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Tetracycline Resistance in Diarrheagenic *Escherichia coli* in Southern Iran

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ABSTRACT

Background: Diarrhea is a predominant contributor to childhood mortality in developing nations, particularly among those under the age of five. Diarrheagenic *Escherichia coli* is one of the main causes of endemic and epidemic diarrhea worldwide. This study aimed to assess the frequency of antibiotic resistance and tetracycline resistance genes in diarrheagenic *E. coli* strains obtained from children under five years of age.
Materials & Methods: 550 pediatric fecal samples were obtained from the laboratory archives of three Shiraz hospitals between April 2018 and December 2019. Bacteria were isolated and identified using standard bacteriological procedures. Antimicrobial susceptibility testing was performed using the disk diffusion method according to CLSI (Clinical and Laboratory Standards Institution) 2019 guidelines. Molecular analysis of tetracycline resistance genes (*tetA*, *tetB*, and *tetC*) was performed using multiplex PCR.
Findings: A total of 112 diarrheagenic *E. coli* strains were isolated. Antibiogram analysis revealed a high antibiotic resistance to tetracycline (57.1%) and the lowest antibiotic resistance to amikacin and nitrofurantoin (3.3%). Ninety-three (83%) isolates showed a multidrug-resistance (MDR) pattern. Of the 64 tetracycline-resistant isolates, 56 (87.50%) contained tetracycline resistance genes. The frequencies of tetracycline resistance genes *tetA, tetB,* and *tetC* in tetracycline-resistant isolates were 58.9, 44.6, and 14.3%, respectively.
Conclusion: Tetracycline-resistance is more common among diarrheagenic *E. coli* strains. Furthermore, resistance to antimicrobial agents is more common in *E. coli* strains isolated from pediatric patients and is correlated with increased antibiotic use among patients with gastroenteritis. However, the influence of unsupervised antibiotic administration on specific antibiotics remains unclear.

Keywords: Diarrheagenic *Escherichia coli*, Enteropathogenic *E. coli*, *E. coli* infections, antimicrobial resistance

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Introduction

Diarrhea stands as a prevalent infectious disease and a significant contributor to illness and death among children under five years old in developing countries [1-4]. Global statistics derived from leading research institutions reveal that nearly one billion cases of diarrhea occur annually among children under five years old, contributing to approximately 4.5 million childhood deaths each year [5]. In Iran, diarrhea has been reported to cause approximately 18 million cases of illness, leading to 12 million medical consultations, one million hospitalizations, and 516 deaths annually in children under five years old [6]. After respiratory infections, diarrhea ranks as the second leading cause of death in children [7]. Among bacterial agents, diarrheagenic *Escherichia coli* is recognized as the major cause of both endemic and epidemic diarrhea globally [8]. *E. coli* has also been reported as one of the most common bacterial pathogens causing acute diarrhea in children under five years of age in Iran and other developing countries, which is associated with significant morbidity and mortality [1, 2]. Tetracycline is a commonly-used antibiotic available in Iran; thus, as a treatment option, it is very important to investigation its resistance genes. Clinicians often evaluate tetracycline resistance in *E. coli* due to its implications for treatment efficacy. Understanding resistance patterns helps in selecting appropriate antibiotics, especially in cases where *E. coli* infections are prevalent. Antimicrobial resistance is a widespread global issue in human and animal populations, and an increasing prevalence of antibiotic-resistant pathogens has been reported worldwide. The main factor behind the increase in antibiotic resistance of pathogenic bacteria is the overuse of antibiotics, which leads to the outburst and dissemination of resistant pathogens and resistance genes [9]. Tetracycline is a cost-effective antibiotic with minimal adverse effects, which is extensively used to treat infections in animals and humans [10]. Tetracycline resistance in bacteria occurs through four mechanisms: energy-dependent efflux pumps, ribosomal protection, effective tetracycline binding, tetracycline-inactivating enzyme production, and target modification [11]. Among antibiotic-resistant *E. coli*, tetracycline-resistant *E. coli* is highly prevalent [10]. In antibiotic-resistant *E. coli* strains, tetracycline resistance is attributed to the presence of *tetA*, *tetB*, *tetC*, *tetD*, and *tetE* genes, among which the frequency of detection of *tetA*, *tetB*, and *tetC* genes is higher than that of other genes [12]. Mobilizable plasmids and conjugative transposons play pivotal roles in the spread of tetracycline resistance genes [13]. Enhancing our comprehension of how resistance genes are acquired and transmitted at the molecular level may aid in mitigating the prevalence of resistant bacterial strains [14]. In recent years, PCR-based techniques have emerged as rapid, specific, and sensitive alternatives for the detection of various antimicrobial resistance genes in clinical samples. Multiplex PCR, an expedient and specific molecular approach, enables simultaneous detection of multiple target sequences in a single assay, which is valuable for epidemiological investigations [15].

