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Original article

ANTIMICROBIAL RESISTANCE PROFILE AND PREVALENCE OF TETRACYCLINE RESISTANCE GENES IN *ESCHERICHIA COLI* ISOLATES FROM BROILER CHICKENS, NORTHWESTERN IRAN

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Summary

Mohammadi, V., A. Ghaniei & P. Sepehrnia, 2018. Antimicrobial resistance profile and prevalence of tetracycline resistance genes in *Escherichia coli* isolates from broiler chickens, northwestern Iran. *Bulg. J. Vet. Med.,* **21**, No 2, 169–175.

Frequencies of tetracycline resistance genes (*tet*A, and *tet*B), and antimicrobial resistance profile of *Escherichia coli* isolated from broilers of West Azerbaijan province were investigated. Concerns have been raised regarding the emergence of antimicrobial resistance in pathogenic organisms and therapy failure in veterinary and human medicine. The resistance profile of 44 *E. coli* isolates recovered from colisepticaemic broilers to five commonly used antibiotics were investigated by disk diffusion method. Tetracycline resistance genes (*tet*A, and *tet*B) also were detected by PCR. The results showed that *E. coli* isolates had the highest resistance rate to tetracycline, sulfadiazine, and florfenicol. Of the isolates 54.5% (24/44) carried tetracycline resistance genes. The positive rates of *tet*(A) and *tet*(B) were 47.7% (21/44) and 9% (4/44), respectively. One *E. coli* isolate carried both tetracycline resistance genes. The finding of this study represented high frequency of resistance to antimicrobial agents used in Iranian poultry industry, especially to tetracycline.

Key words: broiler chickens, drug resistance, *Escherichia coli*, tetracycline resistance genes

INTRODUCTION

Escherichia coli is an important member of the *Escherichia* genus. It is a major pathogen of worldwide importance in commercially produced poultry, contributing significantly to economic losses in both chickens and turkeys. Colibacillosis is one of the most common local or systemic infectious diseases caused by pathogenic *E. coli* in chickens. Overall, colibacillosis begins with infection of the upper respiratory tract, followed by septicaemia. The most common symptoms of colibacillosis are septicaemia, airsacculitis, perihepatitis, pericarditis, vitelline

peritonitis, and salpingitis (Nolan *et al*., 2013). Infectious bronchitis virus, Newcastle disease virus, *Mycoplasma*, and ammonia predispose birds to respiratory colisepticaemia (Nolan *et al*., 2013), which incurs severe economic losses to the poultry industry (Liu *et al*., 2010).

The main preventive measure against colibacillosis is the use of antibacterial drugs. Antimicrobial resistance is a serious emerging public health concern because of the compromised efficacy of antimicrobial agents used in the treatment of infectious diseases (Martinez & Baquero, 2002). Tetracycline is a broad-spectrum antibiotic with bacteriostatic activity acting by prevention of bacterial protein synthesis. It has been widely used in the prevention and treatment of poultry diseases. However, the application of tetracycline directly leads to selection of drug resistance. Tetracycline resistance in bacteria is mediated by four mechanisms including efflux, ribosomal protection, enzymatic inactivation, and target modification. The leading tetracycline resistance mechanism in *E. coli* is the extrusion of drug from the cytoplasm via efflux (Chopra & Roberta, 2001). More than 40 kinds of tetracycline resistance genes have been found and sequenced (Roberts, 2005; Brown *et al*. 2008; Kazimierczak & Rincon, 2008). These genes are transferred easily between bacteria via plasmids or transposons leading to broad drug resistance. Many active efflux genes (*tet*A, *tet*B) were demonstrated by previous studies (Benacer *et al*., 2010). The study on resistance mechanism is of great significance to prevent and control poultry diseases as well as to public health and safety.

