Phenotypic and Genotypic Screening of Extended-Spectrum Beta-Lactamase-Producing Escherichia Coli Isolated from a Sample of Patients with Community-Acquired Urinary Tract Infection

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***Abstract*-The bacteria *Escherichia coli* is the most common community-acquired urinary tract infection (UTI) pathogen, which is responsible for 75%–95% of cases, and due to the extensive use of β-lactam antibiotics in clinical cases, Extended-spectrum beta-lactamase (ESBLs) producing *Escherichia coli* have emerged. This study was done to isolate and identify *Escherichia coli* in a sample of Iraqi patients with a community-acquired urinary tract infection (CA-UTIs), the ESBL-producing *E. coli* was characterized phenotypically and genotypically. About 120 urine samples were collected from September 2022 to February 2023 from UTI patients including males and females. The isolates were tested against 17 antibiotics by using the disc diffusion method. The double disc synergy test (DDST) was used to identify ESBL-producing *E. coli* isolates among the isolates. ESBL-producing *E. coli* isolates were tested to characterize the predominant beta-lactamase genes by real-time PCR using specific primers for encoding genes (TEM, CMY, SHV, and CTX-M). Out of 120 cases, the total isolated *E. coli* was (36) 30%. Sensitivity tests against 17 antibiotics showed high resistance to Rifampin (97.8%), Neomycin (97.4%), Ceftazidime (96.8%), Piperacillin (92.09%), Cefotaxime (91.73%), Ceftriaxone (90.32%) and Ciprofloxacin (89%). Whereas, a high degree of susceptibility among the tested isolates was toward Meropenem (82.73 %), then Imipenem (79.58%) followed by Cefoxitin (79.58%). The double disc synergy test (DDST) was used to identify ESBL-producing *E. coli* isolates. Out of 36 isolates, 9 (25%) showed positive DDST results and were thus identified as ESBL producers. A Real-time PCR assay showed, that 6 (66.6%) isolates out of 9 had bla_{TEM} + bla_{CMY} + bla_{SHV} + $bla_{CTX\text{-}a}$ and $bla_{CTX\text{-}b}$, and 2 (22.2) isolates had had bla_{TEM} + bla_{CMY} + $bla_{CTX\text{-}a}$ and $bla_{CTX\text{-}b}$, and One isolate 1 (11.1%) had bla_{TEM} + bla_{CMY} + $bla_{CTX\text{-}a}$. The drugs of choice among the resistant isolates in our tested group were meropenem, imipenem, and cefoxitin.**

***Index Terms*: antimicrobic resistance, beta-lactamase gene, ESBLs, Iraq, Real-time PCR.**

I. INTRODUCTION

Urinary tract infections (UTIs) are a major worldwide public health problem due to the distribution of multidrug-resistant in hospitals and the community and health costs.[1] About 150 million cases are estimated annually worldwide with UTIs.[2] UTIs are the second most frequently diagnosed infections in communities alarming increases in resistance to last-resort antibiotics. [3,4] They are either hospital-acquired (HA) or community-acquired (CA).5 CA-UTIs occur within a community or not at hospitalization time (within 48 hours of hospitalization).6 The rate of multi-drug resistance (MDR) in pathogens isolated from UTI cases is increased due to inadequate antibiotics without susceptibility testing leading to ineffective UTI treatment.7 Bacteria *E. coli* is the most common cause of UTIs, and the strain uropathogenic *E. coli* (UPEC) is responsible for 65-75% of the cases.[8] Uropathogenic *E. coli* (UPEC) is the most frequent pathogen causing complicated UTIs and 95% of community-acquired infections.[9] Over the last decade, the resistance of *E. coli* to third-generation cephalosporins has increased, e.g. in Spain, it increased from 12.1% in 2010 to 14.1% in 2020, according to the ECDC.[10] ESBL-producing bacteria are considered an important antibiotic resistance type highly distributed among CA-UTIs in many world regions.[11] The distribution of ESBL resistance is not only associated with beta-lactams but also with other antibiotics (like aminoglycosides, sulphonamides, and fluoroquinolones, so the patients need the last resort antibiotics like carbapenems. The main cause of antibiotic resistance is mobile genetic elements which spread easily among bacteria and can cause multiple resistance. However, developing low resistance to carbapenems needs to control the spread of resistance because the carbapenem resistance in Gram-negative bacteria that produce β-lactams will be a major problem.[12] In this case only a few antibiotics like colistin and tigecycline are used to resolve this problem.[13] This study aimed to determine the phenotypic and genotypic characterization of ESBL-producing *E. coli* isolated strictly from a sample of Iraqi CA-UTIs.

