Distribution of *bla*$_{TEM}$ Gene among Escherichia Coli Strains Isolated from Different Clinical Samples in Erbil City

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Abstract:

Background: Emerging antibiotic resistance and Extended-Spectrum Beta-Lactamase (ESBL) producing Escherichia coli causing different nosocomial infections are rapidly increasing at alarming levels and it poses a major health burden in the 21st century globally.

Objective: The aim of this study is to determine the distribution of *bla*$_{TEM}$ gene ESBL-producing E. coli from clinical specimens in different hospitals in Erbil city.

Methods: A total of 200 samples were collected from (sputum, urine, wound) from patients attended public hospitals in Erbil city. The isolation and identification of Escherichia coliand antimicrobial susceptibilitywere performed by using Vitek 2 compact system. Phenotypic screening of Extended Spectrum β-lactamase production in E.coli was confirmed by using both Double disk diffusion and Standard disk diffusion techniques. Moreover, PCR technique was used for genotypic detection of an ESBL gene blaTEMaccording to the standard protocol.

Results: Out of 200 samples 60(30%) of E. coli isolates and the highest rate of E. coli isolates were obtained from the urine samples (20%) and the lowest number was isolated from sputum specimens (2.5%). The ESBL-producing Escherichia coli isolates were detected using double disk synergy test (76.7%) in comparison to standard disk diffusion test (80%). Genotypic screening results confirmed that all ESBL-producing E. coli isolates (66.7%) were carried blaTEM gene (700 bp) in clinical specimens (50% urine, 13.33% wound and 3.33% sputum). All ESBL-positive E. coli isolates showed high rates of susceptibility to Carbapenems antibiotic group including Imipenem (83.3%), Meropenem (81.7%), and Ertapenem (80.0%).

Conclusions: The increased prevalence of TEM β-lactamase gene in ESBL-producing E. coli observed in this study for the first time is considered as alarming because there is a limited treatment options remained for infections. Attempts to reduce the dissemination of multi-drug resistant E. coli through compliance with strict hospital infection control and prevention standards are imperative. Findings of this study may help clinicians selecting appropriate antimicrobial therapy in patients with different infections caused by ESBL-producing E. coli.

Keywords: Escherichia coli, Extended-Spectrum β-lactamase, BlaTEMgene, Antibiotic resistance.

Introduction:

The resistance of antibiotics is rapidly increasing at alarming levels and has currently become a serious nosocomial resistance problems and it poses a major health burden in the 21st century globally [(1)]. In Enterobacteriaceae, the production of β-lactamases by bacteria is the major mechanism of resistance

toward β-lactam antibiotics [2]. β-lactamases are enzymes that are able to hydrolyze β-lactam antibiotics by breaking down the bond in the β-lactam ring, hence rendering them harmless to pathogenic bacteria [3]. One family of β-lactamases, extended-spectrum β-lactamases (ESBLs), which are active site serine Ambler's class A or class D β-lactamases are characterized by having the ability to hydrolyze and cause resistance to 3rd and 4th generation cephalosporins, and monobactams. However this group of β-lactamases are not effective toward carbapenems and cephamycins and are inactivated by β-lactamase inhibitors such as clavulanic acid [4]. ESBLs are encoded by genes carried on the plasmid and are widely recognized as a rapidly evolving family of β-lactamases [5]. Most importantly, point mutations in these genes lead to alteration in the active site and substrate specificity of the β-lactamases to hydrolyze newer generations of β-lactam antibiotics [6, 7]. The most prevalent and variant type of ESBLs is the TEM- β-lactamases which is encoded by blaTEMgene located on the plasmid. This enzyme confers to (90%) of ampicillin resistance and increasingly frequent among Gram-negative pathogens [8]. Escherichia coli is an ESBL-producing organism and the major cause of hospital-associated infections in human to which new antibiotic therapies are urgently needed throughout the world. It is recognized that this enzyme is widely distributed in this pathogen, therefore information on the prevalence of dissemination of blaTEM gene would be valuable in understanding the mechanism of how this important resistant gene is distributed in our region [9]. Since the distribution of ESBL-producing E. coli have been observed in many geographical areas, so this study design to determine the distribution of TEM-type ESBL-producing E. coli strains from clinical specimens in hospitals in Erbil city and to detect the blaTEM gene by genotypic technique, such as Polymerase Chain Reaction.

