Phenotypic And Molecular Screening of Extended Spectrum β-Lactamase ESBLS in MDR- E. Coli Isolated from Urinary Tract Infection Cases

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Abstract

E. coli is a significant infectious agent that causes nosocomial infections in hospitals all over the world. One of the factors that contributes to the development of drug resistance in E. coli is responsible for the production of ESBls enzymes. In this study, bla TEM was documented for the very first time among E. coli in Iraqi hospitals located in the province of Al-Anbar. 100 of the 141 E. coli clinical isolates tested for resistance to cephalosporins were found in urine samples. The VITEK-2 system was used to analyze antibiotic resistance. All of the isolates have been found to produce the cephalosporins gene, as shown by the positive results of the phenotypic detection of Extended-Spectrum β-lactamases enzymes by the Modified Hodge test and the mCIM; 25/25 (100%) of the tests yielded a positive result with the mCIM, and 4.87 % with the modefied Hodge test. Traditional PCR was used to detect the bla TEM gene, and the results showed that 56% of the strains were positive for this gene. These findings demonstrated that TEM was present in MDR-E. coli, indicating that ESBLs were prevalent throughout the province of Anbar in Iraq.

Keywords: BLA TEM, ESBLs, E. Coli, UTIs.

INTRODUCTION

An infection of the urinary tract, also known as a urinary tract infection (UTI), can refer to a wide variety of clinical entities that involve microbial invasion of any tissue of the urinary system, ranging from the renal cortex to the urethral meatus (Najar et al., 2009). Every year, a urinary tract infection (UTI) affects millions of people of all ages, posing a significant risk of morbidity and mortality as well as significant financial burdens on healthcare systems (Orenstein & Wong, 1999). Patients diagnosed with urinary tract infections are more likely than patients with other conditions to have antibiotic resistance. The production of -lactamases enzymes, such as extended spectrum β-lactamases (ESBLs), carbapenemases, and AmpC β-Latamases, is one of the factors that contributes to the development of drug resistance in E. coli bacteria (Shah et al., 2004). Members of the family Enterobacteriaceae, such as E. coli and K. pneumoniae, are a major cause of urinary tract infections (UTIs), and their prevalence is increasing worldwide(Chen et al., 2013). Antibiotic resistance, especially to third-generation cephalosporins, can spread rapidly thanks to these plasmids (Danel et al., 1997).

Since their discovery in 1983, more than 300 different ESBL variants have been found in bacteria outside the family Enterobacteriaceae (Paterson & Bonomo, 2005). Among the many different genotypes of Enterobacteriaceae, those most commonly found in clinical settings are CTX-M, SHV, and TEM, which together confer resistance to β-lactams, fluoroquinolones, and aminoglycosides (Pitout & Laupland, 2008). In this perspective, we aimed to determine the incidence, bacterial etiology of urinary tract infections, phenotyping and genotypes of ESBL-producing multidrug-resistant Escherichia coli at a Ramadi hospitals, Iraq.

MATERIALS AND METHODS

Isolation and identification

In the period between December 2021 to April 2022, specimens of midstream urine was collected from 215 patients of both sexes using the clean catch method recommended by (Vandepitte *et al.,* 2003). Samples were collected from patients who attended Haditha Teaching Hospital and Falluja Hospital for Children & Maternity. Urine specimens were processed within 30 minutes after sample collection in the bacteriology unit lab. **"**The identification and susceptibility profiles of ***E. coli*** were determined using the VITEK 2 method (bioMérieux) in accordance with the Clinical and Laboratory Standards Institute guidelines. (Clinical And Laboratory Standars Institute, 2018) and (EUCAST)(Committee et al., 2020). All

study techniques that involved patients were approved by the Ethical Approval Committee, University of Anbar, Ramadi, Iraq (approval no. 66/ June 2022).

