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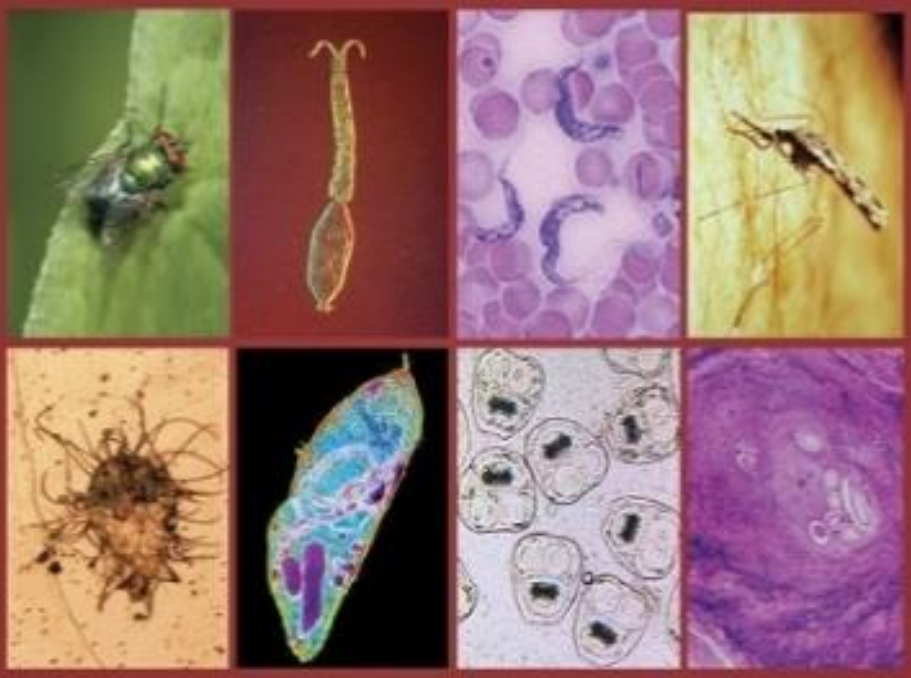

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Molecular Characterization of *Escherichia coli* Isolated from Children Who Have Acute Diarrhea in Different Iraqi Kurdistan Hospitals

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ABSTRACT

Escherichia coli (*E. coli*) is one of the most frequent opportunistic pathogens. We aimed to carry out this study on antimicrobial resistance pattern and phylogenetic background of *E. coli* isolated from children who have diarrhea in different hospitals of the Iraqi Kurdistan region. A total of 200 fecal samples of children (52.5% male and 47.5% female) were included. Stool samples were cultured on selective media to isolate *E. coli* bacteria. The antibiotic susceptibility testing and molecular characterization of the isolates were carried out. Antimicrobial susceptibility testing was performed by disc diffusion test disc PCR was used to detect five antimicrobial resistance genes coding for β-lactamases (*bla*TEM, and *bla*CTX, *bla*OXA, *bla*SHV, *bla*CMY) to study phylogenetic grouping of *E. coli*. The *Escherichia coli* were highly resistant to β-lactam antibiotics, and the highest resistance was seen to Cefotaxime (45.4%). According to the phylogenetic grouping of *E. coli* isolates, group B2 was the dominant group and *bla*OXAgene (57.5%) was the predominant resistance genes among *E. coli* isolates. This study demonstrated the clinical concern of *E. coli* in children. The *E. coli* are highly resistance to common beta-lactam antibiotics and there are high rates of the resistance genes among *E. coli* isolates in children. An antibiotic surveillance would be useful continuously control and prevention of infectious diarrhea in children.

INTRODUCTION

Diarrheal diseases are a worldwide public health problem causing considerable illness and death among infants and children. *Escherichia coli* is found everywhere in the human gastrointestinal tract, can cause disease via multiple mechanisms, and exhibit wide genetic variation within disease phenotypes. (Laupland, 2003). Diarrheagenic *E. coli* strains were among the first pathogens for which molecular diagnostic methods were developed. The molecular methods remain the most popular and reliable techniques for differentiating pathogenic strains from nonpathogenic members of the stool flora and distinguishing one category from another.

