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# Wild birds and urban pigeons as reservoirs for diarrheagenic *Escherichia coli* with zoonotic potential[§]

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**In order to describe the role of wild birds and pigeons in the transmission of shiga toxigenic *Escherichia coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) to humans and other animals, samples were collected from cloacae and oropharynx of free-living wild birds and free-living pigeons. Two STEC (0.8%) and five EPEC strains (2.0%) were isolated from wild birds and four EPEC strains (2.0%) were recovered from pigeons. Serogroups, sequence types (STs) and virulence genes, such as *saa*, *iha*, *lpfA*$_{O113}$, *ehx*A, *esp*A, *nle*B and *nle*E, detected in this study had already been implicated in human and animal diseases. Multidrug resistance (MDR) was found in 25.0% of the pigeon strains and in 57.0% of the wild bird strains; the wild birds also yielded one isolate carrying extended-spectrum β-lactamases (ESBLs) gene *bla*$_{CTX-M-8}$. The high variability shown by PFGE demonstrates that there are no prevalent *E. coli* clones from these avian hosts. Wild birds and pigeons could act as carriers of multidrug-resistant STEC and EPEC and therefore may constitute a considerable hazard to human and animal health by transmission of these strains to the environment.**

***Keywords***: EPEC, antibiotic resistance, STEC, virulence genes, zoonotic pathogens

## Introduction

Enteropathogenic *Escherichia coli* (EPEC) and Shiga toxin-producing *Escherichia coli* (STEC) are foodborne zoonotic pathogens that belong to the category of diarrheagenic *E. coli* that can cause disease in humans. EPEC is responsible for causing severe diarrhea and is characterized by its ability to generate attaching and effacing (A/E) lesions in the intestinal epithelium; the generation of these lesions has been shown to be mediated by the *eae* gene (Kaper *et al.*, 2004). Some EPEC strains contain an EPEC adherence factor (EAF) plasmid and are referred to as "typical EPEC" (tEPEC) strains, whereas EPEC strains lacking this EAF plasmid are referred to as "atypical EPEC" (aEPEC) strains (Trabulsi *et al.*, 2002).

STEC strains are mostly responsible for potentially life-threatening diarrhea with complications such as hemorrhagic colitis (HC) and diarrhea-associated hemolytic-uremic syndrome (HUS). STEC strains produce specific cytotoxins (Shiga toxins) such as Stx1 and Stx2, and some strains can also cause attaching and effacing (A/E) lesions (Kaper *et al.*, 2004). Other putative virulence factors are usually present in pathogenic *E. coli* strains. Some of them, such as the EhxA enterohemolysin, and putative adhesins encoded outside of the enterocyte effacement (LEE) locus, such as lpfA, *nle*B, and *nle*E, have been found in association with severe clinical disease in humans (Boerlin *et al.*, 1999; Afset *et al.*, 2006; Coombes *et al.*, 2008).

Studies reported that the intestinal microbiota of wild birds and urban pigeons includes STEC and EPEC, as well as multidrug-resistant (MDR) strains (Silva *et al.*, 2009; Askari Badouei *et al.*, 2014; Chandran and Mazumder, 2014; Dey *et al.*, 2014; Gargiulo *et al.*, 2014; Chiacchio *et al.*, 2016). Additionally, putative adhesins were detected in STEC and EPEC strains from humans and animals worldwide, including wild birds (Afset *et al.*, 2006; Borges *et al.*, 2012; Cooley *et al.*, 2013; Beraldo *et al.*, 2014; Miko *et al.*, 2014; Akiyama *et al.*, 2016). However, there are no reports of EPEC and STEC strains carrying putative adhesins in pigeons and wild birds from Brazil.

Due to the increased interaction of urban pigeons and wild birds with humans and other animals and the fact that these birds can carry pathogenic microorganisms that pose a risk to public health (Haag-Wackernagel and Moch, 2004; Blyton *et al.*, 2015) we conducted this study to detect and characterize STEC and EPEC strains to determine if those birds can indeed serve as reservoir for diarrheagenic *E. coli* in Brazil.