Objectives: The primary aim of this study was to ascertain the frequency of tetracycline resistance genes (*tetA*, *tetB*, and *tetC*) in diarrheagenic *E. coli* strains obtained from children younger than five years old.

Materials and Methods

Clinical specimens and bacterial isolation: A total of 550 fecal samples were retrieved from the archived laboratory specimens of three hospitals in Shiraz. These samples were collected from the pediatric population under the age of five with acute diarrhea between April 2018 and December

2019. The samples were transferred to the Research Microbiology Laboratory of the Microbiology Department of Islamic Azad University of Shiraz for confirmatory analysis. No sampling was carried out exclusively for this study, and all principles of patient confidentiality were rigorously observed. The research protocol was approved by the Microbiology Department of Islamic Azad University, Shiraz Branch. Diarrhea was characterized as an increase in the fluidity, volume, and frequency of stools compared to the individual's typical defecation patterns. A loopful of each diarrheal sample was streaked onto MacConkey agar and incubated at 37 °C for 24 hrs, which resulted in the development of pink colonies. These colonies were then subcultured on Eosin Methylene Blue (EMB) agar. Colonies that displayed a green metallic sheen were subsequently selected for further analysis using a series of biochemical tests and the API 20 E system (bioMérieux Co., France).

Phenotypic resistance to tetracycline and other antibiotics: *E. coli* strains identified as positive for diarrheagenic *E. coli* were assessed for susceptibility to antimicrobial agents using the disk diffusion method on Mueller Hinton agar (Merck, Germany) in accordance with the guidelines provided by CLSI (Clinical and Laboratory Standards Institution) 2019. [16]. The following antibiotic disks were used: tetracycline (30 µg), cefotaxime (10 µg), ceftriaxone (30 µg), cefixime (15 µg), imipenem (30 µg), ciprofloxacin (30 µg), nalidixic acid (30 µg), chloramphenicol (5 µg), streptomycin (10 µg), gentamicin (30 µg), amikacin (300 µg), ampicillin (10 µg), penicillin (10 U), levofloxacin (5 µg), azithromycin (15 µg), clarithromycin (15 µg), erythromycin (15 µg), and nitrofurantoin (30 µg) (Mast Diagnostics, Merseyside, UK). The standard *E. coli* strain ATCC 25922 was used for quality control. Rigorous criteria were established to define multidrug resistance (MDR), which includes resistance to at least four classes of antimicrobial agents. The minimum inhibitory concentration (MIC) of tetracycline was evaluated using an E-test strip following the instructions provided by the manufacturer (bioMérieux Co., France).

DNA extraction: DNA was extracted from the isolated *E. coli* strains using a DNA extraction kit (Kiagene Co., Iran) and used as a template for PCR.

Multiplex PCR for *tet* gene detection: All *E. coli* strains exhibiting resistance to tetracycline were analyzed for the presence of tetracycline resistance genes (*tetA*, *tetB*, and *tetC*) using multiplex PCR. The primers utilized in this investigation are detailed in Table I. The methodology was optimized with reference strains known to harbor *tet* genes: *E. coli* K-12 NC 50078-02 (*tetA*), *E. coli* K-12 NC 50019 (*tetB*), and *E. coli* K-12 NC 50270-01 (*tetC*), all were sourced from the Public Health Laboratory Service in London, United

Table 1) Amplicons and primers of PCR for diarrheagenic *E. coli*

Target	Sequence (5'→3')	Amplicon (bp)	Reference
tetA	F: AAGCGAGCGGGTTGAGAG R: GCCTTTCCTTTGGGTTCTC	326	This study
tetB	F: ACTTCGGTATCTGTATTATCACG R: TTATCTTTGCTCCTTGGCTTG	415	This study
tetC	F: TTGTTTCGGCGTGGGTATG R: CTGACTGGGTTGAAGGCTCTC	188	This study

Kingdom. Each multiplex PCR reaction was conducted in a 500 µL Eppendorf tube with a total volume of 25 µL consisting of 12.5 µL of PCR master mix, 2 µL of extracted DNA, 0.2 mM of each primer (listed in Table I), 1.5 mM $MgCl_2$, 0.2 mM of a dNTPs mixture, and 1 U of Taq DNA polymerase (Qiagen Co., Iran). Amplification was carried out using a thermal cycler (Genius; Techne, UK). The protocol included an initial denaturation step of 5 min at 95 °C, followed by 30 cycles of amplification for 1 min at 95 °C, 1 min at 58 °C, and 1 min at 72 °C, with a final extension phase of 5 min at 72 °C. The resulting PCR products were analyzed by electrophoresis on a 1.5% agarose gel, visualized by ethidium bromide staining, and examined under UV (ultraviolet) light.

