial isolates obtained as a pure and predominant growth from urine samples were only considered for the present study, and only one isolate per patient from UTI were included in the study. Totally, 455 consecutive nonduplicate bacterial isolates were recovered from urine samples of patients with significant bacteriuria. The isolates were identified by their cultural characteristics, Gram staining, and reactions to standard biochemical tests. Primary tests were carried

out on the isolated colonies of isolates that they behave as a typical *E. coli* on MacConkey agar, and biochemical tests. After confirmation of the presence of suspected *E. coli* isolates, the colony was subcultured on eosin methylene blue (EMB) agar, which is a differential and selective medium to differentiate between different *Enterobacteriaceae* in terms of the morphological characteristics and color on the agar. According to the color of *E. coli* on EMB, a colony was streaked on EMB to obtain pure cultures of *E. coli* colonies. From the pure culture, a distinct *E. coli* colony was screened with additional biochemical tests, the phenotypic characteristics of suspected *E. coli* are listed in Table (3). VITEK 2 system was then carried out for the final identification of nine isolates had a phenotype consistent with production of a CTX-M ESBL.

Table (3): Morphological and biochemical tests of 207 *E. coli* isolated from patients with significant bacteriuria (n= 455)

Test	Result
Gram-negative bacilli	100%
Growth on the EMB agar	Metallic sheen colonies
Indole	100%
Methyl red	100%
Vogas-Proskaur	0%
Citrate utilization	0%
Motility	80%
Acid from glucose	100%
Acid from Lactose	100%
TSI (A/A+ G**)**	100%
H_2S production	0%

EMB, eosin methylene blue; TSI, triple sugar iron; A, acid; G, gas

Phenotypic Detection of β-lactamase Production

All the 207 *E. coli* isolates were tested for ability to produce β-lactamase enzymes. These organisms screened for the presence of ESBLs and then investigated for the presence of CTX-M-β-lactamases. Extended-spectrum β-lactamases-producing

E. coli isolates were detected using an initial screening test according to the CLSI scheme. The isolates were screened for ESBL production by disk diffusion method using ceftazidime, cefotaxime, ceftriaxone and azetreonam (30 µg each) antibiotic disks. According to the CLSI, the isolate is considered to be potential ESBL producers, if the inhibition zone of ceftazidime was ≤ 22 mm, ceftriaxone was ≤ 25 mm, cefotaxime and aztreonam were ≤ 27 mm. The frequency of ESBLs producing isolates by disk diffusion assay are summarized in Table (4). A ceftazidime effect was detected in 205 (99.0%) isolates, decrease susceptibility to cefotaxime was documented in 206 (99.5%) isolates, ceftriaxone influence was observed in 186 (89.9%) isolates, and azetreonam effect phenotype was recognized in all (100%) isolates tested. Of the isolates, 1 (0.5%), 1 (0.5%), 19 (9.2%), and 186 (89.8%) showed reduced susceptibility to 1, 2, 3 and 4 antibiotics tested, respectively. In this study, primary phenotypic test revealed that ESBL phenotype was recognized in all *E. coli* isolates (100%) which were expressed reduced susceptibility to any of the expanded-spectrum cephalosporins and monobactam. Based on this phenotypic detection, the study revealed a high rate of ESBLs-producing *E. coli* recovered from patients with significant bacteriuria in Al-Diwaniya Teaching Hospital.

Double-disk synergy test (DDST) with amoxicillin-clavulanic acid, cefotaxime, ceftazidime, ceftriaxone, and azetreonam disks at a distance of 15 mm (edge to edge) was performed to phenotypic confirmatory detect the ESBL-producing *E. coli* isolates. Since clavulanic acid disks are not available in Iraq, amoxicillin/clavulanic acid disks were used as a source of clavulanic acid. Present results revealed that the isolates did not show any ESBL production as the increased zone size towards the amoxicillin-clavulanic acid disk (Table 4), probably due to the simultaneous production of other type of β-lactamase that could mask the presence of the ESBL **(**AmpC β-lactamases or inhibitor resistant cephalosporinases) . It is noteworthy that the difference in number of the above methods was large. However, the study indicated that no correlation was found between the results obtained with disk diffusion method and DDST.