Substantial progress has been made in the development of nucleic acid-based probe technologies as well as PCR methods (Nataro&Kaper, 1998). With the increase in data transmission speed of sequencing technology, it became possible to sequence several isolates of any type or pathological type, as it became easier to study and distinguishes those (James *et al.*, 2010).

The identification of *E. coli* is necessary the use of molecular methods for the detection of the genes responsible for pathogenicity. This is essential for the identification of pathogenic *E.coli* and is based on the presence of different chromosomal virulence genes that are absent in commensal *E. coli* (Nguyen *et al.*, 2005).

Moreover, the prevalence and other epidemiological features of these pathogens as causative agents of diarrhea vary from one region to the other, and even between and within countries in the same geographical area (Nataro, 2006). Alarming progressive increases have been documented recently in both the incidence of *E. coli*, and the prevalence of antimicrobial resistance. Such a phenomenon also could contribute to the adverse clinical outcomes observed among patients with *E. coli* (MacArthur *et al*., 2004).

The present study aimed to isolate, identify and determine the prevalence, and to study resistance patterns of *Escherichia coli* among children with acute diarrhea in different hospitals in the Kurdistan Region of Iraq.

MATERIALS AND METHODS

Study Population:

From September 2021 to December 2021, samples were consecutively collected from patients whose guardians had agreed to participate in the study. Samples were collected from (200) children patients suffered with diarrhea from different hospitals in the Kurdistan region, (Jamal Rashid Teaching Hospital / Sulaimani, Raparin Teaching Hospital / Erbil, Lalla Private Hospital / Erbil). The study included (105 Male) and (95 Females). All patients were children aged between (1 month to 12 years) suffering from acute diarrhea which is defined as the passage of three or more loose or watery stools per day.

Specimen Collection, Isolation, and Identification of *E. coli*:

A total of 200 stool samples were included in the study. One stool sample was taken from each patient in a sterile container and transported to the microbiology laboratory for bacteriological examination. The samples were examined for consistency, presence of blood, and mucous. The samples were inoculated into MacConkey broth for enrichment at 37 °C for 24 h. After being streaked onto MacConkey agar and incubated for 24 h at 37 °C. After that, lactose ferment in isolates were sub-cultured onto Eosin methylene blue agar (EMB) which is a differential microbiological medium for *E. coli*. Colonies producing greenish metallic sheen on EMB agar was examined by biochemical tests including; IMViC (indole, methyl red, Voges-Proskauer, citrate utilization). Samples containing *E. coli* only were included. After that, *E. coli* was sub cultured again to prepare it for other experiments such as antibacterial susceptibility test and DNA extraction to phylogenetic grouping and genetic tests.

DNA Extraction and Multiplex PCR:

For crude DNA extraction, a typical fresh colony was suspended in 150 ul of distilled water and the mixture was boiled at 100°C for 15 minutes. The boiled mixture was centrifuged at 10,000 RMP and the supernatant containing the DNA template was used for Polymerase Chain Reaction (PCR). The DNA amplification method was performed according to the protocol described by (Heijnen, 2006) as well as by (Chen and Griffiths, 1998).

Antimicrobial Susceptibility Testing: An antibacterial susceptibility test was

performed according to the Kirby-Bauer method (disc diffusion test method) on Mueller-Hinton agar (Modified Kirby-Bauer method: National Committee for Clinical Laboratory Standards, NCCLS, 2016).

Procedure: After completely swabbing the plate of Mueller-Hinton agar with bacteria (*E. coli*), we let the surface dry for 5 minutes before placing antibiotic tablets, five different antibiotics (Table 1). After 24 hours of incubation, we used a metric ruler to measure the area of inhibition and include the diameter of the area of inhibition measured around the disc, and the results were interpreted according to (Clinical Laboratory Standard Institute (CLSI) guidelines 2012).

The antibiotic discs used in this study were: amoxicillin-clavulanic acid (AMC 30 µg), meropenem (MRP 10 µg), Ceftazidime (CAZ 30 µg), cefotaxime (CTX 30 µg), and Piperacillin/tazobactam (PIT 30 µg).

Table -1- Antibiotic used in the study.

