Phenotypic and Genotypic Characterization of IRS-Producing Escherichia coli Isolated from Patients with UTI in Iraq

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Abstract
The aim of this work was to analyze the current of IRT β-lactamas in uropathogenic Escherichia coli. Isolates were prospectively collected in our hospital (2013 and 2014) from urine of hospitalized patients (100%). From a total of 60 E. coli isolates recovered during the study period, 22 showed reduced susceptibility to Beta lactam Beta lactamase inhibitor (BLBLI) combination antibiotics, with 17 of them producing IRT enzymes. These were mostly recovered from urine (100%). A high degree of IRT was detected (TEM-2, CTX-M, bla-SHV, SHV-2, TEM-1, OXA-1, and AMPC). The results of this study are recorded for the first time in Iraq.

Introduction
IRT enzymes represent an adaptive resistance mechanism specifically developed by bacteria to overcome the activity of β-lactamase inhibitors [1]. Resistance to β-lactam–β-lactamase inhibitor combinations in Escherichia coli may be due to different mechanisms, including TEM-1 penicillinase hyperproduction, constitutive AmpC over-production or plasmid AmpC production, OXA-type β-lactamase production, permeability deficiencies involving OmpF and/or OmpC porins, inhibitor-resistant TEM (IRT) and complex mutant TEM (CMT)-like β-lactamase production, and more recently, carbapenemase production [2]. IRT enzymes comprise a group of plasmid-encoding variants of TEM-1 and TEM-2 with decreased affinities for amino-, carboxy-, and ureidopenicillins and altered interaction with class A β-lactamase inhibitors [3]. IRT-producing isolates remain susceptible to cephalosporins, cephemycins, carbapenems, and in most cases, piperacillin-tazobactam. They are usually resistant to ampicillin-sulbactam and intermediate or resistant to amoxicillin-clavulanate combinations. IRT enzymes have previously been reported in different organisms, such as E. coli, Klebsiella spp., Enterobacter cloacae, Proteus mirabilis, Citrobacter freundii, and Shigella sonnei [2]. But there are only a few recent epidemiological studies concerning these enzymes. Moreover, the population structure of IRT-producing E. coli isolates has not been addressed using a multilocus sequence typing (MLST) technique. The results of this study are recorded for the first time in Iraq.
were originally named TRC (TEM enzymes resistant to clavulanic acid) [4], and later TRI (TEM resistant to β-lactamase inhibitors) [5], and were finally named IRT [6].

**Materials and Methods**

**Study design:**
At the beginning of this study, 100 urine samples were collected from patients suffering from significant bacteriuria during the period of November 2013 to the end of January 2014 from three main Hospitals in Hilla city/Iraq (Hilla Teaching hospital, Childhood and gynecology hospital, and general hospital of AL-Hashimiyyeh, in addition to some private clinics. Each sample was immediately inoculated on MacConkey agar plates and EMB agar. The swab has been inoculated on culture media and incubated aerobically for 24 hours at 37°C. Information about age, residence, antibiotic usage, and hospitalization of patients was taken into consideration.

**Bacterial Isolates**
Uropathogenic *E. coli* isolates were recovered and identified based on their morphology, Gram-staining, Indole test, MR-VP test, and motility test [7]. Identification was confirmed using specific 16S rRNA gene by PCR assay.

**Antimicrobial Susceptibility Testing:**
The antimicrobial susceptibility patterns of isolates to different antibiotics were determined using disk diffusion test and interpreted according to CLSI guidelines [8]. The following antibiotics were obtained (from Oxoid, UK, and Bioanalyse, Turkey) as standard reference disks as known potency for laboratory use: Ampicillin, AM (10 µg) Amoxillin, AX (25 µg), Amoxillin/Clavulanic acid, AMC (30 µg), Cefotaxime/Clavulanic acid, CAC (30/10 µg), Ceftriaxone-Tazobactam, CIT (30/10 µg) Cephalothin, KF (30 µg), Cephalxin, CL (30 µg), Cefoxitin, FOX (30 µg), Cefotizoxime, CZX (30 µg), Ceftazidime CAZ, (30 µg), Cefotaxime, CTX(30 µg), Ceftriaxone CRO (30 µg), Cefepime FEP (30 µg), Imipenem IMP (10 µg), Meropenem MEM (10 µg), Aztreonam ATM (30 µg), Gentamicin CN (10 µg), Nalidixic acid NA(30 µg), Ciprofloxacin CIP (30 µg), Tetracycline TE (30 µg), Trimethoprim/ Sulphamethoxazole SXT (25 µg) (1.25/23.75), Nitrofurantoin F (300µg).

