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ORIGINAL ARTICLE

Multidrug-resistant pathogenic *Escherichia coli* isolated from wild birds in a veterinary hospital

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ABSTRACT

Wild birds are carriers of *Escherichia coli*. However, little is known about their role as reservoirs for extra-intestinal pathogenic *E. coli* (ExPEC). In this work we investigated *E. coli* strains carrying virulence genes related to human and animal ExPEC isolated from free-living wild birds treated in a veterinary hospital. Multidrug resistance was found in 47.4% of the strains, but none of them were extended-spectrum beta-lactamase producers. Not only the virulence genes, but also the serogroups (e.g. O1 and O2) detected in the isolates of *E. coli* have already been implicated in human and bird diseases. The sequence types detected were also found in wild, companion and food animals, environmental and human clinical isolates in different countries. Furthermore, from the 19 isolates, 17 (89.5%) showed a degree of pathogenicity on an *in vivo* infection model. The isolates showed high heterogeneity by pulsed-field gel electrophoresis indicating that *E. coli* from these birds are clonally diverse. Overall, the results showed that wild birds can be reservoirs and/or vectors of highly pathogenic and multidrug-resistant *E. coli* that have the potential to cause disease in humans and poultry.

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ExPEC; MLST; multidrug resistance; public health; virulence genes

Introduction

Avian pathogenic *Escherichia coli* (APEC) isolates cause several extra-intestinal infections in birds and are responsible for significant economic losses in the poultry industry worldwide (Barnes *et al.*, 2008). APEC is part of a group of *E. coli* pathotypes known as extra-intestinal pathogenic *E. coli* (ExPEC), which cause infections outside the intestinal tract and can cause diseases in humans and animals, such as urinary tract infections, neonatal meningitis, bloodstream infections and pyometria (Kaper *et al.*, 2004; Maluta *et al.*, 2014a). There are no definitive genetic markers to define an *E. coli* strain as APEC, but Johnson *et al.* (2008) distinguished most APEC from avian faecal *E. coli* by the possession of five genes (*iutA*, *hlyF*, *iss*, *iroN* and *ompT*).

Avian and human pathogenic *E. coli* strains may share common characteristics, such as serogroups, phylogenetic groups, sequence types (ST) and profiles of virulence genes (Moulin-Schouleur *et al.*, 2007; Maluta *et al.*, 2014c), suggesting that a subset of APEC isolates have the potential to be zoonotic pathogens. The increased interaction among wild animals, domestic animals and humans is very important to veterinary and public health because it has been reported that the intestinal microbiota of wild birds may contain multidrug-resistant (MDR) *E. coli* (Guenther *et al.*, 2010; Blyton *et al.*, 2015), including strains carrying genes related to APEC (Ewers *et al.*, 2009). In this context, wild birds may serve as reservoirs and mechanical vectors of antimicrobial-resistant ExPEC and could transmit these infectious agents to humans and commercial poultry (Hubálek, 2004).

Although wild birds have only rare contact with antimicrobial agents, they can be infected or colonized by resistant bacteria through contaminated water or food (Cole *et al.*, 2005). A number of workers have examined *E. coli* associated with human intestinal diseases such as Shiga toxin-producing *E. coli* and enteropathogenic *E. coli* in wild birds (Foster *et al.*, 2006; Ahmed *et al.*, 2007; Farooq *et al.*, 2009). However, only few studies have investigated the presence of *E. coli* harbouring genes related to human and bird ExPEC in these birds (Ewers *et al.*, 2009).

The role of wild birds as reservoirs of APEC may be underestimated. A work presenting a comprehensive characterization of potential APEC isolated from wild birds would be of interest. Therefore, the aim of this work was to characterize potential APEC from wild birds in order to determine if those birds are reservoirs of pathogenic *E. coli* associated with extra-intestinal diseases.

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Supplemental data for this article can be accessed http://dx.doi.org/10.1080/03079457.2016.1209298.

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 No. 22.222/10.

