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Molecular Characterization and Mutational Analysis of Fluoroquinolones and Tetracycline Resistant Genes of *Escherichia coli* Isolated from UTI Patients

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HIGHLIGHTS

- Mutational analysis of antibiotic resistant genes of *E. coli* in UTI Patients.
- These genes were detected by PCR and sequenced using NGS.
- *E. coli* isolates were positive for GyrA gene, GyrB gene, tetB and tetR gene.
- The novel mutation was detected in gyrB gene at codon554.

Abstract: Antibiotic resistance is more challenging in third world countries due to irrational use of antibiotics and poor antimicrobial resistance surveillance. The current study is aimed to determine the molecular characterization and mutational analysis of antibiotic resistant genes of *E. coli* isolated from UTIs patients. A total of 112 *E. coli* isolates were recovered from UTI suspected patients from Khyber Teaching Hospital (KTH), Peshawar. The collected samples were identified phenotypically by API-10S strips and confirmed by Polymerase Chain Reaction. The selected antibiotic resistant genes were detected by PCR and subsequently sequenced by Next Generation Sequencing. The results of antibiogram revealed that the *E. coli* isolates were resistant to various antibiotics; Ampicillin, Cotrimoxazole and Ciprofloxacin while showed good results against Tigecycline, Meropenem and Cefoperazone-Sulbactam. Molecular analysis showed that 72 isolates were

positive for GyrA gene, GyrB gene 46, tetB 28 and tetR gene 6. The results of mutational analysis revealed that gyrA gene have 3 amino acid substitutions (S83L, D87N and A828S), gyrB have 2 amino acid substitutions (E185D and Q554K), tetB also have 2 amino acid substitutions (H204R and R334S) while only one amino acid substitution in tetR gene (L108P) was observed. The current study reported the novel mutation in gyrB gene at codon554, due to which Glutamine (Q) is substituted by Lysine (K). The high frequency of GyrA gene was observed in 72 *E. coli* isolates followed by GyrB gene 46, tetB 28 and tetR gene 6 while novel mutation was detected in gyrB gene at codon554.

Keywords: *E. coli*; antibiogram; USP; GyrA; GyrB; tetB and tetR genes; NGS; novel mutation.

INTRODUCTION

E. coli are Gram-negative rods present in human colon as normal flora. The newborn Gastro-Intestinal Tract (GIT) has been colonized by the organism within hours after birth [1]. Most of the *E. coli* causes infections; Urinary Tract Infections (UTIs), Pneumonia, Diarrhea, Septicemia, Meningitis and Hemolytic Uremic Syndrome (HUS) etc. The UTIs is the most frequent infection (80%–90%) of humans due to uropathogenic E. coli [2, 3].

Antimicrobial Resistance (AMR) is a globally known hazard to health. The impact of primary healthcare is of great consequence as this is where nearly 80% of the entire antibiotics consumed within the health service are advised [4]. Bacterial infections resistant to antibiotics can restrict the accessibility of effective treatment alternatives, making some regularly bacterial infections challenging to handle, with urinary tract infections included [5]. In 3rd world countries, the use of latest broad spectrum antibiotics is restricted by affordability of second line drugs as well as reduced access to healthcare, resulting in rising concerns for amplified morbidity and mortality from antibiotic resistant infections in these nations [6].

Globally, according to estimations, every year antimicrobial resistance is the cause for death of around 700,000 patients. If not dealt with immediately, the numbers are expected to rise upto 10 million by 2050 [7]. Tetracyclines are antibiotics broad spectrum that still have clinical value, though rather limited, nearly 6 decades after their discovery. Primarily two mechanisms facilitate Tetracycline resistance in bacteria: ribosomal protection and drug efflux mechanism. Mobile elements are associated in both of the resistance mechanisms. Resistance against tetracycline can be achieved by enzymatic inactivation. The efflux pump system is the most common resistance mechanism in Gram-negative bacteria. This mechanism is encoded by different genes (tetA, tetB, tetC, tetD, and tetG) i.e. most frequently described genes were tetA and tetB while in Gram-positive organisms, ribosomal protection mechanisms are more common [8]. Quinolones, exclusively fluoroquinolones, are potent, broad spectrum synthetic antibiotics that prevent DNA replication by affecting bacterial DNA gyrase (Gram negative) or topoisomerase II (Gram-positives) [9]. The widespread use of fluoroquinolones due to their efficiency against bacteria, has caused high resistance [10]. Multiple mechanisms necessitate the development of resistance to quinolones. Mutations in the genes of GyrA and GyrB in Gram-negative bacteria is the main cause of quinolone resistance [11]. Quinolone resistance can also be significantly added by the overexpression of MDR efflux pumps particularly in the presence of target mutations. Unpredictably for synthetic antibiotics, horizontal gene transfers as well play a part in quinolone resistance. A resistance in gene qnr encoding a penta peptide repeat protein shields the bacterial topoisomerases from quinolone action [12].