Antibiotic class	Name of antibiotic	Disk code	Ratio
Beta-lactamase inhibitors	Amoxicillin-clavulanate	AMC	30 µg
Carbapenems	Meropenem	MRP	10 µg
Cephalosporins	Ceftazidime	CAZ	30 µg
Cephalosporins	Cefotaxime	CTX	30 µg
Beta-lactamase inhibitors	Piperacillin/tazobactam	PIT	30 µg

Phylogenetic grouping of *E. coli*

Phylogenetic analysis has shown that E. coli strains fall into four main groups (A, B1, B2 and D) Chakraborty *et al.*, 2015). A single-plex PCR was performed to determine the phylogenetic grouping of E. coli according to (Bonacorsi et al., 2000) with some modifications using conventional PCR. Three genes of E. coli, the ChuA, yjaA, and DNA fragment TSPE4.C2 were amplified and a combination of these genes in every strain was used to indicate the phylogenetic grouping of each E. coli isolates. Due to the small size of the genes, PCR products were resolved on 2% DNA agarose gel and the gel was visualized by blue light illumination using Smart Doc 2.0 Imaging System. The primer pairs used for PCR amplification are shown in (Table 2).

Table- 2- The Primer Sequence of Genes for Determination of *Escherichia coli* Phylogenetic Grouping.

Primer name	Direction	Sequence	Size of the Gene in bp
ChuA	Forward	GAC GAA CCA ACG GTC AGG AT	300
ChuA	Reverse	TGC CGC CAG TAC CAA AGA CA	300
YjaA	Forward	TGAAGTGTCAGGAGACGCTG	250
YjaA	Reverse	ATG GAG AAT GCG TTC CTC AAC	250
TspE4.c2	Forward	GAG TAA TGT CGG GGC ATT CA	200
TspE4.c2	Reverse	CGC GCC AAC AAA GTA TTA CG	200

Polymerase Chain Reaction of β-lactamase Resistance Genes:

Five common extended spectrum β-lactamase resistance genes (*blaTEM-1* (1080 bp), *bla*CMY (1000 bp), *bla*Shv (800 bp), *bla*Oxa (610 bp), and *bla*CTX (550 bp)) were PCR amplified using two multiplex PCR. One multiplex was for group *1, blaTEM-1 bla*CMY, and the second PCR reaction was for group 2, *bla*Shv, *bla*Oxa, and *bla*CTX (Table 3).

In this study section, the protocol and all used primers were performed according to (Ahmed*et al*., 2007) with some modification. The final product was visualized under blue light after resolving

on 1% DNA agarose gel using Smart Doc 2.0 Imaging System.

The blast search of the sequences was carried out in NCBI BLASTn search tool (http://www.ncbi. nlm.nih.gov/) to compare them with similar gene sequences available online. A phylogenic tree was made for the local sequences and retrieved sequences of GenBank by the neighbor-joining (NJ) method.

Table 3: List of primer sequences used in this study.

Name	Direction	Sequence	Size of the Gene in bp
*usp*A	Forward	CCGATACGCTGCCAATCAGT	900
*usp*A	Reverse	ACGCAGACCGTAGGCCAGAT	
*uid*A	Forward	TGGTAATTACCGACGAAAACGGC	140
*uid*A	Reverse	ACGCGTGGTTACAGTCTTGCG	
*bla*TEM	Forward	ATAAAATTCTTGAAGACGAAA	1080
*bla*TEM	Reverse	GACAGTTACCAATGCTTAATC	
*bla*CMY	Forward	GACAGCCTCTTTCTCCACA	1000
*bla*CMY	Reverse	TGGAACGAAGGCTACGTA	
*bla*Shv	Forward	TT ATCTCCCTGTTAGCCACC	800
*bla*Shv	Reverse	GATTTGCTGATTTCGCTCGG	
*bla*Oxa	Forward	TCAACTTTCAAGATCGCA	610
*bla*Oxa	Reverse	GTGTGTTTAGAATGGTGA	
*bla*CTX	Forward	CGCTTTGCGATGTGCAG	550
*bla*CTX	Reverse	ACCGCGATATCGTTGGT	
16S-23SITSD	Forward	ATTTGAAGAGGTTGCAAACGAT	130
16S-23SITSD	Reverse	TTCACTCTGAAGTTTTCTTGTGTTTC	

RESULTS

A total of 200 samples from children with diarrhea were included for stool examination, and the most common reason for stool testing is to identify and characterize of *E.coli* because our study is on *E.coli*. If the intestine becomes infected with harmful microorganisms, it can cause problems such as certain types of diarrhea, stool testing can help find out the cause, but to accurately identify the organism, it must be prepared for further tests, and bacteria must be grown in special cultures prepared for this purpose.

