Monitoring the prevalence of the tetracycline efflux genes among *E. coli* isolated from chicken colibacillosis

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Abstract:

BACKGROUND: Avian colibacillosis can lead to important economic losses in the poultry industry. *Escherichia coli*, the causative agent of this disease has acquired resistance to different antibiotics, including tetracycline. **OBJECTIVES:** This study was carried out to detect the distribution of tetracycline Group I efflux genes among *E.coli* isolates from from avian colibacillosis in Iran by PCR assay. **METHODS:** A total of 50 *E. coli* isolates from chicken colibacillosis were characterized by cultural, biochemical and PCR methods. Kirby-Bauer disk diffusion method was used to define the resistance of isolates to tetracycline, then the Frequencies of tetracycline resistance genes (*tetA*, *tetB*, *tetC*, *tetG*, *tetH*, *tetZ* and *tetE*) were also determined using PCR method. **RESULTS:** According to biochemical and molecular experiments, 50 isolates from 237 chicken samples were recognized as *E. coli*. Seventy-six (76%) of the isolates, however, were resistant to tetracycline. The distribution of tetracycline-resistance genes among *E. coli* isolates included *tetB* (34%), *tetA* (26%), *tetE* (16%), *tetC* (15%), *tetH* (12%), *tetG* (12%) and *tetZ* (6%). **CONCLUSIONS:** The present study highlights the prevalence of tetracycline resistant *E. coli* among chickens which is due to extensive use of this antibiotic for growth promotion in poultry industry.

Introduction

Widespread use of antibiotic agents is the most important factor causing emergence and dissemination of antimicrobial resistant bacteria (Van den Bogaard et al., 2001).

Antimicrobial drugs use in poultry production industries as a growth promotion factor, lead to the high resistance to antibiotic agents among avian infectious microorganisms (Romanus et al., 2012). Tetracycline is a broad - spectrum antibiotic that inhibits protein synthesis by binding to the bacterial ribosomes (Chopra and Roberts, 2001). There are four tetracycline resistance mechanisms in microorganisms including: efflux pumps, alteration in target receptors, ribosomal protection and enzymatic inactivation (Tuckman et al., 2007). Efflux pumps are transport proteins which could export antibiotics such as tetracycline from the cells to the outside (Webber and Piddock, 2003). Tetracycline efflux pumps belong to the six groups based on their amino acid sequences. Group 1 includes: *TetA*, *TetB*, *TetC*, *TetE*, TetD, *TetG*, *TetH*, *TetZ*, *TetJ* and *TetI* which are found exclusively in gram negative bacteria (Chopra and Rob-

erts, 2001).

E. coli is an important microorganism among gram - negative bacteria (Sharma et al., 2013). Antibiotic resistance in *E. coli* strains isolated from human, animals and environment, has developed due to widespread use of antimicrobial agents for treatment of *E. coli* caused infections. (Wose Kinge et al., 2010). Among tetracycline resistance mechanisms, efflux proteins are the most important mechanism of resistance in *E. coli* (Tuckman et al. 2007).

Consequently, the increasing rate of antibiotic resistance among *E. coli* isolates complicates treatment of infections (Kibert and Abera, 2011).

Although in some studies the frequency of tetracycline resistance genes (*tetA*, *tetB*) in *E. coli* isolates recovered from commercial broiler chicken in east Azerbaijan were evaluated, there is not any complete study about the prevalence of other tetracycline efflux genes. So this work is the first study that evaluates the frequency of other efflux genes involved in tetracycline resistance in this area. The objective of the present study was to evaluate the occurrence of group I of tetracycline efflux genes in *E. coli* strains obtained from avian colibacillosis in east and west Azerbaijan in Iran.

Materials and Methods

Sample collection: During 2013-2015, a total number of 237 samples from chicken carcasses suspected of colibacillosis were collected from 11 poultry farms located in the provinces of east and west Azerbaijan. All chickens showed clinical signs including watery diarrhea, weakness, anorexia and weight loss before death. A thorough post-mortem examination of all dead birds was carried out. Liver and spleen samples were collected separately in sterile test tubes, then were used for bacteriological studies.

Isolation of *E. coli*: All samples were cultured on Mac Conkey (Sigma Aldrich, USA) and EMB agar (Sigma Aldrich, USA) then incubated for 24h at 37 °C. *E. coli* colonies were examined by biochemical tests like growth on triple sugar iron agar (TSI) or citrate utilization, fermentation of indol, methyl red test, motility and urease test and the *E. coli* isolates were confirmed (Sharma et al. 2013). All isolated bacteria were frozen in trypticase soy broth with 30% glycerol (Sigma Aldrich, USA) at -70 °C (Tadesse et al., 2012).

