Molecular study to detect \textit{bla}_{TEM} and \textit{bla}_{CTX-M} genes in ESβL \textit{Escherichia coli} and their antimicrobial resistance profile

May Abdul Jaleal Raoof¹ and Mohammed A. Fayidh¹∗

¹Department of Biology, College of Education for Pure Science (Ibn Al-Haitham), University of Baghdad, Baghdad, Iraq.

*E-mail: mohammedcbt66@gmail.com

Abstract. The common use of beta-lactam antibiotics resulted in the emergence of bacterial strains capable of spreading and extended-spectrum beta-lactamase (ESβL). Up to 70% of clinical samples, 30 of \textit{Escherichia coli} (E. coli) were investigated as ESβL isolates. These isolates collected from inpatients and outpatients to some hospitals in Baghdad. The samples include urine, pus, stool, and blood from both genders, different age groups. The VITEK2 was used to check production of ESβL and for the automated diagnosis of rapid antimicrobial susceptibility experimentation and to the identification of the target bacteria. Technique of PCR was applied to detect the presence of \textit{bla}_{TEM} and \textit{bla}_{CTX-M} genes. The results demonstrated that 30(43%) isolates of the current study were ESβL producers. The gel electrophoresis of DNA for positive ESβL isolates gives differentiate results of both \textit{bla}_{TEM} and \textit{bla}_{CTX-M} genes were observed in 15(50%) and 29(96%) isolates respectively. The antibiotic resistance pattern to 20 antibiotics were showed majority of isolates have the multi-drug resistant (MDR) phenotypes and susceptible to carbapenems of antibiotic. Furthermore, the results of the genetic analysis, along with the pattern of isolates for antibiotic resistance, it may give an acceptable explanation for the higher presence of the \textit{bla}_{CTX-M} gene compared with the proportion of the \textit{bla}_{TEM} gene in the same isolates.

Keywords. \textit{Escherichia coli}, ESβL, \textit{bla}_{TEM}, and \textit{bla}_{CTX-M} genes, antibiotic resistance.

1. Introduction

\textit{Escherichia coli} is a various group of facultative anaerobic Gram-negative bacilli related in Enterobacteriaceae, and consist of variety strains ranging from commensal organisms to extremely pathogenic variants especially the intestine and urinary tract [1, 2]. Extended-spectrum beta Lactamase (ESβL) has been produced by several bacterial genes and noticed an increased rate after using the β-Lactam group of antibiotics has been extensively applied in human and veterinary medicine against several bacterial pathogenic infections [3]. [4] reported that the unconsidered use of antibiotics led to the evolution of many bacterial strains resistant to beta-lactam antagonists. On the other hand, the excess in the incidence of β-lactam resistance in Gram-negative bacteria has become a major clinical problem worldwide and then limits therapeutic choice, this reduces the chances of identifying appropriate antibiotic options for the resistance of newer isolates of beta-lactam in an Enterobacteriaceae [5]. The ESβLs are a series of enzymes that lead to resistance increase in
Aztreonam, Ceftazidime, Cefotaxime related oxyimino-β-lactams, Cephalosporins, and penicillins, but Clavulanic acid can be effected on them [6]. In the present work, more than 400 different ESβL variants have been identified [7]. Moreover, TEM, SHV, and CTX-M are three main types of ESβLs genes were known to mediate by chromosomes, plasmids, and transposons, which can effortlessly spread from one organism to another [8], and they are sporadically described all over the world, while CTX-M type is the master type in some countries [9] and TEM gene has become more spread than another genes [10]. Also, TEM β-lactamase (“Temoniera”) was isolated from recovered a Greece patient called Temoniera who infected by one strain of E coli and first reported plasmid-mediated β-lactamase which was isolated [11]. Another commonly accruing (ESβL) is the CTX-M was first discovered in an E. coli isolate recovered from Munich, German in1989. Bacterial strains that produce ESβL often demonstrate the resistance to antibiotics belongs to other classes which make strategies of treatment more complex [12], especially prevalent among a wide range of clinically important bacteria Widespread in the world [6]. Due to the lack of studies on the prevalence of beta-lactam genes, the current study was conducted to discuss the range of the prevalence of blaTEM and blaCTX-M genes and the presence of extended-spectrum beta Lactamase in clinical E. coli isolates collected from different hospitals in Baghdad, to determine characteristics and patterns of antibiotic resistance among isolates of E. coli clinical specimens. The aims of the current study to investigate the extent of spread two ESβL genes (blaTEM and blaCTX-M) as well as investigating the antimicrobial resistances in the clinical isolates of E coli producing beta-lactamase.