Materials and Methods:

Bacterial isolates

A total of 200 samples were collected from different clinical specimens (sputum, urine, wound) from patients attended public hospitals in Erbil city with age group up to70 years. The distribution of sources of E. coli isolateswere: urine (n = 120), sputum (n = 20), and wounds (n = 60). These clinical specimens were collected from both inpatients and outpatients over a period of one year from September 2015 to September 2016.

Isolation of microorganisms

Standard microbiological techniques were employed for culture and identification of theE. Coli isolate ,the specimen was inoculated on Blood culture and MacConkey agar plates and were incubated aerobically at 37°C for (24-48) hours, were identified using Vitek 2 system, prior to their testing, all the isolates were stored in (15%) glycerol-supplemented Luria-Bertani medium at –80°C [10]. The study was approved by the local ethics committee of Hawler Medical University- College of Health Sciences.

Antimicrobial susceptibility test by Vitek 2 system

With its ability to provide accurate "fingerprint" recognition of bacterial resistance mechanisms and phenotypes, the antimicrobial susceptibility is a critical component of Vitek 2 technology.Vitek 2 card contains 64 microwells. Each well contains

identification substrates or antimicrobial. Vitek 2 offers a comprehensive menu for the identification and antibiotic susceptibility testing of organisms [11]. The Vitek 2 test card is sealed, which minimizes aerosols, spills, and personal contamination. Disposable waste is reduced by more than (80%) over microtiter methods.

Phenotypic detection of ESBL enzyme

All bacterial species were screened for ESBL enzyme production by the following methods:

Screening test for ESBL (Standard disk diffusion methods

ESBL detection was carried out by standard disk diffusion methods for all Gram negative isolates according to the Standard Institute of Antimicrobial Susceptibility Testing recommendation [12] by using various antimicrobials. ESBL positive meant the organism shows comparatively high level co-resistance to third generation cephalosporin such as; Ceftazidime zone ≤ 22 mm, Aztreonam zone ≤ 27 mm, Cefotaxime zone ≤ 27 mm or Ceftriaxone zone ≤ 25 mm. A laboratory strain of Escherichia coli ATCC (13883) was used as a control as in table (1).

Confirmatory test for detection of ESBL by double disc diffusion test:

A double disc diffusion test was performed with amoxicillin clavulanic acid surrounded by aztreonam and third generation cephalosporin discs cefotaxime and ceftazidime, the standard inhibition zone of amoxicillin-clavulanic acid, ceftazidime, cefotaxime and aztreonam as in table (1) [13].

Table (1): The standard inhibition zone of amoxicillin- clavulanic acid, ceftazidime, cefotaxime and aztreonam**.**

Antibiotic discs	Concentration µg/ml	Resistance mm	Intermediate mm	Susceptibility mm
Amoxicillin-clavulanic acid (AMC)	20-10(30)	< 13	14-17	> 18
Ceftazidime (CAZ)	30	< 14	15-17	> 18
Cefotaxime (CTX)	30	< 14	15-22	> 23
Aztreonam (ATZ)	30	< 15	16-21	> 22

Plasmid DNA extraction

The ESBL-producing E. coli isolates, were cultured overnight on nutrient agar medium at 37^{O}C. Plasmid DNA was extracted by alkaline lysis method with the Prime Prepplasmid isolation kit.