Statistical analysis: Statistical significance of data was assessed using Chi-square and Fisher's exact tests via Statistix 7.0 for Windows (Analytical Software, Tallahassee, FL, USA), with a significance threshold set at $p < .05$.

Findings

Description of sample population: Out of the 550 stool samples analyzed, 112 (20.36%) were found to be positive for diarrheagenic *E. coli*. Of the patients with a positive culture for diarrheagenic *E. coli*, 71 (63.4%) were boys with a mean age of 17 months, and 41 (36.6%) were girls with a mean age of 14 months. The seasonal distribution of patients was as follows: 43 in spring, 45 in summer, and 24 in fall. The most common clinical symptoms were dysentery (20.78%), fever (64.93%), and nausea (67.53%). The majority of diarrheagenic *E. coli* strains were recovered from patients under the age of one year (56.4%), followed by 1–2 years (34.5%), and 3–5 years (9.1%).

Antimicrobial resistance: The antibiotic resistance patterns of 112 diarrheagenic *E. coli* isolates identified in this study are mentioned in Table 2. Additionally, by interpreting the results of the disk diffusion method, 93 (83%) isolates exhibited the MDR phenotype, indicating resistance

Table 2) Antibiotic resistance patterns of diarrheagenic *E. coli*

Antibiotic	Resistance (%)	Antibiotic	Resistance (%)
Cefotaxime	43.3	Ceftriaxone	46.7
Cefixime	61.7	Ampicillin	69.3
Penicillin	100	Imipenem	6.5
Ciprofloxacin	9.7	Levofloxacin	4.8
Nalidixic Acid	37	Chloramphenicol	8.7
Tetracycline	57.1	Streptomycin	56.7
Gentamycin	17.7	Nitrofurantoin	3.3
Amikacin	3.3	Azithromycin	40.3
Clarithromycin	100	Erythromycin	100

to four or more antibiotic classes. The resistance profile of MDR isolates showed that resistance to at least four, five, six, seven, eight, and nine antibiotic classes was 32.4, 17.9, 12.6, 11.7, 4.8, and 4.8%, respectively.

Tetracycline resistance and correlation of *tet* gene with other antibiotic resistances: Sixty-four (57.1%) strains exhibited phenotypic resistance to tetracycline. The tetracycline-resistant strains were further examined for the presence of *tetA*, *tetB*, and *tetC* genes as well as their MICs. The sizes of *tetA*, *tetB*, and *tetC* amplicons were 300, 256, and 280 bp, respectively (Figure 1). Of the 64 tetracycline-resistant isolates, 56 (87.50%) isolates contained tetracycline resistance genes. The *tetA* gene was the most frequently-detected gene, found in 58.9% (33 out of 56) of the tetracycline-resistant strains, followed by *tetB* and *tetC*, which were found in 44.6% (25 out of 56) and 14.3% (8 out of 56) of the tetracycline-resistant strains, respectively. In addition, the results of simultaneous detection of *tet* genes showed that 27.8% of the *E. coli* isolates carried two *tet* genes, of which nine (16.1%) strains harbored *tetA* and *tetB*, four (7.1%) strains harbored *tetA* and *tetC*, and two (3.6%) strains harbored *tetB* and *tetC*. Moreover, only one (1.8%) strain harbored all the three *tet* genes. The MICs of the tetracycline-resistant strains varied between 29 and 243 mg/L, with a median of 128 mg/L. Strains harboring the *tetA* gene exhibited significantly higher MIC values compared to those containing the *tetB* gene (median: 243 mg/L versus 102 mg/L; p= .0001). Among all diarrheagenic *E. coli* strains, 76.8% harbored the *tet* gene. The frequencies of *tetA*, *tetB*, and *tetC* in the strains harboring the *tet* genes were 61.6, 40.2, and 11.6%, respectively. Tetracycline resistance was observed in combination with resistance to cefixime (n=10), ceftriaxone (n=8), cefotaxime (n=7), nalidixic acid (n=4), and streptomycin (n=15). The tetracycline-resistant strains containing *tetA* and *tetB* demonstrated greater resistance to other antibiotics than tetracycline-susceptible strains, with resistance rates of 61 and 42% compared to 13% for susceptible strains, respectively (p= .0003 and p= .002, respectively).

