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# Tetracycline Resistance Genes in *Escherichia coli* Strains Isolated From Biofilm of Drinking Water System in Poultry Farms

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## Abstract

The frequency of resistance genes in clinical and environmental isolates increases the need for molecular knowledge about the antibiotics used in the prevention and treatment of infectious diseases. Hence, in present study, 100 water biofilm samples were collected from poultry water systems from 20 randomly chosen broiler farms in Isfahan province, Iran. *Escherichia coli* strains were identified by conventional microbiological and biochemical characterization. All *E. coli* strains were evaluated for sensitivity to commercially important antibiotics by the disc diffusion method. Furthermore, the tetracycline (TC) resistance genes tetA, tetB, tetC, tetD, tetE, tetG, tetK, tetL, tetM, tetO, tetS were investigated using three multiplex polymerase chain reactions (PCR). The microbiological results showed that 20 samples (20%) yielded *E. coli*. Fourteen isolates (70%) were resistant to TC. The results of PCR revealed that 12 TC-resistant *E. coli* strains (85.71%) harbored the examined tet genes (four, seven and one TC-resistant *E. coli* isolates harbored tetA, tetB, and tetA plus tetB genes, respectively). All the *E. coli* strains that harbored tet genes were resistant to TC. Although these strains did not derive from clinical samples, the prevalence of tet genes among *E. coli* strains in water biofilm suggests the possible transmission of resistance genes from water to the environment, animals, and humans, and can play role in spreading tet genes. Therefore, the sanitation of water in poultry farms can decrease the bacterial infections and antibiotic resistance in poultry and humans.

***Keywords:*** Biofilm, chicken farm, *Escherichia coli*, tetracycline resistance gene

## Introduction

*Escherichia coli* is a common Gram-negative bacterium of the human and animal gastrointestinal tract (Sargeant *et al.*, 2019). Pathogenic and non-pathogenic *E. coli* have been repeatedly detected. Some strains of *E. coli* cause bloody diarrhea, anemia, and kidney failure which can lead to death. Most strains of *E. coli* can produce the shiga toxins that harm the epithelium of the small intestine (Gholami-Ahangaran & Zia-Jahromi, 2014). The *E. coli* infection colibacillosis is characterized by colisepticemia, yolk sac infection, coligranuloma, cellulitis, and swollen head syndrome. The treatment of colibacillosis often requires antimicrobial therapy (Nolan *et al.*, 2020). Improper and repeated antibiotic therapy inevitably leads to antibiotic resistance (Angulo *et al.*, 2004). The transmission of antibiotic-resistant bacteria via the food chain can develop antibiotic resistance in humans (Mainali *et al.*, 2013; Miles *et al.*, 2006). *E. coli* are one of the bacteria of interest in the study of antibiotic resistance. because they are common carriers of resistance genes and are capable of transferring those genes to other bacteria. Animal feces contain *E. coli* which are resistant to a number of different antibiotics important to animal and human health (Deckert *et al.*, 2010).

Antibiotic resistance has raised global concerns (Wright, 2012). In this regard, many governments have put in place programs to control or prohibit the administration of antibiotics to animals raised for food, except in cases of illness (Dai *et al.*, 2020; Muhammad *et al.*, 2020; Nsofor *et al.*, 2013). Accordingly, antimicrobial therapy must be prescribed according to an antibiotic susceptibility test (Sargeant *et al.*, 2019).

Water acts as a passive carrier for numerous microorganisms that cause human illness (Krewski *et al.*, 2004). Water is tested for *E. coli* contamination as an indicator of fecal contamination. Contamination of water is of concern, not just because of the spread of resistant bacteria to humans and other animals, but also due to the horizontal transfer of resistance mechanisms between bacteria in the water

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and the environment (Coleman *et al.*, 2012). Although studies show that ground water close to farms can be contaminated with bacteria carrying resistance genes (Mackie *et al.*, 2006), there are no studies examining what factors are associated with the contamination of private wells with antimicrobial- resistant *E. coli*. *E. coli* have strong ability in biofilm formation. Biofilms are composed of colonies of microbial cells in a matrix which consists of exopolysaccharides, proteins, organic, and non-organic elements, and cell material. The matrix takes about 85% of biofilm. It has been previously reported that inflammation can occur due to bacterial biofilms (Zhao *et al.*, 2013).

