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# Antibiotic Resistance Gene (tetB) in Escherichia coli Isolated from Diarrhea Cases: A physiological and Molecular Study

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## Abstract

**Background:** Escherichia coli (E. coli) is a major cause of diarrheal diseases, with rising antibiotic resistance complicating treatment. This study aims to isolate and identify E. coli from clinical diarrhea samples and to profile the tetB antibiotic resistance gene using PCR, while also exploring genetic polymorphisms within this resistance determinant.

**Methods:** Seventy fresh fecal samples were collected from patients with diarrhea at Al-Hussein Teaching Hospital, Thi-Qar province, Iraq, from February to July 2020. After culture-based identification and verification through the API-20 system, 60 samples (85.71%) were confirmed as E. coli . Analysis by age showed a higher prevalence (40%) in children aged 1-5 years, with lower prevalence (15%) in individuals aged 16-20 years.

**Results:** PCR analysis demonstrated the presence of the tetB gene in 100% of the E. coli isolates. Sequence analysis revealed various mutations within the tetB gene, and phylogenetic trees based on tetB sequences were constructed. These trees accurately placed the E. coli isolates within bacterial taxonomy, offering insights into the relationships between genetic mutations and phylogenetic positioning.

**Conclusions:** This study underscores the utility of tetB amplicons for phylogenetic classification of E. coli isolates. Such methods effectively differentiate antibiotic-resistant strains in clinical samples, contributing to an understanding of their evolutionary traits and potential impacts on public health.

**Keywords:** Antibiotic Resistance, Gene (tetB), E. coli, Diarrhea, Molecular Study

**Introduction:** Antibiotic resistance is a major global health threat, particularly in infections caused by Escherichia coli ( E. coli ), a bacterium commonly linked to gastrointestinal diseases such as diarrhea. [1]The widespread use and sometimes misuse of antibiotics in both clinical and agricultural settings have accelerated the development and spread of antibiotic-resistant genes in E. coli , complicating treatment and increasing the risks of morbidity and mortality [1,2].

Among the many resistance genes identified, the tetB gene is particularly relevant for its role in conferring resistance to tetB acycline. This gene encodes an efflux pump mechanism, effectively reducing the intracellular concentration of tetB acycline and thus decreasing the antibiotic's efficacy [3]. The emergence of tetB in E. coli isolates from diarrheal cases not only presents a direct threat to individual health but also poses a broader risk of horizontal gene transfer, which can further disseminate resistance across bacterial populations [4].

This study aims to molecularly analyze the tetB gene in E. coli isolates obtained from patients with diarrhea. Understanding the prevalence and distribution of this resistance determinant will help clarify resistance trends within clinical settings, ultimately supporting future strategies in antibiotic stewardship and treatment protocols [5].

## Materials and Methods

### Materials

**1 Instruments :** Instruments used are listed in Table 1.

**Table (1): Instruments used and their manufacturers.**

| No | Instruments | Company/Origin |
|---|---|---|
| 1 | Autoclave | 9-Power Supply |
| 2 | Benzene Burner | 10-Refrigerator |
| 3 | Centrifuge | 11-Sensitive Electrical Balance |
| 4 | Electric Hood Chamber | 12-Thermocycler Apparatus |
| 5 | Hot Plate With Magnetic Stirrer | 13-UV Transilluminator |
| 6 | Incubator | 14-Vortex |
| 7 | Light Microscope | 15-Water Bath |
| 8 | Oven | 16-Ph Meter |

**Table ( 2):** The Chemical reagents Culture Media and Kits in the study.

| Chemicals | Media |
|---|---|
| Agar- agar , Agarose | Brain Heart Infusion agar (BHI) |
| Bromothymol blue | Blood base agar (BA) |
| Cider Oil | MacConkey agar |
| Ethanol (absolute) | Nutrient agar (N.A) Oxide (England) |
| Formalin | Nutrient broth (N.B ) Oxide (England) |
| Gene Ruler 100 bp DNA (ladder) | Eosin Methylene Blue (EMB) |
| Gram Stain | Kits |
| Safety dye | Presto™ Mini gDNA Bacteria Kit |
| NaCl | Master mix Kit |
| Nuclease free water | API 20E system |
| 6X Loading dye | Gram stain Kit |

Primers: Primers for gene amplification are provided in Table 3 with sequences and references.

