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Patterns of Efflux Pump Genes Among Tetracycline Resistance Uropathogenic *Escherichia coli* Isolates Obtained From Human Urinary Infections

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Abstract

Background: Uropathogenic *Escherichia coli* is one of the most prevalent infectious agents in humans, but its resistance to commonly used antibiotics is growing rapidly.

Objectives: The aim of this cross sectional study was to investigate the prevalence of tetracycline resistance determinants in urinary *E. coli* isolates obtained from patients in Iran.

Methods: A total of 50 *E. coli* isolates from human urinary infections were characterized by cultural, biochemical, and molecular tests from 2014 to 2015. Isolates were tested for resistance to tetracycline by disc diffusion method. Then, the prevalence of tetracycline efflux genes (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetH* and *tetZ*) was detected by means of molecular polymerase chain reaction method (PCR).

Results: Of the 50 *E. coli* isolates tested, tetracycline resistance was identified in 62% of the strains. PCR analysis revealed that 36% of these isolates contained *tetB* gene, followed by *tetA* determinant with 32% frequency. The prevalence of *tetG*, *tetZ*, *tetC*, *tetE*, *tetH*, and *tetD* were 16%, 14%, 12%, 12%, 11%, and 8% among the isolates.

Conclusions: Tetracycline resistance is widespread among uropathogenic *E. coli* isolates from human infections. Moreover, the distribution of tetracycline resistance determinants among the studied *E. coli* was very similar to the findings of the international sourced gallery of clinical *E. coli* Strains.

Keywords: Antibiotic Resistance, Tetracycline, Urinary Infections, Uropathogenic *E. coli*

1. Background

Escherichia coli is a commensal bacterium living in human and animal intestine, but is also found in many environments such as water, soil, or plants. Moreover, some *E. coli* strains are able to cause disease of the gastrointestinal, urinary, or central nervous system in the hosts. Intestinal pathogenic *E. coli* strains are divided into 6 categories as follow: Enteropathogenic *E. coli* (EPEC); enterohaemorrhagic *E. coli* (EHEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive *E. coli* (EIEC); enteraggregative *E. coli* (EAEC); and diffusely adherent *E. coli* (DAEC) (1).

Among the various extraintestinal infections caused by this bacterium, urinary infections are very important in humans (2). Recent reports have indicated that the rate of antibiotic resistance among *E. coli* isolates is increasing rapidly (3). The main reason for this problem is the widespread clinical use of antimicrobials, which leads to the suppression of susceptible normal flora and rise of resistant strains (4).

Tetracyclines are broad- spectrum antibiotics that act through inhibition of protein synthesis. The suitable antibacterial characteristics of these antibiotics and the absence of any important side effects have led to their widespread use in treating human infections (5). Efflux pumps are transmembrane proteins, which export toxic materials such as antibiotics out of the bacterial cell. Among the prokaryotes, 5 major efflux pumps are detected as follow: MFS (major facilitator super family); MATE (multidrug and toxic efflux); RND (resistance-nodulation-division); SMR (small multi drug resistance); and ABC (ATP binding cassette) (6). Efflux pumps are the most abundant in Gram-negative bacteria, but ribosomal protection mechanisms of resistance is more prevalent in Gram-positive bacteria (7).

Tetracycline efflux pumps belong to FMS group, and most of them export tetracycline from the bacteria but not to minocycline. Only the product of the tetB gene could efflux both antibiotics out of the cell (5). These pumps belong to 6 groups according to amino acid sequence similarities: *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetH*, *tetJ*, *tetZ*, *tetI*, and

tet30 are placed in group 1 (5).

2. Objectives

In the present study, the occurrence of phenotypic tetracycline resistance and carriage of *tet* resistance genes in uropathogenic *E. coli* strains was investigated in Iranian patients.