2. Materials and Methods

2.1. Bacterial isolates

A total 70 bacterial isolates were collected from different clinical specimens includes urine, wound swab, stool, pus and blood samples from both genders patients with different ages and were collected from some Baghdad hospitals between the period 9/10/2019 to 20/12/2019. Standard microbiological techniques were depended for isolation [13] and Conventional microbiological procedures were employed to identify the isolates. The VITEK 2 compact system was used to re-identify them (BioMerieux, France) [14], and applied for the bacterial identification at the species level to determine antimicrobial susceptibility test (AST) of rapid clinically significant human bacterial pathogens.

2.2. Identification of E.coli isolates

All E. coli Isolates were identified and tested for their susceptibility profile using the VITEK 2 compact system by different antimicrobial susceptibility test cards (AST- cards), according to the expected bacterial pathogens. The regarding cards were inoculated and incubated in the instrument according to the manufacturer's instructions. A VITEK 2 susceptibility tests outcome were obtained as MIC values and shown as susceptible, intermediate, or resistant and estimate according to the periodic values of the National Committee for Clinical Laboratory Standard's breakpoint [13]. Final results were explicate according to the instructions of [15] CLSI (2019) using advanced expert system (AES), according to [16]. A total 17 different antibiotics were used and tested in AST-GN71 (bioMérieux) card that included ampicillin (AMP), ampicillin / sulbactam (SAM), aztreonam(ATM), cefazolin (CFZ), cefepime (FEP), trimethoprim / sulfamethoxazole (SXT), ciprofloxacin (CIP), Ceftriaxone (CRO), tigecycline (TGC), imipenem (IPM), meropenem (MEM), tobramycin (TOB), amikacin (AMK), ertapenem (ETP), gentamicin (GEN),moxifloxacin (mxf), nitrofurantoin (NIT)[17].

2.3. Detection of ESβLs

All isolates were tested using the VITEK 2 system with the antimicrobial susceptibility tested AST-GN71 card. This system was used to carry out both screening and confirmatory tests for phenotypic
notice of ESβL at the same plate [18]. All isolates of E. coli were confirmed as ESβL producing using double disc synergy (DDST) test using 30mg of aztreonam, ceftazidime, cefotaxime, ceftriaxone, and 20mg amoxicillin with10M clavulanic acid. Four discs of antibiotic were placed at 15mm apart from each other with amoxicillin/clavulanic acid disc which is placed at the centre (Figure l). Isolates of ESβL production was considered positive when the zone of inhibition around any antibiotic disc was overlap with the amoxicillin/clavulanic acid disc [19].

2.4. Detection of blaTEM and blaCTX-M genes coding for ESβL production

Only the E. coli isolates considered positive for ESβL, were selected for the genotypic study. The detection of blaTEM and blaCTX-M resistance genes were performed after extraction of total DNA of E. coli using the PCR and gel electrophoresis techniques isolated as maintained by the manufacturer's instruction using the ABIQ pure kit (USA). The concentration and the purity of the DNA was determined by a Quantus fluorometer (Promega, USA).

Table 1. Primers details of ESβLs genes used in this study.

| Genes   | Primer name and sequences (5’-3’) | Expected amplicon size (bp) | Annealing temp (°C) | Reference |
|---------|---------------------------------|-----------------------------|---------------------|-----------|
| blaTEM  | TEM. Forward 5‘ATAAAAATTCTTGAAGACGAAA3’ | 1080                        | 50                  | [20]      |
|         | TEM. Reverse 5‘GACAGTTACCAATGCTTAATCA3’ |                           |                     |           |
|         | CTX-M. Forward 5‘GACGATGTACTGGCTGAGC3’ |                           |                     |           |
|         | CTX-M. Reverse 5‘AGCCGCGACGCTAATAAC’ |                           |                     |           |
| blaCTX-M|                                 | 499                         | 55                  | [21]      |

Analysis of PCR technique was applied for encoding two genes of the β-lactamase family: TEM and CTX –M. Primers were supplied by Macrogen Company for the oligonucleotide primer sets (Table 1), Which is used as a specific for the blaTEM and blaCTX-M genes, and the cycling conditions in PCR assay have been adopted and which was described by [22]. The PCR was performed in a PCR Thermal Cycler (BioRad, USA).