**Table (3): Primers sequences used for genes amplification.**

| Gene | Primer Sequences (5´- 3´) | Product Size | Reference |
|---|---|---|---|
| *Tet* (B) | F: (F) CCTCAGCTTCTCAACGC GTG R: GCACCTTGCTGATGACT CTT | 634bp | (Randall *Et Al.*, 2004) |

F : forward, R : reverse, A: adenine, C: cytosine, G: guanine, T: thymine

## Methods:

### Media Preparation

Media were prepared following manufacturer instructions, incubated at 37°C for 24 hours, checked for contamination, and stored at 4°C.

**Stains, Reagents, and Solutions :** Preparation included 70% ethyl alcohol by mixing 70 ml of absolute ethanol with distilled water, and proteinase K (11 mg/1.1 ml) was dissolved in storage buffer. Tris borate EDTA buffer (1X) was prepared by diluting 10X stock with distilled water [(6)].

**Specimen Collection :** Seventy fresh stool samples from diarrheal patients were collected from Al-Hussein Teaching Hospital, Iraq, between February and July 2020, then transferred to the

Microbiology Laboratory. A suspension of each sample was prepared and cultured on blood, MacConkey, and EMB agar plates, incubated at 37°C for 24 hours.

**Isolation and Identification :**MacConkey, Blood, and EMB agar media were used to isolate E. coli through aerobic incubation. Colonies were observed for shape, color, and hemolysis. Gram staining and API 20E biochemical testing confirmed the identification [7].

**Molecular Diagnosis**

**DNA Extraction:** Genomic DNA was extracted using the Presto™ Mini gDNA Bacteria Kit. Following lysis, DNA was purified and stored according to the manufacturer's protocol.

**PCR Amplification:** Amplification for tetB gene was performed with primers listed in Table 5. The reaction mixture was prepared with 5 µl of master mix, 1.5 µl of each primer, and DNA template in a 20 µl final volume, completed with nuclease-free water. Electrophoresis of PCR products was done at 70V for 45-60 min, visualized under UV light [8].

**DNA Sequencing :** Ten PCR products (5 for tetB ) were sequenced at Macrogen, Korea. Sequences were aligned and analyzed using BLAST and BioEdit software (Hall, 1999).

**Phylogenetic Tree Construction :** Phylogenetic trees were constructed based on the neighbor-joining protocol, comparing variants with reference sequences using NCBI-BLASTn. Trees were visualized using iTOL (Letunic & Bork, 2019).

**Statistical Analysis:** Data were analyzed with SPSS (version 16), with p-values $\leq 0.05$ considered significant.

**Results:** The PCR analysis showed that all E. coli isolates contained the tet(B) gene (100%). Additionally, Figures 3.2 and 3.3 display the sizes of these genes, which are approximately 822 bp for sul1 and 634 bp for tet(B).