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II. MATERIALS AND METHODS

A. Bacterial Isolates Collection

About 120 urine samples were collected from September 2022 to February 2023 from males and females within a hospital admission of less than 48 hours (CA-UTI). The age of patients was not considered during collection. Out of 120 urine samples, sixty-three *E. coli* were isolated by cultivation of the samples in nutrient broth at 37°C for 18 to 24 h., then sub-cultured onto EMB agar and identified according to the general culture characteristics including morphology of the colony, Gram Stain, and biochemical /IMViC test).[14]

B. Antimicrobial Susceptibility and ESBL Identification

Kirby-Bauer single disk diffusion method was used to test the susceptibility of the *E. coli* isolates to 17 antimicrobial agents as summarised in Table 1.[15] The zone of inhibition was measured and determined by CLSI.[16] ESBL producer's isolates were determined by a double disc synergy test using Ceftriaxone, Cefotaxime, Ceftazidime, and Amoxycillin/Clavulanic acid (Bioanalyse, Ankara, Turkey). After 24 h incubation, the test was considered positive for a synergistic activity between Cephalosporin antibiotics and the Amoxycillin/Clavulanic acid disc.[17]

C. DNA Extraction and Real-Time PCR

All bacterial genomic DNA was extracted with a Promega Wizard Genomic DNA Purification Kit (Madison, USA) according to the manufacturer's instructions. Extracted genomic DNA was used as a template in Real-Time PCR.[18] The predominant beta-lactamase genes were detected by real-time PCR using specific primers for encoding genes (TEM, CMY, SHV, and CTX-M) (Table 2)19. For Real-Time PCR a master mix was prepared from the GoTaq qPCR master mix (12.5 µl 2x master mix, final concentration 1x, Promega, Madison, USA), nuclease-free water (5.5 µl), the upstream and downstream primer was (1 µl 10 µM solution, final concentration 0.4 µM) for each of which. The mastermix (20 µl) was supplemented with 5 µl DNA template (<250 ng) for a total volume of 25 µl per reaction. PCRs were performed on a Cepheid Smart Cycler real-time PCR instrument (Cepheid, Sunnyvale, USA) using the following PCR reaction conditions: 2 min initial denaturation at 95 ºC, followed by 40 cycles of 15 s at 95 ºC, and 40 s at 60 ºC. Compounds were mixed with an ExiSpin system (Bioneer, Oakland, USA). A positive control sample was amplified with each run, and an isolate that showed sensitivity to all three Cephalosporins by DDST was used as a negative control for the real-time PCR. A melt point analysis was performed after the completion of the 40 cycles using the FAM/Sybr program (excitation at 470 nm and detection at 510 nm) with fluorescence measurement at the end of each extension step.[20]

TABLE 1.
LIST OF ANTIMICROBIAL SUSCEPTIBILITY TESTING DISCS THAT WERE USED THROUGHOUT THIS STUDY

Antibiotics	Discs symbols	Concentration (µg)	Company	Origin
Amikacin	AK	30		
Augmentin (Amoxicillin and Clavulanate)	AMC	30		
Azithromycin	AZM	15		
Aztreonam	ATM	30		
Cefepime	FEB	10		
Cefotaxime	CTX	30	Bioanalyse Ltd.	Ankara, Turkey
Cefoxitin	FOX	30		
Ceftazidime	CAZ	10		
Ceftriaxone	CRO	30		
Chloramphenicol	C	10		
Ciprofloxacin	CIP	5		
Imipenem	IPM	10		
Meropenem	MEM	10		
Neomycin	N	30		
Nitrofurantoin	NI	300		
Piperacillin	PRL	30		
Rifampin	RA	5		