Antimicrobial Susceptibility Testing

"The antimicrobial susceptibility of *Escherichia coli* was determined by the disk diffusion method of modified Kirby–Bauer on the Mueller–Hinton agar (HiMedia Laboratories, India) following standard procedures recommended by the Clinical and Laboratory Standard Institute (CLSI), Wayne, USA" (Abbey & Deak, 2019). Many antibiotics were used in this study including: Cefotaxime, Ceftazidme, Ceftriaxone, Cefepime, Cefoxitin, Piperacillin, and Imipenem.

Screening of ESBLs

Determination of the production of ESBls was carried out by modified Hodge test, modified Cephalosporins Inactivation Method and under the CLSI guidelines (Clinical And Laboratory Standars Institute, 2018) and as described elsewhere"(Pitout et al., 2015). Modified Hodge Test (MHT) was used to identify β-lactamases producing *E. coli* isolates. Briefly, *E. coli* ATCC 25522 suspension of 0.5 turbidity, matching with 0.5 McFarland standard, was prepared by dilution 1:10 using liquid media. Mueller-Hinton agar plates were inoculated with the prepared suspension of the ATCC 25522 and spread all over the plate. Cefotaxime discs were fixed in the center of the Muller plates. Test strains were inoculated in a straight line from the edge of the disc to the edge of the plate, test strains were obtained from an overnight blood agar culture. Finally, the plates were incubated for 18 hr at 37°C. Positive strains were recorded if a cloverleaf-like shape formed that indicates the growth of ATCC 25522 (CLSI, 2021).

The test was performed according to the instructions of CLSI, (2021). A suspension was prepared by dispersing a cultivated colony of the tested strain, full loop, in 2 ml TSA. After that, a disk containing 10 g of cefotaxime or ceftriaxone was submerged in the suspension and incubated at 35 °C for two hours. Then, the disk was taken out of the suspension and fixed on a Mueller-Hinton agar plate containing *E. coli* ATCC 25922, incubated at 35 °C for overnight. After incubation, lack of zone inhibition was an indicator of β-lactamases-positive (enzymatic hydrolysis of β-lactamases), while forming a clear zone inhibition means that the tested strain does not express carbapenemase activity, β-lactamases -negative.

A final concentration of 5 mM EDTA was prepared by mixing 20 µl of 0.5M EDTA and 2ml TSB containing tube. The tube was incubated at 35 oC for 2 h. After incubation, the disk was removed from the suspension and placed on a Mueller-Hinton agar plate that inoculated with Escherichia coli ATCC 29522, incubated at 35oC overnight. Absence of an inhibition zone was recorded as serine β-lactamases. Otherwise, the result was recorded as metalo- β-lactamases, clear zone.

Genotypic Detection of ESBLs

Genomic DNA of *E. coli* isolates was extracted using genomic DNA Purification Kit Promega (USA) following the company instructions, "genomic DNA was extracted from an overnight culture (Promega, USA). The concentration and purity of the DNA extract were determined by measuring absorbance at 260 and 280 nm wavelengths (NanoVue Plus; United States). Electrophoresis was used to assess the integrity of genomic DNA. The primers used in this study were given in lyophilized form and then dissolved in sterile deionized distilled water (Alpha DNA, Canada)". (Table 1).

Table 1: Sequences of bla – TEM

gene	5`- Oligo seq – 3`	Size bp	Tm(ºC)	Refernce
bla – **TEM**	F: ATA AAA TTC TTG AAGAC	1181	50	(Gundran et al., 2019)
	R: TTA CCA ATG CTT AATCA			

*F : forward, R : Reverse.

For PCR program, the initial denaturation phase for each PCR assay with different primers was established on 95°C for 5 min also denaturation was 94°C for 30 sec. The annealing time was 40 sec for all primers and temperature was 50 °C for bla TEM. The extension time was 1 min in 72°C. The final extension for all genes was done at 72°C for 7min".

