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Escherichia coli tetracycline efflux determinants in relation to tetracycline residues in chicken

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ABSTRACT

Objective: To screen for *Escherichia coli* (*E. coli*) resistant to tetracycline, followed by identification of tet efflux genes by polymerase chain reaction (PCR). In addition, detection of tetracycline residues in chicken livers and kidneys were conducted using high performance liquid chromatography-tandem quadrupole mass spectrometry (HPLC-MS-MS). **Methods:** Strains of *E. coli* were isolated from samples of chicken colon and screened for tetracycline resistance. Tetracycline genes conferring resistance (Tc^r) were detected by polymerase chain reaction (PCR). Most of the isolates were resistant to tetracycline (97.9%). **Results:** PCR analysis indicated that Tc^r *E. coli* R-plasmids contained tet(A), tet(B) and a combination of both efflux genes. None of the isolates contained other efflux tet genes tet (C, D, E and Y). High performance liquid chromatography-tandem quadrupole mass spectrometry (HPLC-MS-MS), a sensitive technique, was used to detect residues of chlortetracycline (CTC), oxytetracycline (OTC), doxycycline (DC) in chicken livers and kidneys. The samples containing tetracycline residues were at 0.13-0.65 pg/μL levels. **Conclusions:** Tetracycline and other antibiotics are commonly used in the poultry and meat production industry for prevention of microbial infections. Multiple antibiotic resistant bacteria in Oman have increased to alarming levels, threatening public health, domestic and may have adverse effect on environment.

1. Introduction

The growth promoting effects of antibiotics were first observed in the 1940s when chicken given tetracycline had a faster growth rate than those that did not receive the antibiotic[1]. Among all antibiotics, tetracyclines were used on a large scale to treat a variety of diseases in humans, animals, and plants[2,3]. Some of the most commonly used tetracyclines are tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), and doxycycline (DC).

The overuse of antibiotics has caused the emergence of antibiotic resistant bacteria containing resistance genes found in plasmids or chromosomal DNA[4]. Several studies reported that antibiotic residues were detected in water, soil, wild life, and chicken[5-11].

Several studies reported the presence of tetracycline resistance (Tc^r) in *E. coli*[3,12,13] is an ideal model for studying the ecology of antibiotic resistance. The concept of resistome was introduced to define mechanisms of antibiotic resistance genes which are found in pathogenic or nonpathogenic microbes[14,15]. Tetracyline resistome is considered to be the largest resistome against an individual class of antibiotics. This resistome was reported to have over 189 tet genes in bacteria[16]. More than 26 Tc^r efflux pumps and 11 ribosomal protection proteins have been identified[17,18]. More than 35 tet efflux and ribosomal protection and/or efflux resistome among Gram-negative bacteria. Six of the tet determinants were found in *E. coli* tet (A, B, C, D, E, and Y)[17]. However, the most common types are tet (A) and tet (B)[18]. Ribosomal protection genes are not common in *E. coli*[17].

Antibiotics may have some toxicological effect on the host. For example, tetracycline can be deposited in bones and teeth leading to the inhibition of bone growth[17]. Therefore, maximum residue levels (MRL) have been used to determine

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safety levels in meat containing antibiotics such as livers and kidneys [19-20].

The purpose of this study is to investigate the effect of the tetracycline in chicken feed and its consequent effect on poultry industry.

2. Material and methods

2.1. Isolation of tetracycline resistant E. coli and detection of tetracycline efflux determinants

Colons samples were taken from 30 broiler chicken (6-8 months old) were randomly collected from three poultry farms in Muscat, Oman (10 from each farm). Chicken feed was supplemented with tetracycline. Approximately 1 g of colon was aseptically placed into MacConkey broth (MCB). The samples were then placed in a cool box and brought to the laboratory for analysis. Each colon sample was mixed homogeneously and assayed separately. A loop full from MCB was inoculated and incubated for 18-24 h at 37 ℃ in Endo-agar[21]. Suspected colonies were selected from each plate. The isolates were identified biochemically by API system (API analytical products, New York, NY). The isolates were tested for resistance to tetracycline using the disk diffusion method and the protocols of the Clinical and Laboratory Standards Institute CLSI[22]. A standard *E. coli* (ATCC10536) was used as a control.