It is therefore necessary to determine the antibiogram and molecular characteristics of resistant bacteria in the hospital settings to take infection control measures and empirical treatment for infections. The current study determined the Evaluation of Tetracycline and Fluoroquinolone genes in *E. coli* and their antibiogram isolated from UTIs patients in tertiary care hospital of District Peshawar. This will be helpful for clinicians to overcome the resistance mechanisms.

MATERIAL AND METHODS

The current research work was designed at the research laboratory of Center of Biotechnology and Microbiology, UOP and Department of Pathology, KTH, Peshawar from August 2018 to August 2019. The study was approved by the Institution Research and Ethical Review Board (IREB) of Khyber Medical College, Peshawar (Document No. 122/ADR/KMC).

From urine samples, 112 resistant clinical isolates were recovered from Indoor Patients (IPD) and Outdoor Patients Department (OPD) having UTIs after detailed medical history visiting KTH, Peshawar. The selective media; Cysteine Lactose and Electrolyte Deficient (CLED) and MacConkey agar were used to

inoculate the urine samples. After inoculation, the media plates were kept overnight at 37oC for bacterial growth. These bacterial colonies were then subjected to Gram staining technique to differentiate Gram positive and Gram-negative isolates. Analytical Profile Index (API 10S) strips were used for the identification of urine isolates.

The 48 hours old broth cultures were used for the DNA extraction from well isolated colonies. The extraction was performed by using Thermoscientific Gene JET Genomic DNA Purification kit. After DNA extraction, it was checked through electrophoresis using 1% of agarose gel. Gel documentation system was used for analysis of gel results.

The isolates were confirmed by amplification of Universal Stress Protein (USP) gene using specific primers and conditions (table 1) and the results were observed using Gel Documentation system. The identified isolates were preserved in Tryptone Soya Broth (TSB) supplemented with glycerol (15%) and subsequently stored at -80oC [13].

The antibiotic susceptibility testing against selected antibiotics was performed by disc diffusion method using Muller Hinton agar. These antibiotic discs were placed onto the plates and incubated over night at 37oC. The zones of inhibition were noted after incubation and interpreted as sensitive, intermediate or resistant using Clinical and Laboratory Standards Institute (CLSI) 2019 [14].

The selected antibiotic resistant genes were subjected for amplification by conventional gradient PCR machine (Labnet International, USA), using specific primers under optimized conditions for TetR, TetB, GyrA and GyrB genes (table 1). The reactions for amplification were prepared for each sample by mixing 12.5µL of Taq Master mix (Bioron, life sciences), 0.5µL of each reverse and forward primers (oligonucleotides, Macrogen Korea), 11.5µL of Nuclease-free water and 2µL of sample DNA. The known positive sample was used as positive control whereas water was used as Negative control. The PCR products were subjected to gel electrophoresis (110 volts for 44-60min) on 1.5% agarose suspended in 1X-TAE buffer. Gels were stained with Ethidium Bromide solution and bands were visualized gel documentation system (BIO-RAD Gel Doc™ XR+). The amplicon sizes were determined by matching with DNA ladder (100bp). These PCR products were further subjected to sequencing for mutational analysis after purification.