PCR amplification for *bla*TEM gene

The prepared plasmid DNA extracts from ESBL-producing E. coli isolates were used as template for blaTEMgene amplification. Standard PCR amplification assay was carried out for molecular detection of the blaTEM gene. The oligonucleotides, the PCR conditions, and sizes of the PCR products are shown in table (2). The oligonucleotide primers used for the PCR assays were forward primer blaTEM-SB-F (5'-GGGTGCACGAGTGGGTTACATCG-3'), reverse primer blaTEM-SB-R (5'-CGACGGGGAGTCAGGCAACTATG G-3'). The oligonucleotide primers of blaTEM gene were designed based on the nucleotide sequence of blaTEM gene reported in the National Center forBiotechnology Information (NCBI) Gene Bank database. The amplification reaction mixture, in a final volume of 50µl, contained 10µl of 10x PRC reaction buffer, 5µl MgCl2 (25 mM), 1 µldNTPs (10mM), 2µl (10pmol/ml) of each of forward and reverse

oligonucleotide primers, 5µl of the genomic DNA template, 1µl (500 U/ml) Taq DNA polymerase (Sigma Aldrich, UK) and 24µl of ultra-pure H2O. PCR amplification condition for blaTEM gene was setup on a thermal cycler (Eppendorf, Germany) as follows: initial denaturation step at 95^{O}C for 5 min, 30 cycles of denaturation at 95^{O}C for 30s, annealing at 56^{O}C for 30s and 45s of elongation at 72^{O}C for 45s, followed by a final extension step at 72^{O}C for 5 min. PCR amplicons were analyzed by gel electrophoresis at 20 V/cm for 40 min in (1%) agarose gel containing 0.5 µg/ml of ethidium bromide in borate buffer (TBE, Tris-Borate-EDTA) and visualized under UV transilluminator. A molecular marker (1000 bp DNA ladder) was used to assess PCR amplicon size.

Table (2): Primers used for PCR amplification.

Target	Primer	Nucleotide sequence (5'-3')	Amplicon size (bp)	Annealing temperature (^{O}C)
bla_{TEM}	*bla*TEM-SB-F	GGGTGCACGAGTGGGTTACATCG	700	56
	*bla*TEM-SB-R	CCATAGTTGCCTGACTCCCCGTCG		

Results:

Identification of bacterial isolates from different clinical samples.

In total, 60 (30%) isolates of E. coli were recovered and identified by using Vitek2 identification system from different clinical specimens including urine (n=40, 20%), sputum (n=5, 2.5%) and wound (n=15, 7.5%), as shown in table 3 and figure1.The highest rate of E. coli isolates were obtained from the urine samples (20%) and the lowest number was isolated from sputum specimens (2.5%).

Phenotypic detection of ESBL producing by *E. coli* isolated from different clinical specimens

In the present study, a total of 60 isolates of Escherichia coli were screened for ESBL production by employing two phenotypic methods which are standard disk diffusion methodand double disk diffusion method. Results showed that there was a difference in detection of ESBL between screening test and confirmatory method. As described inFigure 2, lower prevalence level of ESBL-producing E. coli was detectedform different clinical specimens by using double disk synergy test 46 (76.7%) compared to standard disk diffusion test 48 (80%). Moreover, these results reflected significantly higher rate of resistance among ESBL isolates compared to non-ESBL producers ($P < 0.05$), the highest number of ESBL-producing E. coli isolates (66.7%), were detected from urinary tract infection followed by (25%) from wound and (8.33%)from sputum as in table 4.

Antibiotic resistance patterns in ESBL-producing and non ESBL-producing by*E. coli isolates*

AllESBL-positive E. coli isolates showed high rates of susceptibility to Carbapenems antibiotic group including Imipenem (83.3%), Meropenem (81.7%), and Ertapenem (80.0%). Following carbapenems, the lowest rate of resistance in ESBL-producing E. coli isolates were observed for Amikacin (80.0%), Ampicillin-sulbactam (68.3%), and each of Gentamicin, and Aztreonam (25.0%). Whereas all ESBL-producing E. coli isolates showed high rates of resistance pattern toward third-

generation Cephalosporins such as Ceftriaxone (75.0%), and Ceftazidime (73.3%), as shown in table 5and figure3.