Molecular confirmation of *E. coli*: DNA extraction. All *E. coli* isolates were cultured overnight in Nutrient broth media (Sigma Aldrich, USA) and their chromosomal DNA was extracted using bacterial genomic DNA purification kit (INTRON, Korea).

Primers and PCR. *E. coli* 16s rRNA gene was detected through PCR method using universal Primer Sequences used for PCR identification of *E. coli* 16s rRNA gene were 5′- GTA TAG ATA CCC TGG TAG TCCA-3′ as forward and 5′- CCC GGG AAC GTA TTC ACC G-3′ as reverse primers.

The PCR assay was done in a total volume of 25 µl by using INTRON premix. The PCR conditions using DNA thermo cycler (MWG AG BIOTECHTHERMAL CYCLER, USA) were as follow: Three min at 95 °C followed by 26 cycles of 94 °C for 1 min, 55 °C for 1min and 72 °C for 10 min (Sharma et al., 2013). The PCR products were analyzed by electrophoresis in 1% agarose gel.

Antimicrobial susceptibility testing: After identification of *E. coli* isolates by

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	*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′

biochemical and molecular methods, the standard Kirby-Bauer disk diffusion method was used to determine the tetracycline resistance pattern among *E. coli* strains (Sayah et al., 2005).

Distribution of tetracycline resistance genes: All isolates which were resistant to tetracycline were examined further by the use of five PCR assays for the presence of *tetA*, *tetB*, *tetE*, *tetC*, *tetH*, *tetZ* and *tetG* genes. The primer sets used for amplification of these genes were shown in Table 2 (Aminov et al., 2002).

The first PCR assay used for amplification of *tetE*, consisted of initial denaturation at 94 °C for 1 min followed by 25 cycles at 94 °C for 1min, 1min of annealing at 50 °C and 72 °C for 10 min. The second PCR was performed for amplification of *tetB* as described above with the use of specific primers. The third assay was performed for amplification of *tetA* and *tetC* with annealing temperature of 48 °C.

The fourth assay was used to identify *tetG* as described above but the annealing temperature was 46 °C and finally the last amplification was carried out to identify *tetH* and *tetZ* genes using 46 °C as annealing temperature The PCR products were analyzed by agarose gel electrophoresis and the frequency of each tetracycline resistance determinants was determined.

Results

Out of 237 poultry carcasses samples suspected of colibacillosis, 21/09% (n=50) *E. coli* were isolated using culture and biochemical tests. Furthermore, molecular detection of *E. coli* isolates was carried out. 16s rRNA gene of *E. coli* was identified by PCR assay of extracted DNA using 16srRNA universal primers. Figure 1 shows the 16s rRNA gene of *E. coli* strains found at 612bp on 100bp DNA ladder.

As the antimicrobial susceptibility test shows 76% of *E. coli* isolates were resistant to tetracycline. The percentage of isolates susceptible to tetracycline was 24%, none of the isolates showed intermediate suscep-

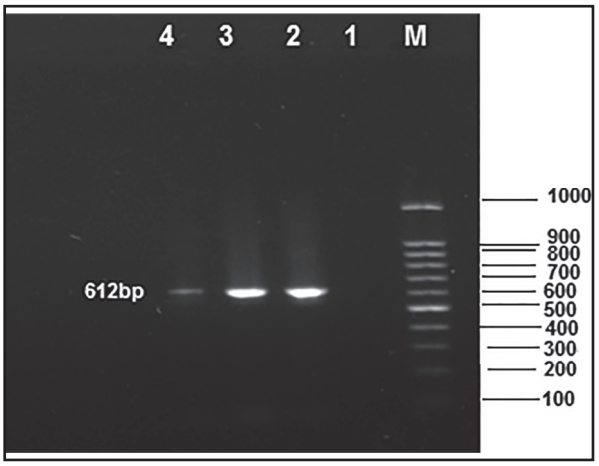

Figure 1. 16s rRNA gene found at 612bp on 1% agarose gel after PCR ampli f cation. Lane M- 100bp ladder marker Lane 1- Negative control. Lane2- Positive control. Lane 3- *E. coli* 16s rRNA gene found at 612bp.

