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**Original Article**

# Molecular Detection of tetA tetB Gene in Escherichia Coli

**Nazeeha Waseem[1], Hafsa Waseem[2], Abid Ali[3], Ihsan Ullah[4] Rabia Sadaf[5] and Momena Ali[6]**

[1] Department of Radiology, University of Lahore, Gujrat. [2]Department of Pathology, Fazaia Medical College, Islamabad, [3]Department of Allied Health Sciences, University of Lahore, Gujrat. [4]Khyber Medical University. Department of pathology, Rawal medical college, (RIHS), Department of pathology, Khyber medical college /Khyber teaching hospital Peshawar.

## ABSTRACT

**Introduction:** Gram negative, rod shaped bacterium *Escherichia. coli (E. coli* )is the most considered microorganism worldwide. The pathogenic variants of *E. coli* cause serious pediatric diarrhea and other diseases. Antibiotic resistance against *E. coli* is increasing step by step in emergency clinics and in developing countries like Pakistan. It is dangerous due to frequent use of antibiotics, un hygienic conditions, food and direct contact with animals.

**Objective:** This study was done to assess the tetracycline-resistant genes among isolated *E. coli* from blood culture received in microbiology laboratory.

**Materials and methods:** study was conducted from January 2020 to January 2021 at the microbiology department of Khyber Medical University. 150 blood samples were inoculated on Blood and MacConkey agar. Identification of *E. coli* was done by Gram staining and biochemical methods. Molecular confirmation of *E. coli* was done using specific primers. Antibiotic sensitivity test was performed to identify the tetracycline resistant isolated *E. coli* phenotypically. Then genotypically, genes conferring tetracycline resistant *tet* (A & B) were detected using PCR amplification. The PCR amplicons were separated electrophoretically and visualized.

**Results:** In result, out of 150 samples 125 isolated *E coli* were detected by Gram staining and using MacConkey agar. 120 isolated *E. coli* were confirmed by API kit biochemically. 114 isolated *E. coli* were detected genotypically by using *uid* gene. 57 isolated were resistant against tetracycline and doxycycline. At last, 21 & 39 isolated *E. coli* were found positive for *tet*A and *tet*B genotypically using specific primers.

**Conclusion:** The study of antimicrobial resistant determinants especially the tetracycline resistant genes in blood samples to set guidelines for the control of these pathogenic microbes and for an effective treatment especially in healthcare facilities in Pakistan.

**Key Words:** Tetracycline, E. coli, Resistance, efflux pump gene

## Introduction

*Escherichia coli(E.coli)* is a rod-shaped, gram-negative, and facultative anaerobic bacterium. *E coli* is commonly found in animals and humans and their pathogenic variations cause extra-intestinal and intestinal contamination such as meningitis, septicemia and peritonitis, urinary tract disease (UTIs), and bloodstream infection [1]. In women, *E. coli*, causing vaginal, endo-cervical establishment and in pregnant ladies cause intra-amniotic and puerperal disease, neonatal infection, for example, early and late infant sepsis .[2]

**CORRESPONDENCE AUTHOR**

**Dr. Nazeeha Waseem**

Department of Radiology

University of Lahore, Gujrat

Ph: 0316-7639781

E-mail: drnazeehawaseem@gmail.com

In the countries, South Asia and geographical area, diarrheal illness caused in youngsters younger than five and because of diarrheal disease children suffer deaths. The largest case-control studies from the World Enteric Multi-Center Study (GEMS) understand a load of pediatric diarrheic illness in South Asia and sub-Saharan Africa. The highest cause of diarrhea is pathogenic *Shigella* and *E. Coli* .[3] Transmission occur through the fecal-oral course, also passed from individual to individual, through defiled food and water and contact with animals. There is no proper vaccine for *E. coli* infection or nor proper medication[4].

Globally antibiotic resistance and the effect of drugs is major cause of sickness and destruction (Laxminarayan et al., 2016). After the survey, we conclude that antibiotic utilization from 2000 to 2015 of every 76 nations and till 2030 it becomes globally. The study reported that in 71 countries antibiotic consumption developed 36% somewhere in the range of 2000 and 2010. Anti-infection utilization

communicated in a characterized day by day portions, between 2000 and 2015 it becomes 65%( 21.1-34.8 billion DDDs) [5].

Tetracycline has some limitations. It cannot be utilized for small children and in pregnant ladies because it causes discoloration of teeth and depression of skeletal development in untimely newborn children [6].

Tetracycline is the most abundant antibiotic resistances amongst commensal microorganisms and clinical and the prevalence of tetracycline has also been increased. Antibiotic medication fix to the 30S ribosomal subunit and causes inhibition of protein production, also by passive diffusion penetrate bacterial chambers [7].