TABLE II
PRIMER PAIRS SEQUENCES WERE USED IN THIS STUDY FOR THE REAL-TIME PCR-BASED DETECTION OF THE BETA–LACTAMASE GENES

TARGET	[1] PRIMER SEQUENCE 5' – 3'
bla_{TEM}	TEM-F: GCATCTTACGGATGGCATGA TEM-R: GTCCTCCGATCGTTGTCAGAA
bla_{CMY}	CMY-F: GGCAAACAGTGGCAGGGTAT CMY-R: AATGCGGCTTTATCCCTAACG
bla_{SHV}	SHV-F: TCCCATGATGAGCACCTTTAAA SHV-R: TCCTGCTGGCGATAGTGGAT
bla_{CTX-M}	CTXa-F: CGGGC**R**ATGGCGCA**R**AC CTXa-R TGC**R**CCGGT**S**GTATTGCC
	CTXb-F: ACCGAGCC**S**ACGCTCAA CTXb-R CCGCTGCCGGTTTTATC

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III. RESULTS

A. *Bacterial Isolates Collection*

In this study, bacteria E. coli is targeted because it is predominant and accounts for up to 80% of community-acquired UTIs.[21] A total of 36 E. coli isolates were collected from 120 patients, including 42 (13%) males and 78 (23%) females. E. coli in females is higher frequency than in males ($p < 0.001$), as represented in Figure 1.

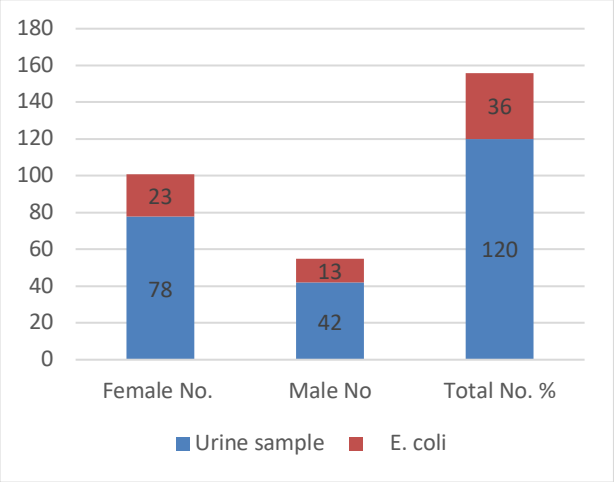
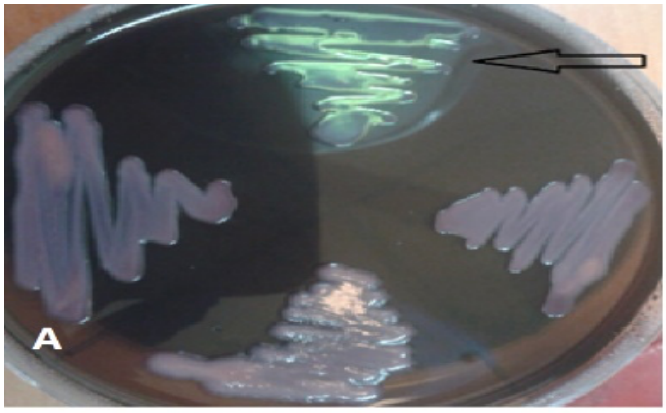


Fig1. Distribution of E. coli isolates from patients with UTI in males and females.

