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Short Paper

The frequency of tetracycline resistance genes in *Escherichia coli* strains isolated from healthy and diarrheic pet birds

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Abstract

Background: Pet birds have close contact to human and resistant bacteria can transfer from birds to intestinal flora of human. **Aims:** This study was carried out to determine the tetracycline resistance genes in *Escherichia coli* strains associated with enteric problem in pet birds. **Methods:** Totally, 295 cloacal swabs were collected from 195 healthy and 100 diarrheic pet birds in Isfahan province, Iran. The presence of *E. coli* was identified by conventional bacteriological, biochemical, and molecular examinations. The presence of tetracycline resistance genes (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, and *tetS* genes) were examined using three multiplex PCR. **Results:** The results showed that 18.9% and 43% of cloacal samples of healthy and diarrheic pet birds contained *E. coli*, respectively. The mean percentage of *E. coli* isolated from cloacal samples of diarrheic birds was significantly higher than the healthy birds (46.6 *vs* 23.1%). In healthy birds, out of 37 *E. coli* isolates, 10 isolates were resistant to tetracycline, harboring *tetA* and *tetB* genes (3 *tetA vs* 7 *tetB*), but in the diarrheic birds, of 26 resistance *E. coli*, 11, 12, and 3 strains contained *tetA* (42.3%), *tetB* (46.15), and *tetA*+*tetB* (11.53%) genes. The percentage of *tet* genes were significantly higher in diarrheic birds than healthy birds (58.9 *vs* 24.0%). **Conclusion:** Both resistant genes of *tet*A and *tet*B were detected in *E. coli* isolates that are related with efflux pump activity. These genes can be transferred between Gram-negative bacteria and they have the potential ability to be transferred to the environment and human flora.

Key words: Diarrhea, *Escherichia coli*, Pets, Tetracycline resistance

Introduction

Tetracycline is one of the broad-spectrum antibiotics that inhibits bacterial protein synthesis by preventing aminoacyl-tRNA from binding to the bacterial ribosome (Chopra and Roberts, 2001). Its low cost, high efficacy, and trivial side effects make it one of the most popular options in avian medicine. Widespread and incorrect use of antibiotics can potentially lead to the emergence of antibiotic resistance in the bacteria (Gholami-Ahangaran *et al*., 2021). Resistance to tetracycline is conferred by one or more of the 38 currently described *tet* genes, which encode one of three mechanisms of resistance: an efflux pump, a method of ribosomal protection, or direct enzymatic inactivation of the antibiotic. Of these mechanisms in Gram-negative bacteria, an efflux pump system is encoded by 32 genes *e.g. tetA*, *tetB*, *tetC*, *tetD*, *tetE*, and *tetG*, while *tetM*, *tetO*, and *tetS* encode ribosomal protection systems (Sigirci *et al*., 2020). Ribosomal protection mechanisms are more common among Gram-positive organisms. Generally, the rapid spread of tetracycline resistance among bacteria is due to the localization of *tet* genes on plasmids, transposons, and integrons (Roberts, 1996). The objective of this study was to evaluate the presence of tetracycline resistance genes in *E. coli* strains isolated from cloacal samples of healthy and sick pet birds and the frequency of *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, and *tetS* genes in the local population of *E. coli.* To the best of our knowledge, this is the first report on the molecular detection of the tetracycline resistance gene in *E. coli* isolated from fecal samples of pet birds in Isfahan, Iran.

Materials and Methods

Sample collection

A total of 295 cloacal swabs were collected from healthy and diarrheic pet birds including parrot, parakeet, budgerigar, lovebird, canary, and finch, from private pet clinics, breeding aviaries, pet shops located in Isfahan province, Iran. The frequency of samples in each healthy or illness pet bird species is listed in Table 1.