**Screening Test for β-Lactam Resistance**
Ampicillin and amoxicillin were added, separately, from the stock solution to the cooled Muller-Hinton agar at final concentrations of 100 and 50 µg/ml, respectively. The medium was poured into sterilized Petri dishes, then stored at 4°C. Preliminary screening of *E. coli* isolates resistant to both antibiotics was carried out using pick and patch method on the above plates [9]. Results were compared with *E. coli* ATCC 25922 as a negative control and *E. coli* ATCC 35218 as a positive control.

**Detection of β-Lactamase Production:**
Nitrocefin diagnostic disk (Fluka, Switzerland) was used to detect the ability of 15 isolates to produce β-lactamase. A number of required nitrocefin disks were placed into sterile empty Petri dish and moistened with one drop of sterile D.W.; then the disk was holed by sterile forceps and wiped across a young colony on agar plate. The development of a red color in the area of the disk where the culture was applied indicated a positive result.

**Determination of MICs of *E. coli* isolates:**
The two-fold agar dilution susceptibility method was used for determination of MICs of β-lactam antibiotics according to CLSI [8].

**Initial screening AmpC β-Lactamase**
All β-lactam resistant isolates were tested for cefoxitin susceptibility by using standard disk diffusion method [8]. The resistant isolates (≤18mm inhibition zone diameter) were consider as initially AmpC β-lactamase producers.

**Initial screening for ESBL production:**
All bacterial isolates that were β-lactamase producing were tested for ESBL production by initial screen test. The isolate would be considered potential ESBL producer, if the inhibition zone of ceftazidime (30 µg) disks was ≤ 22 mm [8].

**Confirmatory test for ESBL production**
All the β-lactamase producing isolates were tested also for confirmatory ESBL production by disk combination test

**Table (1): Primers of monoplex PCR**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer name</th>
<th>Oligo Sequence (5′→3′)</th>
<th>Product Size (Pb)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmpC</td>
<td>AmpC-F</td>
<td>ATC AAA ACT GGC AGC CG</td>
<td>550</td>
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<tr>
<td></td>
<td>AmpC-R</td>
<td>GAG CCC GTT TTA TGC ACC CA</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>TEMU1</td>
<td>ATG AGT ATT CAA CAT TTC CG</td>
<td>867</td>
<td>Reguera et al [40]</td>
</tr>
<tr>
<td></td>
<td>TEML1</td>
<td>CTG ACA GTT ACC AAT GCT TA</td>
<td>867</td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>TEMU2</td>
<td>ACT GCG GCC AAC TTA CTT CTG</td>
<td>374</td>
<td>Kaye et al. [15]</td>
</tr>
<tr>
<td></td>
<td>TEML2</td>
<td>CGG GAG GGC TTA CCA TCT G</td>
<td>374</td>
<td></td>
</tr>
<tr>
<td>SHV</td>
<td>SHV-F</td>
<td>GGT TAT GCG TTA TAT TCG CC</td>
<td>867</td>
<td>Ferreira et al. [41]</td>
</tr>
<tr>
<td></td>
<td>SHV-R</td>
<td>TTA GCG TGT CCA GTG CTC</td>
<td>867</td>
<td></td>
</tr>
<tr>
<td>SHV</td>
<td>SHVU2</td>
<td>CCG CAG CCG CTT GAG CAA A</td>
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<td>Kaye et al. [15]</td>
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<td>SHVL2</td>
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<td>OXA</td>
<td>OXA-1F</td>
<td>ACA CAA TAC ATA TCA ACT TCG C</td>
<td>813</td>
<td>Steward et al. [34]</td>
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<tr>
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<td>OXA-10F</td>
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<td>651</td>
<td>Steward et al [34]</td>
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<tr>
<td></td>
<td>OXA-10R</td>
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<td>651</td>
<td></td>
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<tr>
<td>OXA</td>
<td>OXA-2F</td>
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</tr>
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<tr>
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<td>CGC TTT GCG ATG TGC AG</td>
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<td></td>
<td>CTX-M-R</td>
<td>ACC GCG ATA TCG TTG GT</td>
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</table>

(Recommended by CLSI, 2014) [8] as follows: Cefotaxime alone and in combination with clavulanic acid were tested. Inhibition zone of ≥ 5 mm increase in diameter for antibiotic tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL producing isolate.

**Plasmid DNA extraction and purification:**

A single colony of cultivated bacteria, which had been incubated overnight, transfer to 2 ml of sterile nutrient broth and incubate at 37 °C for 18-20 hours .The DNA extracted and purified using Quick Guide plasmid Mini Prep Kit DNA extraction (SolGent, Korea) according to manufacturer instructions (SolGent, Korea). Plasmid DNA was used to detect TEM-1, TEM-2, SHV, SHV-2, OXA-1, OXA-2, OXA-10, AMPC, and CTX-M.

**Preparing the primers suspension:**

The DNA primers (Table-1) 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.

**Monoplex 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.

### Agarose gel electrophoresis:
The amplified PCR products were detected by agarose gel electrophoresis was visualized by staining with ethidium bromide. The electrophoresis result was detected by using Biometra gel documentation system.