The molecular characterization of ESBL- *bla$_{TEM}$* gene in *E. coli isolates*

Genotyping of ESBL-positive E. coli isolates for the presence of blaTEM gene was studied by using PCR amplification by oligonucleotide primers that specifically detect this gene among different clinical specimens. PCR amplification results demonstrated that 40 (66.66%) of ESBL-producing E. coli isolates carried blaTEM gene (700 bp) in clinical specimens, as shown in Figure 4. Of 30 urine ESBL-positive E. coli isolates, the blaTEM gene was detected in urine samples (50%) ,wound (13.33%) was the common sample for which blaTEM gene in ESBL-producing E. coli isolates were causative agent of wound infection. Following these, blaTEM gene distribution was less commonly detected from sputum samples (3.33%), as described in table (6) and figure (5).

Table (3): Frequency of bacterial isolates from different clinical samples.

Isolated Bacteria	Clinical samples			
	Urine No (%)	Sputum No (%)	Wound No (%)	Total No (%)
Escherichia coli	40 (20%)	5 (2.5%)	15 (7.5%)	60 (30%)
Total	120 (60%)	20 (10%)	60 (30%)	200 (100%)

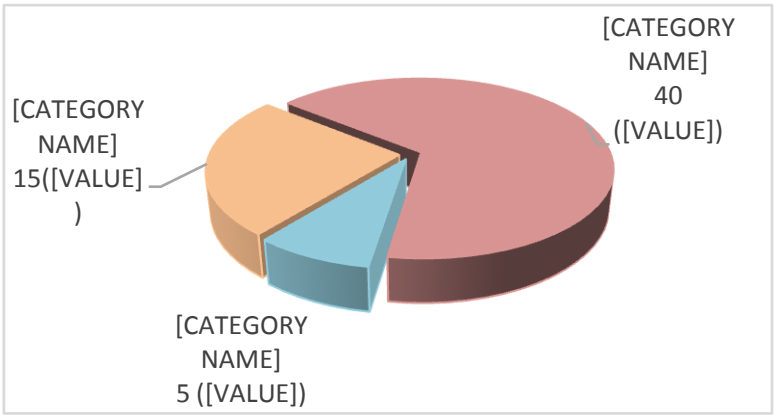

Figure (1): Frequency of *E. coli* bacterium from different clinical samples.

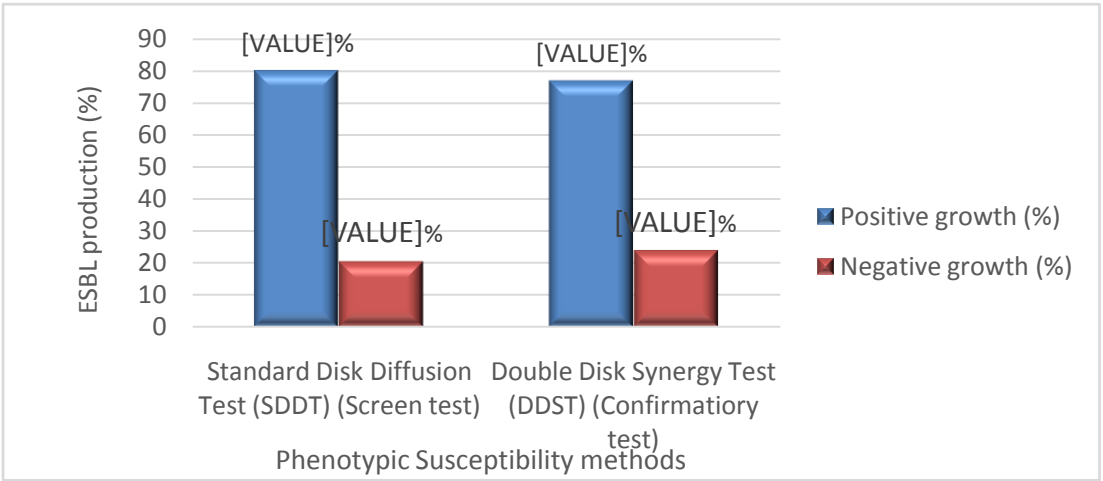

Figure (2): Phenotypic detection of ESBL producing by *E. coli* isolated from different clinical specimens.

