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Diverse Antimicrobial Resistance Profiles Across Phylogroups of Shiga Toxin-Producing Escherichia coli Isolates in Companion Birds

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A B S T R A C T

Escherichia coli (*E. coli*) is an indicator bacteria commonly used to monitor the progression of antibiotic resistance in humans and animals. Shiga toxin-producing *E. coli* (STEC) strains are responsible for severe intestinal diseases in humans, such as hemorrhagic colitis or hemolytic uremic syndrome, and can potentially be transmitted from companion animals, including pet birds. To investigate this potential transmission, 200 fecal samples were collected from birds with varying health statuses, ages, and sexes referred to the Veterinary Hospital at the University of Tehran. Among these samples, 26 isolates of *E. coli* (13%) were found in different bird species. The study identified 9 Attaching-effacing (AEEC) isolates (34.6%), all of which were STEC isolates (9 out of 9 isolates; 100%). Phylogroup analysis showed that 4 isolates belonged to B2, 3 isolates belonged to D, and 2 isolates were untypable. The results of the disk diffusion method indicated that 7 out of 9 STEC isolates (77.7%) were classified as multi-drug resistant (MDR). All 9 isolates (100%) were resistant to penicillin and erythromycin but sensitive to fosfomycin and lincospectin. In the B2 phylogroup, nearly all isolates were sensitive to fluoroquinolones (96.9% sensitivity). However, in the D phylogroup, the results differed, with 87.5% of isolates being resistant or developing resistance against fluoroquinolones. The findings of this study highlight that different species of birds commonly kept as pets in Iran can be affected by STEC strains and can also carry multi-drug resistant *E. coli*. These findings are particularly important for public health implications.

Keywords: *Antibiotic, Escherichia coli (E. coli), Multi-Drug Resistant (MDR), Phylogroup, Shiga-Toxin Producing E. coli (STEC)*

1 Introduction

Escherichia coli (*E. coli*) is a gram-negative, rod-shaped bacteria in the Enterobacteriaceae family. It is commonly found in the gastrointestinal tract of humans and animals. *E. coli* can easily spread through the food chain and water, making it an important indicator bacteria for tracking antibiotic resistance evolution in both humans and animals (1). Based on phylogenetic analysis, *E. coli* strains are divided into nine phylogenetic groups: A, B1, B2, C, D, E, F, Clade I, and recently, Clermont et al. discovered a new phylogroup called G. Distinguishing between phylogroups can provide useful information; for example, phylogroup A primarily consists of commensal *E. coli* strains, while

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external pathogenic strains are more likely to belong to phylogroup B2 rather than D (2). Shiga toxin-producing *E. coli* (STEC) strains, also known as verotoxin-producing *E. coli* (VTEC), are responsible for the worldwide development of human intestinal disease and potentially fatal hemorrhagic colitis or hemolytic uremic syndrome (3). Phylogenetic studies on human diarrheagenic *E. coli* isolates showed that EHEC strains were classified in phylogroups A and B1 (4). STEC strains have been found in fecal samples from healthy birds in several countries. STEC strains produce one or both major Shiga toxin (Stx) types, called Stx1 and Stx2 (5).

The population of companion animals has been increasing worldwide, with pet birds being among the most favorable animals after dogs and cats (6). Pet birds belong to the orders Passeriformes and Psittaciformes, which include canaries, finches, parrots, parakeets, and lovebirds (7). The emergence of multi-resistant *E. coli* has been previously reported in humans and in different animal species including pet birds (8). The administration of antibacterial agents in companion animals is known to lead to the development of resistant bacteria, which can reach humans through direct and indirect pathways. There is evidence that commensal *E. coli* strains from birds have different rates and types of resistance, and can carry and transfer genetic markers related to resistance. On the other hand, STEC is an emerging pathogen with significant clinical and public health concerns that should be investigated in order to be treated and controlled correctly (9, 10). The study aimed to assess the presence and patterns of antimicrobial resistance in different phylogenetic groups of Shiga-toxin producing *E. coli* isolates from pet birds in Tehran, Iran.

2 Methods and Materials

2.1 Sampling and Bacterial Isolates

In August 2020, a total of 200 fecal samples were collected from submitted cases to the Pet Birds Clinic, Department of Avian Diseases, University of Tehran. Birds from 22 different species, mostly Psittaciformes and Passeriformes, with different situations of health status, age and sex were included in this study. Sterile cotton swabs were used for taking fecal samples and the standard methods of isolation and identification were done as described previously (2). Briefly, samples were first cultured in LB (Luria Bertani) broth medium, after 18 hours of incubation at 37°C, the samples were plated on MacConkey agar and incubated at 37°C for 18 hours. All of the possible *E. coli* isolates were stored in LB broth containing 15% glycerol at -20°C for a short time until further processing.