Table (4): Comparative between initial screening and confirmatory test for ESBL production by 207 *E. coli* isolates

Initial screening test No. (%)				Disk approximation test
Ceftriaxone	Ceftazidime	Cefotaxime	Aztreonam	
186 (89.8%)	205 (99.0%)	206 (99.5%)	207(100%)	0(0.0%)

Phenotypic Detection of CTX-M β-Lactamase

All the 207 *E. coli* screened and verified for CTX-M production according to the CLSI criteria. All these isolates were potential ESBL producers as mentioned in Table (4). Primarily detection of CTX-M ESBL-producing isolates was based on their phonotypical properties. In this work, CHROMagar CTX, a novel agar for the selective isolation of *Enterobacteriaceae* expressing the $bla_{\text{CTX-M}}$ gene was used for

the recognition of CTX-MESBL-producing isolates. When the isolate produces CTXM-type enzyme, the color of a colony turns metallic blue (Figure 1). Although all the 207 *E. coli* isolates presented potential ESBL phenotype, only 166 (80.2%) isolates had a phenotype consistent with production of a CTX-M ESBL.

Figure (1): CTX-M ESBL-producing *E. coli* isolate E26 after incubation at 37^{o}C for 24hr on CHROMagar CTX medium. Appearance of *E. coli* isolate displayed the typical metallic blue colonies.

***E. coli* Isolates harboring ESBL Genes**

Of the 166 CTX-M ESBL-producing uropathogenic *E. coli* isolates obtained from patients with significant bacteriuria, a total of 50 isolates was chosen for molecular studies. All the 50 isolates were screened for the presence of $bla_{\text{CTX-M}}$ gene using universal CTX-M primers. The expected size fragment for CTX-M was 550 bp. The results of the PCR with consensus primers for the isolates of *E. coli* showed that all isolates were positive for the presence of $bla_{\text{CTX-M}}$gene (Figure 2). Sensitivity for an a CHROMagar CTX-M medium was calculated as the number of test positives (i.e. all isolates with the $bla_{\text{CTX-M}}$ gene that grew on the medium) divided by the number of 'gold standard' positive isolates (i.e. all isolates positive for the $bla_{\text{CTX-M}}$ gene) times 100. Present study revealed that phenotypic detection of CTX-M β-lactamase on CHROMagar CTX medium corresponded closely with those obtained with PCR using universal CTX-M primers. However, CHROMagar CTX medium showed 100% sensitivity for growth of the $bla_{\text{CTX-M}}$ isolates when compared with the PCR using universal CTX-M primers.

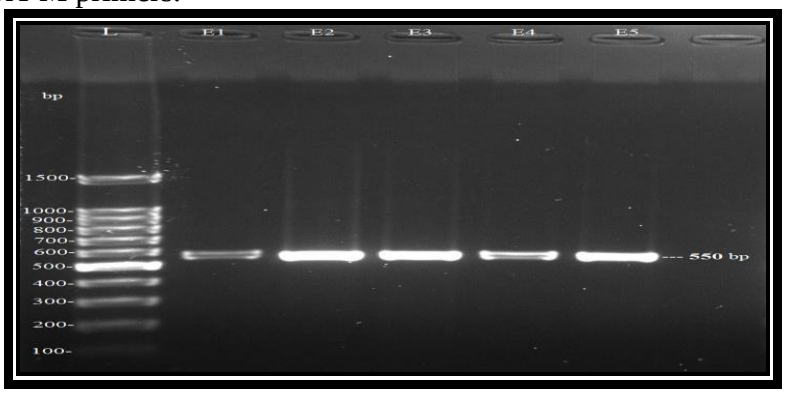

Figure (2): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *E. coli* isolates and amplified with $bla_{\text{CTX-M}}$ gene primers. The electrophoresis performed at 70 volt for 1.5 hr. Lane (L), DNA molecular

size marker (100-1500 bp ladder). Lanes 1-5 showed positive results with *bla*$_{CTX-M}$ gene.

Order ESBL genes in 50 *bla*$_{CTX-M}$ gene carried *E. coli* isolates. The molecular method was used to detect the most common six kinds of ESBLs; TEM, SHV, OXA, VEB,PER, and GES. For this purpose, six types of species primers were used with the help of PCR and electrophoresis systems. Table (5) shows the distribution of ESBL enzymes determined by the consistent results of primers specific PCR. One or two *bla*-genes for ESBL were present in all CTX-M enzyme-harboring isolates. Present results revealed that all isolates (100%) possessed *bla*$_{SHV}$ gene (Figure 3 and table 5) and 49 (98.0%) of isolates positive for *bla*$_{OXA}$ (Figure 4 and table 5), simultaneous appearance of both *bla*$_{SHV}$ and *bla*$_{OXA}$ was observed in only one (2.0%) of isolates. Notable, none of the 50 *bla*$_{CTX-M}$ gene carried *E. coli* isolates had *bla*$_{TEM}$, *bla*$_{VEB}$, *bla*$_{PER}$, or *bla*$_{GES}$ ESBL genes.