3. Methods

3.1. Isolation and Identification of Uropathogenic E. coli

A total of 50 uropathogenic *E. coli* isolates from human urinary samples were obtained from the microbiology laboratory of Azerbaijan hospital, Urmia, Iran, from October 2014 to February 2015. All samples belonged to hospitalized patients aged 20 to 40 years. The urine samples were streaked on to MacConkey agar and incubated at 37°C for 24 hours. Then, the plates were examined for *E. coli* red colonies with a dark red center. Other biochemical tests like indol, methyl red, voges-proskauer, Simmons citrate, and TSI were conducted to confirm the presence of *E. coli* (8).

3.2. Molecular Characterization of Uropathogenic E. coli

Escherichia coli confirmation was achieved by PCR assay (9). *E. coli* cultures were grown overnight at 37°C, and the chromosomal DNA was extracted using bacterial genomic purification kit (iNtRON, Korea). Then, the extracted DNA was used as a template for PCR amplification of uropathogenic *E. coli* 16s rRNA gene by forward primer F: 5/- GTA TAG ATA CCC TGG TAG TCCA-3,/ and reverse primer R: 5/- CCC GGG AAC GTA TTC ACC G-3/. PCR reactions were carried out in the total volume of 25 μL using DNA thermo cycler (MWG AG BIOTECHTHERMAL CYCLER, USA).The conditions used for the PCR assay are as follow: 3 minutes of initial denaturation at 95°C, followed by 26 cycles of 94°C for 1 minute; 55°C for 1 minute, and 72°C for 10 minutes (9). The PCR products were separated by 1% agarose gel electrophoresis stained with red safe (Sigma Aldrich, Germany) and visualized under UV exposure.

3.3. Antibiotic Susceptibility Testing

Tetracycline susceptibility profiles of isolated uropathogenic *E. coli* were determined via the disc diffusion method (10). A 15 mm Muller-Hinton medium plate was swabbed with nutrient broth inoculated with *E. coli* and incubated to a turbidity of 0/5 McFarland standard. Commercially tetracycline disks (30 μg) were placed on the inoculated plates (Padtan Teb, Iran). Then, all the plates were incubated aerobically at 37°C for 18 - 20 hours. The diameters zone of inhibition (mm) around the disks were measured and interpreted by referring to the performance standard for antimicrobial susceptibility testing, as described by the clinical and laboratory standards Institute (CLSI) guidelines, 2015 (11). The percentage of *E. coli* resistant, intermediate and susceptible to tetracycline was determined.

3.4. Identification of Tetracycline Efflux Genes using PCR Assay

3 PCR amplification assays were performed to determine the prevalence of *tetA*, *tetB*, *tetD*, *tetE*, *tetH*, *tetG*, and *tetZ* among all tetracycline resistant *E. coli*. Table 1 demonstrates the sets of primers used to identify these determinants (12). The first PCR assay performed to amplify the *tetB* and *tetE* genes is as follow: Initial denaturation at 94°C for 1 minute, 25 cycles each consisting of minute at 94°C, minute at 50°C, and 72°C for 10 minutes. The second PCR used to detect *tetA* and *tetD* genes was performed as above, but the annealing temperature was 48°C.

Table 1. Primer Sequences Used for PCR Identification of *tetB*, *tetA*, *tetE*, *tetC*, *tetD*, *tetG*, *tetH*, and *tetZ*

