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Molecular Investigation of Some Beta-lactamase Genes by PCR and DNA Sequencing Techniques in clinical *Escherichia coli*

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Abstract

In this study, out of 50 isolates of some nosocomial infections from some Baghdad hospitals, only 13 (26%) were identified as *Escherichia coli*. Depending on selective media, morphological and biochemical tests the species was then confirmed by molecular methods. Later on antimicrobial resistance test was performed by the Kirby-Bauer method. The molecular characterization of *blaTEM* and *blaCTX-M* genes in different clinical isolates of *E. coli* was done through polymerase chain reaction (PCR) by utilizing special primers. These genes were positive to only 4 (30.7%) isolates. The sequence of nucleotides of positive genes was carried out for four isolates. The results showed that there was no variance in the nucleotide sequence between Iraqi isolates compared with the global isolates, and that they were 100% identical to many genera of Enterobacteriaceae. Finally, due to the indiscriminate use of antibiotics, these genes in human strains were likely the source of widespread drug resistance.

Keywords: *Escherichia coli*, *bla*TEM and *bla*CTX-M genes, antibiotic resistance, PCR, nucleotide sequencing.

التحري الجزيئي لبعض جينات بيتا لاكتاميز بواسطة تقنيات تفاعل البلمرة المتسلسل وتسلسل الحمض النووي في الإيشريشيا القولونية السريرية.

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الخلاصة

في هذه الدراسة من بين **50** عزلة من بعض عدوى المستشفيات من بعض مستشفيات بغداد ، تم التعرف على **13** (**26**٪) عزلة على أنها ***Escherichia coli*** بالاعتماد على الوسائط الانتقائية والاختبارات المورفولوجية والكيميائية الحيوية ثم تم تأكيدها بالطرق الجزيئية. تم إجراء فحص مقاومة مضادات الميكروبات بطريقة كيربي باور، وتم التوصيف الجزيئي لجينات ***bla*TEM** و ***bla*CTX-M** في عزلات إكلينيكية مختلفة للإشريشيا القولونية من خلال تفاعل البلمرة المتسلسل (**PCR**) عن طريق استخدام بادئات خاصة. كانت هذه الجينات موجبة فقط لـ **4** عزلات (**30.7**٪). تم إجراء تسلسل النيوكليوتيدات للجينات الإيجابية لأربع عزلات. أظهرت النتائج عدم وجود تباين في تسلسل النوكليوتيدات بين العزلات العراقية مقارنة بالعزلات العالمية ،

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وكانت مطابقة بنسبة**100**٪ للعديد من أجناس البكتيريا المعوية. أخيرًا ، من المحتمل أن تكون هذه الجينات في السلالات البشرية مصدرًا لمقاومة الأدوية على نطاق واسع بسبب الاستخدام الخاطئ للمضادات الحيوية.

Introduction

Beta-Lactamase resistance among Enterobacteriaceae, especially in *E. coli*, is an emerging problem worldwide. In the developing world where apart from the high levels of deficiency lack of knowledge, due to poor health treatments, as well as the spread of poor-quality medicines that are unfit for the body health, gives rise to antibiotoic resistance which has become a serious health issue [1]. E*scherichia coli* is considered to be a multi-drug-resistant pathogen. which has led to the emergence of new strains and is accompanied in Iraq by the lack of molecular studies that can help us understand the mechanism of the disease, its causes and ways to prevent its spread [2 & 3].