Table (5): Frequency of ESBL genes among *E. coli* isolates (n=50)

Type of *bla*-gene	No. (%) of *bla*-gene positive isolates
***bla*$_{CTX-M}$**	50 (100)
***bla*$_{SHV}$**	50 (100)
***bla*$_{OXA}$**	49 (98.0)
***bla*$_{TEM}$**	0 (0.0)
***bla*$_{VEB}$**	0 (0.0)
***bla*$_{PER}$**	0(0.0)
***bla*$_{GES}$**	0 (0.0)

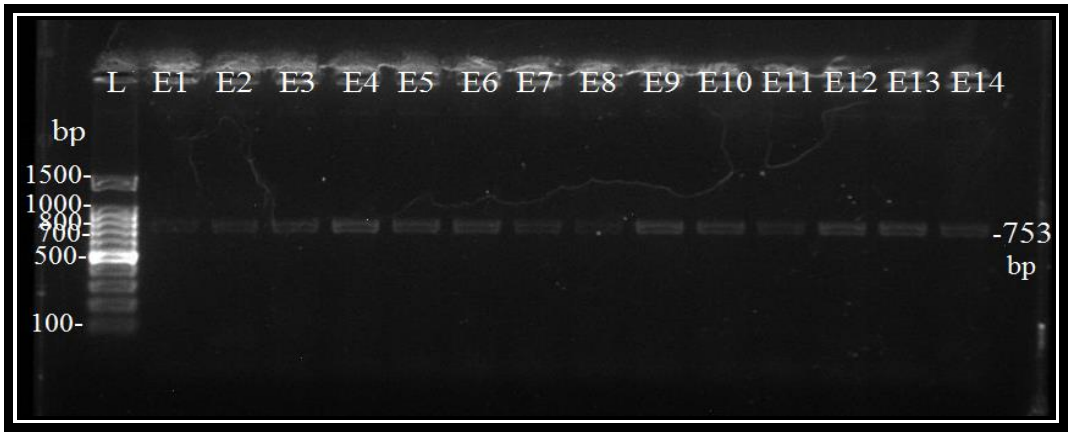

Figure (3): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *bla*$_{CTX-M}$ positive *E. coli* isolates and amplified with *bla*$_{SHV}$ gene primers. The electrophoresis performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (100-1500 bp ladder). Lanes (E1-E14) showed positive results with *bla*$_{SHV}$ gene.

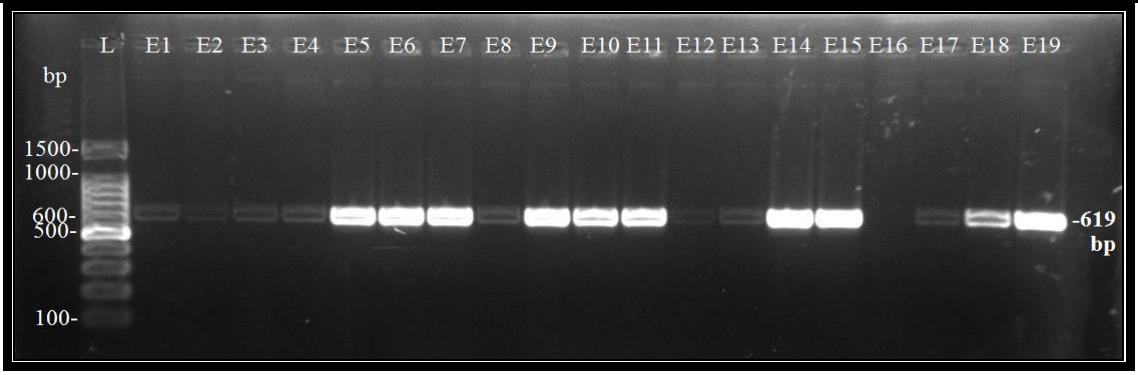

Figure (4): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *bla*$_{CTX-M}$ positive *E. coli* isolates and amplified with *bla*$_{OXA}$ gene primers. The electrophoresis performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (100-1500 bp ladder). Lanes (E1-E15 and E17-E19) showed positive results with *bla*$_{OXA}$ gene, lane (E16) show negative results with *bla*$_{OXA}$ gene.