Table 1. Primer sequences used for the molecular detection of tetracycline and fluoroquinolones genes of *E. coli.*

Targeted Genes	Specific Primers	Product Size (bp)	Annealing Temperature	Cycles
USP	F: ATGCTACTGTTTCCGGGTAGTGTGT R: CATCATGTAGTCGGGGCGTAACAAT	884	56^{O}C for 1 min	35
Tet R	F: AGAATCGGTTATTGATGCGGC R: TCAGCAAAAGGGGATGATAAGT	640 bp	53^{O}C for 30 sec	35
Tet B	F: CCTCAGCTTCTCAACGCGTG R: GCACCTTGCTCATGACTCTT	634 bp	49^{O}C for 30 sec	35
Gyr A	F: ATGAGCGACCTTGCGAGAG R: TCCACTTCCGGAGCGATTTC	2600 bp	59 ^{O}C for 30 sec	35
Gyr B	F: GTAAGCGCCCGGGTATGTAT R: TCGATATTCGCCGCTTTCAG	2350 bp	52^{O}C for 30 sec	35

The PCR products of resistant genes were randomly selected and purified for sequencing process with a PCR Purification Kit (Thermo Scientific, USA). The products were sequenced directly by NGS at Genomic Sciences, Rehman Medical Institute (RMI) Peshawar. The FASTA sequences of PCR products were matched with the original gene sequences of GenBank (NCBI database). The amino acid and nucleotide sequences were analyzed by searching the database with BLAST and BioEdit Software. The IBM SPSS Statistics (version 23.0.0) software was used for the calculation of frequencies and percentages in the current study. The Origin (version 2018) was used for plotting different graphs of frequencies and percentages in different isolates of the present research work.

RESULTS

A total of 112 urine isolates of E. coli were collected and identified using phenotypic method (API 10-S). Furthermore, these identified bacterial isolates were molecularly confirmed by PCR using specific primers of usp gene Figure 2(E).

Out of 112 resistant isolates of E. coli, 49 (43.8%) were collected from male patients while 63 (56.3%) isolates were obtained from females. The current study determined the frequency distribution of E. coli isolates among different age groups. Among 112 clinical isolates of E. coli, the high frequency was recorded in the age group of 21 to 40 (50.0%), followed by the age groups 41-60 years (25.0%), 11-20 years (14.3%) and 0-10 (7.1%). Among all these age groups, the low frequency was observed 61 and above age group (3.6%) as shown in Figure 1.

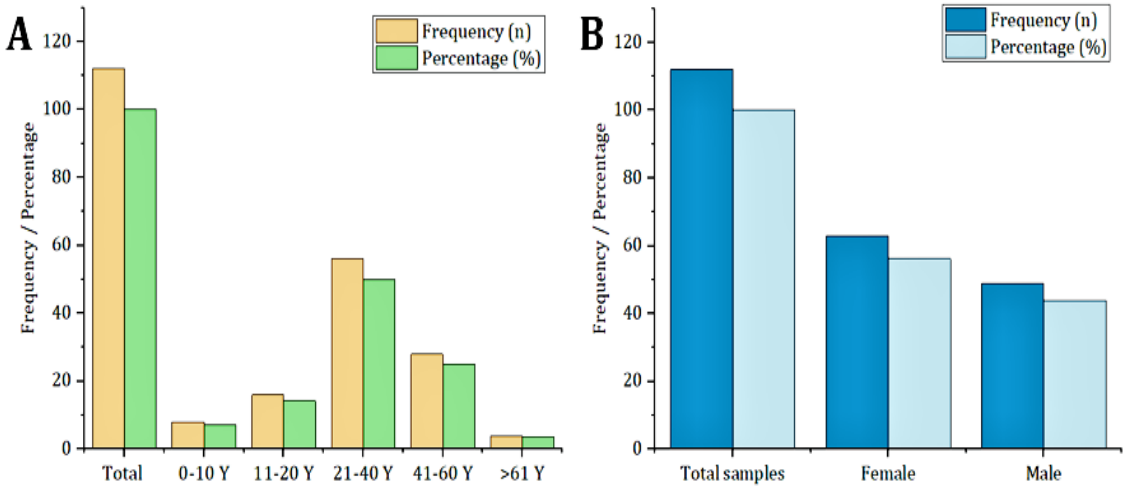

Figure 1. Frequency of A) different age groups and B) gender wise distribution of *E. coli* isolates.

All the *E. coli* isolates were tested against 17 selected antibiotics by disc diffusion method using CLSI-19 guidelines. Most of the *E. coli* isolates were resistant to antibiotics; AMP, DO, CIP, LEV, SXT and C while some antibiotics; AMC, SCF, TZP, FEP, MEM, AK, FOS and TGC were effective against isolates as shown in table 2.

Table 2. Antibiogram of *E. coli* isolated from UTI infected patients (n=112).

