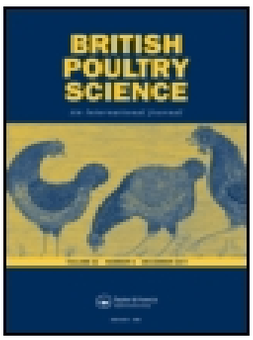

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Prevalence of tetracycline resistance determinants in broiler isolated *Escherichia coli* in Iran

Running title: **TETRACYCLINE RESISTANCE *ESCHERICHIA COLI***

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Abstract. 1. Tetracycline resistance determinants are widespread among bacterial species. Resistance to tetracycline occurs by different mechanisms regulated by various genes.

2. The study was conducted to determine the tetracycline resistance and prevalence of tetracycline resistance determinants among *E. coli* strains isolated from broilers in Northern Iran.

3. Minimum inhibitory concentration (MIC) of tetracycline and susceptibility pattern of the isolates were screened using micro dilution and disk diffusion methods, respectively. The presence of 7 tetracycline resistance genes including *tetA, tetB, tetC, tetD, tetE, tetG* and *tetM* was tested using Polymerase Chain Reaction (PCR).

4. Among 100 strains isolated from broilers, 73% were identified as tetracycline resistant. All isolates showed the presence of tetracycline associated genes. The most prevalent genes were *tetA* (46%) and *tetB* (41%) and totally, 17 different genotypes were recognised according to the presence of tetracycline resistance genes. Statistical analysis revealed that concomitant presence of the resistance genes significantly increased the tetracycline MIC and effectiveness of phenotypic characterisation.

5. The results demonstrated high occurrence of tetracycline resistant *E. coli* and related genes among broilers which presents a risk of increasing these strains in human infections associated with food animals.

INTRODUCTION

Tetracyclines are a group of antibiotics produced by *Streptomyces* spp. and they are active against a broad range of gram-positive and gram-negative bacteria. The wide

spectrum, together with their lack of major side effects, has led to their extensive use for therapy and control of infections in domestic animals (Chopra and Roberts, 2001; Bryan *et al.*, 2004; Miles *et al.*, 2006). Tetracycline resistance genes have been found to be associated with self-transferable plasmids in Gram-negative bacteria (L'abee-Lund and Sorum, 2001; Guerra *et al.*, 2002; Agerso and Sandvang, 2005), and more than 30 different *Tetracycline* genes have been characterised and classified into different groups according to their resistance mechanism, such as the efflux pump, ribosomal protection and production of enzymatic proteins (McMurry and Levy, 2000; Claudio *et al.*, 2003). *Escherichia coli*, which is a significant agent of a number of infections in human and animals, has acquired resistance to many antibiotics, including tetracyclines (Chopra and Roberts, 2001). Like other normal enteric microbiota this microorganism can serve as a reservoir of transferable antibiotic resistance genes to other pathogens (Solway *et al.*, 2003). In this regard, monitoring of resistance patterns of indicator bacteria, such as *E. coli*, to different antibiotics can be an important program for control and management of antimicrobial resistance. Consequently, this study investigates the prevalence, phenotypic features and genetic basis of tetracycline resistance in *Escherichia coli* isolated from broiler chickens in the north of Iran.

MATERIALS AND METHODS

Sample collection

From January to March 2014, a total of 100 faecal samples were obtained from different poultry carcasses on the slaughter line of 2 abattoirs in the Mazandaran province, Northern Iran. Faecal samples were collected in TSB broth tubes using

sterile swabs and taken to the microbiological laboratory, School of Veterinary Medicine, Amol University of Special Modern Technologies, in less than 6 h.

Isolation of *Escherichia coli*

Faecal swabs were cultured on MacConkey and eosin methylene blue agar (Himedia, India) and incubated at 37°C for 18-24 h. Suspected *E. coli* colonies were cultured again on EMB agar and identified by standard methods based on colony appearance and bacterial morphology, followed by biochemical characteristics (CLSI, 2010).