Antibiotic Susceptibility Testing of *E. coli*:

Standard laboratory tests included the Disc Diffusion Method was used to measure the antibiotic susceptibility testing. The aim of this test was to investigate the prevalence of resistant *Escherichia coli* to different antibiotic. The diameter of the inhibition zone was measured around the disc, and the interpretation of the results was amoxicillin-clavulanic acid (AMC 30 µg - 3%), meropenem (MRP 10 µg – 5%), Ceftazidime (CAZ 30 µg – 37.3%), cefotaxime (CTX 30 µg – 45.4%), and Piperacillin/tazobactam (PIT 30 µg – 10.1%). It can be noted that Cefotaxime (54.4%) more Resistance than others, which may be caused by its frequent use and high percentages (Fig. 1.).

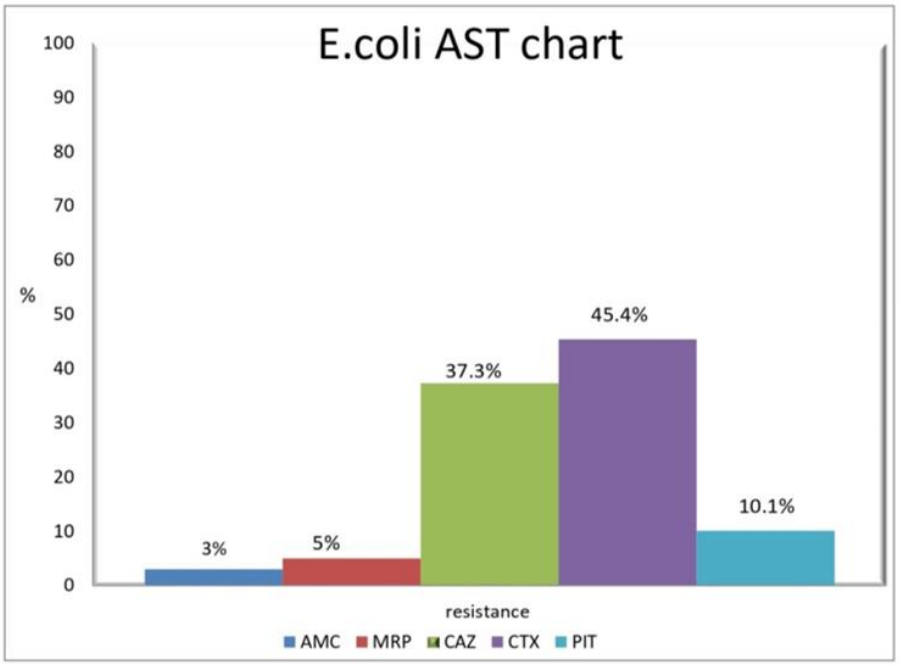

Fig. 1- Antibiotic resistance pattern of *E. coli* antibiotics.

Classification of *E. coli* According to Phylogenetic Grouping:

The phylogenetic grouping of *E. coli* strains was made to four main groups: A, B1, B2 and D. Nonpathogenic strains of *E. coli* usually belong to group A and B1 (Saralaya*et al*., 2015). E. coli strains located in group B2 and to lesser extent group D are usually pathogenic and virulent extra-intestinal strains (Bonacorsi *et al*., 2000).

All the *E. coli* isolates were classified into different phylogenetic grouping of *E. coli* based on the existing and combination of different yjaA, chuA, and TSPE4.C2 genes. Group B2 was the dominant group, with 45.45% followed by group D with, 37.37% and then group A, 11.11%. Group B1 was the least with just 6.1% isolates (6.1%) (Figs. 2 &3).