3. Results

A total 30 isolates of positive ESβL Out of 70 collected isolates of E. coli were identified from different clinical specimens in the patients of both genders and at different ages in all hospitals of Baghdad city. The investigation of isolates was determined up to species level using the VITEK2 automated microbiology system [23]. The isolates were confirmed as ESβL producing E. coli using method of double-disc synergy test (DDST), as outlined in the Figure 1. The results of DDST for 70 isolates showed that 30 isolates (43%) had been ESβL producers and 40(57%) were non-producers.
A total of 30 (43%) clinical isolate of ESβL producing in bacterial isolated from various infections was showing that 18 (60%) isolates in urine tract infections and 2 (6.0%) isolates in stool 4 (12.1%) isolates in pus samples and 6 (18.1%) isolates in the wound swab samples and these results showing in the Table 2.

### Table 2. Type and number of positive samples for ESβLs.

| Samples       | Number of samples | Male%  | Female% |
|---------------|-------------------|--------|---------|
| Urine         | 18 (60%)          | 7 (23.3%) | 11 (36.6%) |
| Stool         | 2 (6.6%)          | 1 (3.3%) | 1 (3.3%) |
| Pus           | 4 (13.3%)         | 5 (16.6%) | 1 (3.3%) |
| Wound swab    | 6 (20%)           | 3 (10%) | 1 (3.3%) |
| Total         | 30                | 16 (53.4%) | 14 (46.6%) |

### 3.1. Sensitivity of antibiotic

The resistant pattern of 30 ESβL producers isolates have been detected with 17 different antibiotic using AST-GN71 card of antimicrobial agent (Table 3). The results were showed that all isolates of the present study were susceptible to imipenem, ertapenem, meropenm, tigecycline, and (96 %) of isolates susceptible to amikacin and nitrofurantion and showed a high of resistance (P value) (100%) to (ampicillin, cefazolin, ceftriaxone and aztreonam) and 96% to cefepime and 90 % to ampicillin/sulbactam whereas resistance to other antibiotic was moderate.

### Table 3. Showing the percentage of antimicrobial resistant for ESβL producing E.coli.

| No. | Antibiotics       | NO. of resistant isolates | Rat of resistant % |
|-----|-------------------|----------------------------|-------------------|
| 1   | Ampicillin        | 30                         | 100%              |
| 2   | Ampicillin/Sulbactam | 27                        | 90%               |
| 3   | Cefazolin         | 30                         | 100%              |
| 4   | Ceftriaxone       | 30                         | 100%              |
| 5   | Cefepime          | 29                         | 96%               |
| 6   | Aztreonam         | 30                         | 100%              |
| 7   | Ertapenem         | 0                          | 0%                |
| 8   | Imipenem          | 0                          | 0%                |
| 9   | Meropenem         | 0                          | 0%                |
| 10  | Amikacin          | 1                          | 3.3%              |
| 11  | Gentamicin        | 13                         | 43.3%             |
| 12  | Tobramycin        | 15                         | 50%               |
| 13  | Ciprofloxacin     | 21                         | 70%               |
| 14  | Moxifloxacin      | 21                         | 70%               |
### Table 1

|   | Drug                  | Resistance |   |
|---|-----------------------|------------|---|
| 15| Tigecycline           | 0          | 0%|
| 16| Nitrofurantion        | 1          | 3.3%|
| 17| Trimethoprim/Sulfamethoxazole | 20  | 66.6%|

#### 3.2. Gel Electrophoresis Analysis of ESβLs gene

Polymerase chain reaction (PCR) technique was conducted to detect the ability to produce and expression on 30 ESβL recognized as positive isolates of *E. coli*, were selected. The blaTEM gene was detected for the 30 ESβL positive isolates shown in (Figure 2 A and B). The bands of the expected size (1080bp) using the TEM-F and TEM-R primers were seen as positive TEM genotype and observed in 15 (50%) of total 30 *E. coli* isolates.

![Figure 2](image)

*Figure 2*. Gel electrophoresis analysis for PCR amplified fragment products of *blaTEM* (1080 bp) genes for 30 bacterial isolates of study using 1.5% agarose and DNA markers (1500 -100bp) are shown in lane M.

On the other hand, results of *blaCTX-M* gene was showing in the (Figure3 A and B). The bands of expected size 499bp using CTX-M-F and CTX-M-R primers were seen as a positive samples the genotype and observed in 29 (96.6%) of a total 30 *E.coli* isolates.
Figure 3. Gel electrophoresis analysis for PCR amplified fragment products of $bla_{CTX-M}$ (ca. 499 bp) genes for 30 bacterial isolates of study using 1.5% agarose and DNA markers (1500 -100bp) are shown in lane M.