The synthesis of enzymes called extended spectrum beta-lactamases (ESBLs) in gram negative bacteria contributes to the multi resistance [4, 5]. Enterobacteriaceae family produces RNA that hydrolyzes the cephalosporin and monobactam species, thus increasing their resistance to antibiotics. Pathogenic bacteria from different sources, like industry, soil and humans, can fuse to hydrous habitants, which causes the high increment in the antibiotic resistance through some genetic mechanism such as transposons and integrons, besides plasmids. In a normal bacterial ecosystem this mutation in bacteria can cause the transformation of bacteria from non-pathogenic to resistant toward antibiotics [6]. Our study aimed to determine the prevalence of some beta lactamase genes (*bla*TEM, and *bla*CTX-M) type ESBL variants among *E. coli* which were collected from some clinical antibiotics resistant isolates. Also, determining the genetic variations of these genes by the nucleotide gene sequence technique to provide beneficial notifications around the epidemiological and evolutional changes of ESBLs in bacteria**.**

Materials and Methods:

Collection of isolates:

Fifty clinical samples were gathered from patients infected with different cases, including urinary tract infections (UTIs) and diarrhea contagions from some hospitals in Baghdad. Samples were collected according to the instructions of the scientific ethics.

Bacterial Isolation and Identification:

All specimens were inoculated on eosin methylene blue and MacConkey agar (Oxoid, UK). The plates were incubated at 37°C for 24 hrs. [7]. Biochemical assays, for pure isolates, were identified through catalase, oxidase and IMViC tests. Next, the confirmation of identified isolates was performed via PCR amplification of *dinB* gene [8].

Antibiotics Susceptibility Test

Antibiotic susceptibility test of various classes of antibiotics (Table 1) was carried out for *E. coli* isolates by disk diffusion method (Kirby-Bauer method), according to Bauer *et al*. in 1966 [9] and Clinical Laboratory Standard Institute recommendations (CLSI, 2021) [10].

Table 1: Antibiotic discs and its concentrations

Classes of Antibiotic	Antibiotics	Disc Concentration (µg)
B-Lactam / (B-Lactamase)	Amoxicillin / Clavulanic acid	75/10
Cephalosporins	Cefotaxime	30
	Cefepime	30
Monobactams	Aztreonam	30
Carbanems	Imipenem	10
Aminoglycosides Aminoglycosides	Gentamicin	10
	Amikacin	30
Macrolides	Azithromycin	15
Quinolones	Ciprofloxcin	5
Tetracyclines	Tetracycline	30

Molecular Detection of Gsenes

Wizard Genomic DNA Purification Kit (Promega, USA) was used to extract DNA from bacterial isolates according to the information of the manufacturing company. The concentrations and purity of DNA were measured using Nanodrop (BioDrop, UK) [11].

Detection of *blaTEM* and *blaCTX-M* Genes:

Determination of *bla*TEM, and *bla*CTX-M genes was done using the primer pair mentioned in Table 2 that was supplied by Macrogen, Korea. Polymerase chain reaction (PCR) was performed to detect the presence of the previous genes using Thermal Cycler (BioRad, USA), according to Kim *et al.* [13]. The amplification was accomplished according to experiment conditions of Shehab *et al.* [11]. The annealing temperature is mentioned in Table 2.

Table 2: Primers used for amplification of genes

Genes	Primer Sequence (5`→3`)	Product Size (bp)	Annealing Temp.	Reference
dinB-f dinB-r	5`-TGAGAGGTGAGCAATGCGTA-3` 5`-CGTAGCCCCATCGCTTCCAG-3`	606	55	(8)
blaTEM -f blaTEM -r	5`-TTTTCGTGTCGCCCTTATTCC-3` 5`-CGTTCATCCATAGTTGCCTGACTC-3`	779	57	New designed in this study
blaCTX –M-f blaCTX- M--r	5`-CGCTGTTGTTAGGAAGTGTG-3` 5`-GGCTGGGTGAAGTAAGTGAC-3`	754	60	New designed in this study

Abbreviations: f, forward primer; r, Reverse primer.

Gel Electrophoresis:

Agarose gel electrophoresis was performed to confirm the amplification. The PCR products were worked on 1% agarose gel soiled with 0.5 µg/ml ethidium bromides in 1X TAE buffer. DNA ladder (100-1500 bp) (Promega, USA) was utilized as a mark of the size of DNA bands. Then, the products were loaded into 1% agarose gel for 65 min at 1watt, spotted with ethidium bromide and detected by UV trans illuminator 320 nm UV light [12].