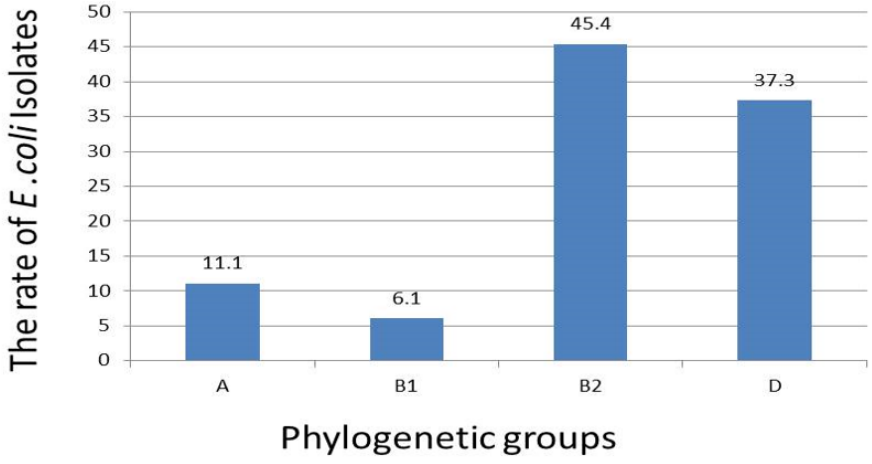

Fig. 2: phylogenetic groups of *E. coli***.**

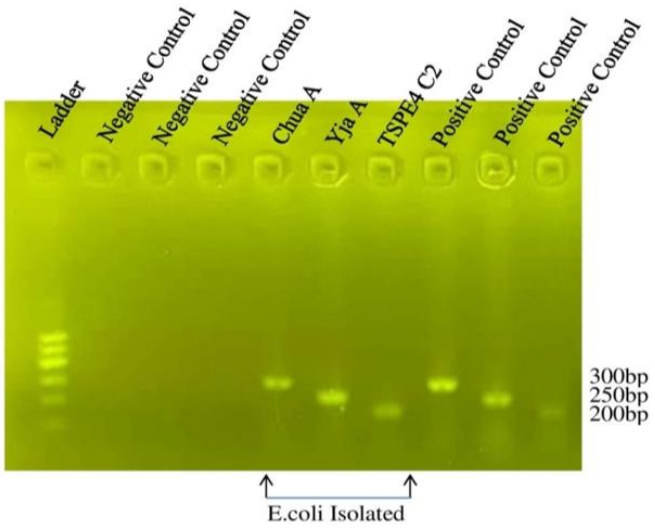

Fig. 3: Phylogenetic grouping based on the different combination of chuaA, yjaA and TspE.C2 genes.

The Rate of Resistance Genes, *bla*TEM, and *bla*CTX, *bla*OXA, *bla*SHV, *bla*CMY, in *E. coli* Isolated from Diarrheic Children:

The class of beta-lactamase enzymes is mostly seen in *E. coli* and *Salmonella* enteric, but they also exist in other members of bacteria (Woodford *et al*., 2004; Hudson *et al*., 2014). In this study, a set of primers was used to amplify each of the five common beta lactamase resistance genes, *bla*TEM, and *bla*CTX, *bla*OXA, *bla*SHV, *bla*CMY, in the study area (Fig. 4). The rate of the beta-lactam resistance genes in the area of the study was clarified in samples (**Fig. 5**), the 57.5% of isolates were positive for the beta lactamase resistance genes, *bla*OXA which the most common resistance genes in this study. It followed by *bla*CTX at the rate of 56.5%. The rate of *bla*OXA and *bla*CTX was remarkably higher than other resistance genes. The lowest number isolates carrying resistance genes was for *bla*TEM at the rate of 4% and the rate of *bla*SHV and *bla*CMY was 8% and 14.1%, respectively.