"After PCR amplification, 5 µL of each reaction was separated by electrophoresis in 1% agarose gel for 90 min at 70 V (7 V/Cm) in 1 × TAE buffer. DNA was stained with ethidium bromide (1 µg/ml), and the amplified DNA bands were visualized using a UV-transilluminator (Cleaver Scientific Ltd). On other hand, reaction of PCR consisted of **12.5 µl Hot PCR Master mix, 1 µl forward an reverse primer, 9.5 µl Free-nuclease water, 1 µl** DNA template to become 25 **µl** as final volume".

RESULTS AND DISCUSSION

Isolation and Identification

During the study period, November 2022 to December 2022, 215 samples were collected from patients attended Ramadi Teaching Hospital and AlRamadi Teaching Hospital for Maternity and children. Our results showed that the distribution of bacterial organisms in UTI samples was as follows: 3 (1.40%) strains of *Streptococcus sp*, 4 (1.86%) *Enterobacter cloacae*, 10 (4.65%) *A. baumannii*, 18 (8.37%) *Staphylococcus sp*, 30 (13.95%) *Pseudomonas aeruginosa*, 50 (23.26%) *Klebsiella pneumoniae*, 100 (46.51%) *E. coli* as shown in Figure 1. Other studies showed same percentage of bacterial distribution with little variations; however, all these studies showed that *E. coli* is the most predominant uropathogens among UTI patients which agrees with our results (Prakash and Saxena 2013; Eriksson *et al.*, 2013; Onuoha and Fatokun, 2014; John *et al.*, 2015; Tessema *et al.*, 2020).

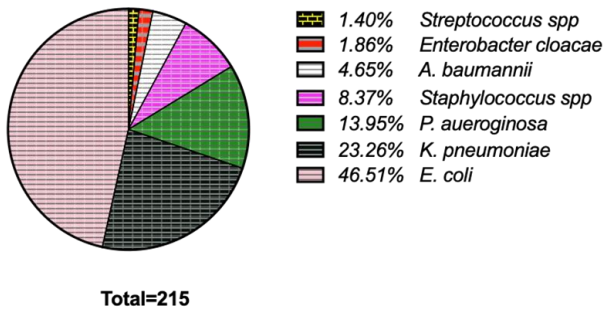|	
1.40%	*Streptococcus spp*
1.86%	*Enterobacter cloacae*
4.65%	*A. baumannii*
8.37%	*Staphylococcus spp*
13.95%	*P. aueroginosa*
23.26%	*K. pneumoniae*
46.51%	*E. coli*

Total=215

Figure 1: Distribution of bacterial isolates among urinary tract infection patients.

Automated diagnosis

Identification of all UPEC strains were accomplished by conventional and automated methods, VITEK-2 compact system, bioMérieux, France, by using ID-GNB cards following the manufacturer's instructions.As shown table 2:

Table 2 Morphological characteristics and biochemical tests of E. coli

Characteristics	E. coli isolates
MacConkey agar medium	Lactose ferment (+)
Gram-stain	Gram negative (-) rods
Catalase test	Positive (+)
Oxidase test	Negative (-)
Indol Test	Positive (+)
Methyl Red Test	Positive (+)
VP Test	Negative (-)
Citrate Utilization Test	Negative (-)
Urease	Negative (-)

Susceptibility to antibiotics

One hundred isolates of *E. coli* that isolated from UTI patients were tested for antibiotic sensitivity using Kirby-Bauer disk diffusion susceptibility test method (figure 2) and confirmed by automated Vitek-2 according to the recommendations of CLSI (CLSI, 2021).

The high percentage of resistant of UPEC to the third generation of β-lactam is due to the excessive uptake of these antibiotics, improper usage of drugs as prescribed by a physician, as well as a lack of personal education exhibited by an incomplete full course of antibiotics to destroy the pathogen in order to increase infection cure rates and avoid the formation of any resistance or treatment failures, and in addition to the fact that the majority of the isolates were beta-lactamase producers (ESBL) (Al-khikani *et al.*, 2020)
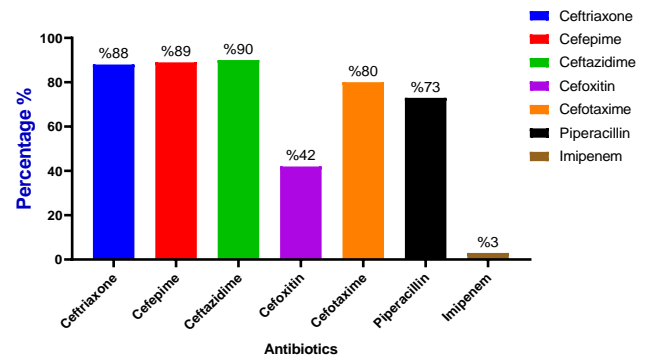