Plasmids were extracted from *E. coli* isolates using GenElute HP Plasmid Midiprep Kit, as recommended by manufacturer (Sigma-Aladrich, UK). Briefly, the cultures were grown overnight at 37 ℃ in orbital shaker in 50 mL of Luria Bertani broth containing 0.25 μL/mL tetracycline antibiotic. Plasmids were then extracted from the cultures. The extracted plasmids were used for PCR amplification using primers as listed in Table 1. The PCR products were then loaded into 0.7% agarose gel wells for electrophoresis. DNA ladder of 10, 25, 50, 100, 200, or 1 000 bp was used as a reference to estimate DNA band sizes (Promega, USA). Forty six *E. coli* strains were examined for the presence of tet (A, B, C, D, E and Y) efflux genes. The PCR was amplified (Table 1). Tetracycline-susceptible *E. coli* (ATCC10536) was used as a negative control. Tetracycline resistant *E. coli* HB101 was used as the positive control.

PCR amplification was performed with Gene Amp PCR system 9700 (ABI, USA) as follows: initial denaturation at 94 ℃ for 5 min. Thirty five PCR cycles were conducted at 94 ℃ for 5 s and at 30 s of annealing. Extension was at 61 ℃ or 68 ℃ depending on the primer used (Table 1). All primers were obtained from Metabion, Germany.

2.2. Detection of tetracycline in chicken kidneys and livers by HPLC-MS-MS

Chicken kidneys and livers (30 each) from the same animals used for colon collection were used to collect kidney and liver samples. The samples were placed in phosphate buffer saline (PBS) containers and stored at -20 ℃ until assay.

An internal standard, TC, was prepared from kidney and liver samples for analysis of OTC, CTC and DC (Sigma Aldrich, UK). The internal standard (120 μL) was added to the homogenized tissue of kidney and liver samples (2 g) in 10 mL of McIlvain-EDTA buffer. The buffer with pH 3.8 consisted of 12 g citric acid monohydrate, 10.9 g Na_2HPO_4 and 37.2 g EDTA in 1 L water. The solution was mixed for 1 min, ultrasonicated for 10 min, centrifuged at 16 000 × *g* for 10 min at 4 ℃ and then filtered. An amount of 10 μL of the filtrate was injected onto analytical separation column (Xterra C18, 2.1 mm×50 mm, 3.5 μm; Waters Corp., MA, USA) via autosampler on a tandem quadruple spectroscopy, High Performance Liquid Chromatography system (HPLC MS-MS) (Quattro Ultima Pt, Waters Corp., MA, USA). The signal intensity was compared with standard solutions of CTC, OTC and DC at concentrations of 1 mg/mL, 100 ng/μL, 1 ng/μL and 100 pg/μL[25].

The TC, OTC, DC and CTC standards were used to produce a calibration line at final concentrations of 1, 5, 10, 50, 100, and 500 pg/μL (Figure 1). Quality control (QC) samples were prepared to assess the precision and accuracy of the analytical method (Figure 2). The final concentrations of

Table 1
PCR primers targeting tet efflux pumps genes.

Resistant determinant	PCR primer	Primer sequence (5′→ 3′)	Annealing temp (℃)[a]	Amplicon size (bp)
tet(A)	F	GCGCGATCTGGTTCACTC	61	164
	R	AGTCGACAGYRGCGCCGGC		
tet(B)	F	TACGTGAATTTATTGCTTCGG	61	206
	R	ATACAGCATCCAAAGCGCAC		
tet(C)	F	GCGGGATATCGTCCATTCCG	68	588
	R	GCGTAGAGGATCCACAGGACG		
tet(D)	F	GGAATATCTCCCGGAAGCGG	68	187
	R	CACATTGGACAGTGCCAGCAG		
tet(E)	F	GTTATTACGGGAGTTTGTTGG	61	199
	R	AATACAACACCCACACTACGC		
tet(Y)	F	ATTTGTACCGGCAGAGCAAAC	68	181
	R	GGCGCTGCCGCCATTATGC		

[a]Two-step PCR conditions are given in materials and methods.

the QC samples were 3, 30, and 300 pg/μL. A 1 ng/μL standard solution of TC, OTC, DC and CTC was used to tune the mass spectrometer for optimum sensitivity. The cone voltage was set to 50 V and the collision energy was set to 18 eV. The multiple reactions monitoring (MRM) transition is summarized in Table 2. The resolution settings were tuned to 0.7 Da at half height with ion energies set to 1.0 V.