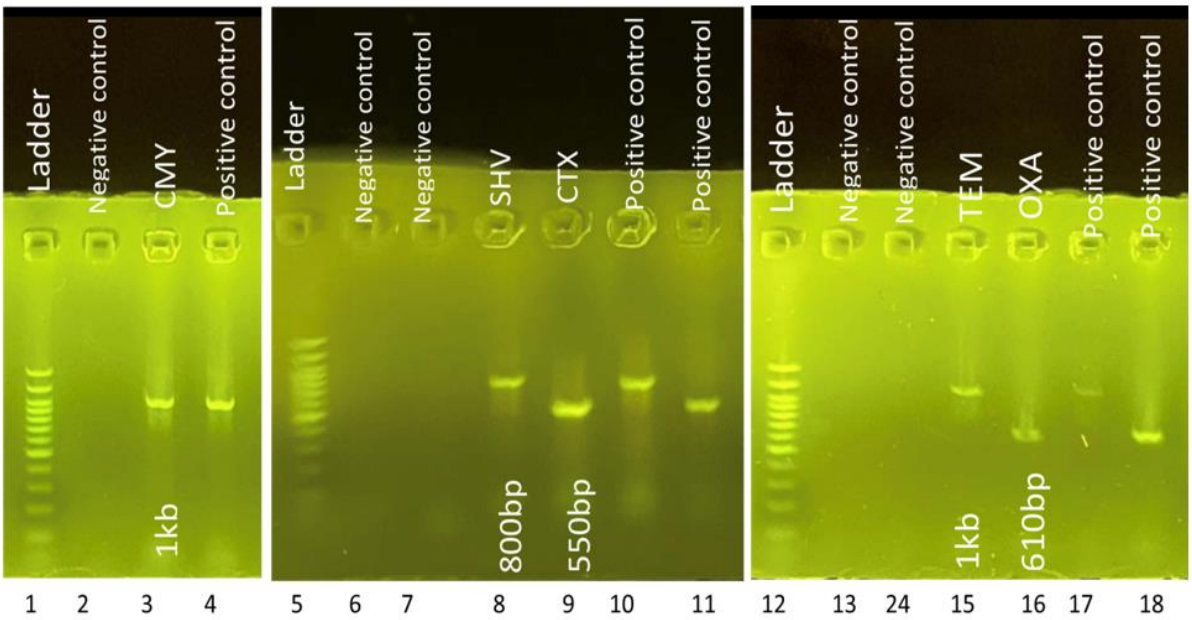

Fig. 4: PCR amplification of different beta lactam resistance genes in *E. coli* isolates. PCR amplification of resistance genes, *bla*TEM, and *bla*CTX, *bla*OXA, *bla*SHV, *bla* CMY

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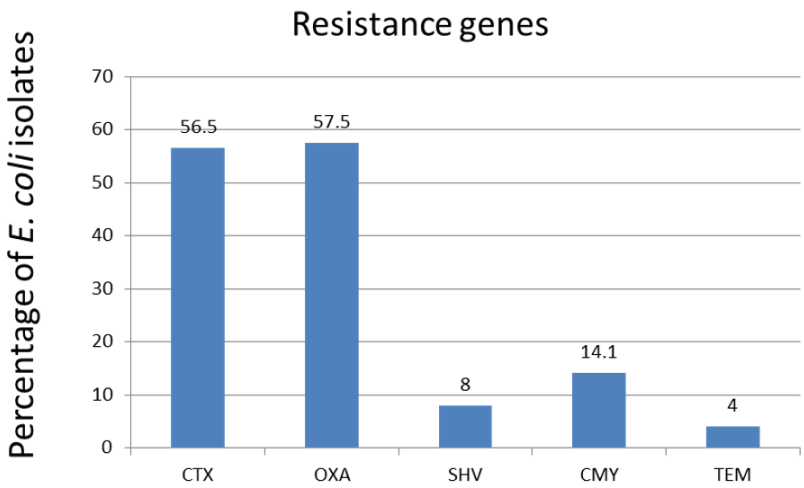

Fig. 5: The rate of different beta lactam resistance genes in *E. coli* isolates.

Sequence Analysis of *bla*TEM:

*bla*TEM gene isolated from *E. coli* in this study were sequenced using sanger sequencing. The results of the sequence of *bla*TEM genes isolated from E. coli in comparison to each other were aligned together (Fig. 6). The results showed that all the sequences of *bla*TEM genes isolated from local *E. coli* isolates and different species of bacteria in different countries were similar to one another with no mutation observed.

The *bla*TEM gene sequences of *E. coli* was also compared to the sequences retrieved from GenBank using BLAST nucleotide search and NCBI nucleotide. The sequence has similarity to available sequences found in the database from other countries. But its different form *bla*TEM isolated from two countries, Taiwan and France in two species of bacteria and it can be seen in phylogenetic tree made in this study (Fig. 7).