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. These wild birds were taken to the veterinary hospital for many reasons, including found as orphans, brain tumour, bone fractures or heavy metal poisoning. None had any known infectious disease. Faecal and oropharyngeal samples were collected from cloaca ($n = 123$) and oropharynx ($n = 123$), respectively, using sterile swabs, as soon as the birds arrived in the hospital, before they received antibiotics. Liver ($n = 14$) and intestinal ($n = 14$) samples were taken from wild birds that eventually died. All samples were immediately cultured into tubes containing 5 ml of brain and heart infusion (BHI) 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.

Obtaining isolates and determining pathotype using PCR

After the sample incubation in BHI broth (37°C for 24 h under static conditions) DNA extraction was performed by the thermal lysis procedure.[1] After preparation of the DNA templates, polymerase chain reaction (PCR) was performed as described previously (Borges *et al.*, 2012) using primers listed in the supplemental table. Each DNA extract was screened for six virulence-associated genes (VAGs) that had been linked to APEC virulence, *iroN*, *iss*, *iutA*, *ompT*, *hlyF* (Johnson *et al.*, 2008) and *cvaC* (Rodriguez-Siek *et al.*, 2005). Two sets of primer pools were used for multiplex PCR: one set contained primers specific for *iroN*, *ompT* and *iss*, and the other contained primers specific for *iutA, hlyF* and *cvaC*. The primers, annealing temperatures and controls are described in the supplemental table. All PCR reactions were carried out using negative (EcL3463) and positive control strains of *E. coli* (supplemental table). Samples that were positive for at least one of the genes by PCR were cultured onto MacConkey agar plates and incubated at 37°C for 24 h. From each plate, 10 randomly selected colonies typical of *E. coli* were tested by PCR as described above. Colonies positive for at least one gene were stored at −80°C as pure cultures for subsequent experiments. This methodology is from the Reference Laboratory for *Escherichia coli* (EcL) Université de Montréal (Maluta *et al.*, 2014b).

Detection of extended-spectrum beta-lactamases (ESBL) and additional virulence genes

All isolates were tested for ESBL and 14 additional virulence genes by PCR amplification. The primers used are listed in the supplemental table. One triplex and four duplex PCR procedures were performed for bulk genotyping studies of the following genes: (1) *irp2* and *fyuA*; (2) *tsh* and *sitA*; (3) *traT* and *iucC*; (4) *fimH* and *iucD* and (5) *papGI*, *papGII* and *papGIII*. The *cnf1*, *papC*, *sfa* and bla_{CTX-M} genes were detected using monoplex PCR assays.

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).

Pathogenicity test in one-day-old chicks

One-day-old chicks were used to determine the pathogenicity of each isolate as described previously (Guastalli *et al.*, 2013). The chicks were observed daily for 10 days, and the strains were classified according to the following mortality index: high pathogenicity (mortality > 80%), intermediate pathogenicity (mortality > 50% but <80%), low pathogenicity (mortality ≤ 50%), and non-pathogenic (no mortality). *E. coli* isolated from broiler chickens with chronic respiratory disease (CRD) – EC55 (serogroup O1, *iss*+ *papC*+ *iucD*+ *cva/cvi*+) was used as a positive control for virulence. The birds used as a negative control were inoculated with BHI broth.

Serotyping

Strains were serotyped at the *E. coli* Reference Center, Pennsylvania State University, USA. The O antigen typing was performed as described previously (Ørskov & Ørskov, 1984). The H antigen typing was performed by the PCR – Restriction Fragment Length Polymorphism of the *fliC* gene which encodes flagella (Machado *et al.*, 2000).

Antimicrobial susceptibility test

Antimicrobial disc susceptibility tests were performed using the disc diffusion method (Clinical and Laboratory Standards Institute [CLSI] 2014). The antimicrobials tested were amikacin (30 µg), amoxicillin-clavulanic acid (30 µg), ampicillin (10 µg), cefoxitin (30 µg), ceftiofur (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), sulfamethoxazole + trimethoprim (25 µg) and tetracycline (30 µg).