Primer	Gene	Sequences
Forward	*tetA*	5/ GCGCGATCTGGTTCACTCG 3/
Reverse	*tetA*	5/ AGTCGACAGYRGCGCCGGC 3/
Forward	*tetB*	5/ TACGTGAATTTATTGCTTCGG 3/
Reverse	*tetB*	5/ ATACAGCATCCAAAGCGCAC 3/
Forward	*tetC*	5/ GCGGGATATCGTCCATTCCG 3/
Reverse	*tetC*	5/ GCGTAGAGGATCCACAGGACG 3/
Forward	*tetD*	5/ GGAATATCTCCCGGAAGCGG 3/
Reverse	*tetD*	5/ CACATTGGACAGTGCCAGCAG 3/
Forward	*tetE*	5/ GTTATTACGGGAGTTTGTTGG 3/
Reverse	*tetE*	5/ AATACAACACCCACACTACGC 3/
Forward	*tetG*	5/ GCAGAGCAGGTCGCTGG 3/
Reverse	*tetG*	5/ CCYGCAAGAGAAGCAGAAG 3/
Forward	*tetH*	5/ CAGTGAAAATTCACTGGCAAC 3/
Reverse	*tetH*	5/ ATCCAAAGTGTGGTTGAGAAT 3/
Forward	*tetJ*	5/ CGAAAACAGACTCGCCAATC 3/
Reverse	*tetJ*	5/ TCCATAATGAGGTGGGGC 3/
Forward	*tetZ*	5/ CCTTCTCGACCAGGTCGG 3/
Reverse	*tetZ*	5/ ACCCACAGCGTGTCCGTC 3/

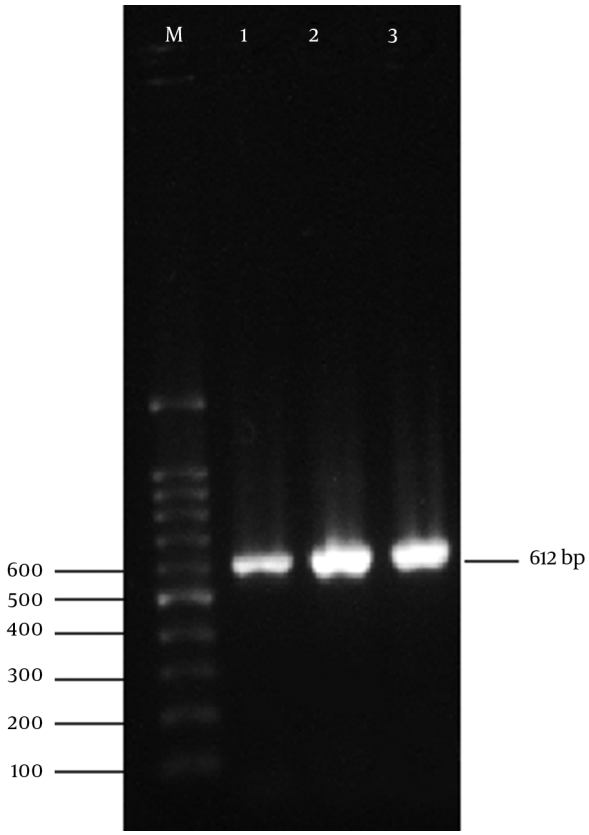
The third PCR regimen used to amplify *tetH*, *tetG*, and *tetZ* was carried out as described above, but the annealing temperature was 46°C. Finally, the reaction products were run on 1% agarose gel, and the prevalence of each tetracycline efflux gene was determined using agarose gel elec-

trophoresis. Descriptive statistics, such as the percentage of tetracycline efflux genes, were done using the statistical package, SPSS, Version 15.0.

4. Results

All 50 isolated bacteria from urinary infections, possessed the cultural, morphological, and biochemical characteristics of *E. coli*. Also, *16s rRNA* gene of *E. coli* were detected in all of the isolates by PCR assay using *16s rRNA* universal primers. Figure 1 displays the product of 16s rRNA gene found at 612 bp using 100 bp DNA marker.