Previous studies have documented the presence of the tetracycline resistance genes *tet*(A) and *tet*(B) in tetracycline-resistant *E. coli* isolated from broiler and layer chickens (Zhang *et al*., 2012; Balasubramaniam *et al*., 2014). In Iran, poultry meat is the most widely produced and consumed meat protein source. Broiler chickens are among the species most affected by the selective pressure of chemotherapeutics; tetracyclines are best example for the results of this selective pressure. Various mechanisms of resistance to tetracyclines in *E. coli* isolates have been found. The tetracycline resistance genes *tetA* and *tetB* are of great importance in tetracycline-resistant *E. coli* isolated from chickens (Momtaz *et al.,* 2012).

The aim of this study was to evaluate antimicrobial resistance profile and the frequency of tetracycline resistance genes (*tet*A, *tet*B) in *E. coli* isolates recovered from commercial broiler chickens in West Azerbaijan province (northwesterm Iran) by PCR.

MATERIALS AND METHODS

Isolation and identification of E. coli

Avian pathogenic *E. coli* (APEC) were isolated from 30 broiler farms in West Azerbaijan province (northwestern Iran). A total number of 44 isolates were collected from 16 (1 isolate from each farm) and 14 (2 isolates from each farm) farms. According to the clinical signs and necropsy findings, the dead birds were preliminarily diagnosed with colibacillosis. Heart blood and liver of chickens with colisepticaemia were aseptically sampled to obtain *E. coli* isolates. Samples were streaked on MacConkey agar (Merck, Germany). A single hot-pink colony was picked on MacConkey agar and purified once again. Biochemical tests were also used for identification of isolates. Indole test, methyl red test, Voges-Proskauer test, citrate utilisation test, urea hydrolysis test, and H_2S production test were per-

formed with the colonies that showed growth features of *E. coli*. By culture and biochemical features, 44 *E. coli* isolates were identified.

Antimicrobial susceptibility

Antimicrobial susceptibility was conducted by disc diffusion method. *E. coli* isolates were uniformly spread on Mueller Hinton Agar (Merck, Germany). Tetracycline (30 µg), enrofloxacin (5 µg), sulfadiazine (300 µg), florfenicol (30 µg), and neomycin (30 µg) discs were placed on the agar surface. These are five commonly used antibiotics in Iranian poultry industry. After incubation for 18–24 h at 37 °C, the diameters of inhibition zones were measured. Drug resistance profile was determined according to the standard recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011).

Amplification of tetracycline resistance genes

E. coli isolates were cultured in Luria-Bertani medium (Merck, Germany) at 37 °C overnight and the genomic DNA was then extracted using a DNA extraction kit (SinaClon Biosciences, Tehran, Iran). In this study, two sets of primer pairs were used for amplification of tetracycline resistance genes (Table 1). The amplification was carried out in a 25 µL reaction volume consisting of 1× PCR buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 1.25 units *Taq* DNA polymerase, 2.5 mM $MgCl_2$ (SinaClon Biosciences, Tehran, Iran), 2 µL DNA, and 16.5 µL dH_2O and programmed in a thermocycler (Mastercycler, Eppendorf, Germany) as followed: 35 cycles of denaturation at 95 °C for 60 s, annealing at 51 °C for 60 s (*tet*A), and extension at 72 °C for 60 s. The annealing temperature was set at 45 °C for 60 s (*tet*B). In all PCR reaction sets, negative controls (dH_2O instead of DNA) were included. The amplification products were detected by gel electrophoresis in 1% agarose gel in a Tris-acetate-ethylenediamine tetraacetic acid buffer.

RESULTS

Antimicrobial susceptibility patterns observed for isolates are shown in Table 2. Among *E. coli* isolates, the highest and the lowest resistance frequencies were observed for tetracycline (79.5%) and neomycin (0 %), respectively. In the present study, multi-drug resistance (MDR) isolates were observed in 45% of *E. coli* isolates. Among MDR isolates, the highest prevalence of resistance to antibiotic was detected for tetracycline (75%).