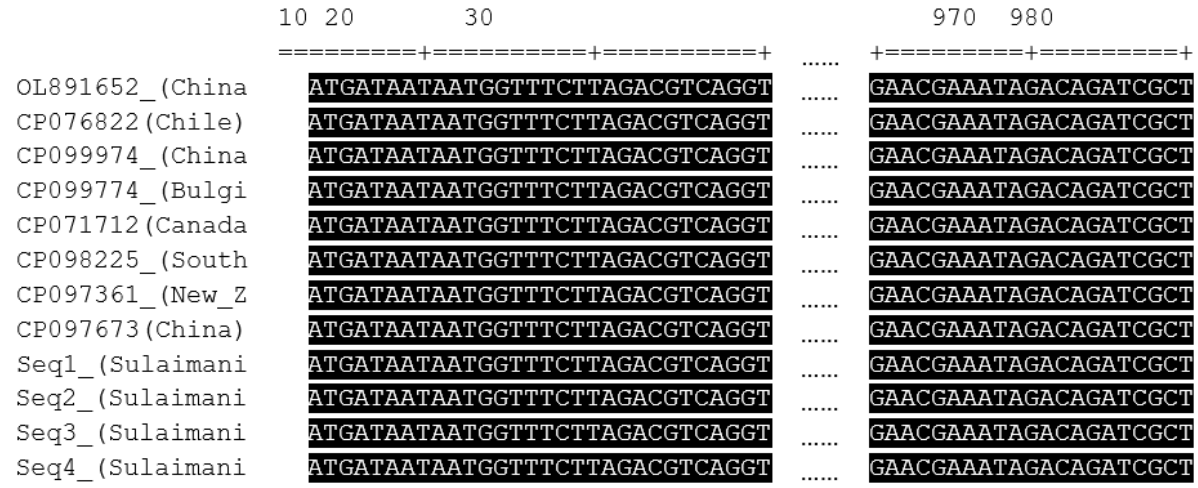

Fig. 6: Multiple sequence alignment of four *bla*TEM genes isolated from four isolates of *E. coli*. The Sequences were aligned with international sequences of *bla*TEM retrieved from NCBI nucleotide using Phylogrny.fr. New number of positions: 985 (selected positions are underlined in blue)

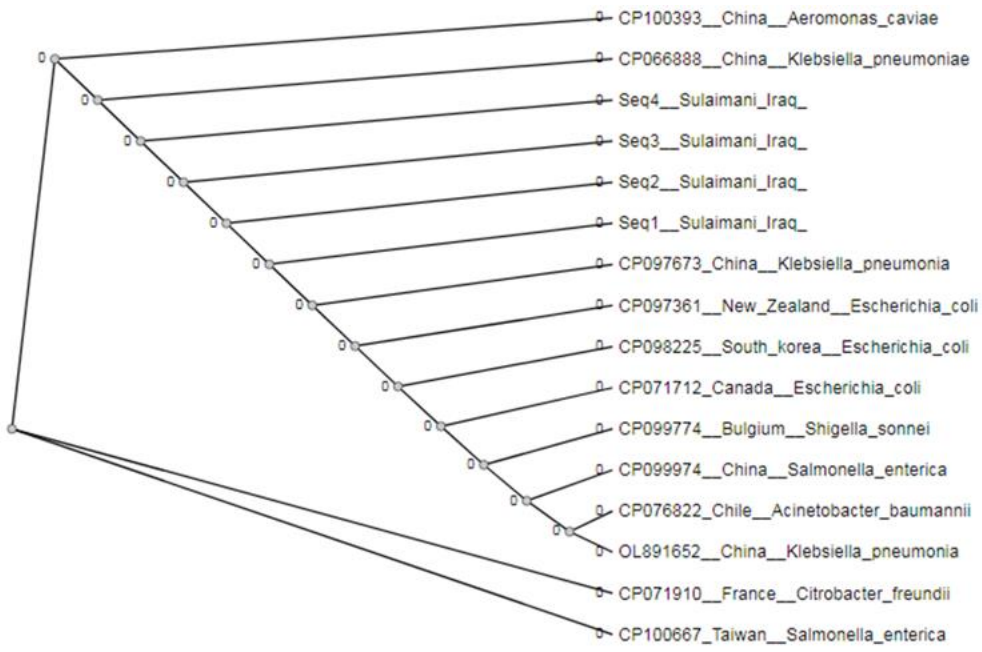

Fig.7: Phylogenetic tree of four *bla*TEM genes isolated from four isolates of *E. coli.* The Sequences was built with international sequences of *bla*TEM retrieved from NCBI nucleotide using Phylogrny.fr.