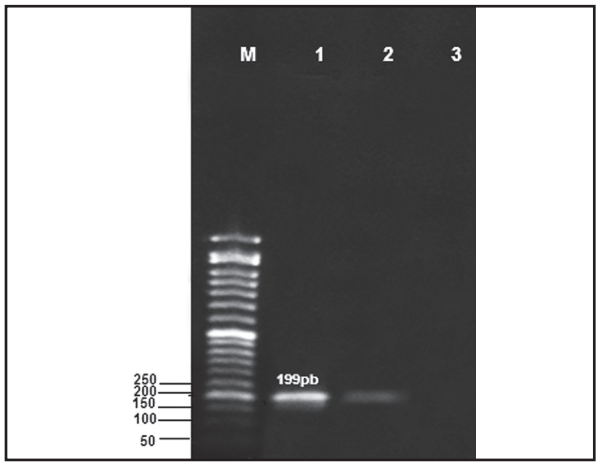

Figure 2. Representing agarose gel of PCR product from *E. coli* isolates, using primers for *tetE*. Lane M- 50bp Ladder marker. Lane 1- *tetE* gene found at 199bp. Lane 2- positive control. Lane 3- negative control.

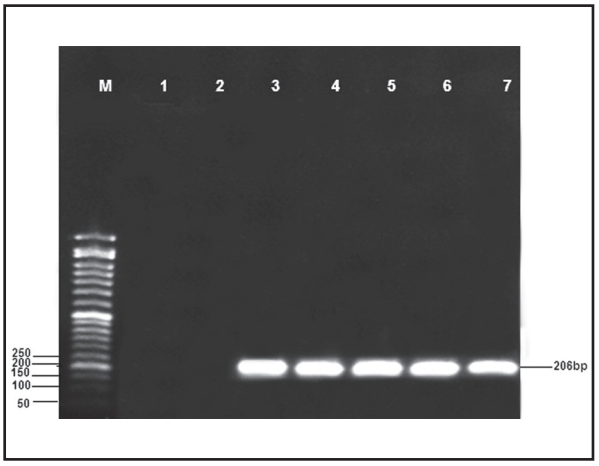

Figure 3. Gel electrophoresis of the PCR products of *tetB* gene. Lane M- 50bp Ladder marker. Lane 1- negative control. Lane 3- positive control. Lane 4, 7- *tetB* gene found at 206bp.

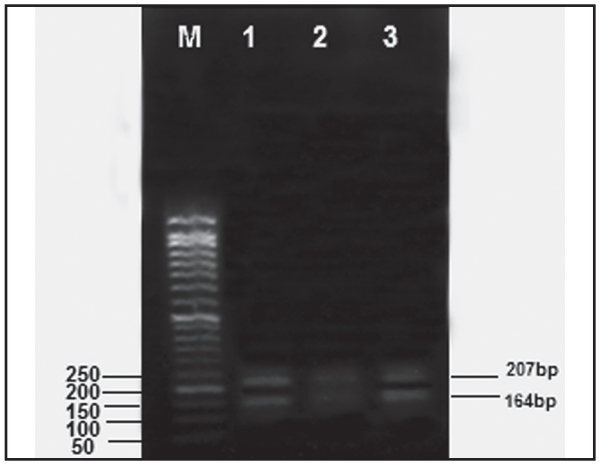

Figure 4. Gel electrophoresis of the PCR products of *tetA* and *tetC* determinants. Lane M- 50bp Ladder marker. Lane 1 to 3- *tetA* (164bp) and *tetC* (207bp).

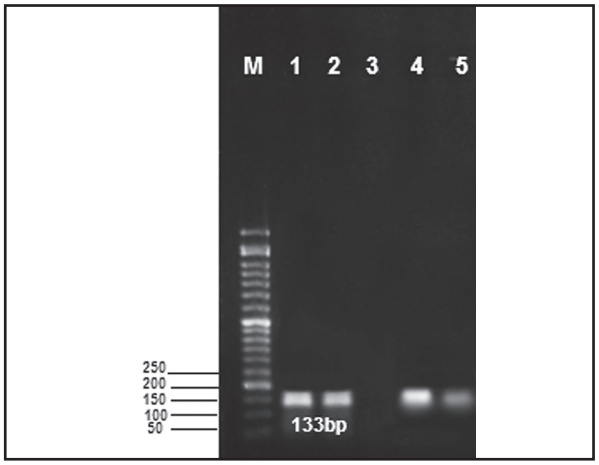

Figure 5. PCR detection of *tetG* marker in *E. coli* isolates Lane M- 50bp Ladder marker. Lane 1- positive control. Lane 3- negative control. Lane 4, 5- *tetG* (133bp).