Efflux force is the utmost widely recognized mechanism of tetracycline opposition. There are 20 different antibiotic medication efflux proteins. For antibiotic obstruction there are numerous qualities, out of 38 *tet* genes eight new *tet* qualities are conveying *tet*(A), *tet*(B), *tet*(G), *tet*(D), *tet*(C), *tet*(H), *tet*(L), or *tet*(K) efflux genes or *tet*(Q), *tet*(O), *tet*(W), *tet*(M), or *tet*(S) ribosomal security genes [8].We amplify the *tet* (B) and *tet* (A) qualities and their molecular function is tetracycline transport activity. The tetracycline genes are identified with the Tn916–Tn1545 group of components [9] and some are identified with the *mef* (D), *msr* (A) components, which programmed the efflux of macrolides [10].

However, this studied related to identified with anti-microbial opposition against *E. coli* is increasing day by day that's why we planned the study to isolate the *E. coli* and see the responsible genes.

## Material and Methods

It was cross sectional study done over a period of one year from January 2020 to January 2021 at the department of Microbiology, Khyber medical university.Blood was cultured on blood agar and MacConkey agar and incubated at 35°C ± 2°C for 24 hours in aerobic conditions. After 24 hours' bacteria was identified according to colony morphology and pigment production. Then Gram staining procedure was performed to identify Gram negative bacteria. To identify organism up to genus and species level API 10S and 20E (Biomerieux, France) were used and antibiotic susceptibility testing was performed by using Kirby- Bauer Disk Diffusion on Mueller Hinton agar according to CLSI 2020 guidelines. After incubation of 18 hours' susceptibility pattern of *Escherichia coli* was noted.

Molecular identification of microscopically and biochemically confirmed *E. coli* clinical isolates was done by utilizing PCR for *uidA* gene. Firstly, DNA of isolates was extracted, then quantified using nanodrop, finally amplification of *uid*A gene was achieved using gene specific primers.

The quantity of extracted DNA was estimated by measuring absorbance at λ = 260 nm, whereas quality was determined by measuring the ratios of 260nm/230nm and 260nm/280nm values. The extracted genomic DNA was separated by horizontal gel electrophoresis and visualized under UV light.

The *uid*A gene is a housekeeping gene of *E. coli* and it encodes for β-glucuronidase enzyme. Using gene specific primers already reported in literature, polymerase chain reaction was conducted to amplify the *uid*A gene. The sequence of forward primer TGGTAATTACCGACGAAAACGGC and reverse primer ACGCGTGGTTACAGTCTTGCG were used and PCR was performed on 16 well platforms with Dream Taq Green Master Mix. PCR reactions contains 1µl of each primer, 1µl of template DNA, 12µl of 2×Dream Taq Green and final volume is adjusted with 10µl of dionized water.

According to manufacturer guidelines PCR was conducted with the following modifications: activation and initial denature at 94°C for 5 minutes. PCR was performed with positive and negative controls for 35 cycles at 94°C for one minute and 50°C for one minute. Annealing at 50°C for one minute, Elongation at 72°C for one minute and final elongation at 72°C for five minutes. Finally, amplicons were separated on 1 % agarose gel and band size was estimated using gene ruler. All the molecular confirmed *E. coli* were stored in the form of 15 % glycerol stocks. The protocol of cell preservation used was and incubated at 37 °C for 6 hours in shaking incubator (speed 150 RPM) and then stored in freezer with temperature -10 °C.

Tetracycline resistance is mediated by multiple systems. In present study, drug exporting systems were screened. The gene *tet*A is an efflux pump protein encoding gene, whereas *tet*B gene is an ABC transporter synthesizing gene. Both genes were screened in the tetracycline resistant strains, using gene specific oligos.

Tetracycline resistant *E. coli* were subjected to *tet*A (efflux pump gene) PCR. The *tet*A gene was amplified using previously reported oligos. The oligos used in study were Forward primer

(GTGAAACCCAACATACCCC) and Reverse primer (GAAGGCAAGCAGGATGTAG)
To amplify *tet*A gene, the recipe of the reaction mixture along with quantity used for amplification was as The PCR profile used for the *tet*A gene amplification was initial denature at 94°C for 6 minutes, annealing at 51°C for 1min, elongation at 72°C for 1 min and final elongation at 72°C for 5 min
Finally, amplified gene was separated on 1 % agarose gel and band size was estimated using gene ruler. Tetracycline resistant *E. coli* clinical isolates were subjected to *tet*B (ABC transporter gene) PCR. The *tet*B gene was amplified using already reported primers. The sequence of oligos used in study were Forward primer (CCTTATCATGCCAGTCTTGC) and Reverse primer (ACTGCCGTTTTTTCGCC)
To amplify *tet*B gene, the reaction mixture consists of 2XDreamTaq green master mix 12µl, primers 2µl, template DNA1µl and distilled water 10µl.
The PCR profile used for the *tet*B gene amplification was as follows denature at 94°C for 6 min, annealing at 52°C for 1 min, elongation at 72°C for 1 min and final elongation at 72°C for 5 min. Finally, amplified gene products were electrophoresed on 1 % agarose gel and band size was determined using gene ruler.

