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

**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; Ghanarbapour 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., 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 mix; 2mM MgCl₂; 2.5μL of 10X PCR buffer; 0.3μM of each oligonucleotide primer; 0.2mM dNTP out in a total volume of 25μL containing: 2μL DNA; 1.5mM MgCl₂; 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., 2001; Lin et al., 1993). Each PCR reaction included: 2.5 μL10X PCR buffer; 1.6 mM MgCl₂; 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* 0157: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. 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 MgCl₂; 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.
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).

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 galusdomesticus        | 8                     |                              |                              |
| Common Buzzard          | Buteo buteo                   | 1                     |                              |                              |
| Common Myna or Indian Myna| Acridotheres tristis          | 34                    | 1                            |                              |
| White-eared Bulbul      | Pycnonotus leucotis           | 2                     |                              |                              |
| Domestic Canary         | Serinucanariadomesticus       | 2                     |                              |                              |
| Common Magpie           | Pica pica                     | 2                     |                              |                              |
| Budgerigar              | Melopsittacus undulatus       | 3                     |                              |                              |
| Blue and Yellow (Gold) Macaw | Ara ararauna                | 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 cachinnanass            | 1                     |                              |                              |
| Orange-winged Amazon    | Amazona amazonica             | 1                     |                              |                              |
| Scaly-breasted Lorikeet | Trichoglossus chlor lepidotus | 1                     |                              |                              |
| Helmeted Guinea Fowl    | Numida meleagris              | 2                     |                              |                              |
| Muscovy Duck            | Carina 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                            |

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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 et al., 2011).

**DISCUSSION**

**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 | Ampliicon Size (bp): | reference |
|------|-----------------------------|-------------|-------------------------|----------------------|-----------|
| SK1  | CCCGAATTCGGCACAAGCATAAGC   | eae         | 94°C 30s;52°C 60s; 72°C 60s (30 cycles): 863 | Schmidt et al. (1994) |
| SK2  | CCCGGATCCTCCTCACGATTTGCG   | eae         | 94°C 30s;45°C 60s; 72°C 60s (33 cycles): 900 | Lin et al. (1993)    |
| Lin-F| GAACGAAATAATTTATATGT       | stx         | 94°C 30s;52°C 60s; 72°C 60s (30 cycles): 225 |                       |
| Lin-R| TTTGATTGTTACAGTCAT          | stx         | 94°C 30s;52°C 60s; 72°C 60s (30 cycles): 384 |                       |
| stx1-F | AAAAAATCCGCACTCCTGAGTACTAC | stx1        | 95°C 60s;65°C 60s; 72°C 60s (first 10 cycles): 95°C 60s;60°C 60s; 72°C 90s (cycles15-25): 255 | Paton & Paton (1998) |
| stx1-R | AGAAACCCGACTGAGTACATC       | stx2        | 95°C 60s;60°C 60s; 72°C 60s (first 10 cycles): 95°C 60s;60°C 60s; 72°C 90s (cycles15-25): 384 |                       |
| stx2-F | GGCACTGTCTGAAAACGTGCTCC     | stx2        | 95°C 60s;60°C 60s; 72°C 60s (first 10 cycles): 95°C 60s;60°C 60s; 72°C 90s (cycles15-25): 255 | Paton & Paton (1998) |
| stx2-R | TGCCCATATGATTGACGATTG       | stx2        | 95°C 60s;60°C 60s; 72°C 60s (first 10 cycles): 95°C 60s;60°C 60s; 72°C 90s (cycles15-25): 384 |                       |
| Eae-F | GACCCCGGCAACAAGCATAAGC      | eae         | 95°C 60s;65°C 60s; 72°C 60s (first 10 cycles): 95°C 60s;60°C 60s; 72°C 90s (cycles15-25): 255 | Paton & Paton (1998) |
| Eae-R | CCACCTGCAAGCAACAAGAGG       | eae         | 95°C 60s;65°C 60s; 72°C 60s (first 10 cycles): 95°C 60s;60°C 60s; 72°C 90s (cycles15-25): 384 |                       |
| Hly-F | GCATCATCAAGCTACGTCC         | Ehly        | 94°C 30s;57°C 60s; 72°C 60s (30 cycles): 428 | Schmidt et al. (2000) |
| Hly-R | ATGACATCAAGCTACGTCC         | Ehly        | 94°C 30s;57°C 60s; 72°C 60s (30 cycles): 428 | Schmidt et al. (2000) |

**Figure 1** – 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) (T3b-Ir strain, accession number KJ397538). G) One of stx2f-positive strains isolated in this study.
Badouei et al., 2014). The prevalence of stx2f+ strains reported in pigeons ranged from 4% to 18.8% 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 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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