Standard Sequencing

PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation – Korea. The results were received by email and were then analyzed using Geneious software. The sequenced DNA was analyzed by the BLAST tool of the NCBI Gene Bank database which is available at the NCBI online at (http:// www.ncbi.nlm.nih.gov).

Results and Discussion:

Bacterial Isolation and Identification

Fifty clinical isolates (urine and stool), collected from hospitals of Baghdad, were examined for *E. coli* antibiotic sensitivity. Among of all tested isolates, 13 proved to be *E. coli* by standard laboratory methods. Only 8 (61.5%) isolates were from urine and 5 (38.4%) from stool, depending on the culture on MacConkey and EMB agar and by performing specific some biochemical test catalase, oxidase and IMViC tests. It followed genotypic identification of bacterial isolates by utilization of housekeeping gene which consider as a confirmatory test to provide a fast diagnostic consistency of bacteria using *dinB* gene and reported a positive result for all isolates. The standard phenotypic methods required several days and had some limitations; genotypic revelation depending on sure housekeeping genes was used as a confirmatory test which provided fast diagnostic consistency of bacteria [13].

Antibiotics Susceptibility Pattern

The antibiogram resistance against the 10 antibiotic (Table 1) by the disc diffusion method showed resistance of all isolates to the various β-Lactam / (β-Lactamase) and cephalosporin antibiotics. So, altogether isolates were resistant to amoxicillin, clavulanic acid and cefepime. The subsequent less active antibiotics were cefotaxime and aztreonam which was exhibited by 11 84.6% of the isolates and were followed by tetracycline with 7 (53.8%) isolates. Whereas ciprofloxacin and azithromycin had 6 (46.1%) isolates. Imipenem was the best antibiotic because all isolates were sensitive to it which was followed by gentamycin and amikacin, meaning that carbanems and aminoglycosides were more effective classes of antibiotics against isolates under study (Table 3).

Table 3: Antibacterial resistance of *E. coli* isolates.

Antibiotic	Mean diameter of inhibition zone for sensitive, intermediate isolates(mm)	Resistant Isolates No. (%)
Amoxicillin / Clavulanic acid	0	13 (100)
Cefotaxime	30	11 (84.6)
Cefepime	0	13(100)
Aztreonam	27	11 (84.6)
Imipenem	31.3	0 (0)
Gentamicin	17.8	2 (15.3)
Amikacin	17	3(23)
Azithromycin	22	6 (46.1)
Ciprofloxacin	31	6 (46.1)
Tetracycline	23	7 (53.8)

The wide spread of third generation cephalosporin resistant indicates a high use of this type of antibiotics. This increase of resistance is worrying as the random use of antibiotics might be the factor of such increase in resistance.

Prevalence of Some Antibiotic Resistance Genes

PCR screening virulence genes of *E. coli* correlates with antibiotic resistance. To establish effective preventive and curative measures, clinical findings and patterns of infection can be useful in in-hospital patients with positive *E. coli* cultures. The frequencies of occurrence of virulence genes in all studied strains (n=13) were as follows. Figure 1 shows positive agarose gel electrophoresis results for *bla*TEM and *bla*CTX-M products (amplified). The results of 13 isolates showed production of *bla*TEM gene 4 (30.7) % and same for the *bla*CTX-M gene. The results of other research were different from our results, such as Hasan Ejaz *et al*. [14]. Our results, however, agree with those of Hano in Baghdad and Dawood in Diyala, Iraq [15, 16].