B. *Identification of E. coli*

Identification and characterization of all E. coli isolates were done according to culture morphology, Gram stain, and biochemical tests. Bacteria E. coli was confirmed by metallic sheen production colonies on EMB agar (Figure 2 A), Gram-negative cocci-bacilli appearance by Gram stain and biochemical tests (IMViC) result showed positive results for the indole and methyl-red test, while Voges-Proskauer and Citrate Utilization Test were negative, as shown in Figure 2 B



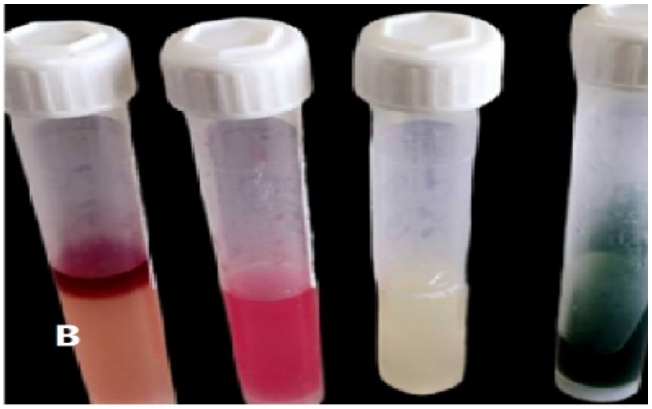


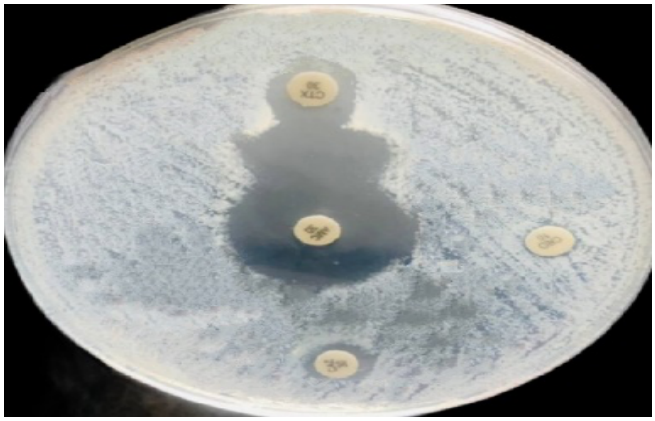
Fig2. E coli detection on EMB agar shows green metallic sheen colonies (A\ arrow). IMViC test (B).

C. *Antimicrobial Susceptibility*

The susceptibility of Seventeen antimicrobial disks including Nine different antimicrobial classes was done to test all *E. coli* isolates (36 isolates). Results in Table 3 showed that most isolates were resistant to Rifampin (97.8%), Neomycin (97.4%), Ceftazidime (96.8%), Piperacillin (92.09%), Cefotaxime (91.73%), Ceftriaxone (90.32%) and Ciprofloxacin (89%). Whereas, Meropenem showed a high degree of susceptibility among the tested isolates (82.73 %), then Imipenem (79.58%) followed by Cefoxitin (79.58%).

Extended-Spectrum Beta-Lactamase (ESBL) Producing D. coli Isolates

The double disc synergy test (DDST) was used to identify ESBL-producing *E. coli* isolates. Out of 36 isolates, 9 (25%) isolates showed positive DDST results and were thus identified as ESBL producers (Figure 3).



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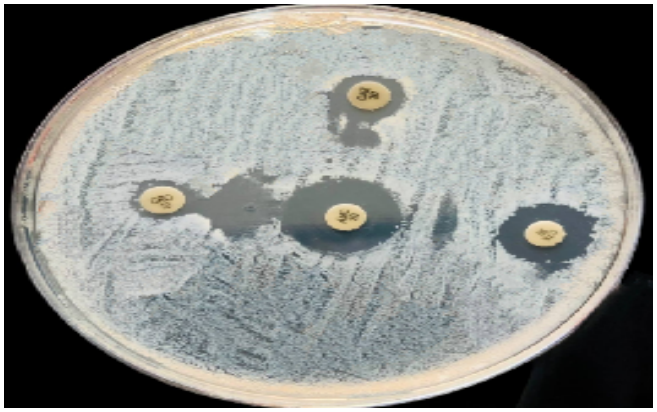

Fig 3. Detection of ESBL production in *E. coli* by Double disc synergy method: Cefotaxime disk (CTX);Ceftazidime disk (CAZ) and Ceftriaxone (CRO) disk around Amoxicillin-clavulanate (AMC) disk in the center. The synergy between the AMC Cephalosporins