Figure 2: Antibiotic resistance profile based on Kirby –bauer disk diffusion

Modified Hodge Test (MHT)

Among phenotypic tests, MHT is a relatively easy and simple test to be performed in a laboratory and described according CLSI for detecting β-lactamases. Out of 41 selected isolates, the results showed that that 2/41(4.87%) were β-lactamases positive, where 39/41 (95.121%) were β-lactamases negative. Other studies showed different results than our results. Azimi *et al.*, (2013) reported that all isolates show β-lactamases positive. In a study that performed an Najaf city, Sadiq and Sehlawi, (2013) reported that all isolates were also show positive result. This could be due to the type of strains isolated, type of infection, and medications history.As shown in figure 3.

Figure 3: Modified Hodge test for UPEC isolates

Modified β-lactam inactivation methods (mCIM) for Suspected β-lactamases Production

mCIM was developed for further detection of β-lactamases production. In our study, all 41 UPEC isolates were positive with ratio 41/41(100%) (Figure 4).

To further differentiate metallo β-lactams (MBL) from serine β-lactams (SBL), the mCIM was adapted by adding ethylenediaminetetraacetic acid (EDTA), a cation chelator and inhibitor of MBLs. Our result showed that 41/41(100%) were positive for SBL as shown figure 5. Differentiating isolated by the previous technique has very important therapeutic implications. The accuracy of the mentioned technique is over 96% in identification (Tenover *et al.*, 2020).
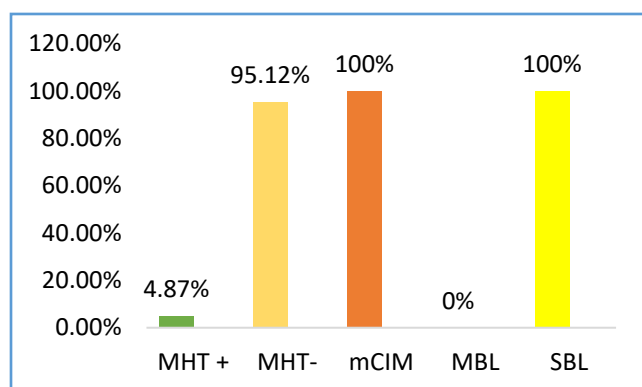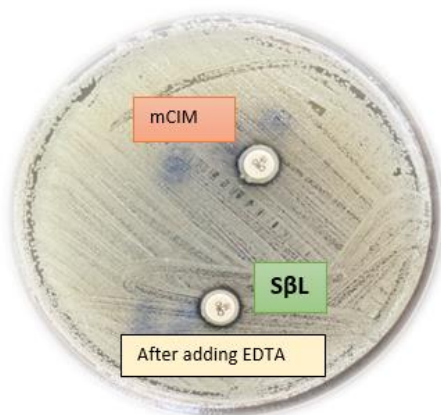

Figure 4: Confirmation tests for β-lactamases production

Figure 5: Confirmation test for antibiotic resistance. A: mCIM, B: Serine β-lactamases

PCR detection of blaTEM

bla-TEM was successfully amplified using the primers mentioned in the methods section and the gel results showed a band with 1181bp when compared with the standard ladder (Picture 6).