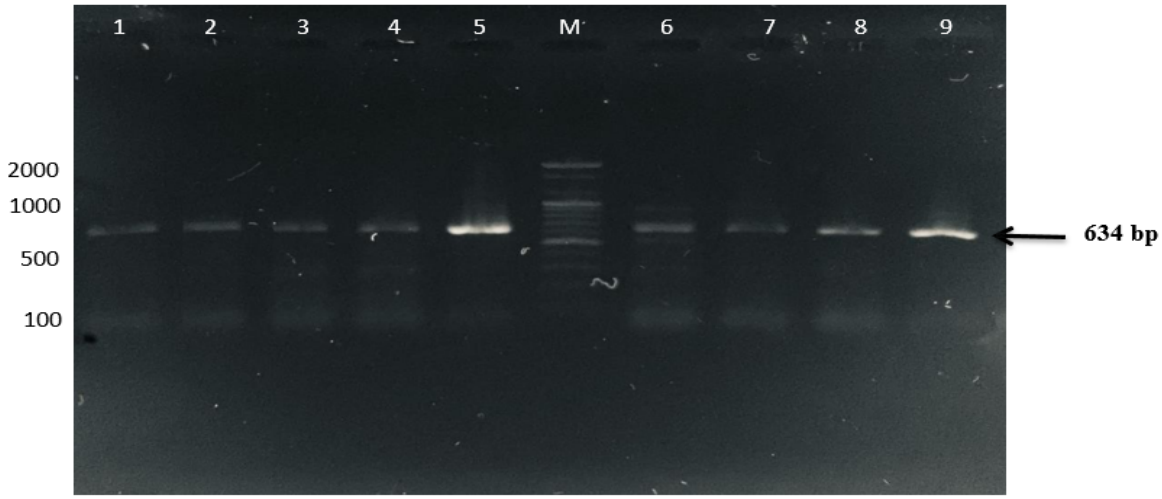


**Fig. (3.2): Agarose gel electrophoresis of *tet* (B) gene amplification, where M: ladder, 1-9: positive results.**

DNA Sequencing of the tetB Gene

This study includes five samples analyzed to amplify the tetB gene sequences. The tetB gene encodes a protein known as tetB acycline resistance transcriptional regulator, which plays a role in tetB acycline resistance within extrachromosomal plasmids. The amplified 634 bp sequences showed approximately 99% similarity to the reference sequences according to NCBI BLASTn results. A comparative analysis between the DNA sequences obtained from the samples and the reference sequences (GenBank accession number CP054215.1) helped to determine the precise positions and details of the PCR fragments (Fig. 3.4).

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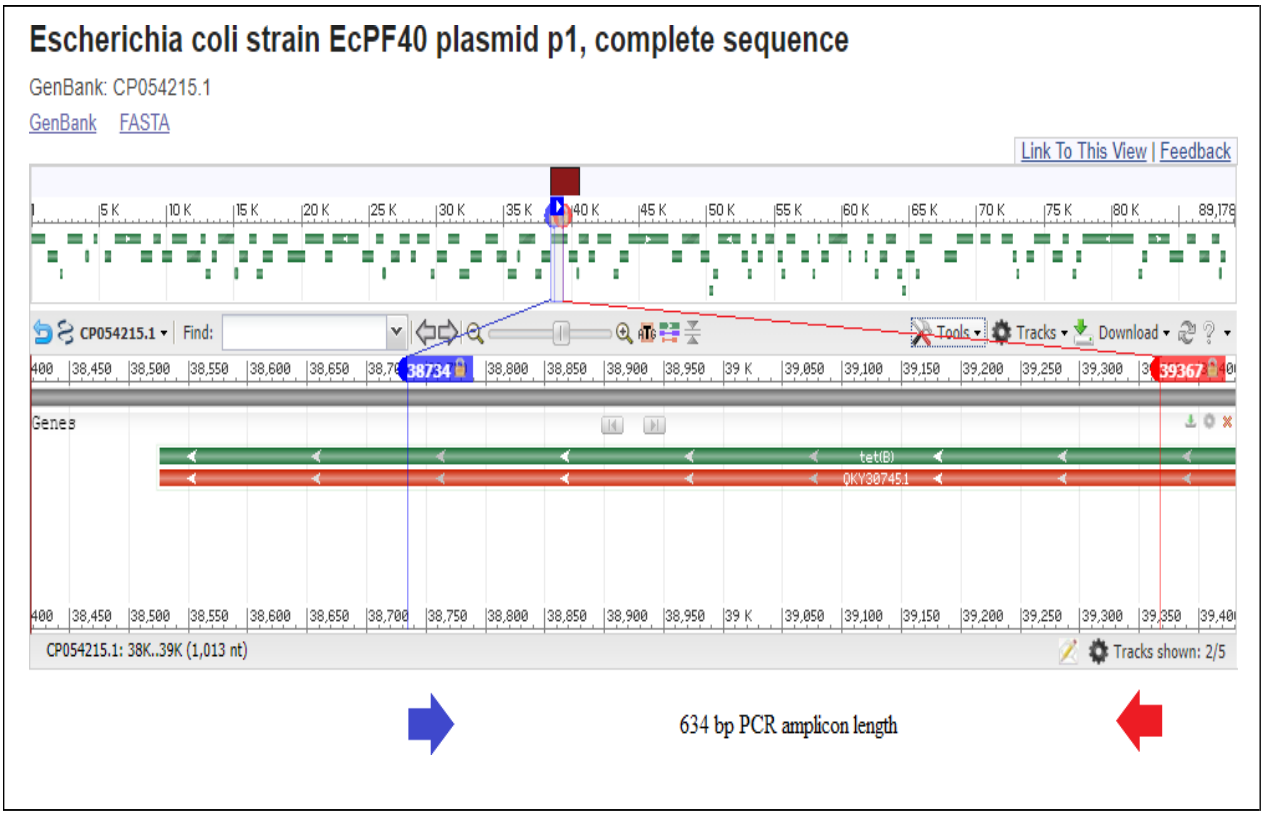