Antibiotics Names	Symbols	Sensitive (n)	Percentage (%)	Resistant (n)	Percentage (%)
Amoxicillin – Clavulanate	AMC	80	71.5	32	28.5
Ampicillin	AMP	14	12.5	98	87.5
Cefoperazone-sulbactam	SCF	93	83.0	19	17.0
Piperacillin-Tazobactam	TZP	86	76.8	26	23.2
Cefepime	FEP	88	78.6	24	21.4
Cefotaxime	CTX	70	62.5	42	37.5
Ceftazidime	CAZ	72	64.3	40	35.7
Aztreonem	ATM	74	66.1	38	33.9
Meropenem	MEM	95	84.8	17	15.2
Amikacin	AK	87	77.7	25	22.3
Doxycycline	DO	40	35.7	72	64.3
Ciprofloxacin	CIP	38	33.9	74	66.1
Levofloxacin	LEV	44	39.3	68	60.7
Cotrimoxazole	SXT	23	20.5	89	79.5
Chloramphenicol	C	64	57.1	48	42.9
Fosfomycin	FOS	80	71.4	32	28.6
Tigecycline	TGC	112	100	0	0

The results of molecular analysis revealed that 72 (64.3%) isolates contained *Gyr A* gene followed by *Gyr B* gene in 46 (41.1%) isolates, *Tet B* gene in 28 (25%). The lowest prevalence was detected in 6 (5.4%) isolates contained *Tet R* gene as presented in Table 3 and Figure 2(A-D).

Table 3. Distribution of different antibiotics resistant genes of *E. coli.*

Antibiotic resistant genes	Frequency (n)	Percentage (%)
Tet R	6	5.4
Tet B	28	25.0
Gyr A	72	64.3
Gyr B	46	41.1