Tetracycline susceptibility and MIC evaluation

Antimicrobial drug susceptibility was assessed by disc diffusion method on Mueller Hinton (MH) agar plates (Merck, Germany), according to the antibiogram standard methods (CLSI, 2010) using tetracycline impregnated discs (30 µg). Quality control was carried out using the reference strain *E. coli* ATCC 25922.The diameter of tetracycline disk inhibition zone was determined and strains divided to resistant, intermediate and susceptible statuses (≤ 11 mm, resistant; 12-14 mm, intermediate; ≥ 15mm susceptible). The minimum inhibitory concentration (MIC) of Tetracycline was determined by the Mueller Hinton broth (Difco Laboratories) micro dilution method (Frech and Schwarz, 2000) on 96-well plates with incubation at 25°C for 24 h.

DNA preparation

A loop of a colony from each isolate on the agar plate was picked and then suspended in 200 µl of distilled deionised water. After vortexing, the suspension was boiled for 5 min, and 50 µl of the supernatant was collected after spinning for 10 min at 18730 *g* in a micro centrifuge. The DNA concentration of boiled extracts was determined with spectrophotometer (Lin *et al.*, 1996).

PCR assay

The presence of 7 tetracycline resistance genes, namely *tetA* (ID: 2716475), *tetB* (ID: 18157916), *tetC* (ID: 6275977), *tetD* (ID: 6276025), *tetE* (GI:12053583), *tetG* (NC_004771) and *tetM* (ID:JF830611.2), were determined by PCR and the primers used for each gene are shown in Table 1. PCR reactions were performed in a total volume of 25 µl, including 2 µl of the DNA template, 2.5 µl10x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 1 µl dNTPs (50 µM), 1 µl (1U AmpliTaq DNA polymerase) (Cinnagen, Iran), 1 µl (25 pmol) from the forward and reverse primers. Amplification reactions were carried out as follows: Three min at 95°C, 33 cycles each consisting of 1 min at 94°C, annealing as shown in Table 1 for 45 sec and 1 min at 72°C, followed by a final extension step of 5 min at 72°C. Amplified samples were analysed by electrophoresis in 1.5% agarose gel and stained with ethidium bromide. A 100 bp, DNA ladder (Jena Bioscience, Germany) was used as molecular size marker (Figure).

Statistical analysis

Statistical analysis was performed using SPSS software. Discrete variables were expressed as percentages and proportions were compared using the Chi-square test with the significance level defined at $P < 0.05$.

Tables 1 and 2 near here

RESULTS

Of the total 100 *E. coli* isolates, 73% exhibited resistance to tetracycline and 10% and 17% were susceptible and intermediately susceptible, respectively. The MIC of the isolates ranged from 4 to > 64 µg/ml (Table 2). All isolates were genotyped according to the presence of one or more tetracycline resistance genes. In total, 17

Figure near here

different genotypes were identified of which the most prevalent genotype was *tetA+tetB* (20 of 100 isolates). One isolate showed presence of 5 tetracycline resistance genes which were resistant and 33 isolates showed the presence of only one gene with 10 (30.3%) of them susceptible. All isolates with two or more tetracycline resistance genes showed resistance to Tet. The most prevalent tetracycline resistance gene was *tetA,* which was found in 46% of the isolates. The other 6 genes including *tetB*, *tetC*, *tetD*, *tetE*, *tetG* and *tetM* were present in 41%, 33%, 39%, 16.8% and 5% of the isolates, respectively. According to the statistical analysis, the MIC of Tetracycline significantly ($P \leq 0.05$) increased in isolates with more tetracycline resistance genes. The *tetD* gene had the least correlation with resistance to tetracycline.