<table>
<thead>
<tr>
<th>Table (2): Programs of Monoplex PCR Thermocycling Conditions</th>
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<tbody>
<tr>
<td><strong>Monoplex gene</strong></td>
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<td><strong>bla-AmpC</strong></td>
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<td><strong>bla-SHV</strong></td>
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<td><strong>bla-CTX-M</strong></td>
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<tr>
<td><strong>TEM-1</strong></td>
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<td><strong>TEM-2</strong></td>
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<td><strong>SHV-2</strong></td>
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<td><strong>OXA-2</strong></td>
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<td><strong>OXA-10</strong></td>
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</table>

**Results and discussion:**

**Isolation and Identification of isolates:**
Out of 100 urine specimens, 90 (90%) showed culture growth positive and yielded 90 bacterial isolates. 60 (60%) were found to be uropathogenic *E. coli*

**Frequency of β-lactam resistant uropathogenic *E. coli***:
The frequency of β-lactam resistance was evaluated when the isolates primarily screened for resistance using ampicillin and amoxicillin [10, 11]. The results obtained in this study revealed that 33 (55%) *E. coli* isolates were resistant to both ampicillin and amoxicillin.

**Production of β-Lactamase by nitrocefin disk method:**
The results revealed that among the 33 isolates tested, 30 (90.9%) produced β-lactamase. This result revealed that β-lactamase producing *E. coli* isolates by nitrocefin method was significant. This may refer to the fact that nitrocefin is more sensitive to be hydrolyzed with all known β-lactamases produced by Gram-negative bacteria [12]. In addition, this method is useful for the detection of β-lactamase patterns from bacterial cell extracts and susceptible for detecting low level of β-lactamases produced constitutively or by induction in enteric bacteria.

Tuwaij [13], found that 21 (84%) and 24 (96%) *Serratia spp.* isolates were identified as β-lactamase producers with rapid iodometric and nitrocefin methods, respectively.

**Susceptibility to β-lactam-β-lactamase Inhibitor (BLBLI) combinations**
The results obtained in this study revealed that only 22 (66.6%) from 33 β-lactam resistant were still resistant or intermediate to one or more of BLBLI combinations antibiotic. In this study 21 (95.4%) of 33 *E. coli* isolates were resistant to ampicillin-sulbactam, 13 (59%) resistant to amoxicillin-clavulanic acid, and 11 (50%) resistant to pipracillin-tazobactam.
Miro et al., [14] found that 7% of 7,252 non duplicated clinical E. coli strains from a Spanish hospital were showed reduced susceptibility to amoxicillin-clavulanate.

Kaye et al., [15] found that out of 283 isolates that tested resistant to ampicillin-sulbactam, 69 unique patient isolates were also resistant to amoxicillin-clavulanate by disk diffusion testing (zone diameter < 13 mm). Among the isolates, 12 were nosocomial (rate of amoxicillin-clavulanate resistance = 4.7%) and 57 were community acquired (rate of amoxicillin clavulanate resistance = 2.8%). No predominant strain was identified.

Leflon-Guibout et al. [16] found that Amoxicillin-clavulanate resistance (MIC >16 mg/ml) and the corresponding molecular mechanisms were prospectively studied in E. coli over a 3-year period (1996 to 1998) in 14 French hospitals. The overall frequency of resistant E. coli isolates remained stable at about 5% over this period. The highest frequency of resistant isolates (10 to 15%) was observed, independently of the year, among E. coli isolated from lower respiratory tract samples, and the isolation rate of resistant strains was significantly higher in surgical wards than in medical wards in 1998 (7.8 versus 2.8%).

**Production of ESBL**

According to CLSI [8] the isolate is considered to be a potential ESBL producers, if the inhibition zone of ceftazidime disks (30 µg) was ≤ 22 mm. The study found that 17 (77.2%) of the 22 E. coli isolates were ESBL positive during the initial screening using ceftazidime disk, which considered as suspected of ESBL-producing E. coli.

Out of the 22 E. coli isolates β-lactamase producers, 18 (81.8%) exhibited zones enhancement with clavulanic acid, confirming their ESBL production.

In this investigation the Vitek2 compact system was also used for detection of ESBLs-production in 22 E. coli isolates. All these isolates were resistant to one or more BLBLI combination antibiotics. 18 (81.8%) of them were found to be ESBL producer.

In this study the Vitek2 compact system detected one isolate (No. 0023) that had positive result for ESBL with susceptible results to new cephalosporins and aztronam (Table 3).