The tetracycline resistance genes were amplified from 24 avian *E. coli* isolates as evidenced by the results of PCR and the positive rate was 54.5% (24/44). Moreover, the *tet*(A) gene had the higher positive rate up to 47.7% (21/44). The positive rate of *tet*(B) was 9% (4/44). One *E. coli* isolate, isolate no. 37, carried both

Table 1. Primers used for amplification of tetracycline resistance genes

Resistance gene	Sequence	Product size	Reference
tet(A)	Forward: TTTCGGGTTCGGGATGGT Reverse: CAGGCAGAGCAAGTAGAGGG	915	Zhang *et al.,* 2012
tet(B)	Forward: GCCCAGTGCTGTTGTTGTC Reverse: TGGTCGTCATCTACCTGC	553	Zhang *et al.,* 2012

Table 2. Susceptibility patterns of 44 *E.coli* isolates to five antimicrobial agents

Antimicrobial agents	Percentage (positive/total isolates number)		
	Susceptible	Intermediate	Resistant
Tetracycline	4.6 (2/44)	15.9 (7/44)	79.5 (35/44)
Enrofloxacin	79.5 (35/44)	13.7 (6/44)	6.8 (3/44)
Sulfadiazine	47.7 (21/44)	6.8 (3/44)	45.5 (20/44)
Florfenicol	86.3 (38/44)	–	13.7 (6/44)
Neomycin	90 (40/44)	10 (4/44)	–

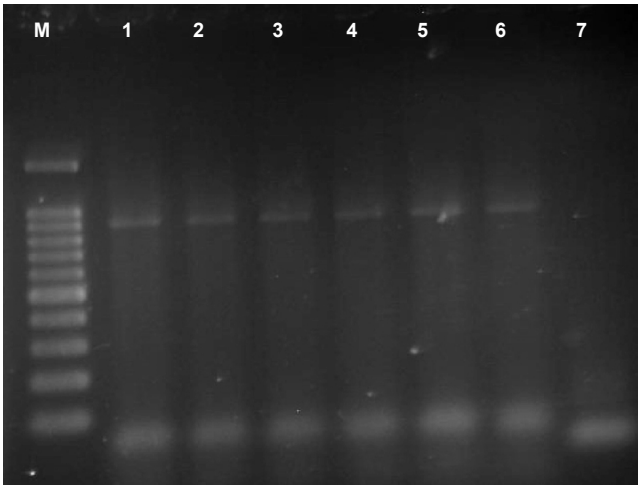

Fig. 1. Gel electrophoresis analysis of PCR product of the *tetA* gene. Lanes 1–6 have the specific bands (915 bp); lane M: 100 bp DNA marker; lane 7: negative control.

tetracycline resistance genes (*tet*A and *tet*B). The resistance genotype results of isolates were consistent with antibiogrammes of isolates. Only one isolate had resistance gene *tet*(B), isolate no. 8, which was intermediate in the antibiogram.

Some *E. coli* isolates which were resistant to tetracycline carried no tetracycline resistance genes, *tet*A and *tet*B. From the isolates with tetracycline resistant phenotype, 60% carried *tet*(A) gene whereas 11.4% carried the *tet*(B) resistance gene.

The agar gel electrophoresis showing the detection of both resistance genes in isolates is illustrated on Fig. 1 and 2.

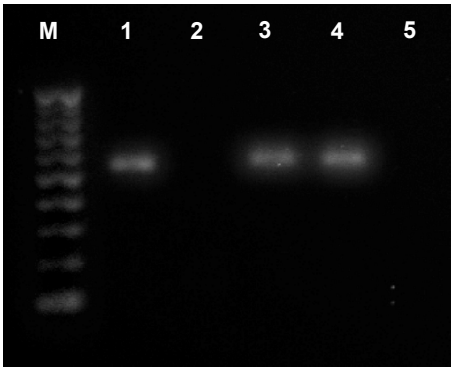

Fig. 2. Gel electrophoresis analysis of PCR product of the *tetB* gene. Lanes 1, 3, and 4 have the specific bands (553 bp); lane M: 100 bp DNA marker; lane 5: negative control.