## Results

In our study, blood origin *E. coli* clinical isolates (n = 70) were cultured on selective and differential agar medium. On MacConkey agar pink colored colonies were observed indicting that our isolates are Gram negative lactose fermenting bacteria. Moreover, on blood agar, our isolates displayed γ-hemolysis (non-hemolytic nature).
Gram negative lactose fermenting colonies were examined by Gram staining. Under the microscope at 100 X using oil immersion the isolates were found pink colored bacilli, the Gram-negative rods.
Microscopically identified isolates (n = 70) were identified by biochemical tests. Our isolates were found oxidase and Voges-Proskauer negative; whereas indole and methyl red positive. Furthermore, the isolates unable to metabolize citrate utilization test. These test results indicated that our isolates are *E. coli*. Finally, the API 20E kit was used for detailed biochemical analysis. According to apiweb™, our isolates were identified as *E. coli*.
Microscopically and biochemically identified clinical E. coli isolates were further confirmed at genetic level. The housekeeping gene *uid*A was amplified using gene specific primers. Band of 162 bp was observed under UV light in ethidium bromide stained 1.5 % agarose gel.
In our study, *E. coli* clinical isolates (n = 70) of blood origin were microscopically, biochemically and genetically identified. Out of 70 isolates, 31 (≈ 44 %) were obtained from male patients, whereas 39 (≈ 56 %) were isolated from female patients. Female patients were more infected with *E. coli* than male patients.
Antimicrobial susceptibility data against tetracyclines of *E. coli* clinal isolates was determined by Kirby-Bauer disc diffusion assay. In our study two drugs tetracycline and doxycycline were selected. Out of 70 isolates 35 (50 %) were resistant against tetracycline and 35 (50 %) were resistant against doxycycline. 5.7 % (n = 04) isolates displayed intermediate level of resistance against tetracycline.

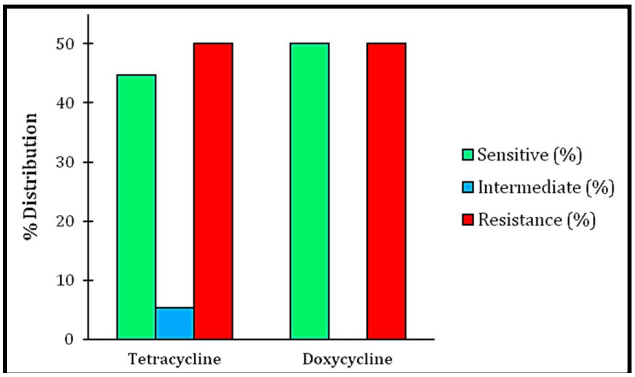


**Figure1: Graphical distribution of tetracycline and doxycycline resistant *E. coli***

According to Kirby-Bauer disc diffusion assay 50 % (n = 35) isolates were screened as resistant against tetracycline and doxycycline antibiotics. Initially, genomic DNA of our isolates was extracted using boiling method, the quantity and quality of extracted chromosomal DNA was estimated using UV spectroscopy. Finally, the genomic DNA of resistant strains was subjected to PCR for the screening of tetracycline resistance genes namely *tet*A and *tet*B. Both genes encode proteins which are part of drug efflux system. The gene *tet*A encodes for efflux pump, whereas *tet*B gene encodes for ABC transporter.
The gene *tet*A (efflux pump) was amplified using gene specific primers. The amplicons were separated on 1 % agarose gel. The size of amplicons was estimated using gene ruler. Ethidium bromide-stained gel showed amplification of 888 bp band corresponding to *tet*A gene.

Molecular detection of tetracycline resistant gene *tet*B (ABC transporter) among isolated *E. coli* was done by using their specific primers. Result of *tet*B PCR represented amplification of 774 bp DNA product corresponding to the size of our desired gene.