**Table (3)** Antibiotic susceptibility of ESBL-producing uropathogenic E. coli isolates detected by Vitek 2 system

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>β-lactamase</th>
<th>CTX</th>
<th>CZ</th>
<th>CAZ</th>
<th>CRO</th>
<th>ATM</th>
<th>IMP</th>
<th>AMC</th>
<th>SAM</th>
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</table>
ESBL extend spectrum Beta-lactams, ATM aztronam, CAZ ceftazidume, CTX cefotaxime, CRO ceftraxone, CZ cefazolin, IMP imipenem, AMC amoxillin-clavulanic acid, SAM ampicillin-sulbactam, TZP pepracillin-tazobactam.

Although strains that produce ESBL are characteristically resistant to new cephalosporins and/or aztronam, many strain producing these enzymes susceptible or intermediate to some or all of these agents in vitro, while expressing clinically significant resistance in infected patients [17]. Such strains are often not recognized as ESBLs producer, placing infected patients at risk of receiving an inappropriate therapy, and also making it difficult to implement effective infection control measures. For these reasons a rapid and accurate detection of ESBL-producing isolates has to become an important issue in clinical laboratories. The detection of organisms producing these enzymes can be difficult [18], because the presence of ESBLs in the bacterial cell does not always produces a resistance phenotype [19]. The majority of ESBL are derived through single amino acid substitutions in three non-ESBL parental β-lactamases enzymes, TEM-1, TEM-2 and SHV-1. Since TEM- and SHV-ESBLs had been uniformly susceptible to β-lactamase inhibitors (e.g. clavulanate, sulbactam, and tazobactam), inhibitor/β-lactam combination were advocated as potential therapeutic alternative [20].

**Production of AmpC β-Lactamase**

Vetik2 compact was applied to detect the production of AmpC β-lactamases in β-lactam resistant *E. coli* isolates. 5 from all 22 isolates showed susceptibility to 3rd generation cephalosporins (ceftazidime, ceftriaxone, cefazolin) and aztronam and 4 of these were recorded as negative for ESBL and one as positive.

This study showed that all 22 uropathogenic *E. coli* isolates were sensitive to cefoxitin and the inhibitor zone was more than 18 µg/ml according to CLSI [8].

Although, some of AmpC types producing Gram-negative bacteria are susceptible to cefoxitin, In general, cefoxitin readily detects hyper-production of AmpC in some *Enterobacteriaceae*. A low level of production yields negative results or marginally positive results. In a previous study, in India, Manchanda and Singh [21], mentioned that 61% of AmpC producers were found to be resistant to cefoxitin and 39% of them were susceptible to cefoxitin antibiotics disk.

The results of Tuwaij [13] revealed that 18 (72%) isolates were cefoxitin resistant while, 9 (28%) and 18 (72%) isolates were confirmed as AmpC producers by rapid iodometric method and nitrocefin disk, respectively.

AmpC β-lactamases are one of the most important β-lactamases in Gram-negative bacteria. Nevertheless, the knowledge about the AmpC β-lactamases is still limited at present. The capability to detect AmpC is important in all hospitals, to improve the clinical management of infections and provide sound epidemiological data. Reduced susceptibility to cefoxitin in the Enterobacteriaceae may be an indicator of AmpC activity, but it should be confirmed by other tests. The detection of AmpC β-lactamase is a challenge for clinical laboratories, and there is no Clinical Laboratories Standards Institute (CLSI) guideline for its detection [22].
AmpC-β-lactamase producing bacterial pathogens may cause a major therapeutic failure if not detected and reported in time. AmpC β-lactamases have been associated with false in vitro susceptibility to cephalosporins. Thus, the type of β-lactamase produced by the organism should be detected along with the antibiogram before administering the β-lactam drug to the patient. The potential benefits would include better patient outcomes in terms of avoiding inappropriate therapy. Also failure to identify AmpC β-lactamase producers may lead to inappropriate antimicrobial treatment and may result in increased mortality. This is alarming and requires urgent action from both a therapeutic and infection control perspective [23].

**Antibiotics susceptibility of E. coli:**

Arrange of antibiotics have been used for the treatment of UTI caused by E. coli in Iraq and other countries. However, the widely spread use of this approach has criticized on the ground of drug toxicity and the risk of an increase spread antibiotic resistance [24].

Antibiotic susceptibility of all 22 E. coli isolates against 20 antibiotics showed multidrug resistance. Bacterial resistance to antibiotic is now widespread and possessed serious clinical threats.