Discussion

Screening for β-lactamase Production

Extended spectrum β-lactamases have evolved remarkably during the last 20 years and the organisms producing these enzymes are responsible for increasing in nosocomial infections, morbidity and mortality (21). The urinary tract of patients has been recognized as the major source of ESBLs producing organisms in Iraqi hospitals (8, 11). This study disclosed an elevated frequency of ESBL presence in 207 *E. coli* isolates cultured from patients with significant bacteriuria, as previously reported in Iraq by quite a few authors (9, 11, 22). Extended spectrum β-lactamases are defined as acquired, transferable β-lactamases that can significantly hydrolyze third generation cephalosporins (e.g. cefotaxime, ceftazidime, and ceftriaxone) and monobactam (aztreonam), but inactive against cephamycins, carbapenems and β-lactamase-inhibitors such as clavulanic acid and tazobactam (23). In recent years, there has been an increased incidence and prevalence of ESBLs in Iraqi hospitals (11, 24). For a number of reasons, the detection of ESBL-producing isolates is of significant importance for all Iraqi hospitals and community. First, these isolates are most likely to be even more prevalent than it is currently recognized, due to the difficulty in their detection by the current clinical methods, many of these isolates have been reported to be susceptible to widely used and tested broad-spectrum β-lactams. Secondly, ESBLs constitute a serious threat to current β-lactam therapy. Treatment of ESBL infection is difficult as the CLSI recommends that all expanded spectrum cephalosporins be taken resistant in ESBL producers (3). Routine antibiotic susceptibility testing methods are not capable of detecting ESBL resistance without modification. Unless specific ESBL screening and confirmation tests are carried out, ESBL producers are likely to be reported falsely susceptible to the cephalosporins this is likely to lead to inappropriate antibiotic therapy. In current study, the isolates were phenotypic screening for potential production of ESBL by an initial screening test followed by a phenotypic confirmatory test. Initial screening for reduced susceptibility to cefotaxime, ceftriaxone, ceftazidime or aztreonam is recommended, as proposed by the CLSI, S100-21 document (25) the use of more than one of these indicator cephalosporins suggested will improve the sensitivity of detection. A decrease in susceptibility to one or more antibiotics tested may indicate production of ESBLs. The high sensitivity of