D. Molecular Characterization of ESBL-Producing Clinical Isolates

Real-time PCR assays have been proven to be effective methods for the detection of various antibiotic resistance genes. Herein, we have further characterization for the 9 (25%) ESBL-producing isolates by real-time PCR as represented in Table 4 and Figure 4. The molecular characterization of these resistant strains revealed the simultaneous appearance of ESBL resistance genes as follows:
blaTEM + blaCMY + blaSHV + bla CTX-a and bla CTX-b was observed in 6 (77.7%) isolates, while 2 isolates showed blaTEM + blaCMY + bla CTX-a + bla CTX-b (22.2%), and one isolate 1 (11.1%) showed blaTEM + blaCMY + bla CTX-a. The p-value (Table 4), represents that there was no significant difference between the ESBL results of antibiotic discs used and the RT-PCR.

TABLE III
ANTIMICROBIAL SUSCEPTIBILITY TEST OF *E. COLI* ISOLATES

Antimicrobial agents	Disc symbols	R%	I%	S%
Augmentin Amoxicillin and clavulanate	AMC	72.8	9.6	17.6
Amikacin	AK	60.78	20.95	18.27
Azithromycin	ATM	67	15.77	17.23
Aztreonam	AZM	5.26	50.21	44.53
Cefepime	FEB	82.71	1.35	15.94
Cefotaxime	CTX	91.73	2.15	6.12
Cefoxitin	FOX	16.8	3.62	79.58
Ceftazidime	CAZ	96.8	3.2	0
Ceftriaxone	CRO	90.32	6.46	3.22
Chloramphenicol	C	25.89	16.28	57.83
Ciprofloxacin	CIP	89	9.19	1.81
Imipenem	IPM	22.73	20.28	79.72
Meropenem	MEM	13.86	3.41	82.73
Neomycin	N	97.2	2.8	0
Nitrofurantoin	NI	58.92	27.02	14.06
Piperacillin	PRL	92.09	4.06	3.85
Rifampin	RA	97.2	2.8	0

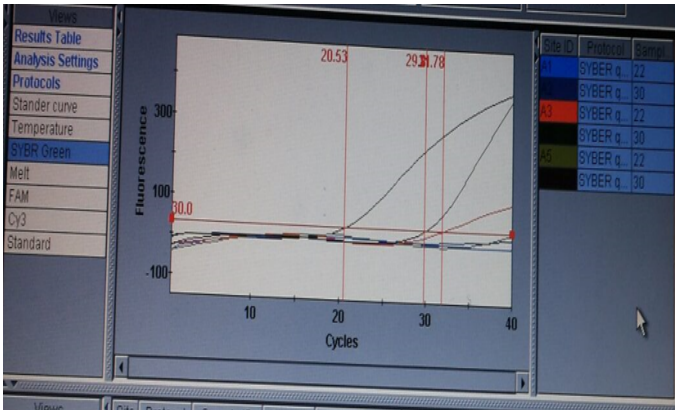

Fig 4. An example of Real-time PCR detection using blaTEM, blaCMY, blaSHV, blaAmpC, bla CTX-a and bla CTX-b ESBL primers. Positive results were represented in Two clinical isolates.

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TABLE IV
COMPARISON OF ANTIBIOTIC SUSCEPTIBILITY DATA AND ESBL GENOTYPES DETECTED BY QPCR OF COLLECTED CLINICAL ISOLATES