The frequency of antibiotic resistance of 22 E. coli isolates that resistant to one or more of BLBL were determined. All these isolates (100%) were found to be resistant to ampicillin and amoxicillin. The susceptibility of 22 E. coli isolates against 20 selected antibiotics was studied. The results in figure-1 represent the antibiogram profile of the isolates, and indicate that isolates varied in their susceptibility to the antibiotics. All isolates were highly resistant (100%) to ampicillin, and amoxicillin. Also the results in this figure showed that all 22 isolates were sensitive to cefoxitin (100%). It was found that 81.8% of the isolates were resistant to cephalixin, cepatholin, and aztreonam. The percentages of resistance to third generation cephalosporins were as follows: 77.2% cefazolin, ceftraxone, and ceftazidime. Additionally, 22.7% of the isolates exhibited resistance to the fourth generation cephalosporin, cefepime. The lowest resistance rate was found against carbapenems. Resistance to carbapeneme antibiotics (represented by imipenem, ertapenem, and meropenem) were 0 (0%), also the lowest rate showed in Nitrofurantoin antibiotic was 0 (0%). Low percentages of resistance to aminoglycoside, gentamicin was detected 45.4%. The most active quinolones against all tested E. coli was levofloxacin, for which isolates had a resistance rate of 27.2% followed by ciprofloxacin which had a resistant rate of 31.8% and nalidixic acid 63.6%. The resistance rate of isolates to the remaining antibiotics was as follows: tetracycline 68.1% trimethoprim-sulfamethoxazole 77.2%, and tobramycin22.7%.
Antibiotics susceptibility profile of β-lactam resistant uropathogenic E. coli isolates by disk diffusion method.

CZ cefazolin, CAX ceftazidime, CRO ceftriaxone, FEP cefepime, CL cephalexin, KF cephalothin, CN gentamicin, TM tobramycin, CIP ciprofloxacin, LEF levofloxacin, AM ampicillin, SXT trimethoprim-sulfamethoxazole, MEM meropenem, NA naldix acid, AT Maztronam, TE tetracycline, IPM imopenem, ETP ertapenem, F nitrofurantoin. Suman et al [25] have reported that 54% of the isolates were sensitive to gentamicin followed by tobramycin (50%), cotrimoxazole (44%) and ciprofloxacin (44%), whereas in the present study, the uropathogenic E. coli isolates were less susceptible to the tested antibiotics.

Supriya et al [26] have reported that 82 and 79.6% of E. coli were resistant to cotrimoxazole, and ampicillin. Similar results were observed in the present study indicating maximum resistance to these drugs. E. coli with integrons are significantly more likely to exhibit MDR to gentamicin, ampicillin, tetracycline and nalidixic acid [27].

In this work, it was found that some of E. coli isolates were resistant to more than six antibiotics, which mean that an alternative choice of antibiotic is needed to eradicate E. coli associated with urinary tract infection.

E. coli as the commonest cause of UTI exhibiting high antibiotic resistance among the strains, so this sure that the need for judicious use of antibiotics. In chronic UTI, a slow growing E. coli with atypical colony morphology and MDR strain was reported by [28].

Penicillins, such as ampicillin and amoxicillin, were used previously as frontline therapies for UTIs. Resistance to these agents is mediated by β-lactamases which degrade them, and these enzymes play an important role in antibiotic-refractory UTIs [29].

The results in the present study were also similar to the results of Aiyegor et al. [24] who found that E. coli was the principal pathogen isolated from patients with significant bacteriuria, showing high resistance to amoxicillin and ampicillin. Other studies from other countries have reported an increasing resistance in E. coli strains to ampicillin [30].

**Determination of MIC of IRs-producing isolates:**

Results of determination of MIC of IRs-producing E. coli isolates revealed that all 22 isolates were highly resistant to ampicillin with concentrations beyond the breakpoint values. The MIC value of ampicillin was 32μg/ml that representing (100%).

The results presented in table 12 evaluate that the MIC of ceftazidime range from 1 to 64 μg/ml; 17% of isolates resistance to ceftazidime, only 5 of isolates
had a minimum MIC values 1 µg/ml; 4 of these with negative ESBL and 1 with positive ESBL; The results presented in table -4 evaluate that the MIC of ceftriaxone range from 1 to 64 µg/ml, 77% of isolates resistance to ceftriaxone also had only 5 isolates with a minimum MIC values 1 µg/ml; also evaluate the MIC of cefazolin range from 1 to 64 µg/ml which had only 3 isolates with a minimum MIC values 4, 86% of isolates resistance to cefazolin; on other hand the isolates had MIC of ampicillin-sulbactam range from 8 to 32 µg/ml with only one minimum MIC values 8 µg/ml, 86% of isolates resistance to ampicillin-sulbactam; also had MIC of pipracillin-tazobactam range from 4 to 128 with only 4 (18%) maximum MIC values 128 µg/ml; and finally the isolates had MIC for amoxcinillin-clavulanis acid range from 4 to 32 only one had minimum values 4 µg/ml, 32% of isolates resistance to amoxcinillin-clavulanis.

The results of this study indicated that only 5 isolates in table-4 were resistant or intermediate to one or more of BLBLI combinations and showed susceptible to cephalosporins so these isolates may had one or more of IRs enzyme such as (TEM, SHV, OXA, CTX-M, and AMPC); On other hand the other 17 isolates in this table had positive results for ESBL may had one or more of IRs that mutated to express ESBL such as (TEM-1, TEM-2, SHV-1, and CTX-M).