Table 1: Characteristic of utilized primers for detection of tetracyline-resistant genes in PCR

tet resistance gene	PCR primer sequence 5´-3´	Amplicon size (bp)
tetA	F: GCT ACA TCC TGC TTG CCT TC R: CAT AGA TCG CCG TGA AGA GG	210
tetB	F: TTG GTT AGG GGC AAG TTT TG R: GTA ATG GGC CAA TAA CAC CG	659
tetC	F: CTT GAG AGC CTT CAA CCC AG R: ATG GTC GTC ATC TAC CTG CC	418
tetD	F: AAA CCA TTA CGG CAT TCT GC R: GAC CGG ATA CAC CAT CCA TC	787
tetE	F: AAA CCA CAT CCT CCA TAC GC R: AAA TAG GCC ACA ACC GTC AG	278
tetG	F: GCT CGG TGG TAT CTC TGC TC R: AGC AAC AGA ATC GGG AAC AC	468
tetK	F: TCG ATA GGA ACA GCA GTA R: CAG CAG ATC CTA CTC CTT	169
tetL	F: TCG TTA GCG TGC TGT CAT TC R: GTA TCC CAC CAA TGT AGC CG	267
tetM	F: GTG GAC AAA GGT ACA ACG AG R: CGG TAA AGT TCG TCA CAC AC	406
tetO	F: AAC TTA GGC ATT CTG GCT CAC R: TCC CAC TGT TCC ATA TCG TCA	515
tetS	F: CAT AGA CAA GCC GTT GAC C R: ATG TTT TTG GAA CGC CAG AG	667

Isolation and identification of *E. coli*

All samples were cultured in peptone water and incubated at 37°C for 24 h. After incubation, 0.1 ml of 1:10 dilution of each sample in sterile water was cultured on three MacConkey's agar plates. The inoculated plates were incubated at 37°C for 24 h. A single predominant and pure colony from each plate was inoculated onto MacConkey's agar plates containing tetracycline (30 µg/ml). The tetracycline resistant growth of suspected *E. coli* colonies was subjected to Gram staining. The presence of *E. coli* was confirmed by growing the isolates on an eosin methylene blue (EMB) agar medium. Gram-negative colonies that grew on this medium were subjected to biochemical tests (IMViC tests: Indole, methyl red, Voges Proskauer, and citrate tests) to confirm the colonies as *E. coli*. The isolates that had typical IMViC patterns (Indol and MR positive, and VP and citrate utilization negative) were considered as *E. coli* (Gholami-Ahangaran and Zia-Jahromi, 2014).

For extraction of DNA, colonies of overnight growth bacteria were used. The colonies were transferred into a microtube containing one ml of distilled water and boiled for 10 min, and then centrifuged for 5 min at 112 g. Five microliters of the supernatant were used for PCR (Gholami-Ahangaran and ZiaJahromi, 2014). After DNA extraction, molecular confirmation of colonies was achieved according to the *16S rRNA* gene of *E. coli* described by Sabat *et al*. (2000). The primer set of ECP79F (forward, targeting bases 79 to 96; 5-GAA GCT TGC TTC TTT GCT-3) and ECR620R (reverse, targeting bases 602 to 620; 59-GAG CCC GGG GAT TTC ACA T-39) were used to identify *E. coli* (Sabat *et al*., 2000).

E. coli ATCC 25922 was used as reference and quality control organisms in the PCR method for *E. coli* band confirmation.

Tetracycline gene resistance detection

The presence of genes associated with resistance to tetracycline (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, and *tetS* genes) were examined using three multiplex PCR. The primers were used according to Ng *et al*. (2001). The characteristics of primers are presented in Table 1. PCR was achieved in three separate categories. In category I, PCR was performed for *tetB*, *tetC*, and *tetD* tetracycline resistance genes. In category II, the *tetA*, *tetE*, and *tetG* genes were amplified, simultaneously. In category III, PCR was performed for *tetK*, *tetL*, *tetM*, *tetO*, and *tetS* tetracycline resistance genes.

The PCR reactions were performed in a total volume of 25 µL, including 3 mM $MgCl_2$, 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.

The amplification reactions were carried out using a DNA thermocycler (Eppendorf Mastercycler, Eppendorf-Netheler-Hinz GmbH, Biorad, Germany) for 5 min of initial denaturation at 94°C, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s. The PCR products were analyzed by gel electrophoresis in 1.5% (w/v) agarose gel and stained with ethidium bromide (Ng *et al*., 2001). A 100 bp DNA Marker (Fermentas, Germany) was used.