Infections with ESBL-producing *E. coli* (ESBL-EC) isolated from livestock and poultry animals are of public health concern because they can result in treatment failure using commonly used penicillins and cephalosporins, increasing

the risk of mortality and delaying appropriate treatment (Gundran *et al.*, 2019). Despite the fact that ESBL-EC is susceptible to some cephalosporins and penicillin/-lactamase inhibitors, these medicines are rarely employed as first-line therapy in *E. coli* infections.

Our results showed that 56% of UPEC having *bla-TEM*; however, the detection of β-lactamase gene (*blaTEM*) in the current investigation did not provide any information concerning the type of β-lactamase enzymes and its correlation with the resistance profile (Table 4-7). The relationships between *blaTEM* and β-lactamase antibiotics used in the study was insignificant. However, to our knowledge, no published data on the frequency of the mentioned or all TEM genes among clinical isolates of UPEC in Iraq were published. There are four TEM lactamase enzymes, TEM1, TEM2, TEM3, and TEM4 and their frequency was investigated by other researchers globally (Bailey *et al.*, 2011; Gundran *et al.*, 2019).

These are by far the most common and have an unknown origin (Wiedemann *et al.*, 1989). Among them, TEM1 is responsible for the majority of ampicillin resistance in 94% of *E. coli* isolates that isolated in Spain, 89% was from Hong Kong, and 78% from London (Livermore *et al.*, 1986). This enzyme can hydrolyze penicillin antibiotics and early cephalosporins like cephalothin and cephaloridine. TEM2 is also common in *E. coli*, but it is substantially less common than TEM1. Moreover, TEM1 and TEM2 enzymes have less activity against newer cephalosporins (Bush and Bradford 2020; Wiedemann *et al.*, 1989).

However, there has been a rising epidemic of ESBLs in the last 20 years, which target several modern cephems and monobactams, as well as third generation cephalosporins and anti-Gram-negative, bacterial penicillins (Bradford, 2001). Although ESBL-producing strains are typically resistant to novel cephalosporins and/or aztreonam, many strains producing these enzymes look sensitive or intermediate to some or all of these drugs in vitro, despite clinically considerable resistance in infected individuals (Paterson and Bonomo, 2005).
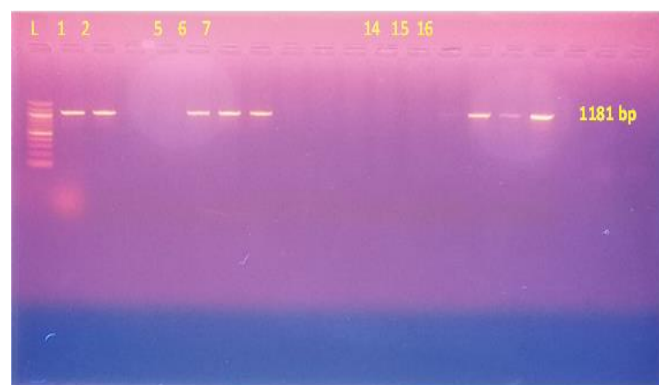

Figure 6: Gel electrophoreses for amplified blaTEM fragment (1181bp). L: 200 bp DNA ladder; Lanes 1-18: PCR products of E. coli DNA using blaTEM primers

Correlation between antibiotic resistance

As shown table table, thes results showed no significant between antimicrobial resistance profile and TEM gene.

Table 4-7 Correlation between antimicrobial resistance profile and PCR virulence genes among UPEC

PCR gene	Antibiotic resistance
bla$_{TEM}$	β-lactam antibiotics (Cefotaxime, Ceftazidme, Ceftriaxone, Cefepime, Cefoxitin, Piperacillin, and Imipenem)
Positive n=23(%)	
Negative n=18(%)	
P value	**NS ***

NS*: more than 0.05.

CONCLUSION

In our investigation, multidrug resistant bacteria were found in more than half of the Gram-negative isolates. Half of the pathogenic E. coli strains were prospective ESBL producers, with half of them carrying the ESBL genes in concern. The outcomes of this study suggest that early suspicion, identification, and AST are critical for optimizing therapy and limiting the spread of AMR.

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