Table (4) MICs of Inhibitor Resistances of uropathogenic E. coli isolates

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>AM* (&gt;32ug/ml)</th>
<th>SAM* (&gt;32/1 6ug/ml)</th>
<th>AMC* (&gt;32/1 6ug/ml)</th>
<th>TZP* (&gt;128/4 6ug/ml)</th>
<th>CAZ* (&gt;64ug/ml)</th>
<th>CRO* (&gt;64ug/ml)</th>
<th>CZ* (&gt;64ug/ml)</th>
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<tbody>
<tr>
<td>002</td>
<td>(-ve) ESBL 32(R)</td>
<td>32(R)</td>
<td>8(S)</td>
<td>4(S)</td>
<td>1(S)</td>
<td>1(S)</td>
<td>4(S)</td>
</tr>
<tr>
<td>003</td>
<td>32(R)</td>
<td>32(R)</td>
<td>16(I)</td>
<td>64(I)</td>
<td>16(R)</td>
<td>64(R)</td>
<td>64(R)</td>
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<tr>
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<td>32(R)</td>
<td>32(R)</td>
<td>16(I)</td>
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<td>32(R)</td>
<td>32(R)</td>
<td>128(R)</td>
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<td>16(R)</td>
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<tr>
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<td>32(R)</td>
<td>8(S)</td>
<td>4(S)</td>
<td>1(S)</td>
<td>1(S)</td>
<td>4(S)</td>
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<td>4(S)</td>
<td>2(R)</td>
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<td>4(S)</td>
<td>1(S)</td>
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<td>16(I)</td>
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<tr>
<td>0027</td>
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<td>32(R)</td>
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<td>0029</td>
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<td>128(R)</td>
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</table>

AM ampicillin, SAM ampicillin-sulbactam, AMC amoxcinillin-clavulanis acid, TZP pepracillin-tazobactam, CAZ cefazidime, CRO ceftriaxone, CZ cefazolin.

*Numbers between brackets refer to break points recommended by CLSIs [8]. R resistant, I intermediate, and S susceptible.
Ampicillin AM, ampicillin-sulbactam SAM, amoxicillin-clavulanic acid AMC, piperacillin-tazobactam TZP, ceftazidime CAZ, ceftriaxone CRO, and cefazolin CZ MICs of the 22 E. coli isolates were established according to clinical and laboratory standards institute criteria [8] by a standard agar dilution method on Muller-Hinton medium containing antibiotics and by Vitek2 compact system. Kaye [15] showed by agar dilution testing, 67 of E. coli isolates were non susceptible (39 resistant and 28 intermediate) to amoxicillin-clavulanate and 37 were piperacillin-tazobactam resistant but only 8 were ceftazidime resistant (ceftazidime MIC > 32 g/ml). Two isolates were susceptible to amoxicillin-clavulanic acid by agar dilution, although they were resistant by disk diffusion testing.

**Molecular screening for IR enzymes**

PCR technique has been used to screen and detect IR genes carrying plasmid primer. The results are illustrated as follows:

**Molecular characterization of TEM-1, TEM-2:**

This molecular method was used to detect the most common kinds of IRs; TEM-1, and TEM-2. Distribution of IRs genes among uropathogenic E. coli isolates is shown in figure-2and-3. One to two genes for IRs were present in some isolates. In this study 3 (13.6%) isolates had TEM-1 (Figure 2) and 7 (31.8%) isolates had TEM-2 (Figure 3).

In this study, results revealed that high percentage of inhibitor-resistant TEM (IRT) isolates were detected. This result is a first record in Iraq.

**Figure (2)** Ethidium bromide-stained agarose (2.1%) gel of PCR amplified products from extracted plasmid DNA of E. coli isolates and amplified with primer TEML-1 forward and TEMU-1 reverse. The electrophoresis was performed at 70 volt for 1.5-2 hr. lane (L), DNA molecular size marker (100bp ladder). Lanes (0015, 0028, and 0029) show positive results with TEM-1 gene (867 bp).

**Figure (3)** Ethidium bromide-stained agarose (2.1%) gel of PCR amplified products from extracted plasmid DNA of E. coli isolates and amplified with primer TEML-2 forward and TEMU-2 reverse. The electrophoresis was performed at 70 volt for 1.5-2 hr. lane (L),DNA molecular size marker (100bp ladder). Lanes (0023, 0029, 0030, and 0032) show positive results with TEM-2 gene (374 bp).
Kaye et al. [15] analysed E. coli isolates in the microbiology laboratory of a US tertiary care hospital, from October 1998 to December 1999, and revealed that the TEM type alone was found in 52 isolates; the TEM type with CMY-2 were found in 2 isolates. Also, there was one isolate had the TEM type and the SHV type. On other hand found one isolate had two enzyme, the first was the TEM type and the second was unidentified.