Pulsed-field gel electrophoresis (PFGE)

The isolates were subtyped by the standardized rapid PFGE protocol used by laboratories participating in the PulseNet scheme as described previously (Ribot *et al.*, 2006). The *E. coli* chromosomal DNA was digested with *Xba*I. Electrophoresis conditions consisted of an initial pulse time of 2.2 s and a final pulse time of 54.2 s at a gradient of 6 V/cm and an included angle of 120° in a CHEF-DRIII PFGE system (Bio-Rad Laboratories, Hercules, CA, USA). 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, Austin, TX, USA).

Multilocus sequence typing (MLST)

MLST was performed following the Achtman 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 (supplemental table). DNA template preparation and PCR were done as described in the previous section. Sequencing was performed at the University of California Berkeley DNA Sequencing Facility.

Results

Isolates harbouring ESBL and virulence genes

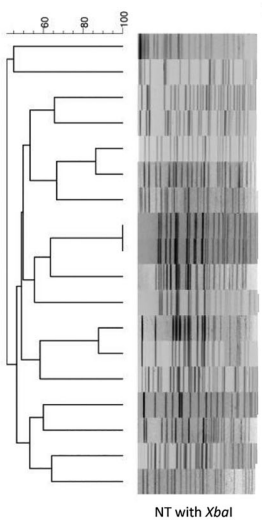
A total of 274 samples from wild birds were tested by PCR for the detection of the APEC VAGs (*iroN*, *iss*, *iutA*, *ompT* and *hlyF*) and an additional gene (*cvaC*); 72 (30.6%) samples were positive for at least one of these six genes (37 from cloaca, 23 from oropharynx, seven from liver and five from intestine). From these 72 positive samples, 19 (6.9%) isolates were obtained (12 from cloaca, four from oropharynx, two from liver and one from intestine). These isolates were subjected to a new round of PCR to detect ESBL and 14 additional virulence genes (Figure 1). Within the isolates, *iss* was the most prevalent gene (94.7%), followed by *sitA* (84.2%), *traT* (63.1%), *ompT* (47.4%), *fyuA* (47.4%), *irp2* (47.4%), *iroN* (42.1%), *hlyF* (42.1%), *fimH* (21.0%), *iutA* (10.5%), *iucC* (10.5%), *iucD* (5.3%), *tsh* (5.3%) and *cvaC* (5.3%). None of the isolates were positive for *papGI*, *papGII*, *papGIII*, *papC*, *cnf1*, *sfa* or bla_{CTX-M} genes.

Determination of the phylogenetic group by PCR

Phylogenetic group testing showed that 8/19 (42.1%) isolates belonged to the B1 group, 8/19 (42.1%) isolates to the B2 group, 2/19 (10.5%) to the D group and 1/19 to the A group (5.2%) groups (Figure 1).

Pathogenicity test in day-old chicks

Pathogenicity testing in one-day-old chicks revealed that six (31.6%), three (15.8%), eight (42.1%) and two