the disk diffusion method by using three or more third generation cephalosporins has been reported by others, (26) recommends testing cefpodoxime or both cefotaxime and ceftazidime as first screening test. Hence, the feasible recommended approach for ESBL detection is to use a screening test, usually the routine susceptibility method used in the clinical laboratory (27). However, the initial screening test revealed that suspicious ESBL phenotype was recognized in all *E. coli* isolates tested (Table 4), which were expressed reduced susceptibility to any of the expanded-spectrum cephalosporins and monobactam. Based on this phenotypic detection, the study exhibited alarmingly high rate of ESBLs-producing *E. coli* recovered from patients with significant bacteriuria in Al-Diwaniya Teaching Hospital. There have been reports of considerable geographical differences in ESBLs worldwide and within countries or different parts of each country and are very much dependent upon antibiotic policies in every region. The hospital-to-hospital variability that occurs possibly reflects the direct proportionality between use and misuse of antibiotics. In Iraq, ESBL producing isolates of *Enterobacteriaceae* and *P. aeruginosa* have emerged as a challenge in hospitalized as well as community based patients (9, 28). Based on phenotypic detection methods, the incidence rate of ESBLs-producing *E. coli* in different province of Iraq varies from 15.8% to 79.7% (8, 10, 11, 22, 29). While, earlier report from Najaf has documented the incidence of ESBLs-producing *E. coli* was 15.8% (8), a recent study from Najaf reported an alarming 79.7% of ESBL-producing isolates (11). In Al-Diwaniya, 62.5% ESBL-producing *E. coli* have been documented (10). It is difficult to make valid comparison of the incidence of ESBLs with other locally reports, because of variations in studies design. Current results in compare with published data from Europeans countries such as France (6), Netherlands (30), and Spain (6), also in Tanzania (31), Thailand (32) and Pakistan (33), showed the high incidence of ESBL-producing isolates in present study. Organisms producing ESBL are typically multi drug resistant and previous exposure to an antibiotic, especially to extended-spectrum cephalosporins is a risk factor for infection with ESBL-producing bacteria (34). Therefore, the very high incidence of ESBL-producing isolates described in this study was probably due to the large amount consumption of third-generation cephalosporins. Regarding the reporting of ESBL producing isolates, the microbiology laboratory report should state that the isolate produced ESBL and should be considered resistant to all penicillins, cephalosporins and aztreonam (3). Initial screening test remains a reliable, convenient and inexpensive method of screening for ESBLs. However, the interpretation of the test is quite subjective. Sensitivity may be reduced when ESBL activity is very low leading to wide inhibition zones around the cephalosporins and aztreonam (35). Several methods have been devised to differentiate between ESBL and non-ESBL producers among which, the DDST has been reported to be the most discriminating (3). In this study, the production of ESBL was also evaluated by the phenotypic confirmatory test using DDST. Interestingly, the presence of an ESBL was not confirmed in all isolates tested, probably due to the simultaneous production of other type of β-lactamase that could mask the presence of the ESBL **(**AmpC β-lactamases or inhibitor resistant cephalosporinases). The CLSI recommended phenotypic confirmatory method fail to detect ESBL in the presence of AmpC, as the latter is resistant to clavulanic acid. Clavulanic acid may induce high-level expression of chromosomal AmpC, masking the synergy arising from the inhibition of an ESBL (36). Several studies have also shown that the DDST failed to detect ESBL producing strains (9, 11, 37). However, the present result was mirrored to the results of (24), who reported that all *K. pneumoniae* isolates which recovered from clinical and hospital environment in Najaf

were negative for this test. Additionally, this test may not be as useful for detection of ESBLs in many cases. If the optimum disc placement is not exactly, the inability of clavulanic acid to inhibit all ESBLs, the loss of clavulanic acid disc potency during storage, and the inability to detect ESBLs isolates which have ability to produce chromosomal and plasmid-mediated cephalosporinases (38).

Incidence of CTX-M-Producing *E. coli* Isolates

Reports of ESBLs become increasingly frequent, and the most common ESBLs that have emerged recently are CTX-M types (24). Since 2000, *E. coli* producing CTX-M β-lactamases have emerged worldwide (39) as an important cause of community onset UTIs and this has been called 'the CTX-M pandemic' (11). In Iraq, a number of studies reported a high incidence of ESBLs producing Gram-negative bacteria (8, 24, 28, 40), however phenotypic characterization of these enzymes to have not been reported so far. The present study investigated the occurrence and description of CTX-M in *E. coli* carried by patients presenting with UTI in Al-Diwaniya. Primarily detection of CTX-M ESBL-producing isolates was based on their phonotypical properties. In contrast with TEM- and SHV-type ESBLs, most of the CTX-M enzymes are much more active against cefotaxime and ceftriaxone than against ceftazidime. Thus, most of the CTX-M producers display levels of resistance to cefotaxime significantly higher than ceftazidime. However, the classical phenotype of resistance conferred by CTX-M β-lactamases is not universal among all CTX-M producers, since many factors, including production of additional β-lactamases (41) or mutations altering the substrate specificity of CTX-M enzymes (42), can mask their presence. In the last few years new CTX-M variants, also able to efficiently hydrolyze ceftazidime, have evolved in this complex selective environment (43). Therefore, phenotypic differentiation of *E. coli* producing CTX-M-β-lactamases from organisms producing other types of ESBLs can be difficult. The difficulty also is due to overlapping phenotypes resulting in interference from other β-lactamases produced by the organism capable of hydrolyzing ceftazidime (44). This difficulty in distinguishing CTX-M isolates from other resistant organisms limits the capacity to carry out large epidemiological studies for the prevalence of CTX-M-producing isolates. In this work, CHROMagar™ CTX, a novel agar for the selective isolation of *Enterobacteriaceae* expressing the $bla_{\text{CTX-M}}$ gene was used for the recognition of CTX-M ESBL-producing isolates in the presence of bacteria expressing AmpC enzymes. The utility of CHROM agar technique for the detection of CTX-M-producing isolates was first documented in Al-Diwaniya. When the isolate produces CTX-M-type enzyme, the color of a colony turns metallic blue. Although, all the 207 *E. coli* isolates presented potential ESBL phenotype, only 80.2% of isolates had a phenotype consistent with production of a CTX-M ESBL. The study revealed that the majority of ESBL-producing *E. coli* had phenotypes consistent with production of a CTX-M enzyme. The present data suggest that the incidence of isolation of ESBLs in *E. coli* has increased as a result of the dissemination of CTX-M enzymes in Al-Diwaniya. In these situations, AmpC producers and other ESBLs that do not belong to the CTX-M group may be inhibited. However, (45) reported that CHROMagar™ CTX had 100% sensitivity and 64.2% specificity for growth of the $bla_{\text{CTX-M}}$ strains. The present study accomplished that CHROMagar™ CTX technique is overall cost per single test, but had the advantage of saving time and materials. Although several studies revealed that CTX-M-type enzymes are becoming the most prevalent ESBL in *E. coli* isolates in different geographic areas (21, 46, 47), it was remarkable that in this study, 80.2% of ESBL-producing *E. coli* isolates had CTX-M β-lactamases.