Statistical analysis

The data were analyzed with SPSS statistical software and method of Chi-square and analysis of variance (One-way ANOVA).

Results

A 544 bp fragment of the *16S rRNA* gene of *E. coli* was amplified in the samples to identify *E. coli* in bacteriological methods (Fig. 1). The microbiological results showed that in healthy pet birds, 37 out of 195 cloacal swab samples (18.9%) were identified as *E. coli.* In healthy pet birds, 5/50 (10%) of the canary, 4/50 (8%) of the finch, 9/22 (40.9%) of the parrot, 6/18 (33.3%) of the parakeet, 8/30 (26.6%) of the budgerigar, and 5/25 (20%) of the lovebird isolates were identified as *E. coli*. In diarrheic pet birds, *E. coli* was isolated from 43 of 100 cloacal swab samples (43%). In diarrheic pet birds, 6/20 (30%) of the canaries, 5/20 (25%) of the finches, 6/10 (60%) of the parrots, 7/10 (70%) of the parakeet, 10/20 (50%) of the budgerigar, and 5/9 (45%) of the lovebird isolates were identified as *E. coli* (Table 2). The mean percentage of *E. coli* isolated from cloacal swabs were significantly different between healthy and diarrheic pet birds (P=0.023). Such that, the isolate frequency from diarrheic pet birds was significantly higher than healthy pet birds (23.1% *vs* 46.6%).

Table 2: The frequency of tetracycline resistance genes in *E. coli* isolates from healthy and illness pet birds

Pet bird species	Healthy birds						Diarrheic birds					
	Total samples	Total *E. coli* isolates (%)	Resistance *E. coli* (%)	*tetA*	*tetB*	*tetA* + *tetB*	Total samples	Total *E. coli* isolates (%)	Resistance *E. coli* (%)	*tetA*	*tetB*	*tetA* + *tetB*
Canary	50	5 (10)	1 (20)	0	1	0	20	6 (30)	3 (50)	1	2	0
Finch	50	4 (8)	0 (0)	0	0	0	20	5 (25)	2 (40)	1	1	0
Parrot	22	9 (40.9)	3 (33.3)	2	1	0	10	6 (60)	4 (66.6)	2	1	1
Parakeet	18	6 (33.3)	2 (33.3)	0	2	0	10	7 (70)	5 (71.4)	2	2	1
Budgerigar	30	8 (26.6)	3 (37.5)	1	2	0	20	10 (50)	7 (70)	3	3	1
Lovebird	25	5 (20)	1 (20)		1	0	20	9 (45)	5 (55.5)	2	3	0
Total	195	37	10	3 (30)	7 (70)	0 (0)	100	43	26	11 (42.3)	15 (57.69)	3 (11.53)

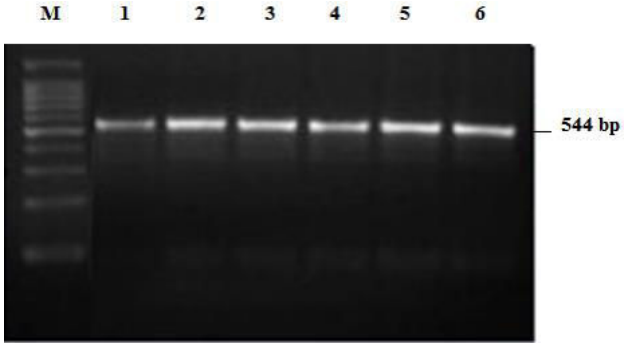

Fig. 1: PCR product electrophoresis (lane M: 100 bp marker, lanes 1 to 5: 544 bp fragment of *16srRNA* gene of *E. coli* in positive samples, and lane 6: 544 bp fragment of *16srRNA* gene of *E. coli* in positive control)

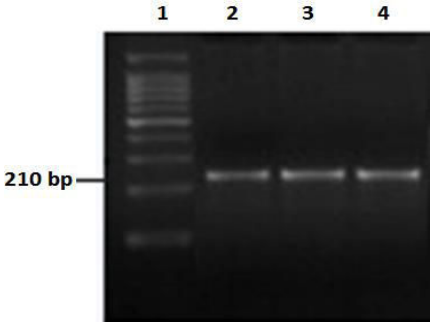

Fig. 2: PCR product electrophoresis (lane 1: 100 bp marker, and lanes 2 to 4: 210 bp fragment of *tetA* gene)