Isolates	Avian host specie	Pathogenicity	Phylogeny	Serotype	ST	Virulence profile
6C	Coscoroba swan	L	B1	ONT:H34	58	*iss+ iroN+ ompT+ hlyF+ sitA+ traT+*
9C	Golden parakeet	H	D	O11:H48	Unknown	*iss+ iroN+ ompT+ iutA+ hlyF+ cvaC+ iucC+ sitA+ traT+ irp2+ fyuA+*
7C	Rufous-bellied Thrush	H	B1	O138:HNT	3310	*iss+ iroN+ ompT+ hlyF+ sitA+ traT+*
8C	American kestrel	NP	A	O106:HNT	5829	*iss+ iroN+ ompT+ hlyF+ sitA+ traT+*
10C	White-eyed parakeet	H	B2	O2:HNT	781	*iss+ sitA+ traT+ irp2+ fyuA+*
10T	White-eyed parakeet	H	B2	O2:HNT	781	*iss+ sitA+ traT+ irp2+ fyuA+*
12C2	Ruddy ground-dove	NP	B1	O106:HNT	Unknown	*iss+ hlyF+*
14F	Crested Caracara	H	B2	O83:H6	2567	*iss+ iucC+ irp2+ fyuA+*
14i	Crested Caracara	I	B2	O83:H6	2567	*iss+ iucC+ irp2+ fyuA+*
15F	White-eyed parakeet	L	B1	O83:H5	Unknown	*iss+ iroN+ ompT+ sitA+ traT+ tsh+*
4C	Crested Caracara	I	B2	O32:H6	Unknown	*iss+ sitA+ irp2+ fyuA+*
3C	Blue-fronted parrot	L	B2	O75:H14	1858	*iss+ sitA+ irp2+ fyuA+*
3T	Blue-fronted parrot	L	B2	O75:H2	1858	*iss+ sitA+ irp2+ fyuA+*
2C	Blue-fronted parrot	I	B1	O79:H14	1287	*iss+ iroN+ ompT+ hlyF+ sitA+*
5T	Crested Caracara	L	B1	ONT:H9	58	*iss+ iroN+ ompT+ iutA+ hlyF+ iucC+ sitA+ traT+ iucD+*
13C	Ruddy ground-dove	L	D	O1:H45	963	*iss+*
1C	Blue-fronted parrot	L	B1	ONT:H14	Unknown	*iss+ iroN+ ompT+ hlyF+ sitA+ traT+*
11T	White-eyed parakeet	H	B2	O2:HNT	Unknown	*iss+ sitA+ traT+ irp2+ fyuA+*
12C1	Ruddy ground-dove	L	B1	O152:H7	156	*ompT+ traT+*

Figure 1. Wild bird paper dendrogram.

Table 1. Antimicrobial resistance phenotypes of *E. coli* isolates from wild birds ($n = 19$).

Isolates	Antimicrobial resistance
1C	NAL + SXT + TET + NIT + GEN + CIP + NOR + FOX + CRO +
2C	NAL + SXT + TET + CHL + CIP + NOR +
3C	No resistance
3T	NAL + TET + GEN + KAN + AMK +
4C	NAL + SXT + TET + AMP + KAN + AMK +
5T	NAL + SXT + TET + NIT + AMP + KAN +
6C	NAL + SXT + TET + GEN + CIP + AMP + NOR + FOX + KAN + AMK + AMC +
7C	NAL + SXT + TET + NIT + CIP +
8C	NAL + CIP + AMP + FOX + AMC +
9C	AMC +
10C	NAL + SXT + TET + NIT + FOX + KAN + CRO +
10T	KAN +
11T	KAN +
12C1	No resistance
12C2	AMK +
13C	TET + AMK +
14F	GEN +
14i	NIT +
15F	NAL + TET + CIP + NOR +

Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; AMK, amikacin; CHL, chloramphenicol; CIP, ciprofloxacin; CTF, ceftiofur; CRO, ceftriaxone; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NIT, nitrofurantoin; NOR, norfloxacin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline.

(10.5%) isolates were classified as highly pathogenic, intermediate pathogenic, low pathogenicity or non-pathogenic, respectively. Of the six highly pathogenic isolates, four belonged to the phylogenetic group B2, one to B1 and one to D. Regarding the intermediate pathogenic isolates, two belonged to B2 and one to B1, while five low pathogenic isolates belonged to B1, two to B2 and one to D. The two non-pathogenic isolates belonged to B1 and A groups (Figure 1).

O and H typing

The serogroups more frequently detected in the *E. coli* isolates were O2 ($n = 3$, 15.8%), O83 ($n = 3$, 15.8%), O75 ($n = 2$, 10.5%) and O106 ($n = 2$, 10.5%). Three strains presented a non-typable O antigen. Serotypes O2:HNT (three isolates), O83:H6 (two isolates) and O106:HNT (two isolates) were detected in more than one strain (Figure 1).