## Materials and Methods

### Ethics statement

The study is in accordance with the Ethical Principles in Animal Experimentation, adopted by the Brazilian College of Experimentation (COBEA) and was approved by Animal Experimentation Ethics Committee (CEUA) from São Paulo State University, protocol n0. 22.222/10 on 22 October 2010.

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### Sampling and initial procedures

Samples were collected from 123 free-living wild birds that were treated at the Wildlife Veterinary Hospital in UNESP-FCAV, Brazil, from September 2010 to April 2012 (*n* oropharynx samples = 123; *n* cloaca samples = 123). The birds did not show clinical signs of infectious diseases. They were distributed in 15 orders: Accipitriformes=2; Anseriformes=1; Cariamiformes=3; Cathartiformes=2; Charadriiformes=2; Columbiformes=14; Falconiformes=9; Galliformes=1; Gruiformes=1; Passeriformes=10; Pelecaniformes=1; Piciformes =6; Psittaciformes=60; Strigiformes=9; Tinamiformes=2. Samples from 100 free-living urban pigeons (*Columba livia*) captured at São Paulo State University (UNESP), Brazil, from February to April 2012 were also collected (*n* oropharynx samples=100; *n* cloaca samples=100). All the 446 samples were immediately cultured into tubes containing 5 ml of BHI (brain and heart infusion) broth and transported to the laboratory on ice in thermal boxes. The tubes were incubated aerobically overnight at 37°C, and an aliquot of each culture was stored at -80°C.

### Detection of STEC and EPEC by PCR

DNA extraction of the 446 samples incubated overnight was performed by thermal lysis procedure, according to a protocol of the OIE Reference Laboratory for *Escherichia coli* (EcL – Faculté de Médecine Véterinaire, Université de Montréal) available at www.apzec.ca/en/APZEC/Protocols/pdfs/ECL_PCR_Protocol.pdf with slight modifications, BHI broth was used instead LB (Luria-Bertani) broth. After preparation of the DNA templates, PCR was performed as described previously (Borges *et al*., 2012) using primers listed in the supplementary table. Each DNA was screened for three genes that identify the STEC (*stx*1, *stx*2) and EPEC (*eae*) pathotypes. The primers, annealing temperatures, and controls are described in Supplementary data Table S1. Samples that were positive for at least one of the genes were subcultured onto MacConkey agar plates and incubated at 37°C for 24 h. From each plate, ten randomly typical colonies of *E. coli* were selected; DNA of each colony was extracted and tested by PCR as described above for the detection of *stx*1, *stx*2, and *eae*. Positive colonies for at least one gene were stored at -80°C as pure cultures for subsequent experiments. This methodology is according to the Reference Laboratory for *Escherichia coli* [EcL] Université de Montréal (Borges *et al*., 2012).

### Detection of additional virulence genes

All isolates were tested for nine additional virulence genes by PCR amplification. The primers used are listed in supplementary table. One triplex (*iha*, *toxB*, *saa*) and six monoplex (*bfp*, *ehx*A, *nle*B, *nle*E, *esp*A, and *lpf*$A_{O113}$) PCR procedures were performed. The detection of *bfp* gene differentiate tEPEC (positive for *bfp*) from aEPEC (negative for *bfp*).

### Determination of phylogenetic group by PCR

Strains were assigned to one of the four main phylogenetic groups of *E. coli* (A, B1, B2, or D) by using the triplex PCR targeting *chuA*, *yjaA*, and the DNA fragment TSPE4.C2 (Clermont *et al*., 2000).