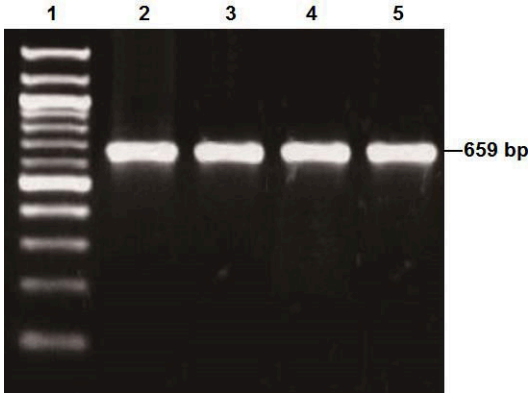

Fig. 3: PCR product electrophoresis (lane 1: 100 bp marker, and lanes 2 to 5: 659 bp fragment of *tetB* gene)

In healthy birds of a total of 37 *E. coli* isolates, 10 *E. coli* were resistant to tetracycline and contained *tetA* and *tetB* resistance genes (3 *tetA vs* 7 *tetB*), but in diarrheic birds of 26 resistance *E. coli*, 11, 12, and 3 strains contained *tetA* (42.3%), *tetB* (46.15%) and *tetA+tetB* (11.53%) resistance genes, respectively. There is a significant difference between the mean percentage of tetracycline resistance gene in *E. coli* isolated from healthy and diarrheic pet birds (P=0.001). The frequency of *E. coli* strains with *tet* resistance genes from cloacal swabs was significantly higher in diarrheic pet birds than healthy birds (24.0% *vs* 58.9%). The relationship between each detected *tet* resistance gene and the pathogenicity of *E. coli* strain (detected from healthy or sick birds) were analyzed with Chi-square and revealed no significant differences between these factors.

None of the isolates contained the *tet*C, *tet*D, *tet*E, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, and *tetS* genes. In PCR, only the *tet*A (210 bp) (Fig. 2) and *tet*B (659 bp) genes were amplified (Fig. 3).

Discussion

In the present study, *E. coli* was identified in 18.9% (37/195) and 43% (43/100) of cloacal samples collected from healthy and diarrheic birds and 27% (10/37) and 60.46% (26/43) of *E. coli* isolates were resistant to tetracycline, in healthy and diarrheic birds, respectively. All of *E. coli* strains resistance to tetracycline pose *tetA*, *tetB* or *tetA+tetB* genes. The mean percentage of *E. coli* isolated from cloacal samples of diarrheic pet birds was significantly higher than healthy pet birds (23.1% *vs* 46.6%). Similar to the present study, other studies have discussed the isolation of *E. coli* from fecal samples of birds (Hidasi *et al*., 2013; Giacopello *et al*., 2015; Horn *et al*., 2015; Beleza *et al*., 2019); their results varied from 3.6% in cloacal swab samples from apparently healthy Belgian canaries (Horn *et al*., 2015) to 62.0% in 50 fecal samples from canaries with signs of illness in Italy (Giacopello *et al*., 2015). Previously, it was declared that the environmental conditions and sanitary status to which pet bids were subjected might influence the isolation rate of *E. coli* (Giacopello *et al*., 2015; Beleza *et al*., 2019).

Various tetracycline resistance rates have been observed in *E. coli* strains isolated from pet birds. For instance, 39.3% of the isolates isolated from canaries of Brazil (Horn *et al*., 2015), and 69% were from fecal samples of Psittacifomes birds (Machado *et al*., 2018). In the present study, the frequency of tetracycline resistance genes in *E. coli* from diarrheic birds are significantly higher than healthy birds. The wide uncontrolled and empirical use of antibiotics in pet birds following

infectious diseases might be the reason for the higher resistance rate in sick birds.