Therefore, present study recommended the highest incidence of CTX-M among ESBL-producing *E. coli* and demonstrates that CTX-M-type ESBL is highly endemic in Al-Diwaniya. However, present findings support the hypothesis that CTX-M is emerging as the dominant ESBL type in uropathogenic *E. coli* isolates. Interestingly, a survey at Najaf hospitals in 2010 revealed that, among 22 isolates of ESBL-producing *E. coli*, SHV enzymes was most predominant (100%), while CTX-M was detected in 77.3% (29). The first detection of CTX-M in Iraq was documented from a study of 47 *Klebsiella* spp. and 42 *E. coli* isolates recovered during 2009 and 2010 from Merjan Teaching hospital in Hilla, where the occurrence of $bla_{\text{CTX-M}}$ was 38.8% (22). CTX-M type enzymes in general the most widely disseminated ESBL enzymes in Najaf hospitals and community (9, 11, 24, 28). The present study reaffirms these findings. CTX-M-types were predominantly found in three geographic areas: South America, the Far East and Eastern Europe (41, 48, 49). However, in few years later, CTX-M-type ESBLs have been reported in Western Europe, North America, China and Japan (50, 51, 52). CTX-M type β-lactamases may be the most frequent type of ESBLs worldwide. In last few years, the worldwide presence of CTX-M-type β-lactamases in *E. coli* isolates is reported to reach a variable ratio up to 80% (53). In a recent study from Turkey, a high prevalence (98%) of CTX-M type β-lactamases was found in ESBL positive *E. coli* strains isolated from UTIs (46). However, the number of CTX-M-type β-lactamases is rapidly expanding. The link between the increase in ESBL-producing *E coli* prevalence and the emergence of CTX-M enzyme is not yet established in Iraq. In the present study, the reasons for the very high detection rate of CTX-M ESBL-producing *E. coli* in urine samples of patients with significant bacteriuria are unclear. A high incidence of ESBL-producing bacterial strains has been reported in fecal samples of healthy persons in several reports (54, 55). Therefore, one of the possible reasons for the high incidence of CTX-M ESBL-producing *E. coli* in patients with UTI may be that these bacteria are intestinal tract origin. Additionally, the high occurrence of ESBL producing *E. coli* isolates in this study may be perhaps due to the large amount of third generation cephalosporins consumption, which has been reported as a risk factor for infection with CTX-M-producing bacteria (56). Furthermore, (57) reported that strains producing CTX-M enzymes are more likely to cause repeated UTIs than strains that do not produce these enzymes, because strains producing CTX-M enzymes commonly contain *iha* gene, which encodes an adhesin-siderophore receptor associated with an increased risk for recurrent UTIs. The high levels of CTX-M ESBL detected in this study are worrisome and warrant special attention by both the clinician and the microbiology laboratory. Until now, all Iraqi clinical laboratories do not routinely screen or detect ESBL production in bacterial isolates. It is possible that these organisms, especially those with CTX-M β-lactamases, may be prevalent in bacterial populations from the hospitals but have not been identified. All clinical laboratories should rule out ESBL-producing organisms and the present study recommends that *Enterobacteriaceae* especially *E. coli*, isolated from community or hospital sources should routinely be screened for ESBL production. The alarming situation with global dissemination of CTX-M-producing *E. coli* isolates highlights the need for their epidemiological monitoring and prudent use of antibiotics in Al-Diwaniya province.