Miro et al. [14] revealed that out of 7,252 nonduplicated clinical Escherichia coli strains from a Spanish hospital showed reduced susceptibility to amoxicillin-clavulanate, 0.8% were probable TEM-1 hyperproducers.

Martín et al. [31] found that from a total of 3,556 E. coli isolates recovered during the study period, 18 of them producing IRT enzymes (0.5%). These were mostly recovered from urine (77.8%). A high degree of IRT diversity was detected (TEM-30, -32, -33, -34, -36, -37, -40, and -54).

The PCR results show that 74 isolates of E. coli (57.8%) had the TEM gene. This study showed that the majority of the ESBL positive clinical isolates of E. coli carried the TEM gene.

Fèria et al [32] showed that the resistance of uropathogenic E. coli isolates from animals to β-lactamase inhibitors was showed in TEM-1 alone (6/26) or together with AmpC (4/26).

**Molecular characterization of bla-SHV**

The study showed that 6 (27.2%) isolates had bla-shv (Figure 4) and 3 (13.6%) isolates had SHV-2 (Figure 5).

![Figure 4](ethidium_bromide-stained_agarose_gel_of_PCR_amplified_products_from_extracted_plasmid_DNA_of_Serratia_spp_isolates_and_amplified_with_primer_bla-SHV_forward_and_bla-SHV_reverse_the_electrophoresis_was_performed_at_70_volt_for_1.5-2_hr_lane(L)_DNA_molecular_size_marker_100bp_ladder_Lanes_003_004_007_009_0013_0027_show_positive_results_with_bla-shv_gene_867_bp.jpg)

**Figure (4)** Ethidium bromide-stained agarose gel (0.7%) of PCR amplified products from extracted plasmid DNA of Serratia spp. isolates and amplified with primer bla-SHV forward and bla-SHV reverse. The electrophoresis was performed at 70 volt for 1.5-2 hr. lane (L), DNA molecular size marker (100bp ladder). Lanes (003, 004, 007, 009, 0013, 0027,) show positive results with bla-SHV gene (867 bp).

![Figure 5](ethidium_bromide-stained_agarose_gel_of_PCR_amplified_products_from_extracted_plasmid_DNA_of_E.coli_isolates_and_amplified_with_primer_SHV-2_forward_and_SHV-2_reverse_the_electrophoresis_was_performed_at_70_volt_for_0.5-1_hr_lane(L)_DNA_molecular_size_marker_100bp_ladder_Lanes_005_0028_and_0029_show_positive_results_with_SHV-2_gene_477_bp.jpg)

**Figure (5)** Ethidium bromide-stained agarose gel (2.1%) of PCR amplified products from extracted plasmid DNA of E. coli isolates and amplified with primer SHV-2 forward and SHV-2 reverse. The electrophoresis was performed at 70 volt for 0.5-1 hr. lane (L), DNA molecular size marker (100bp ladder). Lanes (005, 0028, and 0029) show positive results with SHV-2 gene (477 bp).
In a study from Spain, Miro et al. [14] revealed that out of 7,252 nonduplicated clinical Escherichia coli strains from a Spanish hospital showed reduced susceptibility to amoxicillin-clavulanate, 0.18% were OXA-30.

Soltan et al. [33] showed that PCR was performed for all 128 resistant E. coli isolates, and only seven (5.5%) of the strains tested were shown to express bla-SHV.

Fèria et al. [32] revealed that the resistance to β-lactamase inhibitors uropathogenic E. coli isolates from animals in Portugal was mediated by (OXA, TEM, SHV, and AmpC) and the OXA-1 enzyme was found expressed (2/26).

Kaye et al. [15] studied the molecular epidemiology of amoxicillin-clavulanate-resistant E. coli isolated from a US tertiary care hospital and showed that one isolate in the same time had SHV type and TEM type enzyme.

### Molecular characterization of OXA-1, OXA-2, and OXA-10:

In this study OXA-1 was detected only in 2 (9%) of the isolates (Figure 6). On the other hand, no isolate (of all tested isolates) showed expression of OXA-2 and OXA-10 genes.

![Figure 6](image.png)

**Figure (6)** Ethidium bromide-stained agarose gel of PCR amplified products from extracted plasmid DNA of E. coli isolates and amplified with primer OXA-1 forward and OXA-1 reverse. The electrophoresis was performed at 70 volt for 0.5-1 hr. lane (L), DNA molecular size marker (100 bp laddar). Lanes (0011, and 0029) show positive results with OXA-1 gene (813 bp).

Miro´ et al. [14] found that out of 7,252 non-duplicated clinical E. coli strains from a Spanish hospital showed reduced susceptibility to amoxicillin-clavulanate, 0.18% of isolates were OXA-30.