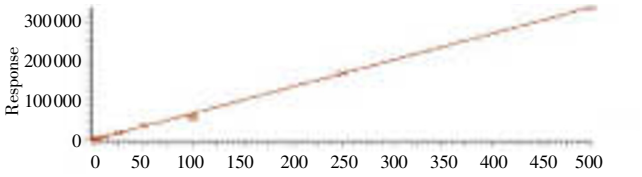

Figure 1. The calculated concentration of the standard and the deviation from the calibration line.

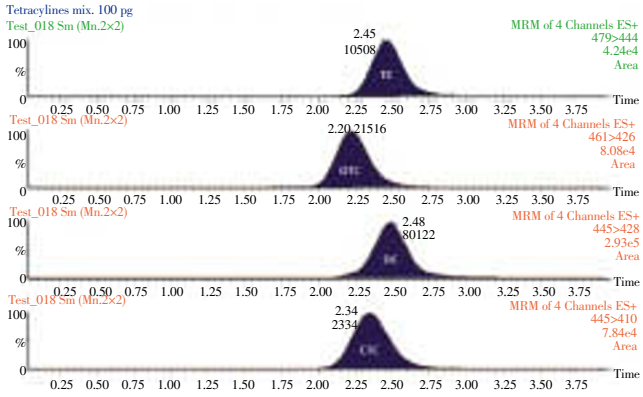

Figure 2. A chromatogram of the method specificity with TC, OTC, DC and CTC using HPLC-MS-MS technique.

X axis represents the retention time (min). Y axis represents 100 pg/μL.

Table 2

A summary of the multiple reactions monitoring (MRM) transition.

Compound	Precursor ion	Product ion	Cone voltage	Collision energy (Ce)
TC	479.1	444.1	50	18
OTC	461.2	426.1	50	18
DC	445.1	428.1	50	18
CTC	445.2	410.1	50	18

The separation of TC, OTC, DC and CTC by HPLC (Agilent 1100 Palo Alto, CA, USA) was used. Acetonitrile/water (70/30, v/v) was used as mobile phase (Sigma Aldrich, UK), at a flow rate of 0.3 mL/min and an Xterra C18, 2.1 mm×50 mm, 3.5 μm column (Waters Corp., MA, USA).

Validation of the HPLC-MS/MS method was made by comparing extraction recoveries. The chromatogram peak area in the 10 pg/mL TC, OTC, DC and CTC standards solutions in water/acetonitrile were compared to a standard, at the same concentration, of spiked solutions. The efficiency of recovery was 98%. The back-calculated concentration from antibiotic extraction method was used to evaluate the tetracycline concentration in actual kidney and liver samples.

2.3. Statistical analysis

Analysis of variance method and comparison of means were used to analyze the data using statistical package SPSS (version 15).

3. Results

3.1. Detection of tet efflux genes

Forty six Tc^r *E. coli* were isolated from colons and were examined for the presence of tet (A, B, C, D, E and Y) efflux genes in their plasmids coded 1-46. Only tet(A) and tet(B) were detected (Figure 3, 4). PCR analysis indicated that the tet(A) was the most frequent followed by tet(B). Few strains contained both genes (Figure 3). Resistance genes were not detected from negative control *E. coli*. Strains 15, 16, 29, 30, 31, 37, 38 and 46 did not contain tet(A). On the other hand, tet(B) was not detected from strains 2, 3, 4, 7, 8, 12, 14, 18, 19, 21, 42, and 46. Both tet(A) and tet(B) were detected from strains 1, 5, 6, 9, 10, 11, 13, 17, 20, 22, 23, 24, 25, 26, 27, 32, 33, 34, 35, 36, 40, 41, 43, 44, 46. Based on our data, some strains were resistant to tetracycline, but they did not contain any of the efflux *E. coli* tet genes.