**Fig. (2): The exact position of the retrieved 634 bp amplicon that partially covered a portion of the *tetB* gene within *E. coli* plasmid sequences (GenBank acc. no. CP054215.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.**

After positioning the 634 bp amplicons' sequences within the chromosome no. 2, the details of its sequences are highlighted, in terms of the positioning of both forward and reverse primers of the 634 bp amplified amplicon .

**Table (4): The position and length of the 634 bp PCR amplicons used to amplify a portion of the *tetB* within the plasmid sequences of *E. coli* (GenBank acc. no. CP054215.1).**

| Amplicon | Reference Locus Sequences (5′ - 3′) | Length |
|---|---|---|
| DNA Sequences Within The *Tetb* Genetic Locus | GCACCTTGCTGATGACTCTTTGTTTGGATAGACATCA CTCCCTGTAATGCAGTAAAGCGATCCCACCACCAGC CAATAAAATTAAAACAGGGAAAACTAACCAACCTTCA GATATAAACGCTAAAAAGGCAAATGCACTACTATCTG CAATAAATCCGAGCAGTACTGCCGTTTTTTCGCCCCA TTTAGTGGCTATTCTTCCTGCCACAAAGGCTTGGAAT ACTGAGTGTAAAAGACCAAGACCCGCTAATGAAAAG CCAACCATCATGCTATTCCATCCAAAACGATTTTCGG TAAATAGCACCCACACCGTTGCGGGAATTTGGCCTA TCAATTGCGCTGAAAAATAAATAATCAACAAAATGGG CATCGTTTTAAATAAAGTGATGTATACCGAATTCGAT TGCGTCTCAA CCCCTACTTCGGTATCTGTATTATCACGTGTATTTTT GGTTTCACGGAACCAAACATAACCACAAGGAAAGCG ACAATATTTAGCAACGCAGCGATAAAAAAGGGACTA TGCGGTGAAATCTCTCCTGCAAAACCACCAATAATAG GCCCCGCTATTAAACCAAGCCCAAAACTTGCCCCTAA CCAACCGAACCACTTCACGCGTTG AGAAGCTGAGG | 634 Bp |

Refers to the reverse primer sequences (placed in a forward direction) Refers to the forward primer sequences (placed in a reverse direction.

The gray- colored sequences referred to the position of the reverse and Forward primers, respectively. Highly interesting differences have been observed in the one nucleic acid substitution as detected in the A2 sample among the other investigated specimens. However, the sequencing chromatogram of the identified variation region, as well as its detailed annotations, are verified and documented, and the chromatograms these sequences have shown according to their positions in the PCR amplicons (Fig. 3).