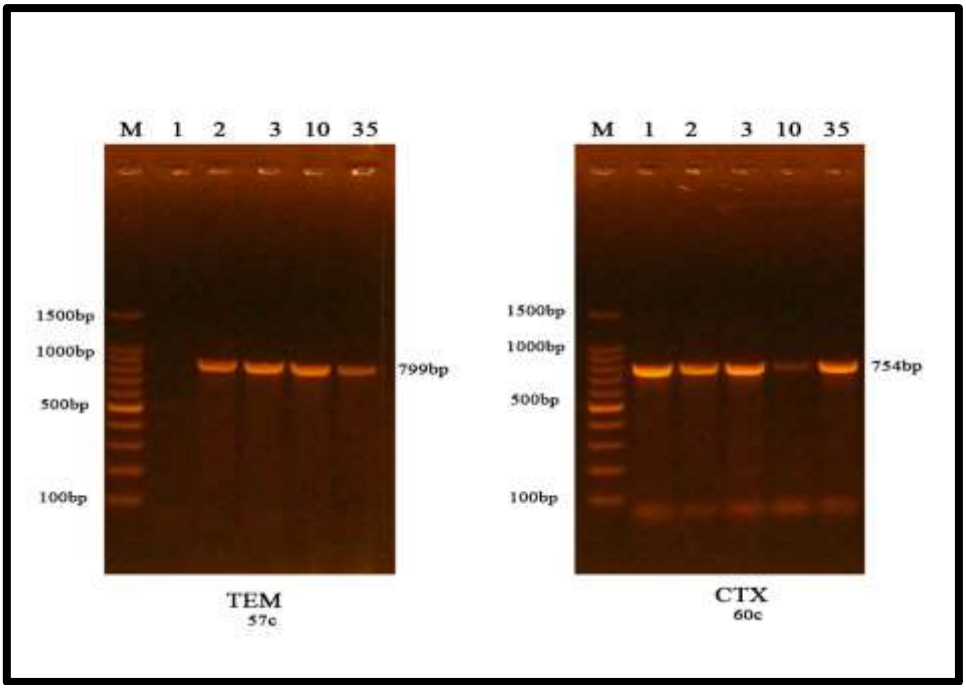

Figure 1: Agarose gel electrophoresis of amplified genes. A 100 bp DNA ladder (M) was used to proximate the gene sizes in agarose gel electrophoresis. The amplification of *bla*TEM was positive for isolates code No. 2, 3, 10 and 35 lanes and *bla*CTX-M was positive for isolates code No. 1, 2, 3, and 35 lanes.

Nucleotide sequence of amplified *bla*TEM and *bla*CTX-M genes

To confirm the identity of *bla*TEM (779 bp) and *bla*CTX-M (754bp) genes detected by PCR for only four isolates from each positive gene which have resistance to more than three classes of the antibiotic under study and to conclude any possible new alleles. The results were analyzed that are ready on the NCBI website to determine nucleotide numbers discovering the mutation numbers and types. And then study our strains sequencing was compared with the original sequence of genes for the global strains of *E. coli* from various parts of the world, as explained in Figures 2 and 3. All these sequences were analyzed by Geneious software. The results showed the absolute identity of all our strains with many global strains with different accession numbers of *E. coli, Klebsiella aerogenes, Enterobacter asburiae, Enterobacter bugandensis* and *Aeromonas sp.* for the *bla*TEM-M gene. While, *bla*CTX gene possesses identical sequences with *E. coli, Klebsiella pneumonia, Enterobacter colaca* and *Enterobacter bugandensis* with percentage that reached 100% for these genes for chromosome and plasmid complete sequences with significant alignments by blast on NCBI (http://www.ncbi.nlm.nih.gov).The results indicate a similarity of resistance for many genera

of Enterobacteriaceae family for beta-lactam genes because they are present in the same environment and are exposed to the same concentrations and types of antibiotics.

Figure 2: Significant alignments of *bla*TEM-M gene by blast program for isolates code No. 2, 3, 10 and 35 compared with sequences available in the Gene Bank.