Emergence *E. coli* Isolates Harboring *bla*$_{CTX-M}$

A total of 50 CTX-M producing *E. coli* isolates was chosen for molecular study using universal CTX-M primer. Production of CTX-M-type enzyme onto CHROMagar™ CTX medium and susceptibility profile to third generation cephalosporins and azetreonam were the criteria used for selecting these isolates. By a PCR analysis with a set of PCR primers specific for *bla*$_{CTX-M}$, a 550-bp fragment was amplified from all isolates that were positive in the CHROMagar™ CTX medium. PCR assay revealed the complete correlation with results of CHROMagar™ CTX medium. This medium showed 100% sensitivity for growth of the *bla*$_{CTX-M}$ isolates. This result agree with those reported by (45) who found CHROMagar™ CTX had 100% sensitivity and 64.2% specificity for growth of the *bla*$_{CTX-M}$ strains. The present results for the second times demonstrate the high proportion of *bla*$_{CTX-M}$ gene among ESBL produced by *E. coli* isolates in Al-Diwaniya. The results support the hypothesis that *bla*$_{CTX-M}$ is represent the dominant ESBL type in *E. coli* isolates. In recent survey in Al-Diwaniya, (10) found high proportion (100%) of *bla*$_{CTX-M}$ gene harboring *E. coli* isolates. Although CTX-M-producing *E. coli* have previously been found in Iraq (9, 11, 24, 28), the descriptions of community-acquired CTX-M-producing *E. coli* infections have not been documented in this country. Interestingly, the present study revealed that the majority of *bla*$_{CTX-M}$ gene harbored isolates had community origins. The results indicate that *bla*$_{CTX-M}$ gene is more frequently detected in *E. coli* from urinary sources in the community (74.0%) than in hospital (26.0%) as shown in Table (6). However, the present data do not allow us to estimate the relative incidence among community- and hospital-acquired isolates due to the denominator data not being categorized according to source. However, the study documented the community emergence of CTX-M as the predominant ESBL type among urinary isolates from patients with UTI. Spread of CTX-M class of ESBL-producing *E. coli* in the community has been also previously described in several countries such as Canada, the United Kingdom, Spain, France and the United States (58, 59, 60). In Al-Diwaniya, β-lactam antibiotics are the most common antimicrobial agents used in the community setting. The documented CTX-M-positive isolates exhibited plasmid-mediated resistance that affected the antibiotic activity of all penicillins and cephalosporins as well as of several alternative antimicrobial agents used to treat community-acquired *E. coli* infections. Although no data about possible previous healthcare contact of the persons infected with CTX-M in the community were available in Al-Diwaniya, the spread of this type of β-lactamases outside hospitals is a matter of great concern. The spread of CTX-M-positive bacteria changes the way the present study think about treating community-acquired infections and limits the antibiotics that maybe administered. The increase in community-acquired ESBL infections in Iraq has not yet been explained; the present study proposed that the reasons are probably the same as for the emergence of CTX-M producers. Recently, a study was published that confirmed that age, female gender, and presence of other medical conditions contributed in increasing the risk for community-acquired ESBL (61). The present investigation revealed that the isolates carrying *bla*$_{CTX-M}$ were detected in all age groups, but maximum number was detected in the age group 11-30 years probably because the maximum number of study groups is also seen in this age group. In the present study, it was observed that *E. coli* isolates having *bla*$_{CTX-M}$ gene were more common among females (68.0%) compared to males (32.0%). This may be because many of the ESBLs isolates from Al-Diwaniya region came from young females with UTI. However, the study found that no indicative significance between age and gender with *bla*$_{CTX-M}$ positive *E. coli* isolated from patients with UTI. In

another study, (62) found that age, and gender was not specifically associated with acquisition of CTX-M positive strains. In contrast, (63) reported that male gender and healthcare facility residency are risk factors for ESBL-producer infections among patients with community-onset bacteremic UTIs.