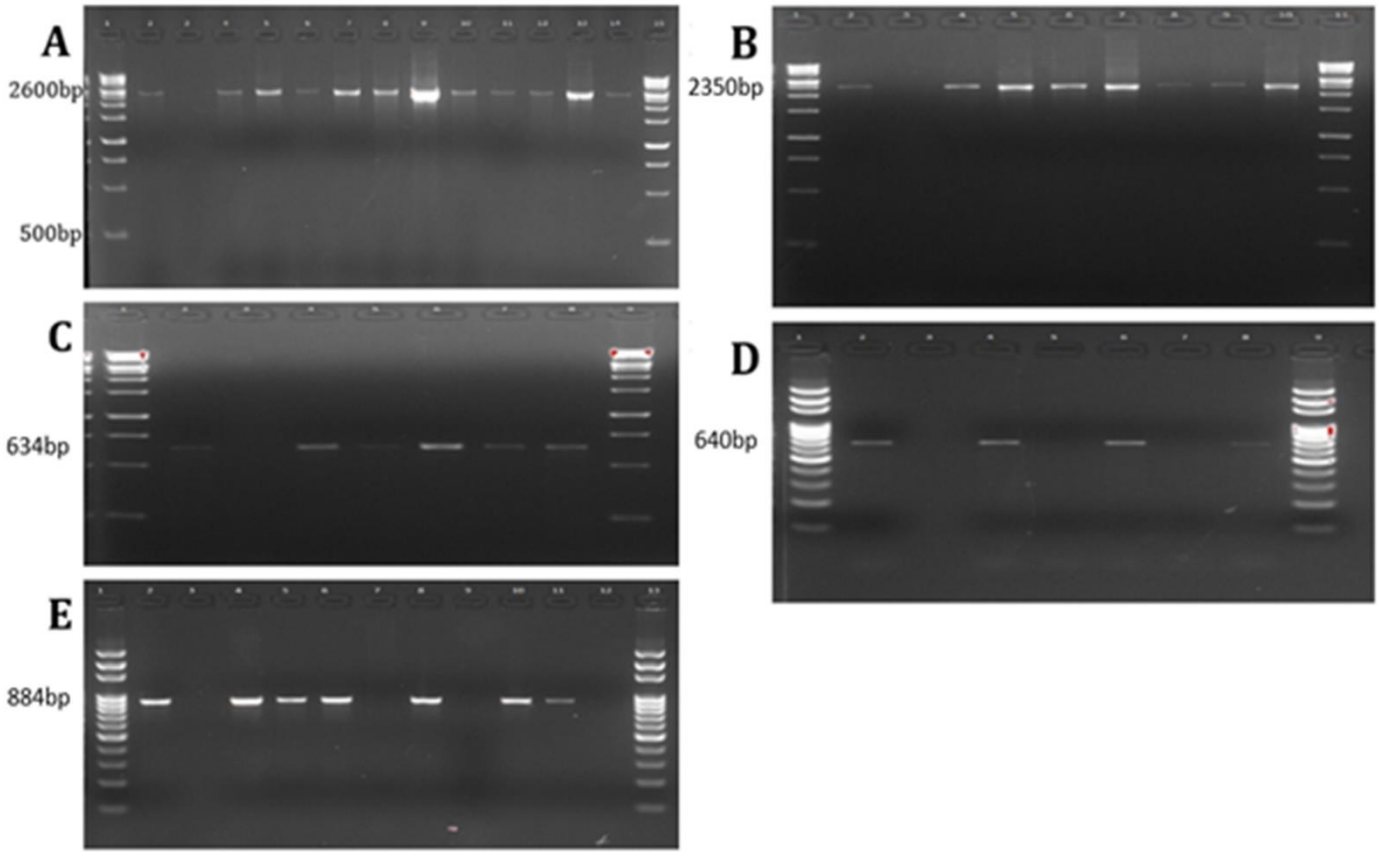

Figure 2. Gel electrophoresis of antibiotic resistant genes of *E. coli*; A) *Gyr A* (2600 bp), B) *Gyr B* (2350bp), C) *Tet B* (634bp), D) *Tet R* (640bp) and E) *USP* (884bp).

The current experimental work was further subjected to NGS sequencing for mutational study. Among tetracycline and fluoroquinolone resistant *E. coli* isolates, two isolates for each gene were randomly selected for genetic characterization of the *TetR, TetB, GyrA* and *GyrB* genes by sequencing process. Finally, these sequences were compared with the published *TetR, TetB, GyrA* and *GyrB* sequence of *E. coli* K-12 genes in the GenBank database (accession numbers: X01083.1, NG_048172.1, NC_000913.3:c2339420-2336793 and NC_000913.3:c3880119-3877705 respectively).

The sequencing results of *GyrA* gene revealed the presence of mutations in all isolates altering amino acid 83, 87 and 828. These substitutions are leucine for serine at position 83, asparagine for aspartate at position 87 and serine for alanine at position 828 (Table 3.7). The mutation at position 83 and 87 has been reported previously in *E. coli* isolates resistant to fluoroquinolones. Apart from this, we identified some isolates with a *GyrA* A828S mutation. Interestingly, many isolates showed *GyrA* sequences which are different from the published *E. coli GyrA* sequence by nucleotide changes at many positions (Table 4 and Figure 3), none of which result in amino acid substitutions and are referred as Synonymous mutation.

Table 4. Synonymous and non-synonymous mutations of tetracycline and fluoroquinolones genes of *E. coli* isolates

Nucleotide position	Reference amino acid	Altered amino acid	Amino acid position
Non synonymous mutation of gene *GyrA*			
248	S (TCG)	L (TTG)	83
259	D (GAC)	N (AAC)	87
2482	A (GCG)	S (TCG)	828
Non synonymous mutation of gene GyrB			
555	E (GAA)	D (GAC)	185
1660	Q (CAG)	K (AAG)	554
Non synonymous mutation of gene TetB			
611	H (CAC)	R (CGC)	204
1002	R (AGA)	S (AGT)	334
Synonymous mutation of gene TetB			
942	CGA – CGG	R	314
Non synonymous mutation of gene TetR			
323	L (CTT)	P (CCT)	108
Synonymous mutation of gene TetR			
279	TCT – TCC	S	93

Figure 3. Sequencing image of *GyrA* gene showing mutations and conserved regions

The sequencing analysis of randomly selected isolates observed the existence of mutation in codon 185 and 554 of *gyrB*. This mutation (GAA to GAC and CAG to AAG respectively) gave rise to an amino acid substitution of Glutamate (E) to Aspartate (D) and Glutamine (Q) to Lysine (K) (**table 4** and **figure 4**). E185D also reported in 2003 while Q554K was reported for the first time. Alterations in the B subunit of DNA gyrase, i.e. less common than those in the A subunit, have been previously observed to confer decreased fluoroquinolone susceptibility.