DISCUSSION

Diarrhea will remain the main life-threatening problem for children, because of the passage of liquid or more frequent stools (watery), more than three times in a day more than 200g/day, with a lack of self-restraint (Valerie and Nicholas, 2021) Most cases are due to ingestion of contaminated food or water with bacteria or its toxin. The most important step in the detection of stool abnormalities and intestinal problems is a microscopic examination of the stool.

E. coli bacteria were chosen as a common opportunistic bacterium in human GIT (Jonathan and Seth, 2020) to characterize antibiotic sensitivity testing. Overall resistance to commonly used antibiotics (amoxicillin-clavulanic acid, meropenem, Ceftazidime, cefotaxime, and Piperacillin/tazobactam) against *E. coli* bacterial isolates was analyzed in this study.

The treatment with antibiotics can lead to shorter disease period in children with diarrhea (Elizabeth *et al.,* 2015). In this study, most antibiotic resistance to *E. coli* was cefotaxime 45.4%, Ceftazidime 37.3 %, close to, (Miad, 2016) reported that most antibiotic resistance was observed to cefotaxime 37.5%, Ceftazidime 30%, And here is a rise in antibiotic resistance to (Zahraa, 2019) where it was ceftazidime،76% and cefotaxime74%.

All *E. coli* isolates were subjected to phylogenetic grouping analysis, based on the existence of one or more genes of the chuA, yjaA and DNA fragment TSPE4.C2, *E. coli* is classified under phylogenetic groups. Most of isolates located at groups B2 and D which is an indicator of pathogenic strains of *E. coli*. Most *E. coli* isolates have β-lactamase resistance genes that are also discovered in *E. coli* phylogenetic groups which are clinically important because most pathogenic *E. coli* and extra intestinal isolates come from these phylogenetic groups, which are similar to (Taib*et al*., 2021).

The result of this study showed a high percentage of fecal *E. coli* bacteria carrying antibiotic resistance genes, and the highest percentage of antibiotic resistance genes was for *bla*OXA which is 57.5%. The lowest percentage of resistance genes was

*bla*TEM.A number of studies have also reported that ESBL resistance genes are harbored by *E. coli* strains from food-producing animals, meat products (Carattoli, 2008).The proportion of ESBL resistant gene determinants reported among bacteria isolates may be dependent on differences in geographical locations, the sensitivity of the methods used to either isolate bacteria or detect resistance genes as well as the source and nature of specimens analyzed (Kotsoana*et al.*, 2019).

According to the phylogenetic grouping of *E. coli* isolates, group B2 (45.45%) was the dominant group followed by group D with, and then group A, and Group B1 was the lower.

(Duriez, 2001) who observed that group B2 was rare among commensal isolates while groups A and B1 were the most common, and this does not agree with our result. The group of *E. coli*isolates varies greatly depending on their regions of occurrence and coexistence (Duriez*et al*., 2001).

Although not absolute, some preferential associations exist between certain phylogenetic groups and pathovars. As an example, extraintestinal pathogenic *E. coli* (ExPEC) strains belong more frequently to the B2 and D phylogroups, while enterohaemorrhagic *E. coli* (EHEC) strains most often belong to the E group, but also belong to B1 and A groups (Perna, 2011)

The branching pattern of the phylogenetic tree reflects how other species or groups evolved from a series of common ancestors and graphs represent evolutionary relationships among microorganisms (Rodrigo,2009).We infer from the tree a common relationship between *Escherichia coli* in deferent country and they have common genes and thus a common characteristic. The sequence of *bla*TEM in the study area is intact and it doesn't contain any mutations. Therefore, it's clustered with the same gene sequence in different countries. But its different form *bla*TEM isolated from two countries, Taiwan and France in two species of bacteria and it can be seen in phylogenetic tree made in this study.

CONCLUSION

We found from this study that E. coli had a higher resistance to cefotaxime than other antibiotics used in the study, and it was found that the dominant group for the development of E. coli is the B2 group. As for the rate of beta-lactamase resistance, blaOXA was the most common resistance gene. Finally, it was found from the Phylogenetic tree of E. coli that no mutation was observed among a group of different countries at least so far. The sequence has similarities to available sequences found in the database from other countries.

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