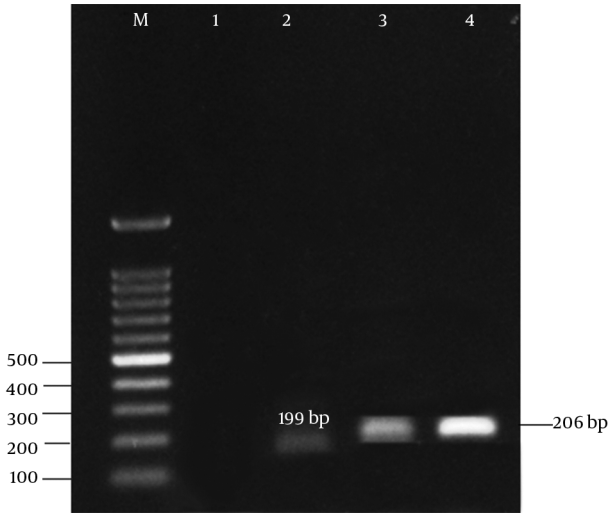
Figure 1. Gel Electrophoresis of the PCR Products of *16s rRNA* Gene



Lane M, 100 bp ladder marker; lane 1, positive control; lane 2, 3, *E. coli* 16s rRNA gene gound at 612 bp.

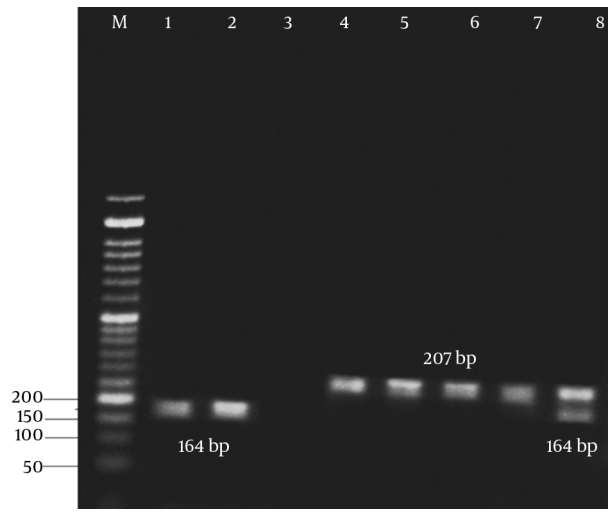
The results of antibiotic susceptibility testing in *E. coli* isolates showed that 31 (62%) of them were phenotypically resistant to tetracycline, 16 (32%) were susceptible, and 3 (6%) showed intermediate susceptibility to tetracycline. Three multiplex PCR assay were developed to monitor the prevalence of resistance determinants for tetracycline. The results are presented in Figures 2 - 4.

Figure 2. PCR Identification of *tetB* and *tetE* Genes with 206 and 199bp on 1% Agarose Gel



Lane M, 50 bp ladder marker; lane 2, *tetE* gene found at 199 bp; lane 3, 4, *tetB* gene found at 206 bp.

Figure 3. *tetA* and *tetC* Genes Found at 164 and 207 bp on Agarose Gel after PCR Amplification



Lane M, 50 bp ladder marker; lane 8, *tetA* (164 bp) and *tetC* (207 bp).

The frequency and distribution of tetracycline resistance genes among *E. coli* strains used in the presented study are demonstrated in Table 2.

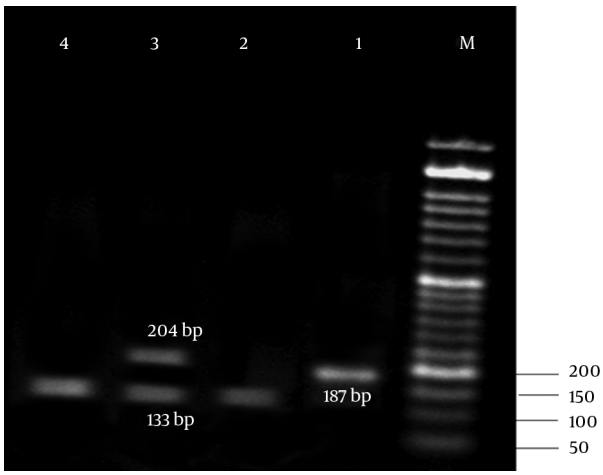
The results of the present study revealed that all tetracycline resistant isolates carried at least 1 of the *tet* determinants examined; *tetB* was the most prevalent gene detected in 36% of the resistance isolates, followed by *tetA*

Table 2. The Frequency of Tetracycline Efflux Genes in *E. coli* Obtained from Patients with Urinary Infections

Sample	*tetZ*	*tetG*	*tetH*	*tetD*	*tetC*	*tetA*	*tetE*	*tetB*
Frequency[a]	14	16	8	11	12	32	12	36

[a]Values are expressed as %.