Antimicrobial susceptibility test

All the isolates in this study were tested against 15 antimicrobial agents. Multidrug resistance, which was defined as resistance against three or more classes of antimicrobials, was associated with 47.4% of the *E. coli* isolated from wild birds. Two isolates were susceptible to all antimicrobials. The isolates were more resistant to tetracycline (52.6%), nalidixic acid (52.6%), sulfamethoxazole/trimethoprim (36.8%), kanamycin (36.8%), ciprofloxacin (31.6%), amikacin (26.3%), nitrofurantoin (26.3%), ampicillin (21.0%), cefoxitin (21.0%), gentamicin (21.0%), norfloxacin (21.0%), amoxicillin-clavulanic acid (15.8%), ceftriaxone (10.5%), chloramphenicol (5.2%) and ceftiofur (0%). Table 1 shows the resistant profile of each isolate.

Pulsed-field gel electrophoresis (PFGE)

Genetic diversity was analysed in the 18 strains of *E. coli* that were isolated from wild birds by PFGE (one strain was non-typable with *XbaI*), which revealed 17 distinct restriction patterns. Accordingly, the 18 typable isolates were clustered into 15 groups and demonstrated high heterogeneity. Isolates 14F and 14i shared the same PFGE fingerprint pattern and represented one cluster, whereas two other clusters contained two closely related isolates each. The remaining 12 *E. coli* isolates demonstrated distinct PFGE profiles (Figure 1).

Multilocus sequence typing (MLST)

MLST was performed with 19 *E. coli* isolates and revealed nine distinct STs. Those more frequently found were ST58 ($n = 2$), ST789 ($n = 2$), ST1858 ($n = 2$) and ST2567 ($n = 2$), while ST156, ST963, ST1287, ST3310 and ST5829 were detected in single isolates. Five isolates contained unknown STs.

Discussion

Due to the limited information about the characteristics of ExPEC isolated from wild birds, we performed this study where a number of virulence genes and the patterns of antimicrobial resistance were determined. Wild birds are reservoirs of poultry pathogens, such as avian influenza A (H5N1) and Newcastle disease (Cardenas Garcia *et al.*, 2013; Gerloff *et al.*, 2013). The presence of virulence genes that are reported to be frequent in APEC from commercial poultry and free-ranging frigates (Rodriguez-Siek *et al.*, 2005; Johnson *et al.*, 2006; Zhao *et al.*, 2009; Saviolli *et al.*, 2016) associated with the pathogenicity test suggest that wild birds are a reservoir for *E. coli* that is pathogenic to poultry. Moreover, the VAGs found in this study were also detected in wild mammals and human ExPEC (Frömmel *et al.*, 2013; Maluta *et al.*, 2014c), suggesting that wild birds might be reservoirs for virulence genes that are transmissible to other ExPEC strains.

The majority of *E. coli* isolates of high and intermediate pathogenicity from the present study were found in birds of prey and psittacine species, belonging to the B2 phylogroup, similar to other studies where B2 strains from frigate birds, seagulls and broiler chickens with colisepticemia had the highest pathogenicity score (Simões *et al.*, 2010; Barbieri *et al.*, 2014; Saviolli *et al.*, 2016) agreeing with studies that proposed that B2 is the most predominant and virulent in most cases of ExPEC

infections (Clermont *et al.*, 2000; Smith *et al.*, 2007). Most of the low pathogenic strains belonged to the B1 group, the non-pathogenic strains belonged to A and B1 groups, while the D group contained one highly pathogenic isolate and one of low pathogenicity. Based on these results, we can infer that the ECOR typing system also gives a good correlation between phylogenetic background and virulence for *E. coli* from wild birds.

All B2 phylogenetic group isolates showed pathogenicity in the *in vivo* infection model and belonged to O2:HNT, O83:H6, O32:H6, O75:H2, and O75:H14 serotypes. This fact is of great importance because these serogroups presented zoonotic potential and were implicated in extra-intestinal human diseases such as urinary tract infections, newborn meningitis and septicaemia (Ewers *et al.*, 2007; Maluta *et al.*, 2014c). Besides that, O2 is one of the serogroups most associated with APEC strains worldwide (Moulin-Schouleur *et al.*, 2007) and this serogroup was also found in free-ranging frigates belonging to the B2 phylogroup (Saviolli *et al.*, 2016), suggesting that either wild birds are reservoirs for *E. coli* pathogenic for poultry or that poultry could transmit this pathogen to wild birds, since there is environmental pollution with MDR bacteria from poultry farms (Ferreira *et al.*, 2014).