Tetracycline (TC) is a wide-range antibiotic that prevents aminoacyl-tRNA from binding to the bacterial ribosome, inhibiting the synthesis of protein (Chopra & Roberts, 2001). Its beneficial properties of lower cost, greater efficiency, and fewer adverse effects have made it a popular antibiotic in poultry farms. Studies have shown that resistance to TC is more likely due to its long-term use (Dai *et al.*, 2020; Koo & Woo, 2011). Molecularly, the resistance determinants are the *tet* genes, which encode mechanisms of inactivation of TC comprising the efflux pump system and ribosomal protection (Roberts, 1996). In Gram-negative bacteria, the efflux pump system is encoded by the *tet*A, *tet*B, *tet*C, *tet*D, tetE, and *tet*G genes, while in Gram-positive bacteria the *tet*M, *tet*O, and *tet*S genes contribute to ribosomal protection (Wright, 2012). The *tet* genes can localize on plasmids, transposons, and integrons, and easily spread among bacteria (Angulo *et al.*, 2004; Dai *et al.*, 2020). Therefore, in our study, we evaluate *tet* resistance genes in *E. coli* strains detected from biofilm originating from drinking water systems in poultry farms in Iran. The aim of this study is to describe the proportion of biofilms in closed water supply systems as a cumulative locus of microorganisms and other elements involved in the spread of waterborne infection in poultry farms.

## Method

### Sample Collection

Between 2019 and 2020, one hundred samples were collected from biofilms of water systems from 20 randomly chosen broiler chicken farms with a capacity of 5000–15,000 chickens, in the Isfahan province of Iran. The samples were collected from chicken farms that had mortality related to colibacillosis.

### Isolation and Identification of *E. coli*

All samples were diluted in sterile water (1:10), cultured on MacConkey's agar (Merck, Germany) (0.1 mL), and inoculated (37°C for 24 hours). After the Gram-staining test and growth on Eosin methylene blue agar (Merck, Germany), the confirmed bacteria were examined with biochemical tests (the IMViC test: Indole, methyl red test, Voges–Proskauer test, and citrate utilization test). Isolates that had typical IMViC patterns (Indole and MR positive, and VP and citrate utilization negative) were considered to be *E. coli* (Feng *et al.*, 2002). Colony confirmation was achieved according to the 16S rRNA gene amplification of *E. coli* (Sabat *et al.*, 2000).

### Antibiotic Sensitivity

To evaluate the susceptibility of *E. coli* strains to antibiotics, we utilized the disc diffusion method (Kirby–Bauer method) using a commercial antibiotic disc (Padtan Teb; Iran) comprising Imipenem (10 µg); Gentamicin (10 µg); Ampicillin (10 µg); Cefixime (5 µg); Ciprofloxacin (5 µg); Furazolidone (100 µg); TC (30 µg); Amoxicillin (25 µg); Amikacin (30 µg), and Nalidinxic acid (30 µg). For achievement of experiment, the criteria proposed by the Clinical and Laboratory Standards Institute (CLSI) were used (CLSI, 2018). For this purpose, a suspension of pure bacterial culture equivalent to 0.5 McFarland ($1.5\times10^8$ CFU/mL) was prepared. The bacteria were then cultured on Müller–Hinton agar medium after disc placement and incubated at 37°C for 24 hours. The diameter of the growth inhibition zone was measured and interpreted according to the standard antibiogram protocol.

### TC Resistance Gene Detection

In this study, the TC resistance genes comprising *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetO*, *tetS*, *tetG*, *tetK*, *tetL* and *tetM* were amplified by the polymerase chain reaction (PCR) method. The sequence of specific primers was synthesized according to the sequences published by Ng *et al.* (2001). The multiplex PCR was achieved in three separate categories. In category I, the PCR was performed for *tetB*, *tetC*, and *tetD* TC resistance genes. In category II, the *tetA*, *tetG*, and *tetE* genes were amplified. In category III, the PCR was performed for *tetK*, *tetL*, *tetM*, *tetO*, and *tetS* TC resistance genes, simultaneously.

PCR was achieved in 25 µL, including 3 mM MgCl2, 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µm of each dNTP (Fermentas, Germany), 1 µm primers, 2.5 IU of Taq DNA polymerase (Fermentas, Germany), and 5 µL (200 ng/µL) of DNA.

Amplification reactions were carried out using a DNA thermal-cycler (Eppendorf Mastercycler, Eppendorf-Nethel-Hinz GmbH, Biorad, Germany) according to Ng *et al.* (2001) temperature program. The amplification products were analyzed by gel electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and photographed at UV exposure (Chopra & Roberts, 2001). A 100 bp Marker (Fermentas, Germany) was utilized. Sterile double-distilled water was used as negative control. The *E. coli* strains that harbored the *tetA* and *tetB* genes identified in a previous study were utilized as positive control (Gholami-Ahangaran *et al.*, 2021).

## Results

The microbiological and biochemical results showed that 20 samples (20%) yielded *E. coli.* According to antibiogram results, 14 isolates (70%) were resistant to TC. The percent of antibiotic resistance in *E. coli* strains isolated from biofilm samples in the drinking water systems are presented in Table 1.

