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Prevalence of Shiga Toxin-Producing and Enteropathogenic Escherichia coli in Wild and Pet Birds in Iran

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ABSTRACT

The aim of this study was to investigate the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) strains and to identify the *stx* gene types in wild captive and companion birds. In total,657 *E. coli* isolates from 219 birds belonging to 38 different species were investigated for the presence of STEC and EPEC strains. It was shown that five birds (2.28%) carried strains positive for one or more of the virulence factors investigated. The results indicated that 1.8% (n=4) and 0.45% (n=1) of the birds carried STEC and EPEC strains, respectively. All STEC strains harbored the *stx2f* and *eae* genes and this finding reveals the role of other birds, in addition to pigeons, as reservoirs of STEC. The only EPEC strain in this study was isolated from a Myna. Based on our knowledge, this is the first report of Stx2f-producing STEC in Geese, Duck and Lesser kestrel. In conclusion, the results indicate a low frequency of STEC carriage in wild and companion birds, and point out the need of additionally screening for the presence of *stx2f* in all the *eae*-harboring strains from birds.

INTRODUCTION

Escherichia coli belongs to the intestinal bacterial flora in most animal species. Although most *E. coli* strains are nonpathogenic, some strains may cause diarrhea and other intestinal diseases (Law, 1988). For instance, enteropathogenic *E. coli* (EPEC) have been considered as one of the most important strains that cause diarrhea in humans (Norazah *et al*., 1998). EPEC strains may express the outer membrane protein intimin (94-97 KDa), which is encoded by the *eae* gene and causes the attaching and effacing lesions in the epithelial cells of the intestine and resulting diarrhea in humans (Adu-Bobie *et al*., 1998). Some studies have shown the carriage of EPEC strains in birds (Kobayashi *et al*., 2009; Oh *et al*., 2011).

Shiga toxin-producing *Escherichia coli* strains (STEC) harbor Shiga toxin (*stx*) genes (Kobayashi *et al*., 2002) and are also able to cause diarrhea in humans and some animal species. They are linked to hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in humans, which require hospitalization and intensive care with considerable mortality in children and elderly patients (Gyles, 2007). The ability of STEC strains to cause serious diseases in humans is related to the production of one or more Shiga toxins (Stx1, Stx2, or their variants), which inhibit protein synthesis in host cells leading to cellular damage (O'Brien *et al*., 1992).

While ruminants are the main reservoir of STEC, other domestic animals such as cats, dogs and pigs may also carry STEC and EPEC strains (Beutin *et al*., 1995; Zahraei Salehi *et al*., 2011). Moreover, some studies have also investigated STEC strains in wild birds and poultry in different countries (Kobayashi *et al*., 2002; Schmidt *et al*., 2000;

Morabito *et al*., 2001; Ghanbarpour *et al*., 2011). More recently, a new subtype of *stx*, called *stx2f*, has been described in STEC in pigeons (Schmidt *et al*., 2000). Strains harboring the *stx2f* gene have been considered as emerging pathogens (Prager *et al*., 2009). Various methods have been applied for identification of STEC strains in birds, but most of them were unable to target the *stx2f* subtype (Askari Badouei *et al*., 2014; Ziebell *et al*., 2002; Feng *et al*., 2011).

Due to the wide geographical distribution, migratory habits, and the great diversity of avian species, the role of different bird species in carriage of *eae* and *stx* possessing *Escherichia coli* is poorly understood. Nevertheless, most birds, including pet birds, domestic fowl, and even raptors kept by humans may be potential unnoticed reservoirs of these enteric pathogens. To our knowledge, there are no studies on the prevalence and molecular characteristics of STEC and EPEC strains derived from pet and wild birds in Iran. Therefore, the aim of this study was to assess the role of birds as STEC and EPEC reservoirs in Iran.

MATERIALS AND METHODS

Sample collection and culture

A total number of 219 birds belonging to 38 different species were sampled in pet shops, zoological parks (Saei park) and birds referred to veterinary clinics (Table 1). The samples were collected from fresh droppings, or directly from the cloacae, using sterile swabs (Table 1). The samples were transported in Amies transport media (BBL, USA) to the laboratory and immediately streaked on MacConkey agar (Merck, Germany). After overnight incubation at 37°C, up to four well-separated lactose-fermenting colonies were picked from each plate. The confirmation of the suspected isolates was performed by biochemical tests, including conventional lactose and glucose fermentation (using TSI medium), urease, indol, methyl red, Voges Proskauer, citrate and lysine decarboxylase (Quinn *et al*., 2011).