In resistance *E. coli* strains in healthy birds, 30% and 70% of the isolates contained *tet*A and *tetB* genes, and in diarrheic birds, 42.3%, 46.15%, and 11.53% of *E. coli* isolates contained *tetA*, and *tet*B and *tetA*+*tetB* genes. With regard to tetracycline resistance genes in birds, there are some limited reports. Noori-Gharajari and Shahbazi (2020) demonstrated that *tetB* (43.3%) and *tetA* (30%) are the common resistance genes in fecal *E. coli* isolates (Nouri-Gharajalar and Shahbazi, 2020). Zibandeh *et al*. (2016) reported *E. coli tetA* resistance gene in 32.5%, 65%, and 72.5% of one-day-old chicks, 30 days old chickens, and chickens at the slaughterhouse (Zibandeh *et al*., 2016). Seifi and Khoshbakht (2016) showed that 73% of *E. coli* isolates from fecal samples of poultry exhibited resistance to tetracycline and the most prevalent genotype was *tetA*+*tetB* (20 of 100 isolates) (Seifi and Khoshbakht, 2016). In our study, there were no significant differences between the frequency of *tetA* and *tetB* in *E. coli* isolates. Some previous studies showed an increase in the *tetB* prevalence of isolated *E. coli* (Bryan *et al*., 2004; Wilkerson *et al*., 2004), while others have reported an increase in the prevalence of *tetA* (Nsofor *et al*., 2013; Seifi and Khoshbakht, 2016). Moreover, Koo and Woo (2011) reported that *tetA* and *tetB* were the most frequent genes in tetracycline resistant *E. coli* strains in Korea (52.4% and 41.3%, respectively) (Koo and Woo, 2011). Most reports have attributed the tetracycline resistant mechanism of *E. coli* to efflux pump-related genes (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, and *tetG*) (Bryan *et al*., 2004; Wilkerson *et al*., 2004). However, distribution and incidence of antibiotic resistance mediated by efflux genes depend on the geographical location, species, origin of the isolate (Kang *et al*., 2005; Miles *et al*., 2006), time of sampling (Rahimi *et al*., 2012) and even virulence of isolates (Sengeløv *et al*., 2003). Sengelov *et al*. (2003) showed that the frequency of *tetA* gene in pathogenic *E. coli* isolated from broilers was significantly higher than other *tet* genes, while the *non-tetA* genes was higher in non-pathogenic *E. coli* in comparison with pathogenic *E. coli* (Sengeløv *et al*., 2003). However, lower frequency of *tetA* in *E. coli* isolated from healthy birds could be related to the number of samples or different virulence of *E. coli* isolated from healthy birds. Therefore, the monitoring of tetracycline resistance genes in samples revealed the new knowledge about resistance situation in one state or country that can transfer to animal or human.

None of the *tetC*, *tetD*, *tetE* or *tetG*, *tetK*, *tetL*, *tetO* and *tetS* genes were detected in any of our *E. coli* isolates. These findings are in accordance with results by Skockova *et al*. (2012) that they found only *tetA* and *tetB* in 100 *E. coli* stains isolated from ill and healthy pigs, cattle, and chickens. They tested five tetracycline resistance determinants but only detected *tetA* and *tetB* genes (Skočková *et al*., 2012). In the present study, 3 *E. coli* isolates simultaneously showed the *tetA* and *tetB* genes. The potent selection pressures that provided by environments containing high levels of tetracycline can lead to the acquisition of more than one tetracycline gene in a given strain. The results of Bryan *et al*. (2004) also showed that 22.2% and 1.9% of the isolates contained two and three *tet* genes, respectively (Bryan *et al*., 2004). However, the prevalence of tetracycline resistance genes among *E. coli* strains isolated from pet birds suggests that these same resistance determinants may also be present in animal and human pathogens and it is supposed to investigate in future.

In conclusion, it was demonstrated that *E. coli* strains can be detected from healthy and diarrheic pet birds and can harbor tetracycline resistance genes. The frequency of *tet* resistance genes of *E. coli* strains were significantly higher in diarrheic birds than healthy birds ($P<0.05$). Both *tet*A and *tet*B resistant genes of the *E. coli* isolates are related to efflux pump activity which can be transferred among Gram-negative bacteria and have the potential ability to be transferred to the environment and human flora.

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Conflict of interest

The authors declare no conflict of interest.

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