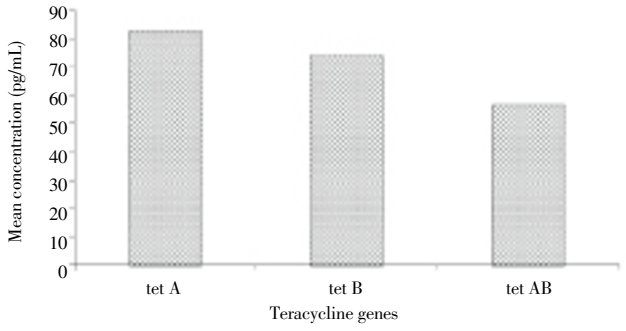

Figure 3. Percentage frequency of *E. coli* plasmids with tet(A) and tet(B) and a combination of both tet genes.

3.2. Residues of tetracyclines in chicken kidneys and livers

The relationship between the peak area ratio of TC, OTC, DC and CTC concentrations were linear (mean r = 0.999 n=5) over the calibration range (1 to 500 pg/μL) (Figure 1). The calibration line and the chromatogram specificity of the method with TC, OTC, DC and CTC shows no interfering peaks at the same retention time (Figure 2).

All collected chicken samples (30 livers and 30 kidneys) were positive for the three tetracycline's residues. The detected ranges in liver were: DC 0.299 (±0.015 1) pg/μL; OTC 0.317 (±0.015 2) pg/μL; CTC 0.317 (±0.015 2) pg/μL. The detected ranges in kidneys were: DC 0.320 (±0.013 8) pg/μL; OTC 0.267 (±0.012 3) pg/μL; CTC 0.286 (±0.016 5) pg/μL. The mean concentration of the three antibiotics in kidney and liver samples are shown in Figure 5. CTC values in liver were the highest followed by DC and OTC. However, in kidney, DC was the highest followed by CTC and OTC. The three tetracycline values from kidneys and livers were not

significant different (Figure 5 and Table 3). During the field observation it was observed that chickens were fed with different type of tetracyclines in the three farms.

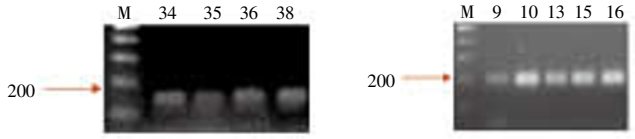

Figure 4. Example of some strains with tet(A) and tet(B) determinants on agarose gel.
An approximate 164 bp band size represents tet(A), (gel a), and 206 bp represents tet B (gel b). Lane M represents a molecular weight marker.

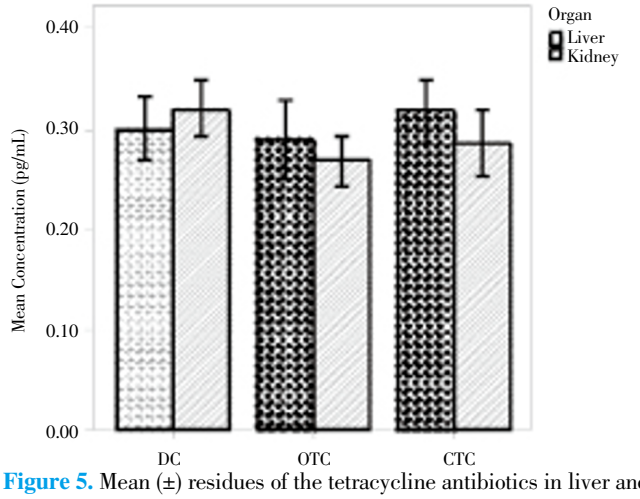

Figure 5. Mean (±) residues of the tetracycline antibiotics in liver and kidney tissues.
DC = doxycycline, OTC = oxytetracycline and CTC = chlortetracycline.

Table 3

Analysis of variance of three tetracycline antibiotic residues in liver and kidney.