Almost 50.0% ($n = 9$) of the *E. coli* strains presented MDR, they were obtained from psittacine species ($n = 4$), birds of prey ($n = 3$), passerine species ($n = 1$) and waterfowl ($n = 1$); however studies conducted in Australia and Bangladesh detected only 1.5% and 22.7% of MDR *E. coli* from wild birds, respectively (Hasan *et al.*, 2012; Blyton *et al.*, 2015). Diet seems to be the main factor influencing the exposure of these birds to MDR bacteria, for example birds of prey may eat animals that are in close contact with humans, while the other birds may ingest contaminated food and water in landfills, livestock farms, wastewater treatment facilities or sewage systems (Allen *et al.*, 2010).

Wild birds should have limited contact with antimicrobial agents, but due to the expansion of urban areas and loss of wildlife habitats the amount of contact that wild birds have with contaminated environments increases, favouring the transfer of antimicrobial-resistant bacteria not only to wild birds, but also to other wild animals, since studies in Europe found wild mammals carrying antimicrobial-resistant *E. coli* (Costa *et al.*, 2006; Literak *et al.*, 2010b; Smith *et al.*, 2014).

Although MDR *E. coli* were found in the present study, none of them were ESBL-producers. There are no scientific reports from Brazil in the available literature with which to compare our data; however, the same results were reported in Nigeria where they did not detect ESBL-producing *E. coli* isolates from wild birds and bats (Iroha *et al.*, 2015). These results contrast with studies conducted in Germany, Mongolia, Russia, Poland and the USA that reported ESBL-producing *E. coli* in wild birds (Hernandez *et al.*, 2010; Literak *et al.*, 2010a; Poirel *et al.*, 2012; Guenther *et al.*, 2013).

MDR *E. coli* from the present study belonged to ST58 (phylogroup B1), ST1287 (phylogroup B1), ST1858 (phylogroup B2) and ST789 (phylogroup B2), which have already been found in wild, companion and food animals, environmental and human clinical isolates in different countries (Simões *et al.*, 2010; Dierikx *et al.*, 2012, 2013; Vredenburg *et al.*, 2014; Manges *et al.*, 2015; Hertz *et al.*, 2016).

STs found in *E. coli* that did not show MDR, such as ST963 (phylogroup D), ST156 (phylogroup B1) and ST2567 (phylogroup B2), were also detected in *E. coli* from wild birds in North America and Europe, in broiler chickens from Brazil and in human clinical samples in Korea (Poirel *et al.*, 2012; Maluta *et al.*, 2014c; Vredenburg *et al.*, 2014; Alcalá *et al.*, 2015; Yun *et al.*, 2015). The present results highlight the difficulty in determining the human or animal origin of the *E. coli* strains infecting wild birds.

The PFGE results and profiles of virulence genes demonstrated high heterogeneity among the isolates, indicating the lack of a predominant clone. Some isolates recovered from the same bird (10C/10 T, 3C/3 T and14F/14i) but at different body sites (cloaca and oropharynx or liver and intestine) showed more than 80% similarity and had the same virulence profile. This correlation indicates that *E. coli* may have migrated from the intestine into other body sites, supporting the idea that the avian gut may be a potential ExPEC reservoir.

In conclusion, our data show that wild birds can be a reservoir for MDR ExPEC that are potentially pathogenic for humans and poultry. This is even more important considering the fact that these birds can shed the bacteria with the faeces intermittently for months or years, thus transmitting this pathogen within the environment and posing a risk to human and bird health.

Note

1. See: http://www.apzec.ca/en/APZEC/Protocols/pdfs/ECL_PCR_Protocol.pdf (accessed 9 June 2015).

Disclosure statement

No potential conflict of interest was reported by the authors.

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