### Detection of extended-spectrum β-lactamases (ESBLs) genes and antimicrobial susceptibility test

*E. coli* isolates were screened for ESBL-genes using PCR as described previously for $bla_{CTX\text{-}M}$ genotype groups 1, 2, 8, 9 and 25, $bla_{TEM}$, $bla_{SHV}$ (Dallenne *et al*., 2010). Sequencing was done at the University of California Berkeley DNA Sequencing Facility (Berkeley, USA). The sequences were compared with those deposited in the National Center for Biotechnology Information (NCBI) database by an updated version of the BLAST program. Antimicrobial disk susceptibility tests were performed using the disk diffusion method (CLSI, 2015). The antimicrobials tested were amikacin (30 μg), ampicillin (10 μg), cefotaxime (30μg), cefoxitin (30μg), ceftiofur (30 μg), ertapenem (10μg), imipenem (10μg), fosfomycin (200 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30μg), nitrofurantoin (300 μg), norfloxacin (10 μg), sulfamethoxazole + trimethoprim (25 μg), and tetracycline (30 μg).

### O and H typing

Strains were analyzed for O and H antigens at the *E. coli* Reference Center (The Pennsylvania State University, University Park, USA). The O antigen typing was performed by using antisera produced against serogroups O1–O181 with the exceptions of O14, O31, O47, O72, O93, O94, and O122 since these serogroups have not yet been designated. The H antigen typing was performed by the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of *fliC* gene which encodes flagella.

### Pulsed-field gel electrophoresis (PFGE)

The isolates were subtyped by the standardized rapid PFGE protocol used by laboratories in PulseNet (https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf). The *E. coli* chromosomal DNA was digested with *Xba*I. Electrophoresis conditions consisted of an initial pulse time of 2.2 sec and a final pulse time of 54.2 sec at a gradient of 6 V/cm and an included angle of 120° in a CHEF-DRIII PFGE system (Bio-Rad Laboratories). The gels were electrophoresed for 22 h. The similarities of fragments were compared using a Dice coefficient at 1% tolerance and 0.5% optimization, and Dendrograms were constructed using the UPGMA clustering method using the BioNumerics version 7.1 (Applied Maths).

### Multilocus sequence typing (MLST)

MLST was performed following the Achtmans's scheme (http://mlst.ucc.ie/mlst/dbs/Ecoli). This scheme is based on the sequencing of the PCR amplification products of *adk*, *fum*C, *gyr*B, *icd*, *mdh*, *pur*A, and *rec*A (Supplementary data Table S1). DNA template preparation and PCR were conducted as described in the previous section. Sequencing was performed at the University of California Berkeley DNA Sequencing Facility.

## Results

### Isolates harboring virulence genes

A total of 246 samples from free-living wild birds and 200 samples from free-living urban pigeons were subjected to PCR screening for the detection of *stx*1, *stx*2, and *eae* genes. The number of wild birds samples positive for STEC genes (*stx*1 and/or *stx*2) and EPEC gene (*eae*) were 24 (9.8%) and 31 (12.6%), respectively. From these positive samples two (0.8%) STEC (*stx*2+*eae*-) and five (2.0%) EPEC isolates were obtained from wild birds, being two aEPEC (*eae*+*bfp*-) and three tEPEC (*eae*+*bfp*+). Regarding the urban pigeons, the number of samples positive for STEC genes (*stx*1 and/or *stx*2) and EPEC gene (*eae*) were 1 (0.5%) and 24 (12.0%), respectively. From these positive samples, four (2.0%) aEPEC (*eae*+*bfp*-) isolates were obtained. These isolates were subjected to a new round of PCR to detect additional virulence genes. The *saa* gene was the most prevalent in EPEC isolates (100.0%), followed by *iha* (81.8%), *espA* (45.5%), *lpfA*$_{O113}$ (45.5%), *nleE* (45.5%), and *nleB* (27.3%). The most prevalent genes in STEC isolates were *saa* (100.0%), *lpfA*$_{O113}$ (100.0%), *nleE* (100.0%), *iha* (50.0%), and *ehxA* (50.0%). All STEC and EPEC isolates were negative for *toxB* gene and all STEC were negative for *nle*B and *esp*A. Figure 1 shows the virulence profiles of the tested isolates.

### Phylogenetic typing

Phylogenetic group test showed that one aEPEC and three tEPEC belonged to B2 (44.5%), three aEPEC belonged to A (33.3%) and two aEPEC belonged to B1 (22.2%) groups, while STEC strains belonged to A (1/50.0%) and B1 (1/50.0%) groups. None *E. coli* belonged to D group (Fig. 1).