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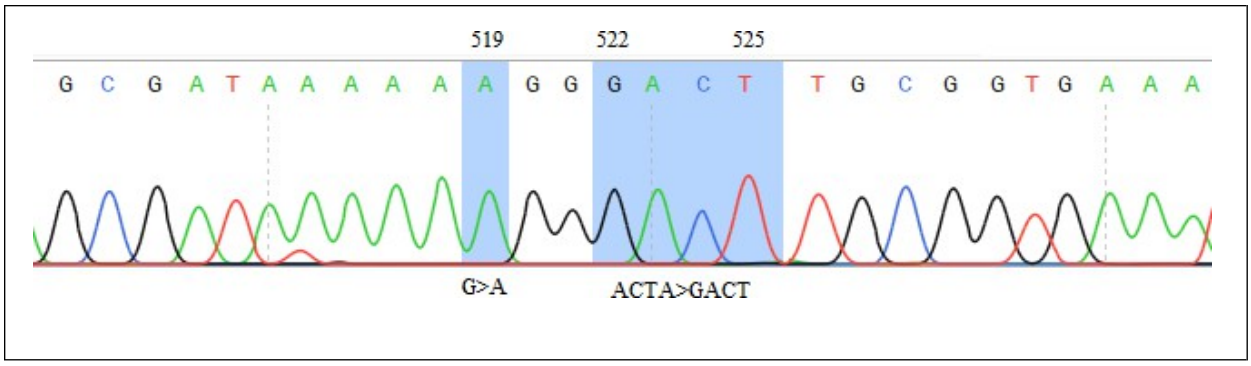


**Fig. (3): The pattern of the detected mutations within the DNA chromatogram of the targeted 634 bp amplicons of the *tetB* gene. The identified substitution mutations are highlighted according to their positions in the PCR amplicons. The symbol ">" refers to "substitution" mutation.**

The observed mutations have further analyzed to identify whether such substitutions induce possible alteration in their corresponding positions in the encoded tetB acycline resistance transcriptional. All nucleic acid sequences are translated to their corresponding amino acid sequences using the Expasy translate suite. The total number of the detected nucleic acid substitutions is only five, in which only two missense mutations were detected, namely His>Gln and Ser>Val, while the other variations are found to had given silent effects on the resulting protein (Fig. 4).

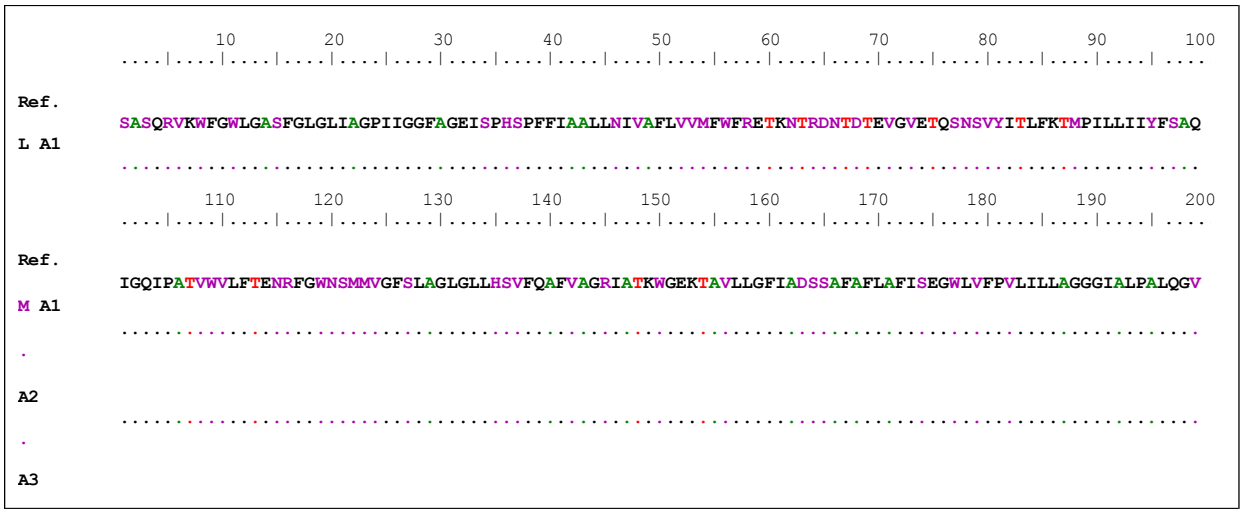


**Fig. (4): Amino acid residues alignment of the detected variations within the investigated samples of *E. coli* samples.**

The missense mutations localization within the encoded tetB acycline resistance transcriptional. The positions of the amino acid substitutions are highlighted according to their corresponding position within the amplified locus. The highlighted colors refer to the identified amino acid substitutions.

To summarize all the results obtained from the sequenced 634 bp fragments, the exact positions and annotations of the observed nucleic acid substitution mutations are described in the NCBI reference sequences as shown in (Table 3.5).