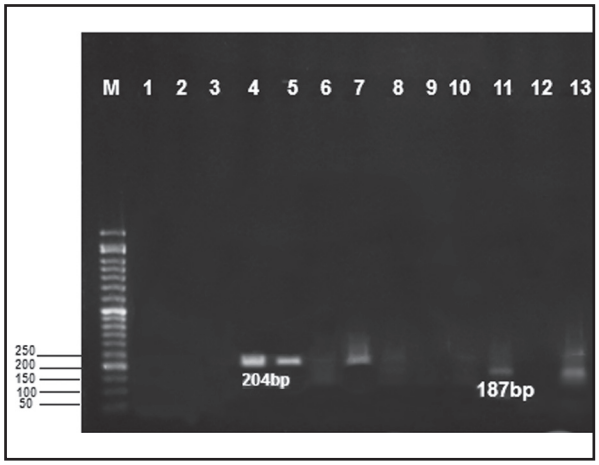

Figure6. PCR detection of tetH and tetZ markers in *E. coli* isolates.

tibility to the drug.

For detection of the tetracycline efflux genes frequency, among isolates, five PCR assays were used and results were shown in Fig. 2 to Fig. 6.

The most common determinants were *tetB* (34%) followed by *tetA* (26%). However, *tetH* (12%), *tetG* (12%), *tetC* (15%) and *tetE* (16%) were also found with different frequencies. The lowest Frequency among studied genes belonged to *tetZ* (6%).

Discussion

Among fifty *E. coli* isolates from chicken colibacillosis, 38 isolates were resistant to tetracycline. Study of the occurrence of tetracycline efflux genes showed that the *tetB* gene had the highest frequency among studied genes.

Antibiotics are usually used for treatment of bacterial infections in humans and animals. They are also used in commercial livestock and poultry food for promotion of growth. As a result, antimicrobial resistance among bacteria will be increased (Sayah et al., 2005).

Tetracyclines are broad spectrum antibacterials that have been used for a long period of time in humans and animals. But nowadays a widespread range of bacteria are showing resistance to tetracycline which encodes by several resistance genes (Trzcinski et al., 2000). Pathogenic strains of *E. coli* are common infectious microorganisms among gram negative bacteria, causing intestinal and extra intestinal infections. Many times, antibiotic therapy is needed for treatment of *E. coli* infections both in humans and veterinary medicine (Sharma et al., 2013). A great deal of evidence indicates that antimicrobial resistance in *E. coli* is the highest among other bacteria and tetracycline resistance is a very common resistance pattern in animal's coliforms (Taddesse et al., 2012).

Advances in molecular bacteriology lead to detection and use of new techniques for describing the genetic determinants responsible for antibiotic resistance in bacteria. Also, designing of specific primers have provided accurate and rapid detection of antibiotic resistance genes (Blake et al., 2003). According to prior studies, tetracycline efflux pumps are important resistance mechanisms in *E. coli* and among these determinants *tetB* gene was the most prevalent, responsible for tetracycline resistance (Tuckman et al., 2007). Our results also confirm the prevalence of tetracycline resistance and the predominant role of *tetB* resistance gene among *E. coli* isolated from animals.

In 2004 distribution of tetracycline resistance genes in *E. coli* strains from diverse human and animal sources was examined by Brayan et al. The most common genes found in their isolates were *tetB* and *tetA*. *tetC* and tetD were also found with lower frequencies. Also, Tuckman et al in 2007 monitored the prevalence of tetracycline resistance genes in *E. coli* strains by PCR assay. They revealed that *tetB* and *tetA* markers had the highest frequency among studied determinants. Our results are in accordance with their findings.

Momtaz et al in 2012 evaluated the distribution of antibiotic-resistant genes in *Escherichia coli* isolates from slaughtered commercial chickens in Iran by PCR. The distribution of *tetA* and *tetB* genes in the *E. coli* isolates were 52.63%. Also, Wang et al in 2013 investigated the prevalence of antimicrobial-resistant chicken *Escherichia coli* strains and the resistance genes in *E.*

coli. The detection rates of *tetA*, *tetB* genes were 95/3% and 97/4%. Here, we also used molecular PCR technique to determine the occurrence of tetracycline resistance genes in *E. coli* bacteria. Our results also indicate that *tetB* and *tetA* genes were the most frequent determinants for tetracycline resistance. Relative to the results obtained in the detection of antimicrobial resistance genes, in a study of *E. coli* isolated strains from broiler chickens in Brazil, Santos et al. (2014) observed the concomitant presence of *tetA* and *tetB* and genes. This is consistent with the detection of two resistance genes in the strains of *E. coli* in the present study.

The high percentage of phenotypes of *E. coli* isolates that were resistant to tetracycline, suggested that the widespread use of tetracycline in poultry creates the reservoir of resistant bacteria and resistance genes and may shorten the time that this antimicrobial agent will be available for effective treatment of infections in poultry.

Examining the frequency of tetracycline resistance genes among *E. coli* isolated from chicken colibacillosis, confirmed the presence of tetracycline resistance genes in most of the *E. coli* isolates and pictured the distribution pattern of these determinants which is very similar to the prior findings about these determinants.

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