The *tet*A and *tet*B are members of drug exporting system. Specifically, TetA protein is an efflux pump, whereas TetB protein is part of ABC transporters. 50 % (n = 35) *E. coli* isolates of our study were tetracycline and doxycycline resistant. Out of 70 E. coli studied in present work, 13 (18.6 %) isolates contained *tet*A gene, whereas 22 (31.4 %) isolates harbored *tet*B gene. Isolates in our study harbored *tet*B tetracycline resistance gene more frequently.

## Discussion

It is becoming up progressively evident that once *E. coli* strains arrive at the internal organ, to colonize; they should enter the bodily fluid layer and use supplements there for development. It is likewise many pathogenic strains of *E. coli* caused meningitis, septicemia, neonatal sepsis, acute renal failure in children, food poising, and seriously caused diarrheal disease in children under the age of 5 and also cause death in neonatal. Shiga toxin-producing *E. coli* is a bacterium that suffers the patient with severe foodborne illness. Virulence factors are pathogenic or microbe's ability to infect or damage the host includes adhesion (Pilli. Fimbriae), aerobactin system, hemolysin, K capsule and resistance to serum killing. Antibiotic resistance occurs worldwide and influences the patient to causes illness and destruction. Antibiotic resistance genes (ARGs) mostly present in hospitals, clinics, sewage, the surface of soil water and animal trash and help to study the disease transmission. Mostly we studied the cheap, easily available anti-infection agents is tetracycline. Side effects of tetracycline are not used pregnant women and little children because it depressed the skeletal development and discoloration of teeth. Three mechanisms of tetracycline resistance are efflux pumps, ribosomal dysfunction, and enzymatic inactivation, through these first two are common. There are 20 *tet* genes that are observed but we focus on the two 35% *tet* (A) and *tet* (B) 63%. Tetracycline binds to the 30S ribosomal subunit and stops the protein synthesis. Some antibiotics also bind to the 50S subunit restrain protein synthesis by either disturbing the official of aminoacylated-tRNAs at the A or P site. Efflux system present in both gram-positive and negative bacteria.

In this study, 50% frequency of tetracycline resistance in E coli is also indicating towards worsening situation due to injudicious use of antibiotics. When we compared our findings with studies done in other countries, we have found more or less the same situation. A study done in seven developing countries of South Asia and Sub Saharan Africa in 2018 to investigate the AMR pattern of EPEC in children showed the tetracycline resistance of 56% [12]. Another study done in Egypt in 2021 on urine samples of hospitalized patients calculated the 53% resistance to tetracycline [10]. Similar results are shown in a research done in Kenya on stool samples of asymptomatic school going children i.e. 55% tetracycline resistance rate [13] All these findings depicts that tetracycline resistance has developed worldwide, mostly in under developed countries. Two studies done in different regions of Iran witnessed more frequent resistance to tetracycline, i.e. 68% and 62% respectively [15, 17]

In our study we have also determined the frequency of tet A and tet B genes which are mostly responsible for tetracycline resistance. Our results showed the presence of tetA gene in 18.6% isolates and tet B gene in 31.4% isolated E coli. A study done in Iran in 2017 demonstrated the tet B (36%) as more frequent resistance gene than tet A (32%) which is in accordance to our results[17] Another study done to detect the tet genes distribution in enterobacteriaceae has found 14.4% tet A and18.4% tet B gene [18] A research done in Bangladesh to detect MDR Escherichia coli isolated from patients of diarrhea showed the same ratio of tet genes i.e. tet A gene as 35% and tet B gene as 57% [16] A study done in southwest Iran in 2021 to detect resistance genes of E. coli also demonstrated the more frequency of tet B gene (66.7%) than tet A gene (59.7%) [11]

However, some studies reported more frequent presence of tet A gene than tet B as depicted by a study done in India in 2019 in which frequency of tet A is 20% and tet B is 10% [19] Another study done on uropathogenic E coli isolates showed prevalence of tet A as 86.27% and tet B as 81.37% [15] A study from Iraq also detected higher prevalence of tet A gene as 77.4% in *Escherichia coli* isolates collected from patients of urinary tract infection [14]

A study done to detect AMR in intestinal E coli from stool samples also witnessed the frequency of tet A gene as 34% and tet B gene as 27% [12]

## Conclusion

This study of antimicrobial resistant determinants especially the tetracycline resistant genes may be helpful to set guidelines for the control of these pathogenic microbes.

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| CONTRIBUTION OF AUTHORS | |
|---|---|
| **Author** | **Contribution** |
| Nazeeha Waseem | A,B,C |
| Hafsa Waseem | B,C |
| Abid Ali | C |
| Ihsan Ullah | C |
| Rabia Sadaf | A,C |
| Momena Ali | B,C |

**KEY FOR CONTRIBUTION OF AUTHORS:**

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C. Interpretation/ Analysis and Discussion