ESBL producer Isolate number	Resistance phenotype			Resistant genes
	R	I	S	
1, 2	CTX, CAZ, N, PRL, RA, CIP, AMC, CRO	AZM, NI	ATM, MEM, IMP, FOX, AK, FEP, C	blaTEM + blaCMY + blaSHV + bla CTX-a + bla CTX-b
3	CTX, CAZ, N, PRL, RA, CIP, FEP	AZM, AMC, NI	FOX, C, AK, CRO, MEM, ATM, IPM	blaTEM + blaCMY + blaSHV + bla CTX-a + bla CTX-b
4, 5	CTX, CAZ, N, AZM, PRL, RA, FOX, IPM, CIP	FEP, C	NI, MEM, AK, CRO, AMC ATM	blaTEM + blaCMY + bla CTX-a + bla CTX-b
6	AK, CTX, CAZ, N, PRL, RA, AMC, FEP, ATM, CRO	AZM	C, IPM, MEM, FOX	blaTEM + blaCMY + blaSHV + bla CTX-a + bla CTX-b
7	CTX, CAZ, N, PRL, RA, AMC, CRO, MEM, CIP, NI	AZM	AK, C, IPM, FEP, ATM, FOX	blaTEM + blaCMY + bla CTX-a
8	AZM, CTX, C, CAZ, N, PRL, RA, AMC, FEP, ATM, CRO, NI, CIP	AK	IPM, MEM, FOX	blaTEM + blaCMY + blaSHV + bla CTX-a + bla CTX-b
9	AK, AZM, CTX, C, CAZ, N, PRL, RA, IPM, AMC, FEP, ATM, CRO, MEM, FOX, NI, AMC			blaTEM + blaCMY + blaSHV + bla CTX-a + bla CTX-b

	Coeffi cients	*Stan dard Erro r*	*t Sta t*	*P-valu e*	*Low er 95%*	*Upper 95%*	*Low er 95.0 %*	*Uppe r 95.0 %*
Inter cept X	1.692 3077	2.42 033	0.6 992	0.51 5605	-4.52 9344	7.9139 592	-4.52 9344	7.913 9592
Vari able 1	0.692 3077	0.52 285	1.3 241	0.24 2768	-0.65 1723	2.0363 38024	-0.65 1723	2.036 3380

1V. DISCUSSION

The frequency of isolated *E. coli* in females is higher than in males ($p < 0.001$), this may be due to the women having a shorter urethra than men, and this anatomical difference increases the risk of bacterial colonization in the gastrointestinal periurethral region rising into the bladder, the same result reported by Gu and his colleagues.[22]

Isolation of *E. coli* was confirmed by metallic sheen production colonies on EMB agar. *E. coli* is a lactose fermenter with a green-metallic sheen formation. The production of metallic sheen is due to the metachromatic characteristics of the dyes. The formation of green-metallic sheen is due to the presence of pH indicators (methylene blue dye and eosin Y), which form a green-metallic precipitate at acidic pH and inhibit Gram-positive bacteria, Gram stain represents the characteristics of gram-negative cocci-bacilli appearance, and biochemical tests (IMViC) which is a differential test for *Enterobacteriaceae* members, and showed positive results for the indole and methyl-red test, while Voges-Proskauer and Citrate Utilization Test were negative, but an opposite result observed *in Klebsiella* spp. and *Enterococcus faecalis.* Indole test: using Kovac's reagent to detect the indole-producing bacteria by forming a red ring which indicates a positive result, while non-producing indole bacteria showed a brown ring which indicates a negative result. The Methyl red test detected the fermentation of glucose. The color changed to red due to the methyl red as an indicator of acid production and a low pH of less than 5. Vogas-Proskauer test positive result showed a pink-red color at the surface due to glucose fermentation and production of acetone, the color changed by the presence of an indicator at a low pH of less than 5. Simmon citrate test, Simmon citrate is a carbon source that differentiates bacteria according to their ability to hydrolyze and change the color from green to blue due to the presence of the indicator Bromothymol blue. [23]

According to the antibiotic sensitivity results, the drugs of choice for *E. coli* isolates, are Meropenem, Imipenem, and Cefoxitin. Our study also revealed high susceptibility rates towards chloramphenicol (57.83), but chloramphenicol is not advisable because of its severe side effects. A study by Hala and her colleagues showed the same result (high sensitivity rate) regarding Meropenem and Imipenem (82.30%), (69.91%) respectively, but had a susceptible rate reached to (69.91%) towards Piperacillin-tazobactam.[23] Such local epidemiology data of antimicrobial resistance patterns are important resources for clinicians to make the right therapy decision for bacterial infections treatment such as *UTIs*.