**Table (5): The pattern of the observed mutations in the 634 bp amplicons of the *tetB* gene in comparison with the NCBI referring sequences (GenBank acc. no. CP054215.1).** The symbol "A followed by a number refers to the investigated sample numbers.

| Sample No. | Native | Allele | Position In The Pcr Fragment | Position In The Reference Genome | Amino Acid Position | Type Of Mutation |
|---|---|---|---|---|---|---|
| A2 | G | A | 519 | 39252 | 160 Pro | One Silent Mutation (160 Pro=) |
| A2 | Acta | Gact | 522 – 525 | 39255 - 39258 | 158 His 159 Ser | Two Missense Mutations (158 His>Gln) (159 Ser>Val ) |

A comprehensive phylogenetic tree has been generated in the present study, which is based on the observed nucleic acid variations. This phylogenetic tree is contained A1 to A5 samples alongside with other relative DNA sequences. A total number of the aligned nucleic acid sequences in this comprehensive tree was 107. In addition to *E. coli*, which represents the main incorporated organism within the tree, other highly related organisms are also included within the present inclusive tree to represent an outgroup for the current tree, namely *Acinetobacter baumannii*. Noteworthy, the investigated samples have been clustered into only one clade within the *E. coli* sequences (Fig. 3.8).

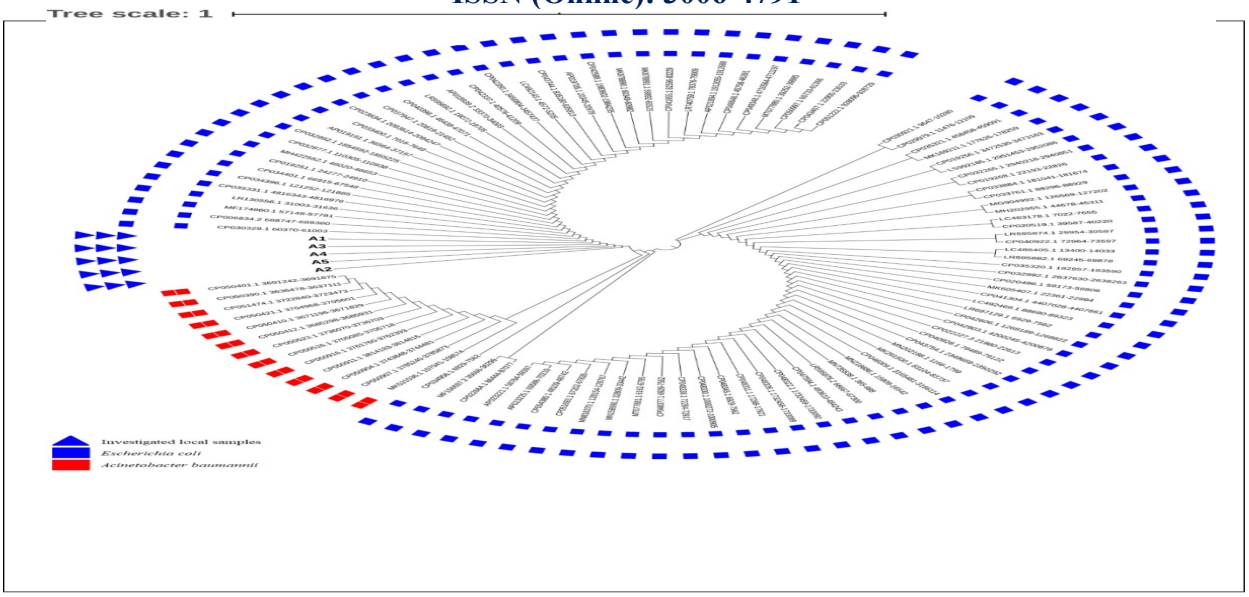


**Fig. (5): The comprehensive phylogenetic tree of genetic variants of the *tetB* gene fragment of five *E. coli* local samples.**

The cyan triangles color refers to the analyzed variants, while other colors refer to the related referring NCBI deposited species. All the mentioned numbers referred to Genbank acc. no. of each referring species. The number "1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The letter "A#" refers to the code of the investigated samples.