Figure 4. Representative image of NGS *GyrB* gene Showing novel mutation at position 1660 (C replaced by A)

Out of total 112 clinical isolates, only 28 isolates carried *TetB* resistance gene as shown in Table 3.4. From these 28 *TetB* positive isolates, 2 isolates were randomly selected for mutational study. The results of analysis showed that both isolates have an amino acid substitution at position H204R and R334S as shown in Table 4 and Figure 5. Another nucleotide change was also observed at position 942 as shown in Table 3.14. This alteration has no effect on amino acid substitution, which was referred as synonymous mutation.

Figure 5. Sequence of *TetB* gene Showing mutations and conserved regions

The results revealed that out of 112 clinical isolates only 5.4% were positive for *tetR* gene as shown in Table 3.4. The results of mutational analysis showed that both isolates had an amino acid substitution at only position L108P as shown in Table 4 and Figure 6. Synonymous mutation was also found at nucleotide 279 as shown in Table 3.17, which have no effect on amino acid substitution.

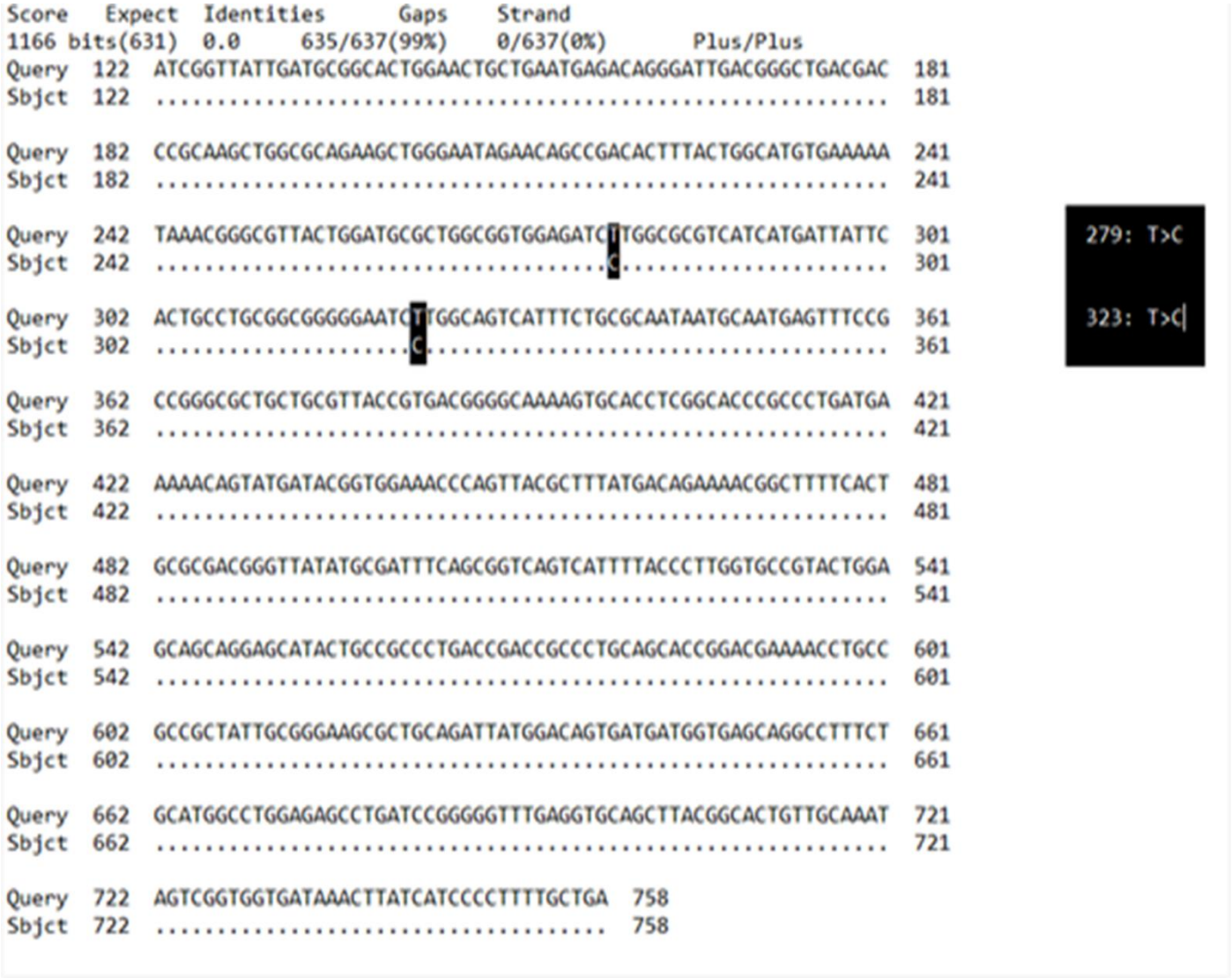

Figure 6. Showing nucleotides changes in sequence of *TetR* gene.

DISCUSSION

In humans, *E. coli* is the main cause of UTI, enteric, extra-intestinal and systemic infections worldwide. The Uropathogenic *Escherichia coli* (UPEC) predominantly causes nosocomial (30-50%) and community acquired (80-90%) UTIs [15]. The drug resistance in uropathogenic *E. coli* is spreading globally which is an alarming situation [16]. The transmission of antibiotic resistance among uropathogens causing UTIs is life threatening worldwide. This study determined the etiology of UTIs, antibiogram and mutational changes in antibiotic resistance genes. The present study reported *E. coli* in urine isolates. The same results were obtained in Saudi Arabia [17] and other countries [18] in which *E. coli* as the most common uropathogens (93.55%) recovered from urine samples. The other study recovered *E. coli* (75%) from urine samples [19]. It was reported in a study that *E. coli* was the most frequent pathogen found in urine [20]. *Ejrnaes K* reported that 89-90% of community acquired UTIs were due to *E. coli* [21]. The high frequency of UTIs was reported in female 63(56.3%) as compared to male patients 49(43.8%). The same results were observed in which the prevalence was high in female than male [22]. The results reported in other study also confirmed our findings in which high incidence