2.2 DNA extraction

The study utilized a routine boiling method, as described by Zahraei Salehi et al., for DNA extraction. The process involved harvesting 6 to 8 typical colonies from each isolate's culture, suspending the colonies in 100 µl of sterile deionized water, incubating the suspension at 100°C for 10 minutes to release the DNA contents, and centrifuging the suspension at 6000g for 5 minutes. The supernatant was then used as the template DNA in the PCR reaction(11).

2.3 PCR Assays

In order to confirm *E. coli* strains, isolated samples were examined for the presence of the uspA (universal stress protein A) gene based on Chen & Griffiths' study (12). Then, positive samples were tested for detection of eae, bfpA, stx1, and stx2 virulence genes (13, 14). Techniques from Clermont et al. (15, 16), were utilized for phylogenetic analysis of isolated AEEC strains. *E. coli* strains were assigned to one of the phylogroups A, B1, B2, C, D, E, F, clade I, or G based on the results (2).

For PCR procedures in this study, the positive control was an *E. coli* O157:H7 strain which had already been isolated and identified (ATCC 35150,(17)), and sterile deionized water was used as the negative control. All PCR reactions were containing: 12.5 µl 2x master mix (Ampliqon, Denmark), 0.5 µl of each forward and reverse primers (10 pmol/ µl), 9.5 µl nuclease-free water and 2 µl of DNA sample. All the PCR products were separately run on 1.5% agarose gel (Yekta Tajhiz Azma, Iran) in TBE buffer (Tris Base, Boric Acid, EDTA, pH 8, 0.5M), dyed with Safe Stain (SinaClon, Iran) and viewed under UV light illumination (Kiagen, Iran). The primer sequences, target genes and amplicon sizes are described previously (2).

2.4 Antimicrobial Susceptibility test

Isolates which considered as AEEC according to PCR results, were investigated for their antibiotic resistance characteristics. For this Purpose, disk diffusion (DD) method, based on the Clinical Laboratory Standard Institute (CLSI, 2020) standard was used. In brief, a suspension of overnight growth of bacteria in LB broth, with turbidity equivalent to a 0.5 McFarland standard was inoculated on Mueller-Hinton (MH) agar by sterile cotton swabs. After 15 minutes, the antibiotic disks (29 antibiotics used for DD method, listed in Table 1) (Padtan Teb®, Iran) were placed on MH agar and subsequently incubated at 35°C ± 1 for 24 hours. After that, based on CLSI instructions, the results were charted in excel sheets

Table 1. The list of 28 used antibiotics in disk diffusion method and the susceptibility test results of 9 STEC isolates

Antimicrobial class	Antimicrobial agent	Disk Content (µg)	e.118	e.122	e.135	e.156	e.158	e.162	e.165	e.170	e.171
β-lactamase Inhibitors	Amoxiclav	30	R	R	R	R	I	R	S	R	R
Penicillins	Ampicillin	10	R	R	R	R	S	R	S	S	S
	Penicillin	1 IU (0.6 µg)	R	R	R	R	R	R	R	R	R
Cephems (parenteral)	Cefotaxime	30	R	I	I	I	I	R	S	I	I
	Ceftriaxone	30	S	I	S	S	S	I	I	S	S
	Cefixime	5	I	I	I	I	I	R	S	R	I
	Ceftazidime	30	S	S	S	S	S	S	S	S	S
Fluoroquinolone	Ciprofloxacin	5	R	R	R	I	S	S	S	S	S
	Danofloxacin	10	R	I	S	I	S	S	S	S	S
	Difloxacin	10	R	I	I	I	S	S	S	S	S
	Enrofloxacin	5	R	I	I	I	S	S	S	S	S
	Levofloxacin	5	R	I	I	I	S	S	S	S	S
	Norfloxacin	10	R	I	S	I	S	S	S	S	S
	Ofloxacin	5	R	I	I	I	S	S	S	S	S
	Flumequine	30	R	I	S	I	S	R	S	S	S
Phenicols	Chloramphenicol	30	R	R	R	R	S	S	R	R	S
	Florfenicol	30	S	R	R	S	S	S	S	S	S
Tetracyclines	Doxycycline	30	R	R	R	R	I	R	I	S	S
	Oxytetracycline	30	R	R	R	R	I	R	S	S	I
	Tetracycline	30	R	R	R	R	I	S	R	I	I
Macrolides	Erythromycin	15	R	R	R	R	R	R	R	R	R
Fosfomycins	Fosfomycin	200	S	S	S	S	S	S	S	S	S
Aminoglycosides	Gentamicin	10	S	R	S	S	S	S	R	S	S
	Neomycin	30	R	S	S	I	I	R	R	S	S
	Streptomycin	10	S	R	R	R	I	R	R	R	I
Folate synthesis inhibitor	Trimethoprim + Sulfamethoxazole	1.25 + 23.75	R	R	R	R	S	S	S	S	S
Quinolone	Nalidixic acid	30	R	R	R	R	S	S	S	S	S
Lipopeptides	Colistin	10	I	S	S	R	S	I	S	S	S
-	Lincospectin	15/200	S	S	S	S	S	S	S	S	S