3.3. Genotype patterns of ESβLs

Analysis of the PCR amplified DNA fragment of 30 $E. coli$ ESβLs producers using the specific primers revealed that the two genotype patterns were obtained in (Table 4). These results also showed that predominant genotype rate was CTX - M (50%) then genotype amalgamation of TEM + CTX - M (46.6%). The commonness rate of the two different ESβL genes in the present study demonstrated that $bla_{CTX-M}$ gene was the most predominant type (29/30; 96.6%) while TEM gene was less dominant type (15/30; 50% respectively).

Table 4. Showing ESβLs genotype patterns among $E. coli$ isolates.

| ESβLs Genotype       | No. of isolates | Percentage (%) |
|----------------------|----------------|----------------|
| $bla_{TEM}$ only     | 1              | 3.3%           |
| $bla_{CTX-M}$ only   | 15             | 50%            |
| $bla_{TEM}$-$bla_{CTX-M}$ | 14             | 46.6%          |
| Total                | 30             | 100            |

4. Discussion

The increasing rate with the progression of ESβL producing by Enterobacteriaceae has threatened the entire world. However, nowadays the main challenge to infection control is entailing continuous monitoring systems for the emergence and spared isolates of Enterobacteriaceae that produce of ESβLs. In the present study, a total 30 bacterial isolates of $E. coli$ from total samples (43%) were identified as ESβL producers. These results give a dangerous indication of public health in Iraq and the need to take caution from the excessive use of antibiotics. Also, $E. coli$ and other genera of gram-negative bacteria have possessed a naturally occurring, chromosomally mediated β-lactamase and plasmid-mediated of β–lactamase [24]. The unconstrained use of these antibiotics in developing countries, poor dosing, ineffective empiric antibiotic therapy, prolonged antibiotic treatment, and
antibiotic misuse are the most reasons for the development and high resistance rates in bacterial infections [25]. Although Phenotypic tests, such as DUST method it’s commonly used, for detection of ESβLs and confirm whether an ESβL is produced by bacterial isolates but cannot detect the ESβL subtype easily. Furthermore, [26] reported that although molecular methods appear sensitive, but the time consuming, expensive and require specialized equipment and expertise. Definitive identification is possible only by molecular detection methods. On the hands, the techniques, that are necessary for the task of the exact ESβL subtype, are available only in research facilities. Also, PCR fragments products for blaTEM and blaCTX-M genes produce expected bands of 1080 bp and 499 bp respectively. Moreover, using the same primers in the present study, the amplified DNA products of comparable molecular weight were obtained in the different worldwide studies and showed the same results of [22]. Also, some studies performed in some countries, such as Iraq, Iran, and Turkey to detect other ESβLs genes using different primer sequences gave amplified products with various molecular weights [27], which confirms that there are many genes responsible for producing of ESβLs. The covariance in the molecular weights of the amplified products could be indicating the differentiation in primer sequences or in the type of the gene that detects, currently, more than 150 gene TEM types are recognized are there [28]. For example, CTX-M gene is divided into five subgroups that have more than 80 enzymes. This variety supplies an auxiliary way to follow the prevalence of individual resistance genes. Interestingly, that 96.6% of ESβLs producer E. coli have sheltered the CTX - M gene. Furthermore, TEM gene was found in 50% of bacterial isolates and that consistent with the present study and with diffusion worldwide including the Middle East area, where CTX- M type has replaced, and therefore the TEM type and became the predominating ESβL among isolates of Enterobacteriaceae [29]. Different studies that conducted in Iraq and adjacent countries have declared that the CTX-M type was the predominant gene type in E. coli [30], while the studies in Turkey and India showed that the TEM type was the dominant type [31]. Data analysis revealed that there are six genotype patterns of ESβLs exist among the 70 isolates. CTX - M was the most prevalent genotype (50%) followed by the genotype combination TEM + CTX - M (46.6%). This genotype combination has also been published to be the most dominant genotype in Japan, India, and Arabia Saudi kingdom [32, 33] said that in some isolates means that the ESβL producing strains may be regarding to a complex antimicrobial resistance. Finally, the results were demonstrated that the TEM gene is a broad spectrum β- lactamase that is always concerted with CTX-M on the same plasmid. The release of TEM+CTX-M collection can cause resistance to carbapenems; this is worrisome and more serious for the community [34].

5. Conclusion

In the present study, a 43 % of the uro-pathogenic isolates of E. coli were identified as ESβLs producers. All isolates were certain by PCR to have one or more ESBL genes. Type of CTX - M was the dominant ESβL in the isolated E. coli and followed by the genotype of TEM+CTX-M combination. So, it is very important to highlight on antimicrobial resistance must be perceiving as an healthy as well as ecological problems and increase efforts to monitor and control the spread of antimicrobial-resistant strains in hospitals and communities.

6. References

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