The results of PCR revealed that only *tet*A (210 bp) and *tet*B (659 bp) genes were amplified in samples (Figure 1). Of the total isolates, 33.33% possessed the *tet*A gene, whereas 58.33% of *E. coli* isolates possessed the *tetB* gene and 8.33% of the *E. coli* isolates contained the *tet*A and *tet*B gene simultaneously (four, seven, and one, respectively). On the other hand, 12 TC-resistant *E.* strains (85.71%) harbored *tetA*, *tetB,* and/or *tetA* plus *tetB* genes. The other examined *tet* genes were not identified in any *E. coli* isolates.

The TC resistance was found in all *E. coli* strains that harbored *tetA*, *tetB,* and *tetA* plus *tetB* genes. The antibiotic resistance characteristics in *E. coli* strains that harbored *tetA* or *tetB* are presented in Table 2.

## Discussion, Conclusion, and Recommendations

TC is a wide-range antibiotic that prevent aminoacyl-tRNA binding to the bacterial ribosome and thus inhibits protein synthesis (Chopra & Roberts, 2001). In our present study, of the 70% of *E. coli* isolates

**Table 1**
*The Percent of Antibiotic Resistance in Escherichia coli Strains Isolated From Biofilm Samples in Water Systems of Poultry Farms*

| Antibiotics | Frequency (%) of Resistance in *E. coli* Isolated from Biofilm Samples |
|---|---|
| Imipenem (10 µg) | 0 (0) |
| Gentamicin (10 µg) | 0 (0) |
| Ampicillin (10 µg) | 5 (25) |
| Ciprofloxacin (5 µg) | 6 (30) |
| Furazolidone (100 µg) | 4 (20) |
| Tetracycline (30 µg) | 14 (70) |
| Amoxicillin (25 µg) | 6 (30) |
| Amikacin (30 µg) | 3 (15) |
| Nalidixic acid (30 µg) | 4 (20) |
| Cefixime (5 µg) | 3 (15) |
| Oxytetracycline (30 µg) | 8 (40) |
| Doxycycline (30 µg) | 5 (25) |
| Trimethoprim+Sulfamethoxazole | 7 (35) |
| Penicillin (10 µg) | 0 (0) |
| Amoxicillin+Clavulanic acid | 2 (10) |
| Chloramphenicol (30 µg) | 4 (20) |

which were identified as resistant to TC, 33.33% of the isolates contained the *tet*A gene, and 58.33% of the isolates contained the *tet*B gene. There are some limited reports on the TC resistance genes in chickens in Iran. Zibandeh *et al.* (2016) reported that the *tet*A resistance gene was present throughout the grower period, at 32.5% in one-day old chicks, and at 72.5%. in chickens at slaughter. In this study, the *tet*M, *tet*O, or *tet*S resistance genes were not detected in any strains. Seifi and Khoshbakht (2016) stated that 73% of *E. coli* isolates from fecal samples of poultry exhibited resistance to TC, and that *tetA* was the predominant *tet* gene (46%), the *tetA* plus *tetB* was the predominant genotype (20%). There is a contradiction in the most prevalent *tet* genes in *E. coli* isolates; *tet*A (Nsofor *et al.*, 2013; Seifi & Khoshbakht, 2016) or *tet*B (Bryan *et al.*, 2004; Wilkerson *et al.*, 2004). Moreover, Koo and Woo (2011) from Korea reported that *tetA* and *tetB* are the most frequent TC resistance genes in *E. coli* strains (52.4 and 41.3%). However, in most reports, there is a common result that the TC resistance mechanism in animal *E. coli* isolates is associated with efflux pump-related genes (*tetA, tetB*, *tetC*, *tetD*, *tetE*, *tetG*) (Bryan *et al.*, 2004; Wilkerson *et al.*, 2004), and that the distribution and incidence of the TC resistance genes mediated by efflux genes depends on the environment and characteristics of the isolate (Kang *et al.*, 2005). Furthermore, the frequency of TC resistance genes in clinical and environmental strains can change over time. In Czech, in 2005–2006, the frequency of the *tet*A gene was changed from 8.1 to 81.3% and that of *tet*B genes was changed from 86.5 to 18.8% within five years (Skočková *et al.*, 2012). Additionally, (Sengeløv *et al.*, 2003) showed that the frequency of *tet*A resistance gene in pathogenic *E. coli* isolated from broilers was significantly higher than other *tet* resistance genes, while the *non-tetA* resistance genes were higher in non-pathogenic *E. coli*. However, the lower detection of *tetA* in the present study could be related to the frequency of samples or different pathogenicity of *E. coli* in biofilm isolates. Therefore, the

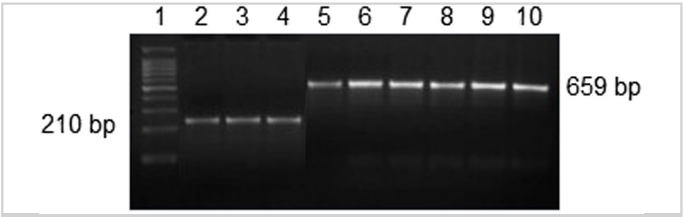


**Figure 1**
*PCR Product Electrophoresis (Left-to Right Side: 1: 100 bp Marker; 2: Positive Control for tetA Gene, 3-7: tetA Positive Samples; 8: Positive Control for tetB Gene, 9-16: tetB Positive Samples, 17: Negative Sample).*

monitoring of TC gene resistance in samples revealed new knowledge about antibiotic resistance in one state or country that can be transferred to animals or human.