3 Results

Out of 200 fecal samples, we found 26 isolates of *E. coli* (13%) from pet birds as described in our previous study (2). Briefly, the presence of *E. coli* in different species of birds varied and included: white-eared bulbul (3 isolates out of 5 samples, 60%), duck (3 isolates out of 8 samples, 37.5%), canary (1 isolate out of 5 samples, 20%), mynah and rose-ringed parakeet (4 isolates out of 22 samples and 2 isolates out of 11 samples respectively, 60%), budgerigar (1 isolate out of 7 samples, 14.3%), African grey parrot and lovebirds (1 isolate out of 8 samples and 2 isolates out of 16 samples respectively, 12.5%), and cockatiel (9 isolates out of 89 samples, 10.1%) (Table 2). The identity of the *E. coli* isolates was confirmed by detection of uspA gene and based on the presence of eae gene, 9 AEEC isolates were found (34.6%). After that, because of the absence of bfpA gene, and the presence of stx1, stx2 and/or both of them in the isolates, all the AEEC isolates were considered as STEC (9 out of 9 isolates).

Table 2. The list of bird species included in this study, and their detailed results of PCR assays

Birds species	Sample size	*Usp*A +	*bfp*A +	*eae* +	*stx*1 +	*stx*2 +	Phylogroup
Cockatiel	89	9	-	3	3	3	D, (untyped), B2
Mynah	22	4	-	2	1	2	D, B2
Lovebirds	16	2	-	-	-	-	-
Rose-ringed parakeet	11	2	-	1	-	1	B2
Green-cheeked parakeet	8	-	-	-	-	-	-
Duck	8	3	-	1	-	1	(Untyped)
African grey parrot	8	1	-	-	-	-	-
Budgerigar	7	1	-	-	-	-	-
White-eared bulbul	5	3	-	2	1	2	D, B2
Monk parakeet	5	-	-	-	-	-	-
Canary	5	1	-	-	-	-	-
Finch	5	-	-	-	-	-	-
Old World sparrows	2	-	-	-	-	-	-
Conures	1	-	-	-	-	-	-
Grass Parakeets	1	-	-	-	-	-	-
Eclectus parrot	1	-	-	-	-	-	-
Iraq babbler	1	-	-	-	-	-	-
Wrens	1	-	-	-	-	-	-
Common swift	1	-	-	-	-	-	-
Starling	1	-	-	-	-	-	-
Common buzzard	1	-	-	-	-	-	-
Amazon parrot	1	-	-	-	-	-	-
Total	200	26 (13%)	0 (0%)	9 (4.5%)	5 (2.5%)	9 (4.5%)	-

In order to analyze the phylogenetic groups of STEC isolates, Clermont et al.'s upgraded approach (2019) was used (16). The results determined 4 phylogroup B2 (isolated from Cockatiel, Mynah, Rose-ringed parakeet and White-eared bulbul), 3 phylogroup D (isolated from Cockatiel, Mynah and White-eared bulbul) and 2 un-typed isolates. Clermont et al.'s phylogenetic analysis could not determine the phylogroups of these 2 mentioned isolates (isolated from Duck and Cockatiel) (Table 3).