Frequency of *bla*-Genes among bla_{CTX-M} Positive *E. coli*

Resistance to β-lactam antibiotics by *E. coli* is a major public health issue worldwide (64), and is often caused by the production of β-lactamase enzymes. Specifically, ESBLs, which problematic diagnosed by phenotypic tests recommended by the CLSI, are often produced. In this study, the use of designed primers with high ability to detect the ESBL genes coding bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{VEB}, bla_{PER}, and bla_{GES} was preferred. Even though there are some limitations as there are many more ESBL genes, which were not included in this study. The present study has selected only these families, because they are reported to be disseminated in Iraq, whereas the other gene families are reported in other geographic regions. As shown in Table (٥), all isolates harboring the CTX-M enzyme were found to carry SHV β-lactamases. Moreover, all isolates but one was found to express OXA β-lactamases. These genes are frequent and have already been described in *Enterobacteriaceae* and *P. aeruginosa* in Iraq (9, 11, 24, 29). *bla* genes are often associated with transferable plasmids, and some of them are parts of transposons or constitute cassettes in integrons. The association of insertion sequences such as IS*Ecp1* that mobilize β-lactamase-encoding genes with these ESBL genes may be involved in their dissemination and expression (65). In this study, the current spread of bla_{SHV} may be explained in part by the ability of some insertion sequence elements to mobilize and promote the expression of these β-lactamases. However, PCR analysis was negative for other ESBL genes tested. Dissemination of SHV has been described previously in Iraq among community and hospitalized patients, the studies showed variable incidences of bla_{SHV} gene in *E. coli*, 75%in Al-Diwaniya (10) and100% (29), 30% (9), and 3.1% (11) in Najaf. The variation in present study results compared with others about incidence rate of SHV may be due to different reasons such as difference in type and volume of consumption of antibiotics, difference in time that the isolates were collected, and difference in type and source of isolates. The OXA-type enzymes are another growing family of ESBLs in Iraqi hospitals and communities (11, 28). These β-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d (66). Present data also confirm the frequent association of OXA and CTX-M in *E. coli*. This result demonstrated that OXA comprise the third largest family of β-lactamases after CTX-M and SHV (Table 5), 98.0% of the isolates harbored bla_{OXA} gene, which is not a very common ESBL in *Enterobacteriaceae*. In fact, there are limited epidemiologic data on dissemination of OXA-type ESBLs among *E. coli* in Iraq (10, 11, 22). On the other hand it is well documented these type ESBLs are highly incident among *P. aeruginosa* isolates in Najaf (28).

Conclusion

Screening and confirmatory tests considered the most accurate method for detection of ESBL-producing isolates, comparative with disk approximation test. There are alarming spreads of the most common ESBL responsive genes (bla_{CTX-M}, bla_{SHV} and bla_{OXA}) in Al- Diwaniya city.

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*وجود إنزيمات البيتالاكتاميز واسعة الطيف في بكتريا القولون لمرضى خمج المسالك البولية

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الخلاصة

هدفت الدراسة إلى عزل وتشخيص بكتريا *E. coli* من المرضى المصابين باخماج المسالك البولية والتحري عن إنتاج إنزيمات البيتالاكتاميز واسعة الطيف مظهريا وجزيئا. جمعت 2000 عينة إدرار من المرضى المصابين باخماج المسالك البولية المراجعين إلى مستشفى الديوانية التعليمي للفترة من آذار 2011 إلى ايار 2012، شخصت العزلات (٢٠٧ عزلة) اعتمادا على الفحوصات البايوكيمياويه التقليدية وتقنية الفايتك Vitek -2 . اختبرت قابلية العزلات على إنتاج إنزيمات البيتالاكتاميز واسعة الطيف مظهريا باستخدام فحص التقصي الأولي والذي اظهر نسبة إنتاج تراوحت ,186(89.8 %) / 207(100%) والفحص التأكيدي باستخدام الأقراص المتراصة وتقنية CHROMagar التي عدت الأكثر دقة ١٦٦ (80.2%) مقارنة بطريقة الأقراص المتراصة التي لم تعط أية نتيجة موجبة 0(0.0%). أظهرت نتائج الفحص الجزيئي بان ٥٠ (١٠٠%) و ٤٩(٩٨.٠ %) من العزلات الموجبة لمورث $bla_{\text{CTX-M}}$ كانت حاملة لمورثات bla_{SHV} و bla_{OXA} على التوالي فيما لم تعط العزلات أية نتيجة تجاه مورثات bla_{TEM} ، bla_{PER} ، bla_{VEB} و bla_{GES}.

*البحث مستل من أطروحة الدكتوراه للباحث الأول