Table (4): Frequency of *ESBL producers* in *E.coli* isolated from different clinical specimens by using standard disk diffusion.

Isolated Bacteria	ESBL producers			Total
	Urine No (%)	Sputum No (%)	Wound No (%)	*No* *%*
Escherichia coli	38 (66.7%)	2 (8.33%)	8 (6%)	48 (80%)
Total	40(66.7%)	5 (8.3%)	15 (25%)	60 (100%)

Table (5): Antibiotic resistance patterns in ESBL-producing and non ESBL-producing by*E. coli isolates*.

Antibiotics	Resistant		Intermediate		Susceptible	
	No.	%	No.	%	No.	%
Ciprofloxacin (CIP)	48.0	80.0	2.0	3.3	10.0	16.7
Amikacin (AK)	11.0	18.3	1.0	1.7	48.0	80.0
Levofloxacin(DO)	43.0	71.7	10.0	16.7	7.0	11.7
Ceftriaxone(CRO)	45.0	75.0	1.0	1.7	14.0	23.3
Meropenem(MPM)	5.0	8.3	6.0	10.0	49.0	81.7
Imipenem(IPM)	3.0	5.0	7.0	11.7	50.0	83.3
Ceftazidime(CAZ)	44.0	73.3	5.0	8.3	11.0	18.3
Gentamicin(CN)	15.0	25.0	9.0	15.0	36.0	60.0
Piperacillin(PRL)	35.0	58.3	5.0	8.3	20.0	33.3
Aztreonam(AX)	15.0	25.0	10.0	16.7	35.0	58.3
Etrapenem(EPM)	2.0	3.3	10.0	16.7	48.0	80.0
Tobromycin(TBM)	39.0	65.0	0.0	0.0	21.0	35.0
Ampicillin-Sulbacam(AMP-SbM)	10.0	16.7	9.0	15.0	41.0	68.3

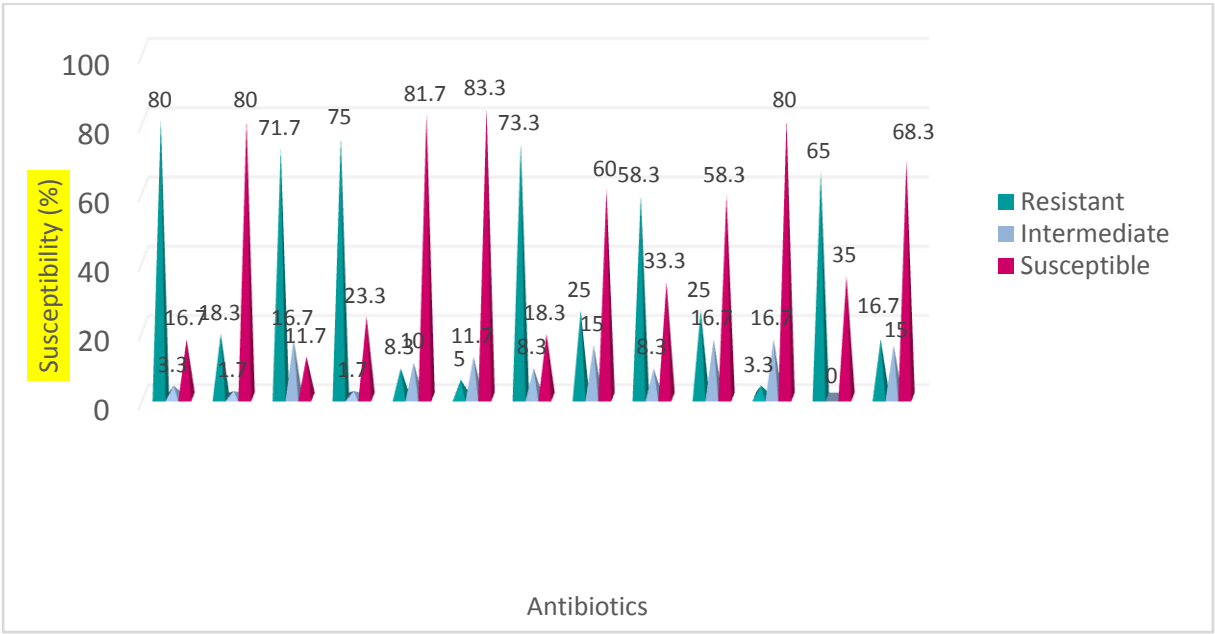

Figure (3): Antibiotic resistance patterns in ESBL-producing and non ESBL-producing by *E. coli isolates.*