Table 3. The detailed information of STEC isolates found in pet birds, and their assigned phylogroups

Isolate Laboratory Code	Avian Species of Origin	Phylogroup	STX Gene Status
E.118	Cockatiel	D	Stx2, Stx1
E.122	Mynah	D	Stx2, Stx1
E.135	White-eared bulbul	D	Stx2, Stx1
E.156	Duck	-	Stx2
E.158	Cockatiel	-	Stx2, Stx1
E.162	White-eared bulbul	B2	Stx2
E.165	Cockatiel	B2	Stx2, Stx1
E.170	Mynah	B2	Stx2,
E.171	Rose-ringed parakeet	B2	Stx2

Based on the results attained from DD method, 7 out of 9 STEC isolates showed resistance against at least 3 different antimicrobial drug classes and 7 multi-drug resistance (MDR) isolates were detected in this study (77.7%). In the evaluation of STEC isolates, 9 out of 9 (100%) isolates were resistant to penicillin and erythromycin, and sensitive to fosfomycin and lincospectin. In B2 phylogroup, almost all the isolates were sensitive to fluoroquinolones (96.9% sensitive) and only one isolate was resistant to flumequine (3.1% resistance). In D phylogroup, the results were different, the rate of sensitivity was 12.5% and 87.5% were resistant or they were developing resistance (intermediate). The un-typed isolates, one isolate was totally sensitive and the other one was developing resistance against fluoroquinolones.

D phylogroup isolates were 58.4% resistant (or intermediate) to cephems, while in B2 phylogroup, 50% were sensitive and 50 % were resistant (31.25% intermediate and 18.75% resistant). In D phylogroup, isolates were 83.4% resistant and 16.6% sensitive to phenicols, and in B2 phylogroup, 75% were sensitive and 25% were resistant. D phylogroup isolates, were 100% resistant to tetracyclines, and in B2 phylogroup,41.7% were sensitive, 25% were resistant and 33.3% were developing resistance. D phylogroup isolates were 55.5% resistant to aminoglycosides, and B2 phylogroup isolates were 58.3% resistant and 41.7% sensitive. For colistin, in D phylogroup the rate of resistance was 33.3% and the isolates were developing resistance (intermediate), in B2 phylogroup, the isolates were developing resistance in the rate of 25%.

Against nalidixic acid and TS, D phylogroup isolates were all resistant and B2 phylogroup isolates were all sensitive to these two mentioned antimicrobial drugs.

4 Discussion

Companion animals, such as pet birds, often have close contact with humans, creating a great potential for interactions between themselves and humans. Transmission of pathogens, including antimicrobial resistance genes, has always been a notable issue that could have interrelated effects on both humans and animals(6). Bacteria that cause urinary tract infections, sepsis, respiratory infections, and food poisoning have been found in the feces of all birds(18). It has been suggested that food could be the most common route of transmission(19). Animals can serve as a reservoir of pathogens and also, antibiotic-resistant bacteria(20). As a fact, the main habitat of *E. coli* is the intestine of animals and this bacterium could be found in vast amounts in feces of different species (21). STEC strains, as an important zoonosis have been studied multiple times worldwide. In Iran, different studies found different incidence ranges from 1.1% to 7.4% in humans with diarrhea. Rates of incidence varied in different studies due to different age of patients and the size of taken samples (22-26). Alizade et al. in 2014 found 36 STEC isolates out of 117 samples from immunocompromised (HIV or Thalassemia) cases (30.7%) (27) which is far more than other studies, comparing with 34.6% of incidence in pet birds included in our study. These results show the significant incidence of STEC strains, especially in patients with gastroenteritis(28). Recent discoveries in the field of phylogenetic analysis have shown the possibility of transmission between humans and animals. The phylogenetic typing method provided by Clermont et al. (15, 16)was used to identify the phylogroups of STEC isolates. Out of 9 isolates, 4 (44.4%) belonged to the B2 phylogroup, while 3 isolates (33.3%) were classified as D phylogroup. Gioia-Di Chiacchio et al. (29) also detected B2 phylogroup in AEEC isolates from Psittacine birds, they also found phylogroup F and clade I but they did not detect any isolates from D phylogroup. It is worth mentioning that we found 2 STEC isolates, which this typing method was unable to distinguish and classify as certain phylogroups; which illuminates the necessity of further investigations in the field of phylogroup analysis. Understanding the scale of antimicrobial resistance among STEC isolates from companion birds' origin was the main focus of this study. Most of the antimicrobial agents included in this study are commonly used in the treatment of human infections, and high resistant rates could be considered an alarm to pay more attention to this phenomenon. A variety of resistance rates against different antimicrobial agents has been reported worldwide in different time periods (30, 31). Primarily, chloramphenicol and tetracycline, followed by erythromycin, streptomycin, ciprofloxacin, gentamicin, and ampicillin are considered "very important antimicrobials" for use in human medicine by the World Health Organization (WHO). The acquisition of resistance to these antibiotics in the bacteria of the human environment and microflora is a very important issue (32).

In the past years, high levels of resistance against tetracycline, sulfamethoxazole, ampicillin, and streptomycin were found in *E. coli* isolates (33). Zarei et al. (34). examined the prevalence of STEC in 257 samples of raw chicken meat and found high rates of resistance to some antibiotic agents such as nalidixic acid (91.4%), tetracycline (89.8%), ampicillin (82.8%), and sulfamethoxazole-trimethoprim (71%) using the DD method.