DNA extraction

Isolates confirmed as *E. coli* were sub-cultured on LB Agar. After an 18-20 hours incubation at 37°C, DNA was extracted of the strains by boiling method, as described previously (Zahraei Salehi *et al*., 2007).

Screening PCRs for *eae* and *stx*

The presence of the *eae* gene was screened using SK1 and SK2 general primers (Table 2; Schmidt *et al*., 1994). The PCR protocol was conducted using 2.5 µL10X PCR buffer, 2mM $MgCl_2$, 0.2mM dNTP, 1 unit *Taq* DNA polymerase enzyme (Cinnagen, Iran),0.4 µM of each primer working stock and 2 µL boiled lysate as template DNA. Molecular grade distilled water was added to make the final volume of 25µL.

In order to detect STEC strains, Lin-F and Lin-R primers (Table 2) that can detect all *stx* subtypes and variants, were used (Ziebell *et al*., 2002; Lin *et al*., 1993). Each PCR reaction included: 2.5 µL10X PCR buffer; 1.6 mM $MgCl_2$; 0.2mM dNTP; 1 unit *Taq* DNA polymerase enzyme; 0.4 µM of each primer; 3 µL DNA; and ultrapure water up to 25 µL (Table 2).

Amplification cycles for both protocols are summarized in Table 2. Positive control (*E. coli* O157:H7 Isolate No. 295) and negative control (sterile water) were included in all PCR reactions. To observe results, the PCR products were visualized on 1.2% agarose gel after electrophoresis and staining with ethidium bromide.

Multiplex-PCR for *stx1*, *stx2*, *eae*, *Ehly*

All *stx* harboring *E. coli* isolates were further screened by a multiplex-PCR using four pairs of specific primers (Table 2) for *stx1, stx2, eae* and *Ehly* as described by Paton and Paton (1998). Amplification was carried out in a total volume of 25µL containing: 2µL DNA; 0.3µM of each oligonucleotide primer; 0.2mM dNTP mix; 2mM $MgCl_2$; 2.5µL of 10X PCR buffer; 1 unit *Taq* DNA polymerase (Cinnagen, Iran); and PCR grade water up to 25µL. Samples were subjected to 35 cycles of touchdown PCR (Table 2) according to Paton and Paton (1998). The PCR products were submitted to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. Positive PCR reactions were recorded by comparing the specific bands with 100bp-plus molecular size marker (Fermentas, Lithuania). Positive controls and negative controls (sterile water) were included in all PCR reactions.

stx2f gene detection

In order to detect *stx2f* gene in *stx* positive strains that yielded negative result in Multiplex-PCR, another PCR was conducted with *stx2fF* and *stx2fR* primers (Table 2) as described previously (Schmidt *et al*., 2000). Each PCR reaction included: 2.5 µL 10X PCR buffer; 1.5mM $MgCl_2$; 0.2mM dNTP; 1 unit *Taq* DNA polymerase; 3 µL DNA; 0.1 µM of each primers; and molecular grade water. The applied thermal cycles are summarized in Table 2. T5b-Ir strain (Accession number KJ397538) was used as positive control.

Table 1– Fecal samples obtained from various birds in Iran assessed for the presence of *Escherichia coli* harboring *eae* and *stx* genes.