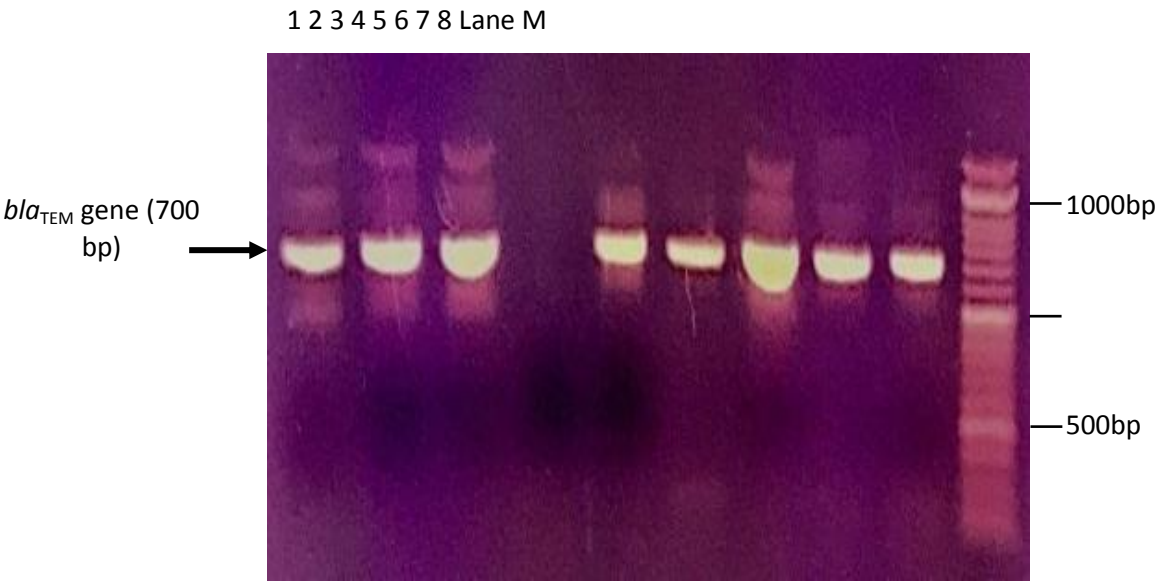

Figure (4): Confirmatory PCR screening analysis of the presence of *bla*$_{TEM}$ gene among ESBL-positive *E. coli* clinical isolates. Lane M. DNA ladder (BioLabs); Lanes 1-3 and 5-9; ESBL-producing E. coli clinical isolates; Lane 4. Negative control (no template DNA added). Agarose gel showing polymerase chain reaction amplified product of blaTEM gene (700 bp).