## Discussion:

### Distribution of E. coli in Diarrheal Samples:

In this study, Escherichia coli ( E. coli ) was identified in 85.71% of diarrheal samples, with growth confirmed on selective media and only 14.29% of samples harboring other bacterial species. This finding is consistent with Buxton and Fraser's early work on bacterial identification in diarrheal diseases, as well as more recent research highlighting E. coli 's significant prevalence in diarrhea cases worldwide, especially in developing areas where high morbidity and mortality rates persist due to diarrheal infections [9-12]; Bonyadian et al., 2019[13] show a compatable results. E. coli 's antibiotic resistance patterns in these isolates align with studies by Shehabi et al. 2006)([14, 15], who identified similar resistance profiles in stool isolates. However, our results differ from studies by Virpari et al. (2013) and Adnan et al. (2017)([16], who reported lower rates (59–60%) of E. coli in stool samples. These variations may reflect differences in geographic location, patient age, and sample collection techniques.

**Distribution of E. coli by Age**

Age-based analysis revealed that E. coli prevalence was highest in children aged 1-5 years (40%) and significantly lower in older age groups (15%). This trend is well-documented, as E. coli is frequently isolated in diarrheal cases among young children in developing regions, where factors such as environmental exposure and hygiene play a role (Wilson et al., 2006). Other studies, such as those by Bonkoungou et al. (2012)[17] and Heidary et al. (2014)[18], report a similar prevalence of E. coli infections in younger children, corroborated by research from, Wilson et al. (2016) in Jordan Albert et al. (1995[20]) in Bangladesh, and Jafari et al. (2009)[21] in Tehran. These findings suggest that age remains a significant factor influencing E. coli infection rates, highlighting the need for age-targeted interventions.

These sources support the age-related findings and geographical factors affecting *E. coli* prevalence and reinforce the importance of targeted interventions for younger children in regions with high exposure to diarrheal pathogens

**Molecular Diagnosis :**PCR analysis in this study showed the presence of the tetB resistance gene in all E. coli isolates. This gene, associated with resistance to tetB acyclines, is part of a plasmid-borne mechanism that can facilitate horizontal transfer of resistance genes across bacterial populations (Thungt al., 2018; Tamma, 2021)[22,23]. Studies by Shehata et al. (2017)[24] and Lien et al. (2017)[25] have similarly identified high rates of multidrug-resistant E. coli in clinical samples, emphasizing the clinical significance of plasmid-mediated antibiotic resistance. Identifying these genes is critical in guiding treatment and understanding the local resistance landscape, particularly for commonly prescribed antibiotics such as tetB acyclines.

molecular diagnosis of the *tetB* gene in *E. coli* isolates and its implications for antibiotic resistance, focusing on studies that highlight the significance of plasmid-mediated resistance and the high prevalence of multidrug-resistant strains

**Phylogenetic Analysis of tetB Gene :** Phylogenetic analysis of the tetB gene sequences demonstrated limited genetic variation among isolates, indicating minimal evolutionary divergence. The close clustering of tetB sequences suggests a stable, conserved resistance pattern across isolates, which may be due to selective pressure from antibiotic use (Gibson & Eyre-Walker, 2019)[26]. This genetic conservation underlines the gene's value as a marker in studying E. coli resistance patterns. Barroso-Batista et al. (2015)[27] and Adrian et al. (2019)[28] have noted that minor genetic variations, even in highly conserved genes, can impact bacterial pathogenicity and antibiotic response. Phylogenetic analysis provides insights into evolutionary dynamics and may aid in the development of targeted interventions to mitigate the spread of resistant E. coli strains.

## Conclusions:

1. The highest incidence of E. coli infections is found among children aged 1-5 years.

3. Emerging E. coli strains are likely to appear in the coming years.

4. Rising antibiotic resistance poses a growing threat to public health.

## Recommendations:

1. Perform antibiotic susceptibility testing before administering antibiotic treatments.

2. Conduct further studies on resistance genes in pathogenic bacteria.

3. Limit the use of tetB acycline and sulfonamides in treating diarrheal cases

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