Figure 4. Gel Electrophoresis for PCR Product of *tetH*, *tetG* and *tetZ* Markers in *E. coli* Isolates



Lane M, 50 bp ladder marker; lane 1, *tetH* (187 bp); lane 3, *tetG* (133 bp and *tetZ* (204 bp).

(32%), and tet A + B (18%). Moreover, *tetC*, *tetE*, *tetG*, *tetD*, and *tetZ* were found to have lower frequencies. However, the lowest prevalent gene among the studied determinants belonged to *tetH* (8%).

5. Discussion

Among 50 *E. coli* strains isolated from patients with urinary infections, 31 strains showed resistance to tetracycline. Monitoring the distribution prevalence of tetracycline resistance genes, we found that the *tetB* gene had the highest frequency among the studied determinants. *Escherichia coli* is a facultative Gram-negative bacterium, which can cause many infections, like diarrhea, urinary tract infections, meningitis, peritonitis, and septicemia in humans (13). However, the most prevalent form of the extraintestinal infection caused by *E. coli* is urinary tract infection (14).

Antibiotic resistance in *E. coli* has emerged and increased in many countries and led to the difficult treatment of infectious bacteria (2). In the present study, 62% of human *E. coli* strains were resistant to tetracycline, which supported the above hypothesis. Tetracyclines are broad-spectrum antibiotics that are used for treatment and prevention of human infections (15). Chlortetracycline was the first tetracycline identified in the late 1940s (16). However, heavy clinical use of tetracyclines has a major role in the emergence and dissemination of tetracycline resistance among infectious bacteria (17).

Different tet genes are responsible for tetracycline resistance in Gram- negative bacteria like *E. coli* (15). However, the most common *tet* resistance mechanism in *E. coli* is tetracycline efflux pumps, which exports the drug out of the cell (17). These proteins belong to 6 groups: *tetA*, *tetB*, *tetC*, *tetD*, *tetG*, *tetH*, *tetZ* , *tetE*, *tetI*, and *tet30* that are placed in Group 1. Several investigations have studied tetracycline resistance in bacteria by examining their ability to grow in the presence of drugs, but these studies are not useful for determining the types of tetracycline resistance determinants responsible for antibiotic resistance in bacteria (7). On the Other hand, molecular microbiology techniques can accurately describe genetic basis of antibiotic resistance in bacteria (18).

In the present study, the molecular PCR assay was also used to determine the tetracycline resistance genes prevalence among *E. coli* isolates. In 2006, Karami et al. studied the prevalence of tetracycline resistance genes in intestinal *E. coli* strains and identified *tetB* and *tetA* as the most common tetracycline resistance genes in human *E. coli* (10). Our findings about the frequency of tetracycline resistance genes in urinary *E. coli* are in agreement with their results.

In 2007, Tuckman et al. examined the occurrence of tetracycline resistance genes among *E. coli* isolated from patients and found that *tetA* and *tetB* had the highest frequency among the studied determinants. In the present study, the same determinants had the highest occurrence among tetracycline efflux genes belonging to Group 1. The results of monitoring the prevalence of tetracycline resistance genes in *E. coli* strains isolated from human urinary infections, confirmed the emergence and spreading of the resistant *E. coli* in human populations.

Footnotes

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Conflict of Interest: This work was financially supported by Tabriz University.

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