DISCUSSION

Tetracycline is a broad spectrum antibiotic that constrains bacterial protein synthesis by preventing aminoacyl tRNA from binding to the bacterial ribosome (Roberts, 1996). Studies have shown differing prevalence of tetracycline resistant *E. coli* in humans and domestic animals. For example, Bryan *et al.* (2004) found a 47% prevalence of tetracycline resistance in chicken isolates which was lower than in isolates from pigs, whereas Miles *et al.* (2006) reported that 82.2% and 43.8% of avian and human *E. coli* isolates were resistant to tetracycline, respectively. Mainali *et al.* (2013) showed that tetracycline was the most common resistance antibiotic in *Escherichia coli* isolates from broiler chickens at slaughter in Alberta, Canada (69.2%). Nevertheless, results of the present study are in accordance with most tetracycline investigations (Bryan *et al*., 2004; Miles *et al*., 2006). Tetracycline is not an expensive antibiotic for the poultry industry, so the high presence (73%) of

resistant strains among avian isolates can be due to incorrect use and over-administration of tetracycline in flocks. Tetracycline resistance relies on genes that encode energy-dependent pumps to transport tetracycline outside the cell, or make a ribosomal protection protein, which relocates tetracycline from the ribosome and the genes responsible for producing tetracycline inactivating enzymes (Chopra and Roberts, 2001). Six of the genes examined were efflux genes (all genes except *tetM*) that are widely distributed and are associated with large plasmids of which most of them are conjugative (Chopra and Roberts, 2001). This plasmid genes can be transmitted inter and intra species and spread the resistance to tetracycline among gram negative bacteria. Most studies indicate that the tetracycline resistant mechanism in animal *E. coli* isolates is associated with efflux pump related genes (Bryan *et al.*, 2004; Wilkerson *et al.*, 2004). A total of 6 genes were selected that were related to this category of Tet-resistance and one (*tetM*) related to a ribosomal protection protein mechanism (Benacer *et al.*, 2010). Surprisingly all isolates were genotyped and showed the presence of at least one of the 7 genes. The most prevalent tetracycline resistance gene in present study was *tetA*. This result differs from other studies that reported the *tetB* gene as the main gene for responsible for tetracycline resistance (Bryan *et al.*, 2004; Wilkerson *et al.*, 2004). Recent studies similar to the present study have shown an increase in prevalence of *tetA* in animal isolates of *E. coli* (Momtaz *et al.*, 2012; Nsofor *et al.*, 2013), and that the distribution and incidence of tetracycline resistance mediated by efflux genes depends on the geographical location, species and origin of the isolate (Kang *et al.*, 2005; Miles *et al.*, 2006).

Of the 27 susceptible and intermediate susceptible isolates, 19 had only one tetracycline resistance gene, suggesting that some resistance genes in the *E. coli*

isolates were non-functional or provided intermediate or low-level resistance. Furthermore, the *tetD* and *tetE* genes showed minimum association with resistance when alone in comparison to their combination with other genes (*tetA* and *tetB*). In addition, the present study tetracycline MIC was estimated and all resistant isolates showed the MIC ≥ 32µg/ml. Tetracycline MIC of the isolates was increased significantly among isolates with more tetracycline genes and it seems that there is a direct correlation between the presence of more tetracycline resistance genes and increased resistance in *E. coli* isolates. In summary, the results of this work indicate that poultry *E. coli* strains in Northern Iran have various tetracycline resistance gene properties that can cause extensive resistance of isolates to tetracycline.

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FIGURE LEGEND

Figure. *Agarose gel electrophoresis of the PCR products of tetracycline resistance genes. Lanes 1: tetB; Lane 2: tetC; Lane 3: tetD; Lane 4: tetA; Lane 5: tetE; Lane 6 tetG; Lane 7: tetM; Lane M: 100bp DNA marker.*