Bird (Common Name)	Bird (Scientific name)	No. of samples tested	No. of *eae*-positive isolates	No. of *stx*-positive isolates
Sulphur-crested Cockatoo	*Cacatua galerita*	2		
Green-winged Macaw	*Ara chloropterus*	1		
Lesser Kestrel	*Falco naumanni*	7	1	1
Alexandrian Parrot	*Psittacula eupatria*	1		
Eurasian Eagle-Owl	*Bubo bubo*	1		
Fischer's Lovebird	*Agapornis fischeri*	3		
Chukar Partridge	*Alectoris chukar*	5		
African Grey Parrot (AGP)	*Psittacus erithacus*	18		
Pet Chicken	*Gallus gallusdomesticus*	8		
Common Buzzard	*Buteo buteo*	1		
Common Myna or Indian Myna	*Acridotheres tristis*	34	1	
White-eared Bulbul	*Pycnonotusleucotis*	2		
Domestic Canary	*Serinuscanariadomestica*	2		
Common Magpie	*Pica pica*	2		
Budgerigar	*Melopsittacus undulatus*	3		
Blue and Yellow (Gold) Macaw	*Ara ararauna*	1		
Eastern Rosella	*Platycercus eximius*	1		
Cockatiel	*Nymphicus hollandicus*	1		
Domestic Duck	*Anas platyrhynchos domesticus*	30	1	1
Domestic Pigeon	*Columba liviadomestica*	6		
Hooded Crow	*Corvus cornix*	8		
Saker Falcon	*Falco cherrug*	1		
Steppe Eagle	*Aquila nipalensis*	2		
Eurasian Sparrowhawk	*Accipiter nisus*	2		
Eurasian Woodcock	*Scolopax rusticola*	1		
Caspian Gull	*Larus cachinnanas*	1		
Orange-winged Amazon	*Amazona amazonica*	1		
Scaly-breasted Lorikeet	*Trichoglossus chlor lepidotus*	1		
Helmeted Guinea Fowl	*Numida meleagris*	2		
Muscovy Duck	*Cairina moschata*	5		
Common Pheasant	*Phasianus colchicus*	7		
Black Swan	*Cygnus atratus*	2		
Blue Peafowl	*Pavo cristatus*	4		
Japanese Quail	*Coturnix japonica*	1		
Ring-necked Parakeet	*Psittacula krameri*	31		
Domestic goose	*Anser anser domesticus*	21	2	2
Total		219	5	4

RESULTS

Among the 657 *E. coli* isolates investigated for the presence of the *eae* gene, five isolates, which were originated from five different birds belonging to four different species, resulted positive (Figure 1; Table 1). In screening PCRs for *stx*, four birds belonging to three different species carried STEC strains. The evaluation of the STEC isolates using a multiplex PCR for *stx1*, *stx2, eae, Ehly* only yielded the *eae* amplicon, but not *stx1* and/or *stx2*. All of these strains were shown to be positive for *stx2f* as demonstrated using the specific primers (Figure 1). In fact, except for one isolate, all *eae*-harboring isolates were STEC and carried *stx2f* gene. In total, five birds (2.28%) carried strains positive for one or more of the virulence factors tested. Four *E. coli* strains were isolated from four birds belonging to three different species including (goose, duck and lesser kestrel) harbored both *stx2f* and *eae* genes, while one isolate obtained from a Myna harbored only the *eae* (Figure 1; Table 1).

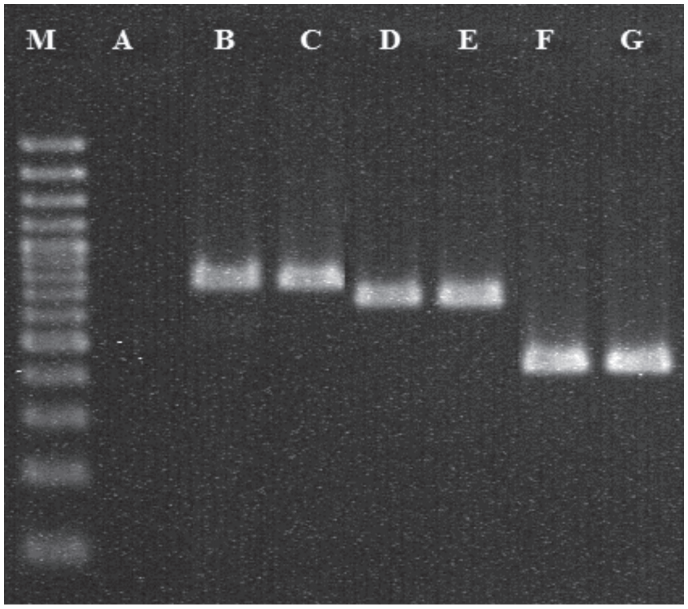

Figure1 – Different PCR assays for the detection of *eae*, *stx* and *stx2f* genes. **M**) Marker 100bp. **A**) Negative control. **B**) Positive control for *stx* gene (900bp) (*E. coli* O157:H7, Isolate No. 295).**C**) One of *stx* positive strains isolated in this study. **D**) Positive control for *eae* gene (863bp) (*E. coli* O157:H7, Isolate No. 295). **E**) One of the *eae* positive strains isolated in this study. **F**) Positive control for *stx2f* gene (428bp) (T5b-Ir strain, accession number KJ397538).**G**) One of *stx2f*-positive strains isolated in this study.