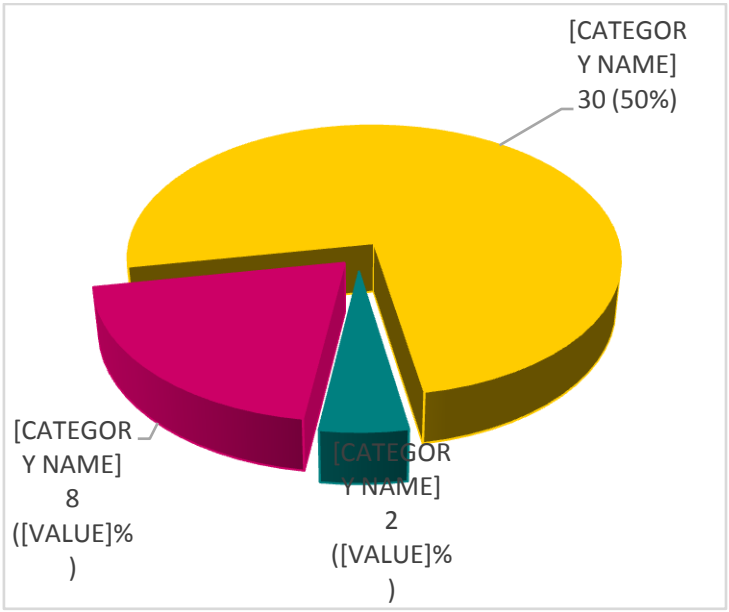

Figure (5): Distribution of *bla*$_{TEM}$ *gene*in *E.coli* isolated from various clinical specimens.

Table (6): Distribution of *bla*$_{TEM}$ *gene*in *E.coli* isolated from various clinical specimens.

Isolated Bacteria	PCR amplification results			
	Urine No (%)	Sputum No (%)	Wound No (%)	Total No (%)
Escherichia coli	30 (50%)	2(3.33%)	8 (13.33%)	40 (66.66%)
Total	40(66.7%)	5(8.3%)	15(25%)	60(100%)

Discussion:

The alarming increase of β-lactam antibiotic resistance, fueled by infections caused particularly by ESBL-producing E. coli in recent years imposes a significant and increasing burden on patients and limiting therapeutic options. In Iraq, one leading factor of resistance problem is the over consumption of antibiotics and their over prescription by the clinicians. This has led to emergence of multi-drug resistance patterns in our region. Therefore, it is essential to report trends in antimicrobial resistance of E. coli regularly.

The result of this study indicate that the high percentage of E. coli was 60 (30%) distributed between 40(20%) urine, 15(7.5%) wound, 5(2.5%) sputum. These results were in agreement with the results obtained from other studies conducted worldwide which approved that E. coli is the major pathogen that cause UTIs. These result were in agreement with finding of Al-Jebouriand Mdish [14] inTikrit, Iraq who found E. coli was the most prevalent in urine samples (31%) but not concordance with a study by Banadkaret al. [15] from India reported prevalence rate of E. coli was (37.5%) in post-operative wound infections.

Extended-spectrum beta lactamase enzyme is increasingly expressed by E. coli isolates with rapid widespread among hospitalized patients that represent a major infection threat through their ability to hydrolyze β-lactam antibiotics including third-generation cephalosporin. Moreover, the production of this enzyme presents diagnostic challenges to the clinical microbiology laboratories. The emergence of ESBL has become a matter of serious problem for the therapeutic treatment with posing significant burden on patients in Iraq. Although various bacterial infections such as urinary tract infection, wound infection and respiratory infection caused by ESBL-producing. A number of studies have reported that the prevalence of ESBLs among clinical isolates varies and patterns are rapidly changing over time worldwide as well as across the Middle Eastern countries.

In the present study, ESBL-producing E. coli was found to be (66.66%) isolated in the clinical specimens. This finding is alarming for clinicians as this rate is considered to be very high in comparison to prevalence of ESBL production in previous studies as reported in Iran (41.7%) [16], the United Arab Emirates (62%) [17], Lebanon (13.3%) [18], Kuwait (11.7%) [19], Jordan (10.8%) [20], and the Saudi Arabia (9.6%) [21], (61.6%) in Maternity Teaching Hospital in Erbil city [22]. Furthermore, the results of the present study showed that the majority of isolates were resistant to third-generation cephalosporins and none of them were sensitive to all tested antibiotics. These findings concludes emergence of a high prevalence rate of ESBL-producing E. coli strains circulating hospitalized patients in this region, which may have been caused by the heavy usage of broad-spectrum of antibiotics such as β-lactam antibiotics and third-generation cephalosporin and conferred the highest resistance percentage. A recent study of European antimicrobial resistance surveillance system reported a significant increases in third-generation cephalosporin resistance and aminoglycoside resistance in over 800 laboratories from 30 countries for the period 2013- 2016 [23]. Meanwhile, the