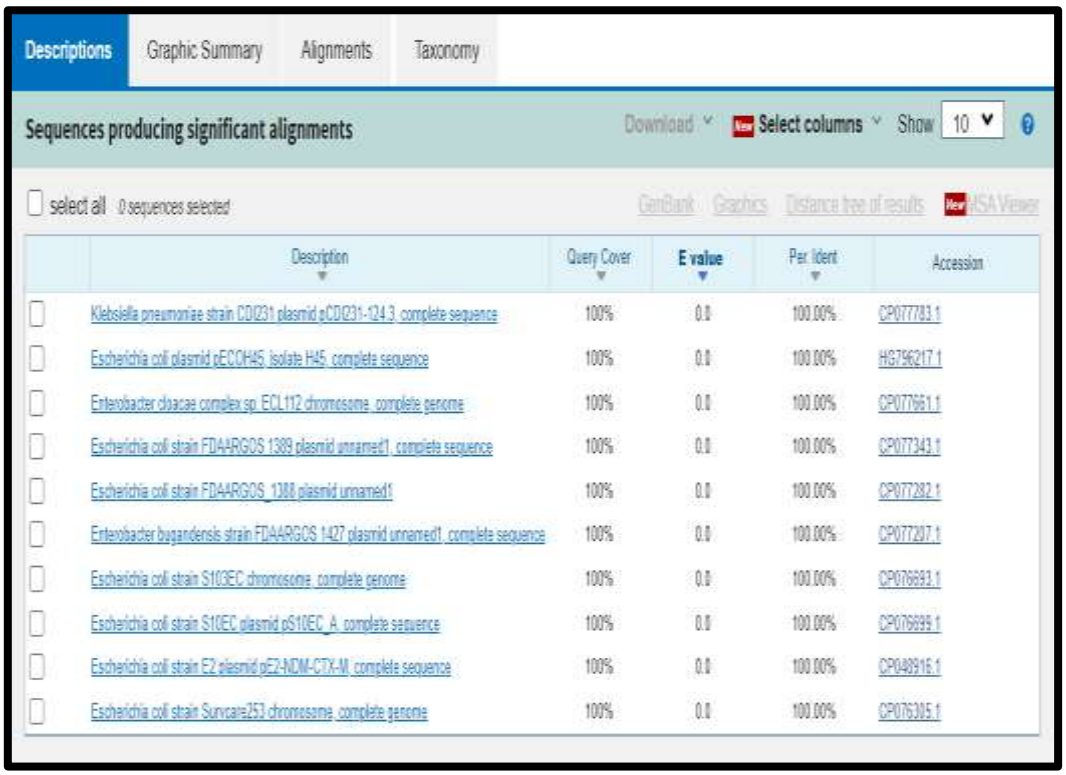

Figure 3: Significant alignments of *bla*CTX gene by blast program for isolates code No. 1, 2, 3, and 35 compared with sequences available in the Gene Bank.

In conclusion, the prevalence of *bla*CTX and *bla*TEM-M genes among *E. coli* isolates may contribute to high resistance to multiple antimicrobial agents, also for many genera of

Enterobacteriaceae leading to a rise of Multi-Drug Resistance phenotype, due to the use of plentiful antibiotics, though many of them without the requirement, consumption of antibiotics when not prescribed by the physician, use of antibiotics in animal fodder, and the molecular methods that are not possible to carry out routinely in the laboratories of developing countries. Some extra-efforts such as PCR should be carried out for the correct identification of the genes involved in antibiotic resistance [17]. Treating of the Enterobacteriaceae family, including *E. coli*, is growingly difficult via appearance of resistant strains to most first-line antibiotics. Where the ESBL enzymes break down and destroy some commonly used antibiotics, including penicillin and cephalosporin, and make these drugs ineffective for treating infections [18]. Resistance is important in intestinal organisms as it leads to the failure in treating infections caused by pathogenic bacterial strains. Gut contains many diverse and competing organisms with complex resistance in which gene transfer can occur horizontally in the strains of Enteropathogenic *E. coli* (EPEC). Researchers have documented a significant prevalence of antibiotic resistance worldwide [15, 19]. We recommend carrying out susceptibility tests before prescribing any antibiotic in order to prevent the growing trend of resistance against beta-lactam drugs.

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