DISCUSSION

The result of the current study showed a low prevalence of STEC in wild and pet birds in Iran. The prevalence of STEC has been investigated in different bird species in other geographical regions. Farooq *et al*. (2009) found 5% and 1% of *E. coli* strains positive for *stx1* and *stx2* in pigeons, respectively. In broilers, the *stx2* gene was detected in 4.5% of the isolates in Iran (Ghanbarpour *et al*., 2011). On the other hand, some studies found no Shiga toxin genes in *E.coli* strains from poultry (Wani *et al*., 2004; Farooq *et al*., 2009). Similarly, *stx1* or *stx2* genes were not detected in *E. coli* from wild birds (Kobayashi *et al*., 2009), which is in agreement with the findings of the present study. As reported previously (Zeibell *et al*., 2002), the multiplex-PCR was not able to identify *stx2f* subtype in the mentioned study.

In our study, the combination of *stx2f* and *eae* genes were detected in *E. coli* strains isolated from four birds (1.8%) belonging to three different species. In general, pigeons are known as natural reservoirs of *stx2f*-harboring STEC strains (Kobayashi *et al*., 2002; Schmidt *et al*., 2000; Kobayashi *et al*., 2009; Askari

Table 2 – PCR primers and conditions for the amplification of *stx* and *eae* genes in this study.

Name	Primer Sequence(5'to 3')	Target Gene	Amplification condition	Amplicon Size (bp)	reference
SK1 SK2	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	*eae*	94°C 30s;52°C 60s; 72°C 60s (30 cycles)	863	Schmidt *et al*. (1994)
Lin-F Lin-R	GAACGAAATAATTTATATGT TTTGATTGTTACAGTCAT	*stx*	94°C 30s;45°C 60s; 72°C 60s (33 cycles)	900	Lin *et al*. (1993)
Stx1-F Stx1-R	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	*stx1*		180	
Stx2-F Stx2-R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	*stx2*	95°C 60s;65°C 120s; 72°C 60s (first 10 cycles) decreasing to 60°C (cycles 10-15) 95°C 60s;60°C 120s; 72°C 90s (cycles15-25) 95 °C 60s;60°C 120s; 72°C 150s (cycles25-35)	255	Paton & Paton (1998)
Eae-F Eae-R	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	*eae*		384	
Hly-F Hly-R	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	*Ehly*		534	
Stx2f-F Stx2f-R	AGATTGGGCGTCATTCACTGGTTG TACTTTAATGGCCGCCCTGTCTCC	*stx2f*	94°C 30s;57°C 60s; 72°C 60s (30 cycles)	428	Schmidt *et al*. (2000)

Badouei *et al*., 2014). The prevalence of *stx2f*+ strains reported in pigeons ranged from 4% to18.8% in different studies (Askari Badouei *et al*.,2014; Schmidt *et al*., 2000; Farooq *et al*.,2009). Additionally, Wen-Jie *et al*. (2008) study showed the presence of *stx2f* gene in avian pathogenic *E. coli* (APEC) strains in China. Similar to our observation, previous studies showed that *stx2f*-harboring strains lack other *stx* subtypes and mostly possess the *eae* gene (Askari Badouei *et al*., 2014; Schmidt *et al*., 2000; Morabito *et al*., 2001). The strains possessing the *stx2f/eae* genes in this study isolated from a duck, two geese and a lesser kestrel. Previously, *eae+/stx2f+ E. coli* strains were detected in barn swallows in Japan (Kobayashi *et al*., 2009). However, the low prevalence of *stx2f*-harboring STEC in the current and previous studies suggests that these strains are only part of the transient gut microflora. In this sense, wild and pet birds may have a minor epidemiologic role in comparison with Columbiformes as carriers of *stx2f+/eae+ E. coli*.

In the present study, only one EPEC strain was identified. Farooq *et al*. (2009) concluded that all of the ducks and chickens sampled in their study were reservoirs of EPEC strains, while in another study only 8.7% of the birds harbored EPEC strains (Kobayashi *et al*., 2009).

According to the results of the present study, wild and pet birds may carry STEC and EPEC strains. Although all STEC strains in this study only possessed the *stx2f* subtype, the public health significance of these strains should not be overlooked, because the *stx2f+ E. coli* strains have also been isolated from humans with diarrhea (Prager *et al*., 2009; Isobe *et al*., 2004). Recent evidences also show the particular importance of *stx2f*-STEC as an emerging unnoticed human pathogen (Friesema *et al.,* 2014). Since the *stx2f* is not easily identified using most routine diagnostic procedures (except using appropriate general primers), all of the *eae*-harboring strains from birds should be checked for the presence of this particular Shiga toxin subtype. Additionally, the role of pet birds in epidemiology of STEC infection should not be underestimated.

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