In this study, all *E. coli* strains that harbored *tet* genes were resistant to TC, while 85.71 of TC-resistant *E. coli* strains (12 resistant *E. coli* strains) harbored the *tetA*, *tetB,* or *tetA* plus *tetB* genes. This finding demonstrated that in addition to the examined *tet* genes, other determinants are needed for TC resistance in *E. coli* strains, and expression of *tetA* or *tetB* genes alone cannot appear as the TC resistance phenotype. Moreover, the evaluation of frequency and antibiotic resistance patterns in *E. coli* strains revealed that there is no specific antibiotic resistance pattern in *E. coli* strains that harbored *tetA* or *tetB*. On the

**Table 2**
*The Percent of Antibiotic Resistance in Escherichia coli Strains That Harbored tetA or tetB Genes*

| Antibiotics | Frequency (%) of Antibiotic Resistance in *E. coli* Strains that Harbored *tetA* | Frequency (%) of Antibiotic Resistance in *E. coli* Strains that Harbored *tetB* |
|---|---|---|
| Imipenem (10 µg) | 0 (0) | 0 (0) |
| Gentamicin (10 µg) | 0 (0) | 0 (0) |
| Ampicillin (10 µg) | 2 (66) | 1 (16.66) |
| Ciprofloxacin (5 µg) | 2 (66) | 2 (33) |
| Furazolidone (100 µg) | 1 (33) | 1 (16.66) |
| Tetracycline (30µ g) | 5 (100) | 8 (100) |
| Amoxicillin (25 µg) | 2 (66) | 1 (16.66) |
| Amikacin (30 µg) | 1 (33) | 0 (0) |
| Nalidixic acid (30 µg) | 2 (66) | 1 (16.66) |
| Cefixime (5 µg) | 1 (33) | 1 (16.66) |
| Oxytetracycline (30 µg) | 3 (100) | 2 (33) |
| Doxycycline (30 µg) | 2 (66) | 1 (16.66) |
| Trimethoprim+ Sulfamethoxazole | 2 (66) | 2 (33) |
| Penicillin (10 µg) | 0 (0) | 0 (0) |
| Amoxicillin+ Clavulanic acid | 1 (33) | 0 (0) |
| Chloramphenicol (30 µg) | 2 (66) | 1 (16.66) |

other hand, there is no correlation between the antibiotic resistance pattern and the presence of *tetA* or *tetB* genes in *E. coli* strains. The results demonstrated that *E. coli* strains which harbored *tetA* or *tetB* showed, beyond TC resistance, aminoglycoside and sulfonamide resistance, which are antibiotics extensively used in veterinary treatment.

The present study demonstrated that more than one *tet* gene could be found within the same strain. The simultaneous detection of *tet*A and *tet*B gene from one strain was supported by (Koo & Woo, 2011). In our present study, the other TC genes *tet*C, *tet*D, *tet*E or *tet*G, *tetK*, *tetL*, *tetO and tetS* genes, alone or in combination, were not detected in *E. coli* isolates. Our results are aligned with findings by (Skočková *et al.*, 2012). Sengeløv *et al.* (2003) reported the higher occurrence of TC resistance genes *tetA* and *tetB* in diseased pigs, cattle and chicken compared to healthy animals (71% vs. 25%). Sengeløv *et al.* (2003) could not find any *tet* genes other than *tet*A and *tet*B in any tested *E. coli* strains. Moreover, Bryan *et al.* (2004) found the double *tet* resistance genes. In our present study, one *E. coli* isolate contained the *tet*A and *tet*B, together. It seems that potent pressure provided by environments containing elevated levels of TC can lead to the attainment of more than one TC gene.

In conclusion, this study demonstrated that *E. coli* strains can be detected from biofilms of water systems in poultry farms and can contain TC resistance genes. Molecular approaches are very sensitive and accurate methods for the identification of microorganisms and resistance genes. That both the resistance genes of *tet*A and *tet*B were detected in *E. coli* isolates is related to the efflux pump activity that can be transferred between Gram-negative bacteria. Therefore, monitoring antibiotic resistance genes in *E. coli* is warranted, along with other pathogens of different origins.

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