Fèria et al. [32] revealed that the resistance to β-lactamase inhibitors uropathogenic E. coli isolates was mediated by (OXA, TEM, SHV, and AmpC) and the OXA-1 enzymes was found to expressed (2/26).

The molecular epidemiology of amoxicillin-clavulanate-resistant E. coli isolated in the microbiology laboratory of a US tertiary care hospital was studied by Kaye et al. [15] and showed that the OXA enzyme type was found in 1 isolate.

Steward et al. [34], studied the presence of ESBLs in K. pneumoniae, K. oxytoca, and E. coli in Spain. using isoelectric focusing (IEF), and they showed that 7 of the 23 isolates contained a β-lactamase with a pI of >8.3 suggestive of an AmpC-type β-lactamase; 6 of the 7 isolates were shown by PCR to contain both bla-OXA and ampC-type genes.

### 3.10.4. Molecular Characterization of CTX-M enzymes:

In this study CTX-M enzymes were detected in 6 (27.2%) uropathogenic E. coli isolates (Figure 7) which had positive results for ESBL and resistance for cefotaxime and ciprofloxacin.
In study from 28 Russian hospitals, Edelstein et al. [35] revealed a total of 904 consecutive nosocomial isolates of *E. coli* and *K. pneumoniae* were screened for production of ESBLs. The ESBL phenotype was detected in 78 (15.8%) *E. coli* and 248 (60.8%) *K. pneumoniae* isolates. 115 isolates carried the genes for CTX-M-type β-lactamases, which, as shown by PCR-RFLP analysis.

A previous study [36] reported higher (43/84, 51%) urinary *E. coli* ESBL producers harboring both *bla*-CTX-M, and *bla*TEM, and the *bla*-SHV gene detected in one of their isolates was not detected in our isolates.

Nimri and Azaizeh [37] out of the 165 isolates, 83 (50.3%) were ESBL-producing isolates, 67 (80.7%) of these had at least one ESBL gene (either *bla*-CTX-M or *bla*-TEM, or both), 16 (19.3%) isolates didn’t have any of the three ESBL genes, and *bla*-SHV was not detected in any of the isolates. Out of the 67 isolates 47(70.1%) had either *bla*-CTX-M (28 isolate), or *bla*-TEM gene (19 isolates), while 20 (29.9%) isolates had both *bla*-CTX-M and *bla*-TEM genes.

Khosravi, et al [38] revealed that out of 500 tested *Entrobacteriaceae* isolates were identified as *K. pneumoniae* possessing 26 (47.27%) ESBL positives amongst them. Also found that the prevalence of SHV-1, TEM-1 and CTX-M-1 genes among ESBLs-positive isolates was 12 (46.15%), 9 (34.61%), and 7 (26.92%) respectively.

### Molecular Characterization of AmpC

Only 1 (4.5%) of uropathogenic *E. coli* isolates had AmpC enzyme (Figure8). This isolate was designated as negative for ESBL expression in phenotypic assays (Index-1; 0023, Table 3-6) and found to express the TEM-2 gene.

**Figure (7)** Ethidium bromide-stained agarose gel (0.7mg) of PCR amplified products from extracted plasmid DNA of *E.coli* isolates and amplified with primer *bla*-CTXM forward and *bla*-CTXM reverse. The electrophoresis was performed at 70 volt for 1.5-2 hr. lane (L), DNA molecular size marker (100 bp ladder). Lanes (003, 004, 007, 009, 0013, 0027) show positive results with *bla*-CTXM gene (550 bp).

**Figure (8):** Ethidium bromide-stained agarose (0.7) gel of PCR amplified products from extracted total DNA of *E. coli* isolates and amplified with primer *bla*-AmpC forward and *bla*-AmpC reverse. The electrophoresis was performed at 70 volt for 1.5-2 hr. lane (L), DNA molecular size marker (100 bp ladder). Lane (0023) shows positive result with *bla*-AmpC gene (550 bp).
Coque et al. [39] revealed that two isolates of E. coli isolates were designated as negative for ESBL expression in phenotypic assays were found to express the TEM gene, possibly due to expression of novel β-lactamase enzymes, such as AmpC. Therefore, the use of molecular methods coupled with phenotypic tests is essential for the definitive identification of these types of β-lactamase enzymes.

Fèria et al. [32] showed that the resistant to β-lactamase inhibitors was mediated mainly by (AmpC, OXA, TEM, and SHV) and the AmpC type alone (1/26) or together with TEM-1 (4/26).

Kaye et al. [15] showed that E. coli isolates resistant to amoxicillin-clavulanate in a tertiary care hospital was mediated by (TEM, SHV, OXA, and AmpC), AmpC enzyme was found in 4 isolates (2 identified as containing CMY-2).

References
uropathogenic *E. coli* isolated from dogs in Portugal., 49:77-85.