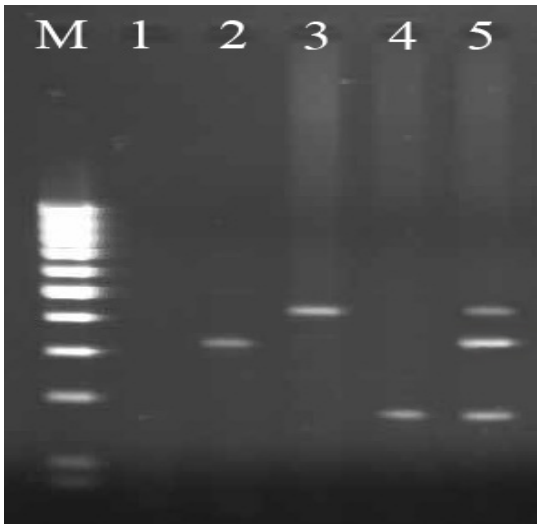

Figure 1) Agarose gel electrophoresis of *E. coli* tetracycline resistance genes. Lane M: 1000 bp DNA ladder; lane 1: negative control; lane 2: *tetA* (300 bp); lane 3: *tetB* (256bp); lane 4: *tetC* (280bp); lane 5: each *tetA*, *tetB*, and *tetC* by multiplex PCR

Discussion

Diarrhea is one of the most prevalent infections in human populations and is the leading cause of mortality among children under five years of age in developing countries [4, 7, 17, 18]. Among microbial agents, *E. coli* is the most important cause of gastroenteritis and diarrhea in infants and children under five years of age [4, 19]. Numerous investigations have been conducted to survey the prevalence of intestinal pathogens causing diarrhea worldwide, and comparing the results shows different prevalence rates in different regions [19]. In this study, 112 (20.36%) diarrheagenic *E. coli* strains were isolated from a total of 550 stool samples collected from children experiencing diarrhea. A prior study conducted in Tehran, Iran, reported a higher isolation rate of 38.8% [10]. Conversely,

Albert et al. (2009) found that diarrheagenic *E. coli* was not the primary cause of diarrhea in hospitalized children in Kuwait. They suggested that diarrheagenic *E. coli* was not epidemiologically linked to Kuwaiti children admitted for diarrhea [20]. Hien et al. (2008) reported that the prevalence of diarrheagenic *E. coli* strains isolated from stool specimens was 25.7% among Vietnamese children under five years of age [21]. Medina et al. (2010) reported that diarrheagenic *E. coli* was the most commonly-isolated pathogen from diarrheal patients [22]. Also, the study by Theresa et al. (2009) indicated that the prevalence of diarrheagenic *E. coli* was 29% among children diagnosed with gastroenteritis [23]. Antibiotic therapy is a treatment strategy for infectious diarrhea in patients. Cephalosporins, quinolones, and aminoglycosides are the most commonly-prescribed antibiotics for the treatment of diarrhea caused by *E. coli* [24]. Due to the excessive use of antimicrobial drugs, the prevalence and spread of resistant strains and resistance genes have increased in hospitals [4, 9]. Thus, bacterial resistance to antibiotics has become a major health challenge in this century [9]. Although the intestinal flora is composed of many bacterial species, *E. coli* is more resistant to antibiotics than other *Enterobacteriaceae*, and the prevalence of antibiotic resistance among commensal bacteria is increasing in both developing and developed countries [25]. In this study, a high level of resistance was observed to tetracycline (57.1%), and the lowest resistance was against amikacin (3.3%). Djie-Maletz et al. (2008) reported a high drug resistance rate in children with diarrhea. Their study results showed that diarrheagenic *E.coli* was resistant to ampicillin (approximately 81-91%), trimetoprim (76-88%), and chloramphenichol (41-44%) [17]. In another study by Theresa et al. (2009), diarrheagenic *E. coli* strains demonstrated significant resistance to ampicillin (85%), co-trimoxazole (79%), tetracycline (65%), and nalidixic acid (28%) [23]. Garcia and colleagues (2011) showed that the highest antibiotic resistance rates in diarrheagenic *E. coli* were against tetracycline (39.7%), ampicillin (30.9%), and sulfamethoxazole-trimethoprim (30.9%). In addition, they reported antimicrobial resistance against ceftazidime, ceftriaxone, imipenem, and piperacillin-tazobactam [4]. In this study, *E. coli* strains showed an incidence of tetracycline resistance similar to that reported in recent surveillance studies worldwide (50–65%) [4, 23]. However, Okeke and colleagues (1999) observed that 100% of human *E. coli* isolates were resistant to tetracycline [26]. The high prevalence of tetracycline resistance genes in diarrheagenic *E. coli* strains is likely attributable to the indiscriminate use of this antibiotic. In terms of aminoglycosides, low levels of resistance have been reported to both amikacin and gentamicin, indicating that these antimicrobials are still effective against diarrheagenic *E. coli*. In this study, approximately 83% of the strains were found to be multidrug resistant. Usein et al. (2009) studied antibiotic resistance patterns of diarrheagenic *E. coli* isolates from children and found that the prevalence of multidrug resistance in isolates was 56.4%, and 49% of isolates demonstrated resistance to approximately three antibiotics, including ampicillin, trimethoprim, and streptomycin [3]. Wilkerson et al. (2004) also reported that 52% of diarrheagenic *E. coli* isolates were simultaneously resistant to two or more different classes of antibiotics, such as ampicillin and tetracycline [15]. Multiplex PCR indicated that 87.5% of the tetracycline-resistant *E. coli* isolates harbored a single *tet* gene. The frequency of tetracycline resistance genes may indicate the widespread distribution of tetracycline-resistant strains among other diarrheagenic