The results of our study revealed that7 out of 9 STEC isolates were resistant to at least 3 different classes of antimicrobial drugs and thus, 7 multi-drug resistant isolates were detected and identified in this study (77.7%). This finding is consistent with previous studies conducted by Sigirci et al. (6). In this regard, Sigirci et al. (2020) reported a rate of 67% multi-drug resistance (MDR) in *E. coli* isolates from companion birds. Additionally, Hidasi et al. (35) found a MDR incidence of 33.8% in *E. coli* isolates from parrots seized from the illegal wildlife trade (35). Horn et al. (36) found that 55.7% of members of Enterobacteriaceae family isolated from canaries were MDR (36). Furthermore, Pontes et al. (37) measured the total rate of MDR incidence in *E. coli* and salmonella spp. isolated from cockatiels to be 59%(37). Regarding resistance against penicillin and erythromycin, both of which are common antibiotics for various human infections, 100% of STEC isolates in the study showed resistance, which should be considered an alarm. High rates of resistance have also been reported in other studies in Iran, such as Zarei et al. (2019) from chicken meat *E. coli* isolates (34) and Tavakoli and Pourtaghi (38) from STEC isolates of clinical mastitis in dairy cows (38), but Mohammadi et al. (39) did not detect any MDR isolates from STEC isolated from raw milk (39).

In phylogroup D isolates, all of them (100%) were resistant to nalidixic acid and sulfamethoxazole-trimethoprim, this resistance may be the result of arbitrary usage of antibiotics without proper veterinary supervision.

In the studies of Sigirci et al. in 2019 (40) and 2020 (6), respectively, 38% of cloacal swab isolates of synantropic birds and 46% of companion bird isolates were resistant to sulfamethoxazole-trimethoprim and Pontes et al. (2018) found 41% resistance to nalidixic acid in clinically healthy cockatiels in captivity (37). In the present study, 55.5% of phylogroup D isolates and 58.3% of phylogroup B2 isolates were resistant to aminoglycosides. In the study of Sigirci et al. in 2020, three parakeet isolates sampled from one breeder were resistant to all classes of aminoglycosides, and in general, 34% of isolates were resistant to streptomycin, 25% resistant to kanamycin, and 7% were resistant to gentamicin (6). Also, in the study of Horn et al. (36) and Pontes et al. (37), 40% and 67% of the strains isolated from canaries and cockatiels kept in captivity were resistant to streptomycin, respectively. In our study, the resistance rate of streptomycin was measured to be 66.66% (6 out of 9 isolates).

The isolates of phylogroup B2 were 25% and phylogroup D isolates were 100% resistant to tetracyclines. In this case, confirmative results have been reported in several other studies, such as Pontes et al. (2018) with 41% resistance rate in captive cockatiels (37) and Machado et al. (2018) with 28.6% resistance from free-ranging gray-breasted parrots (41). Collectively, the irresponsible use of antimicrobial compounds in human infections and veterinary purposes, beside the recent increases in food demands -livestock animals as a source of human food supply- from growing populations, and the void of proper laboratory facilities to help in diagnosis and choosing suitable treatments could be responsible for the emergence and spread of resistant and MDR strains, and this can create some risks for human and animals (42).

5 Conclusion

The study revealed that different species of birds commonly kept as pets in Iran can be affected by STEC strains and identified as multi-drug resistant *E. coli*, which doubles the importance of these findings for public health. While there are different studies that can reinforce the attained data in the field of resistance against antimicrobial agents, there are also some studies whose findings seem to be otherwise. However, the facts that STEC isolates are potentially dangerous for both animals and humans and multi-drug resistance is an increasingly risky factor for human health are still clear and undeniable. Actions should be undertaken to fight against it, and the prevalence of STEC isolates and their complementary information (especially the resistance against different antimicrobial agents) in different animals should be investigated more deeply to achieve a better understanding and to control/treat this issue.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contributions

MRP participated in coordinating the study, sampling, data analysis, and drafting the manuscript. MA provided the isolates and collected data, and also contributed to drafting the manuscript. JR & SMP participated in the study design, analysis, and contributed to drafting the manuscript. All authors have read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data are available from the first author upon reasonable request.

Ethical Considerations

All ethical principles were considered in this work according to the principles outlined by the ethical committee of the Faculty of Veterinary Medicine, University of Tehran. Project Number: 7508007-6-42-291227/76.

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