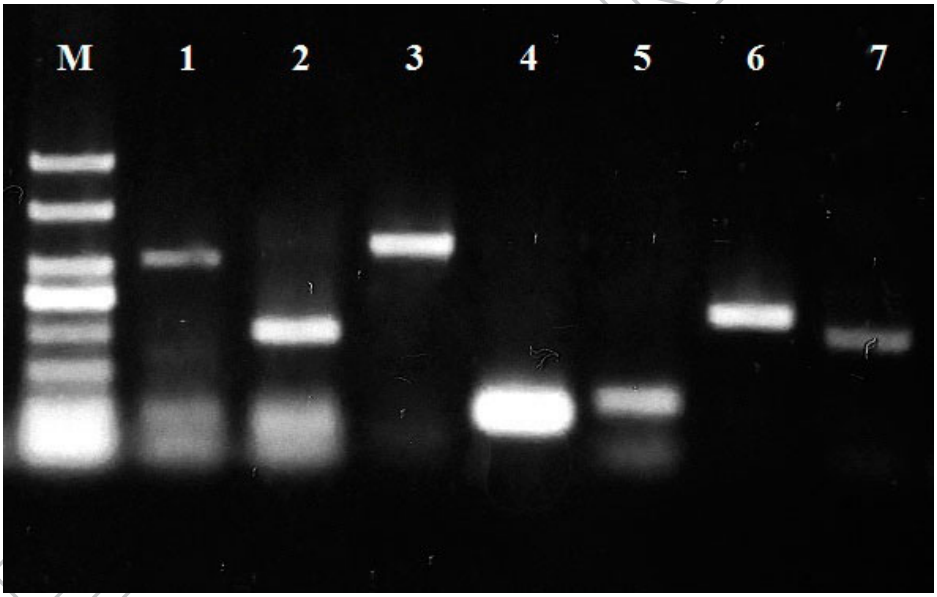

Table 1. *Primers used in PCR for detection of tetracycline resistance genes*

Target gene	Primer sequence (5` to 3`)	Annealing temperature	Product size (bp)	Reference
tetA	Forward: GCT ACA TCC TGC TTG CCT TC Reverse: CAT AGA TCG CCG TGA AGA GG	50	210	Mendez et al., 1980
tetB	Forward: TTG GTT AGG GGC AAG TTT TG Reverse: GTA ATG GGC CAA TAA CAC CG	50	659	Marshal et al., 1983
tetC	Forward: CTT GAG AGC CTT CAA CCC AG Reverse: ATG GTC GTC ATC TAC CTG CC	49	418	Marshal et al., 1983
tetD	Forward: AAA CCA TTA CGG CAT TCT GC Reverse: GAC CGG ATA CAC CAT CCA TC	48	787	Marshal et al., 1983
tetE	Forward: AAA CCA CAT CCT CCA TAC GC Reverse: AAA TAG GCC ACA ACC GTC AG	49	278	Marshal et al., 1986
tetG	Forward: GCT CGG TGG TAT CTC TGC TC Reverse: AGC AAC AGA ATC GGG AAC AC	49	468	Zhao and Aoki, 1992
tetM	Forward: GTG GAC AAA GGT ACA ACG AG Reverse: CGG TAA AGT TCG TCA CAC AC	46	406	Warsa et al., 1996

Table 2. *Tetracycline associated genotypic and phenotypic characteristics of the isolates*

Tetracycline resistance gene status (genotype)	No. of isolates	Antibiotic susceptibility status			MIC (µg/ml)
		Susceptible	Intermediate	Resistant	
tetB	9	3	-	6	4 - 32
tetD	10	-	9	1	8 - 32
tetC	8	2	-	6	8
tetE	6	5	-	1	4 - 32
tetA+tetB	20	-	8	12	16 - >64
tetA+tetC	8	-	-	8	16 - 64
tetA+tetD	4	-	-	4	32
tetB+tetD	6	-	-	6	32
tetC+tetD	4	-	-	4	64
tetC+tetE	4	-	-	4	32
tetD+tetG	5	-	-	5	32
tetA+tetB+tetC	3	-	-	3	>64
tetA+tetB+tetM	2	-	-	2	>64
tetA+tetC+tetD	5	-	-	5	64
tetD+tetE+tetM	2	-	-	2	32
tetA+tetD+tetE+tetG	3	-	-	3	64
tetA+tetB+tetC+tetE+tetM	1	-	-	1	>64
Total	100	10	17	73	4 - >64