E. coli strains. Among all *E. coli* strains harboring *tet* resistance genes, tetracycline resistance was associated with the presence of *tetA* (58.9%), *tetB* (44.6%), *tetC* (14.3%), *tetA* and *tetB* (16.1%), *tetA* and *tetC* (7.1%), *tetB* and *tetC* (3.6%), and all the three *tet* genes, which were also found in one strain (1.8%). Sandalli and colleagues (2010) characterized tetracycline resistance genes in tetracycline-resistant *Enterobacteriaceae*. Among 52 tetracycline-resistant isolates, *tetA* and *tetB* genes were detected in 15.3 and 19.2% of strains, respectively, while *tetC* was found in 1.9% of isolates [18]. In another study, Schwaiger and colleagues (2010) found that *tetB* was the most common *tet* gene (56%) in human strains, and the frequency of this gene was significantly higher than that of other genes [12]. Ahmed et al. (2010) reported that among all tetracycline-resistant *E. coli* isolates, 86.8% tested positive for *tet* genes via PCR, with *tetB* being the most prevalent gene (71%), followed by *tetA* (18%) and both *tetA* and *tetB* (11%) [14]. Tuckman et al. (2007) found that among tetracycline-resistant strains, *tetA* and *tetB* were responsible for tetracycline resistance of 58% of all isolates, with 26% of strains harboring the *tetA* gene and 32% possessing the *tetB* gene [11]. Additionally, Karami et al. (2006) concluded that all tetracycline-resistant strains harbored at least one *tet* gene, with *tetB* being the most common (51%), followed by *tetA* (49%), while *tetC* was identified in only one strain [27]. This study results showed that among tetracycline-resistant *E. coli*, *tetA* was more prevalent than other *tet* genes. This result is similar to that of Sianglum et al. (2009), who reported that among 15 of 18 tetracycline-resistant strains, *tetA* was the most prevalent (77.7%) compared to other *tet* genes [28]. Furthermore, the co-occurrence of *tetA* and *tetB* in *E. coli* isolates was highly prevalent in the present study. Additionally, various combinations of *tetA*, *tetB*, and other *tet* genes have been frequently identified, particularly in *E. coli* strains. This study results indicated that 27.8% of *E. coli* isolates carried simultaneously two *tet* genes, which is in agreement with the results of another study by Schwaiger and colleagues (2010), who found two *tet* genes in 17% of human isolates [12].

Conclusion

The prevalence of antibiotic resistance in diarrheagenic *E. coli* among children under five years of age with diarrhea in southwestern Iran is notably high. This is likely attributable to inadequate diagnostic practices and inappropriate use of antibiotic regimens. Therefore, the detection of resistance genes in tetracycline-resistant *E. coli* should be considered; in addition, it is essential to implement strategies for the appropriate use of antimicrobial drugs in Iran. It is concluded that the advancement of multiplex PCR techniques for simultaneous detection of resistance genes in a single PCR reaction significantly reduces the time and effort required to analyze multiple resistance genes. This approach aids researchers in elucidating the role of antibiotic resistance in diarrheal diseases.

The primary limitations of this study were the small sample size, the lack of detailed patient information, and the inability to validate gene sequences through sequencing. These constraints were due to administrative challenges and financial resource limitations.

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