Recently, Ciprofloxacin has been utilized to treat *E. coli* infections, but the present study showed a high resistance rate for Ciprofloxacin (89%), this agreed with the study results by Hala and her colleagues who showed that the rate reached (84.07%).[24] This may be due to the increase and extensive use of this drug to treat UTI, Guanyu and Xiaoju showed that Ciprofloxacin resistance in Hospitals is more than community-acquired *E. coli* UTI.[25] Regarding Neomycin, the current study is consistent with a study by Asma and Afram,[26] who showed a

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high resistance rate but with less frequency (77%), this may be due to the differences in the size of the studied population. Rifampicin is a broad-spectrum antibiotic that is the basis of antituberculosis as monotherapy or in combination with another drug, it can also be used in UTI treatment, so it is important to make a routine sensitivity test against it, in the current study it showed high resistance rate (97 %), which may be due to rifampicin widespread misuse or using the substandard drug as reported by Weinstein and Zaman.[27] The observed ESBL rate of 25% is low compared to an ESBL rate of 48 % observed in a study by Ehsan and his colleagues.[28] The spread of ESBL-producing bacteria is rapidly increasing worldwide; therefore, continuous monitoring systems, and effective infection control measures, prevent the prolonged and widespread usage of these antibiotics on a global scale, and a decrease in the use of cephalosporins with an oxyimino side chain is essential to decrease the transmission of ESBL-producing bacteria in community and hospitals. Production of β-lactamase especially, ESBL is the most important cause of resistance to β-lactams antibiotics (Penicillin and Cephalosporin). The ESBL producer *E. coli* is resistant to a wide range of β-lactams antibiotics, and non-β-lactams like Nitrofurantoin, Fluoroquinolones, Aminoglycosides, etc.[29] Results of RT-PCR showed promising agreement within phenotypic AST which could be used as an accurate predictor of AMR in bovine *E. coli* isolates. The CTX-M beta-lactamases are the most prevalent ESBLs in many parts of the world. Most isolates harboring *bla* $_{CTX-M}$ and *bla* $_{CMY}$ were multi-resistant to many antibiotic classes because they spread rapidly among Enterobacteriaceae. β-lactamase (*bla*) genes results showed that bla_{SHV} and bla_{CMY} had lower frequency than bla_{TEM} and bla_{CTX-M} genes, the same result observed by Fsharikhah *et al*.[30] The CMY-2 gene related to plasmid-mediated AmpC family. It is the most common worldwide, and the most frequent plasmid-mediated AmpC beta-lactamase in hospitalized patients.[31]

In practical terms, detection of an ESBL in a clinically significant isolate, whether mediated by TEM, SHV, or CTX-M genes, should mean therapeutic resistance to all extended-spectrum cephalosporins (indeed, to all cephalosporins, aztreonam, and penicillin). The drug of choice in such a case will then be either carbapenems or cephamycin, depending on the sensitivity result. As mentioned previously the drug of choice for *E. coli* isolates in the current setting in Iraq based on the outcome of our study is meropenem in addition to imipenem and cefoxitin.

Our study was based on isolates limited to community-acquired *E. coli* isolates of UTI in Baghdad province. Thus, much remains to be learned about the clonal diversity of UTI-causing *E. coli* isolates, including those circulating in another province.

CONCLUSION

Most E. coli isolates collected from patients showed highly antimicrobial-resistant patterns and most of them were classified as MDR, which revealed a severe health problem in the community. Attention should be considered to prevent the spread of antibiotic-resistant bacteria, by stopping the misuse of antimicrobial agents, physicians should prescribe antibiotics to patients after antimicrobial susceptibility tests and patients should complete their prescribed antibiotic treatment cycle. Also, the increase in community-associated ESBL-producing *E. coli* infections has the potential effect on empiric management of community-associated *E. coli* infections is suspected. Continued surveillance and investigation among healthy persons are needed to prevent further spread in the community.

ETHICAL APPROVAL

Ethical approval was obtained from the patients and a consent form for specimen collection was filled out.

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