rate of UTIs in females was reported [23]. The present research work reported the high incidence rate of UTIs (50%) in age group 21-40 years followed by 41-60 years (25%) and 14.3% (11-20 years). Apart from this, the low incidence (3.6%) was observed in age group above 61 years. This shows that infection is more common among low and middle aged groups, which was similar in earlier studies [24].

This study reported the antibiotics resistance in *E. coli* isolates ranging from 65 to 90% to majority of the antibiotics; AMP, SXT, DO, CIP and LEV. The antibiotic resistance was also observed in USA that *E. coli* exhibited 97.8% resistance to AMP, 92.8% to SXT, and 38.8% to CIP [25] which are on same line with our findings. The high rate of resistance to AMP (55%) and SXT (40%) were also observed in *E. coli* isolates in UK [26]. Another study [17] also reported resistant *E. coli* to antibiotics; CIP (27.27%), AMC (27.27%), AMP

(82.76) and 59.09% to cotrimoxazole. Ramirez-Castillo and coauthors also observed the 40% resistance of *E. coli* to antibiotics CIP and LEV and more than 70% of resistance to SXT [27]. The results of antibiogram in our study showed that 60% of the *E. coli* isolates were found resistant to CIP. A study also reported that nearly half of urinary *E. coli* were resistant to LEV or CIP [23] which confirms our findings. The resistance to quinolones varied from country to country but significantly less, from region to region. A study conducted in Panama reported the same results of 70% resistance while low prevalence of 37.8% in Latin America. India had the highest fluoroquinolone resistance rate with 75% non-susceptible UTIs isolates. The rate of resistance for fluoroquinolones at United States and Canada were 24% and 22% respectively in *E. coli* [28]. Fluoroquinolone resistance is increasing in UTIs pathogens worldwide [29]. The present study reported that (100%) of the *E. coli* isolates were found sensitive to TGC. Other antibiotics also showed good results; MEM(84.8%), SCF(83%), FEP(78.6%), AK(77.7%) and TZP(76.8 %). A study reported that MEM, TGC and AK have shown >95% of susceptibility against *E. coli* isolates [30]. *Rossi et. Al* also observed the same findings in which all the *E. coli* isolates were susceptible to antibiotics l; AK (97.3%), FEP (80.4%) and TZP (91%) [31]. Another study reported the best activity of TGC and MEM which are similar to our findings [32]. Furthermore, *Castillo et. Al* also observed good results of TZP against *E. coli* isolates [27].