Source	Sum of squares	*df*	Mean square	*F*	Sig.
Corrected model	0.060	5	0.012	1.672	0.144
Intercept	15.802	1	15.802	2200.796	0.000
Organs	0.005	1	0.005	0.718	0.398
Antibiotics	0.032	2	0.016	2.244	0.109
Organs antibiotics	0.023	2	0.011	1.577	0.209
Error	1.249	174	0.007	–	–
Total	17.112	180	–	–	–

4. Discussion

In this study, Tc^r in *E. coli* from chicken colons were found in the majority of the isolates. Earlier investigations conducted in Oman revealed that there was a low prevalence of bacteria resistant to tetracycline[5,26]. However, in this investigation, prevalence of resistance to Tc was significantly higher. This may be related to the misuse of Tc in the poultry industry. Although regulations in Oman limit the use of antibiotics, the results of this study suggest that there is an overuse of Tc in the chicken feed.

Only two tet genes were identified in this investigation. The majority of the isolates contained tet(A) followed by tet(B) which is in agreement with Roberts findings[18]. The two identified tet efflux genes belong to a family of genes detected from *E. coli*. Most of the tet determinants are associated with either conjugative or mobilized elements, which explains their wide distribution among bacteria[16,27]). Some of the isolates in this study carried either tet(A) or tet(B) genes, with some strains containing both. Most of the isolates contained tet(A) determinant. This is in agreement with Miranda who reported that most of Tc^r Gram negative bacteria carried tet(A) while few isolates contained tet(B)[11]. Others reported that 60% of the Tc^r *E. coli* strains in bovines carried tet(B) gene only[28]. Another study reported that tet(B) was the most common (80%) in *E. coli* isolates[13].

In this study, the combined presence of tet(A), tet(B) in *E. coli* isolates was the highest reported. Based on other studies, there is a significant variation of tet(A) and tet(B) combination. Only 2% of *Shigella* spp and *E. coli* strains isolated from a variety of geographical locations had both tet(A) and tet(B)[1]. However, Dolejska reported that none of the isolates carried a combination of tet(A) and tet(B)[29].

Tetracycline efflux genes were not detected from some isolates although the strains were resistant to tetracycline. This could be that some of the isolates contained other types of resistant tetracycline genes probably belong to the ribosomal protection genes which have been identified by Chopra and Roberts[17,18].

The majority of *E. coli* strains in this study were resistant to tetracycline. Therefore, tetracycline residues were screened in chicken livers and kidneys using a sensitive and precise HPLC-MS/MS method.

The purpose of MRL was to determine safe concentration levels in meat before animals could be slaughtered for food. Data obtained from this study indicated that farmed chickens showed no significant difference in Tc levels of liver and kidney samples. Specifically, DC, OTC and CTC showed no significant differences between the liver and kidneys throughout the study period. Also, all three types of tetracycline residues were detected below the MRL. However, in a related study, excessive use of tetracyclines was detected in chicken tissue and 95.5% of the antibiotic-residue-positive liver had mean concentrations of at least one tetracycline compound above the MRL[19].

During the last few years in Oman, there has been an alarming rise of multiple antibiotic resistant bacteria, and specifically to tetracycline. Resistant bacteria in chickens were reported to be 26.04% in 1999 but rose significantly to 90% in 2007[5,26,30]. In sewage treated effluent some of resistant bacteria to tetracycline rose from 30% to 100%[30,31]. Resistant bacteria to tetracyclines have been transported through sewage to the environment and have infiltrated the marine life and were isolated in marine fish near the dumping site of treated sewage effluents[6]. Interestingly, tetracycline resistant bacteria were also isolated from turtle eggs and oviductal fluids[7,8,32].

In Oman, antibiotics particularly tetracyclines must be used cautiously to avoid medical and environmental problems, which in the long run may affect the productivity of the poultry, meat industry and fisheries, as well as being

a serious public health issue. Strict regulations must be implemented to control the usage of antibiotics in order to stop the emergence of resistant strains. Public education and awareness of the risk of misuse of antimicrobial drugs is crucial to decrease the overuse of antibiotics and to avoid damaging effect on human health and environment.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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