### Detection of ESBL genes and antimicrobial susceptibility test

The isolates were subjected to PCR to detect ESBL genes. Sequence analysis of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes identified one isolate from wild bird carrying both *bla*$_{CTX-M-8}$ and *bla*$_{TEM-1}$, while none of them presented *bla*$_{SHV}$ gene. Furthermore, all the isolates were tested against 15 antimicrobial agents. Multidrug resistance, which was defined as resistance against three or more classes of antimicrobials, was associated with 25.0% of the *E. coli* isolated from urban pigeons and 57.0% of the *E. coli* isolated from wild birds. The isolates from wild birds were more resistant to ceftiofur (71.4%), nitrofurantoin (57.0%), kanamycin (42.8%), tetracycline (42.8%), ampicillin (28.6%), cefotaxime (14.3%), fosfomycin (14.3%), nalidixic acid (14.3%), norfloxacin (14.3%), and sulfamethoxazole/trimethoprim (14.3%). All isolates from wild birds were susceptible to amikacin, cefoxitin, gentamicin, ertapenem, and imipenem. On the other hand the isolates from urban pigeons were more resistant to ceftiofur (100.0%), cefoxitin (25.0%), kanamycin (25.0%), and nitrofurantoin (25.0%). All isolates from urban pigeons were susceptible to ampicillin, amikacin, cefotaxime, ertapenem, imipenem, fosfomycin, gentamicin, nalidixic acid, norfloxacin, sulfamethoxazole + trimethoprim, and tetracycline. Figure 1 shows the resistant profile of each isolate.

### O and H typing

The only serogroup detected in the *E. coli* isolates from urban pigeon was O184; three strains presented a non-typable O antigen. Regarding the *E. coli* isolates from wild birds the serogroups detected were O6, O48, O110, and O137. Three strains presented a non-typable O antigen (Fig. 1).

### Pulsed-field gel electrophoresis (PFGE)

Genetic diversity was analyzed in the 11 strains of *E. coli* that were isolated from free-living wild birds and urban pigeons. PFGE revealed 11 distinct restriction patterns using a difference of a single band as a basis for discriminating between isolates. All isolates were grouped into unique pulsotypes demonstrating a high degree of heterogeneity among STEC and EPEC examined in the study (Fig. 1).

### Multilocus sequence typing (MLST)

MLST was performed in the 11 *E. coli* and revealed six distinct sequence types (ST). The STs found in pigeons were ST28 (*n*=1), ST40 (*n*=1), and ST1296 (*n*=1) and the STs detected in wild birds were ST28 (*n*=1), ST1423 (*n*=1), ST2101 (*n*=1), and ST2678 (*n*=1). One isolate from pigeons and two isolates from wild birds contained unknown STs (Fig. 1).

## Discussion

In this work it was determined the prevalence of STEC and EPEC strains in wild birds and urban pigeons. The characterization of these strains allowed us to show that these birds can carry human MDR diarrheagenic *E. coli*.