The results of molecular characterization of antibiotic resistance genes revealed that high prevalence of *tetB* gene (25%) was observed than *tetR* (5.4%) in *E. coli*. the high prevalence of *TetB* gene was also reported in a study conducted by [33]. The same results of high prevalence for *tetB* gene was also observed in *E. coli* isolates origin [34]. Different studies reported that tet(B) gene has ability to transfer among bacteria species and genera [35]. This resistance is mainly due to different mechanisms; efflux pumps, ribosomal protection genes, plasmids and transposons. The efflux genes are the most commonly found tet genes in aerobic and facultative gram-negative bacteria [36].

In order to know the high antibiotic resistance mechanisms, the *E. coli* isolates were sequenced to find the novel mutations in resistance genes for fluoroquinolone and tetracycline. The Ojdana and coauthors reported that Quinolone resistance is due to mutations either in *gyrA* or *gyrB* gene [37]. Though, *gyrA* gene mutation is more common in quinolone resistant *E. coli* isolates [38]. The analysis of DNA sequence revealed that mutations has mostly occurred in the first half of *gyrA* gene known as Quinolone Resistant Determining Region (QRDR). The QRDR is in close relation with *gyrA* active site (Tyr-122), that interacts with quinolone and DNA [39]. However, mutation are also reported outside this region [40].

All randomly selected isolates have mutation in *gyrA* gene, resulting a change of an amino acid at codon83 from serine to leucine; so, this seems to be an initial step in attainment of an in vivo resistance to fluoroquinolones. It was proposed that substitution of hydrophobic amino acid (Leu, Trp, Ala or Pro) for hydrophilic amino acid (Ser) at codon 83 leads to stimulation of local conformation change of subunit A. Furthermore, our results reported that the deletion of codon 83 leads to CIP resistance which reconfirms the importance of this site and suggests that the deletion or substitution at this site will leads to enzyme-drug interaction. Moreover, mutation at codon87 in *GyrA* results in amino acid substitution of asparagine for aspartate which is a basic amino acid. In our results alanine is substituted by serine at position 828 which is also reported by Heisig and coauthors in 1993 [41] and Phan and coauthors in 2015 [42]. Another study reported that 5 strains of *E. coli* isolates had *GyrA* mutation, 2 had *gyrB* mutation while unidentified mutations were also observed in two isolates [11]. The alteration in *GyrA* gene at codon83 were also observed in another study [43]. Heisig and coauthors*,* 1993 and Vila and coauthors*,* 1994 also observed mutations at codon83 and 87. Two mutations (Asp-87 to Val-87) and (Asp-87 to Asn-87) has also been observed in another study at amino acid 87 of *GyrA* gene, which shows similarity with our results [44, 45].

It has been reported that mostly A subunit of DNA gyrase is targeted but decreased susceptibility of quinolones resistance can also be due to mutations in *gyrB* gene [46]. The finding in the present study reported mutation at amino acid 185 and 554. Ruiz, 2003 reported E185D mutation [43] while Q554K has not been reported yet i.e., first time observed in the current study. A study reported that *E. coli* K12 mutants has mutations at codon426 and 447, which leads to amino acid change of aspartate to asparagine and lysine to glutamate respectively [46], however, our isolates were negative for this known mutations.

CONCLUSION

The high spread of resistance mechanism among the bacteria is an issue of concern for clinicians during treatment management. The multidrug resistant *E. coli* is highly spreading among the hospitalized and non-hospitalized patients resulting life threatening infections. The current study documented that the *E. coli* isolates were highly resistant to AMP, SXT and CIP. The antibiotics like TGC, CO, MEM and SCF showed good results against *E. coli* isolates. In resistant isolates, 72 were positive for *GyrA*, 46 *GyrB* gene, 28 *tetB* and 6 isolates were positive for *tetR* gene. The results of mutational analysis revealed that *gyrA* gene have

3 amino acid substitutions (S83L, D87N and A828S), *gyrB* have 2 amino acid substitutions (E185D and Q554K), *tetB* also have 2 amino acid substitutions (H204R and R334S) while only one amino acid substitution in *tetR* gene (L108P) was observed. The novel mutation was detected in *gyrB* gene at codon554, due to which Glutamine (Q) is substituted by Lysine (K).

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