DISCUSSION

In the present study, twenty four (54.5%) *E. coli* isolates showed the presence of *tet*(A) and *tet*(B) genes and the percentage of tetracycline-resistant isolates was 79.5%. It must be noted that new generations of tetracycline were not included in this study for evaluation of susceptibility. The results of such evaluations may be somewhat different. Some isolates with no resistance genes were detected to be resistant to tetracycline. It can be presumed that tetracycline resistance in *tet*(A) and *tet*(B) negative isolates might be encoded by some other resistance genes such as *tet*(C) and *tet*(D) or ribosomal protection encoded by *tet*(M), *tet*(O), *tet*(Q) and *tet*(S) genes than the genes investigated in this study. Interestingly, one isolate carried both *tet*(A) and *tet*(B) genes. Occasionally, some isolates had more than one tetracycline resistance gene (Sengelov *et al*., 2003; Bryan *et al*., 2004; Zhang *et al*., 2012). The *tet*(A) gene had the highest positive rate. Our results showed that the *tet*(A) gene was the most important resistance gene related to tetracycline antibiotics in northwestern Iran. The positive rates of the *tet*(A) and *tet*(B) genes in our study were different from those reported in *E. coli* isolated from chickens in North China (Zhang *et al*., 2012). Wang *et al*. (2013) investigated resistance phenotype and genotype of *E. coli* isolates in Hebei province (China). Their results showed that 74.2% of isolates were resistant to tetracycline and that the *tet*(A) genotype was the most prevalent one (52.3%). In the study carried out in Thailand on broilers, all *E. coli* isolates were resistant to tetracycline in disk diffusion method. In 90% of isolates, *tet*(A) was detected (Mooljuntee *et al.,* 2010). Guerra *et al*. (2003) characterised the antimicrobial resistance of German *E. coli* strains isolated from cattle, swine, and poultry. Resistance to tetracycline was one of the most prevalent resistance patterns by broth microdilution assay. Frequencies of *tet*(A) and *tet*(B) among *E. coli* isolates were 66% and 42%, respectively. A study conducted on *E. coli* isolated from healthy layer flocks in India showed that 88% of isolates were resistant to tetracycline and *tet*(A) was found in 29% of isolates (Balasubramaniam *et al*., 2014).

Resistance to tetracycline was the most common finding (79.5%), followed by resistance to sulfadiazine (45.5%), and florfenicol (13.7%). The rate of resistance to tetracycline in avian *E. coli* isolates in our study was lower compared to results of others (Zahraei Salehi & Farashi Bonab, 2006). The results of resistance to tetracycline in *E. coli* were consistent with the report of Haghighi Khoshkhoo & Alinezhad (2010). Momtaz *et al*. (2012) studied resistance profile in *E. coli* isolates recovered from slaughtered chickens. Resistance to tetracycline was the most common finding (91.22%), and *tet*(A) and *tet*(B) were detected in 53.63% of isolates. Based on several reports, resistance rate to tetracycline antibiotics is high in Iran (Zahraei Salehi & Farashi Bonab, 2006; Haghighi Khoshkhoo & Alinezhad, 2010; Momtaz *et al*., 2012; Ghaniei & Peighambari, 2012). This might be explained by the fact that tetracycline antibiotics are extensively used in the Iranian poultry industry. However, administration of antimicrobial agents provides a selective pressure which causes selection of resistant bacteria. Therefore, the antibiotic selection pressure for resistance in bacteria in poultry is high and thus their faecal flora contains a relatively high proportion of resistant bacteria (Zahraei Salehi & Farashi Bonab, 2006). There is also a concern that antimicrobial use in food animals

can lead to the selection of antimicrobial drug-resistant zoonotic enteric pathogens which may then be transferred to people by the consumption of contaminated food or by direct animal contact. Hence, resistant faecal *E. coli* from poultry can infect humans both directly and via food. These resistant bacteria may colonise the human intestinal tract and thus bring resistance genes to human endogenous flora.

In conclusion, our study on the resistance to tetracycline and antibiotic-resistant genotype of avian *E. coli* could be of importance in clinical medication and prevention of avian colibacillosis in northwestern Iran. It also provides reference for studies on resistance genes presence in bacteria and their transfer between bacteria. The results of this study showed high resistance rate to tetracycline among poultry *E. coli* isolates. Therefore, prudent use of antimicrobials and development of alternatives to antimicrobials are vital to protect animal and human health against *E. coli* infections.

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