highest rates of susceptibility were observed for Imipenem (83.3%), Ertapenem (80%), and Amikacin (80%), suggesting as antibiotic-choice of treatment. Our findings are similar to a previous study reported from Iran [24]. It is noteworthy that the highest number of ESBL-producing E. coli isolates (66.7%) were detected from urinary tract infection. This is attributed to the fact that E. coli is the major causative agent for more than (80%) of infections [25]. This finding was in agreement with previous studies reported that urine was the major source of ESBL-producers [26]. An earlier study conducted in Baghdad in 2015 [27] showed that about (52%) of all uropathogenicE. coli isolates were ESBL producers. These findings indicates that the level of ESBL-producing E. coli strains has dramatically increased in our region. In addition, it was observed that wound infection (53.3%) was the second major source of ESBL-producing E. coli isolates followed by respiratory tract infections (40%). However, another studycarried out in Erbil by Bakirand Ali [28], observed that the main source of ESBL-producing E. coli strains was respiratory tract samples. On the other hand, a study carried out in the Burn and Plastic Surgery Hospital in Sulaimani city showed lower prevalence rate of ESBL-producing E. coli isolated from wound specimens (4.52%) [29].

Genotypic detection and characterization of β-lactamase genes would be necessary for a reliable analysis of antibiotic resistance. The prevalence of TEM-type may vary from one geographic region to other. To our knowledge, this is the first study that reports the distribution frequency of blaTEM gene among ESBL-producing isolates caused various types of bacterial infections. Clearly, TEM-ESBL type is the most prevalent among ESBL types produced by E. coli in our study, accounting for (66.6%) as PCR analysis confirmed that all ESBL-producing E. coli isolates were positive for blaTEM gene. In our region, this type of ESBL gene is the most widespread in particular among uropathogneic E. coli strains 30(50%) that caused urinary tract infection, followed by wound infection 2(3.33%) and respiratory tract infection 8(13.33%). These findings corroborates with studies from India in which TEM-type β-lactamase gene was the predominant (93.5%) [30], Iran (83.33%) [16], Turkey (72.7%) [31] and Italy (45.4%) [32], also these results are similar to previous work in Trinidad and Tobago which showed that (100%) of E. coli ESBL strains carried the blaTEM genes [33]. This gene distribution varies according to geographical region. Indeed, no ESBL was detected among 267 strains of E. coli and 53 strains of K. pneumoniae in a study in China [34]. The alarming increase of β-lactam antibiotic resistance, fueled by infections caused particularly by ESBL-producing E. coli in recent years imposes a significant and increasing burden on patients and limiting therapeutic options. In Iraq, one leading factor of resistance problem is the over consumption of antibiotics and their over prescription by the clinicians. This has led to emergence of multi-drug resistance patterns in our region. Therefore, it is essential to report trends in antimicrobial resistance regularly.

Conclusions:

In nutshell, findings of this study report for the first time that there is an alarming of rapid dissemination and dominance of TEM β-lactamase gene carried on plasmids from E. coli strains

and the rising problem of multidrug resistance occurs in our region. Consequently, this has led to a decrease in clinical therapeutic treatment options and to an increase in hospital burden. These strains are considered as causative agents of infections in the hospital and a risk factor for community. The most effective antibiotics for treating E. coli isolates that expresses ESBL in this region are Imipenem, Meropenem, and Ertapenem. It is necessary to be updated with the prevailing resistant pattern of any locality which will help in appropriate antimicrobial therapy. Infection control and prevention standards are important to be applied to restrict the use of antimicrobials. Therefore, ESBL producing organisms should be promptly reported to prescribe appropriate antibiotic treatment and proper implementation of infection control measures. Appropriate and careful prescription of broad-spectrum antibiotics is an important factor to prevent the emergence of yet another occurrence of antibiotic resistance in our region. Moreover, updates of trends for epidemiological investigation data on antimicrobial resistance are crucial in our region to promote appropriate antibiotic therapy as well as an effective infection control and clinical care management.

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