The prevalence of EPEC and STEC in wild birds and urban

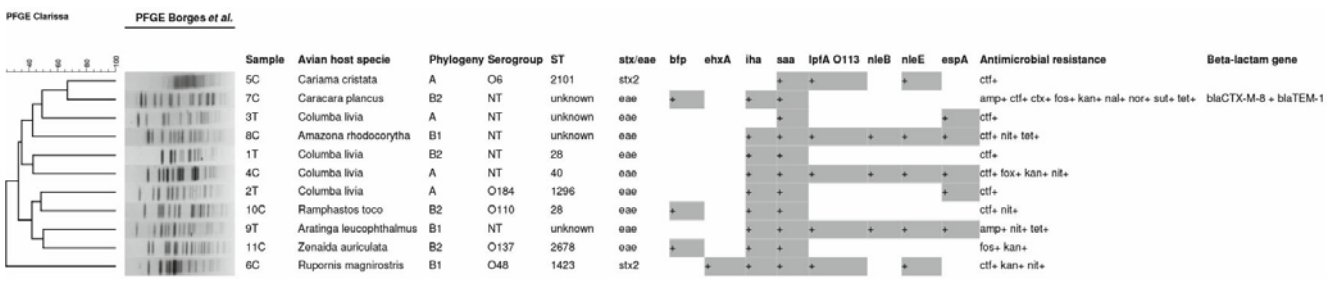


**Fig. 1. Dendrogram showing similarity relationship established by PFGE based in the Dice coefficient and clustering by UPGMA.** C, cloaca; T, oropharynx; NT, nontypeable.

pigeons are similar to the ones reported in other studies conducted in these free-living avian species, where frequencies ranged from 0% to 1.8% for STEC strains (Kobayashi *et al.*, 2002; Wani *et al.*, 2004; Silva *et al.*, 2009; Caballero *et al.*, 2015; Koochakzadeh *et al.*, 2015) and from 2.8% to 4.9% for EPEC strains (Hughes *et al.*, 2009; Silva *et al.*, 2009; Oh *et al.*, 2011; Sacristán *et al.*, 2014).

Genetic analyses showed that the strains in the present study harbored other genes that contribute to virulence, such as *saa*, *iha*, $lpfA_{O113}$, *ehx*A, *esp*A, *nle*B, and *nle*E. These genes had previously been detected in *E. coli* isolated from humans with diarrhea, hemorrhagic colitis, and HUS. Specifically, the *nle*B and *nle*E genes have been statistically associated with EPEC strain that causes diarrhea in humans (Jenkins *et al.*, 2003; Afset *et al.,* 2006; Garrido *et al.*, 2006; Bielaszewska *et al.*, 2011; Coombes *et al.*, 2016). To the best of our knowledge, this is the first report of the presence of the $lpfA_{O113}$, *saa*, *esp*A, *nle*B, and *nle*E genes in STEC and EPEC strains isolated from wild birds and urban pigeons from Brazil.

Phylogenetic typing demonstrated that all tEPEC strains from the present study were classified as B2 and one of them was found to harbor the ESBL gene $bla_{CTX\text{-}M\text{-}8}$ in association with a TEM-1-type penicillinase. It had previously been shown that tEPEC strains might be more virulent than aEPEC strains due to the presence of the EAF plasmid. Since humans are the major natural reservoir for tEPEC (Trabulsi *et al.*, 2002), it suggests that these birds may constitute a reservoir for pathogenic *E. coli* with zoonotic potential.

The fact that MDR strains were found in these birds could be explained by their eating habits, as they can acquire pathogens through food and/or water contaminated with human feces and farm waste. Furthermore, strains of the present study belonged to STs and serogroups that were previously implicated in animal and human diseases (Blanco *et al.*, 2006; Persson *et al.*, 2007; Coque *et al.*, 2008; Dallman *et al.*, 2012; Kang *et al.*, 2014; Maluta *et al.*, 2014; Ferdous *et al.*, 2016), indicating that transmission of *E. coli* between birds and humans might occur and reinforce that wild birds and pigeons may be a reservoir of pathogenic *E. coli* for humans.

PFGE analysis showed that *E. coli* strains isolated from wild birds and pigeons exhibit high heterogeneity. Similar to this result, a number of studies have reported the occurrence of diverse *E. coli* populations in different hosts and environments (Afset *et al.*, 2006; Ibekwe *et al.*, 2011; Kemmett *et al.*, 2013). The high variability shown by PFGE and the difference in serogroups, virulence genes and antimicrobial profiles demonstrate that there are no prevalent STEC and EPEC clones from these avian hosts.

The results presented here suggest that wild birds and pigeons could act as carriers of multidrug-resistant STEC and EPEC and therefore may constitute a considerable hazard to human health by transmission of these strains to the environment. Besides that, the fact that these pathogens were found in bird species living in rural areas indicates that human activities are disturbing natural environments, representing also a risk to animals in the wild.

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