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Short communication

Shiga toxin-producing *Escherichia coli* (STEC): Zoonotic risks associated with psittacine pet birds in home environments

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ABSTRACT

Psittacidae are frequentely bred as pets worldwide, but little is known about the zoonotic risks of these animals. The objective of this study was to investigate the presence of Shiga toxin-producing *Escherichia coli* (STEC) in the feces of psittacine birds housed as pets. A total of 171 fecal samples (67 cockatiels, 59 budgerigars, and 45 agapornis) were cultured. Forty-two (*E. coli*) strains were identified, and the presence of the *eae*,*stx*1, and *stx*2 genes was determined using PCR. The antimicrobial resistance profiles of the STEC strains were determined using the disk diffusion method and phylogenetic analysis according to the new Clermont phylotyping method. Using these methods, 19.4% (8/42) of the STEC strains were determined to be positive for the *eae* and *stx*2 genes. The results revealed a STEC frequency of 4.6% in the birds (8/171), with a percentage of 8.47% in budgerigars (5/59), 4.47% in cockatiels (3/67), and 0% in agapornis (0/45). None of the STEC isolates belonged to the O157 serogroup. Most of the strains were classified as sensitive to the 18 antibiotics tested. None of the strains exhibited a multiresistance profile. In the phylogenetic analysis, two strains were classified as non-typeable, three were classified as B2, two were classified as F, and one was classified as Clade I. Seven of the eight STEC strains showed a clonal profile using AFLP. *E. coli* strains that are *stx*2$^+$ plus *eae*$^+$ are usually associated with severe human diseases such as hemorrhagic colitis and hemolytic-uremic syndrome. The STEC-positive results indicate the zoonotic risk of breeding psittacidae in home environments.

1. Introduction

Psittacidae are frequentely bread as pet worldwide, but little is know about the zoonotic risks of these animals (Mattes et al., 2005). Cockatiels (*Nymphicus hollandicus*), agapornis (*Agapornis* spp.) and budgerigars (*Melopsittacus undulatus*) are exotic species that are kept as pets in Brazil because they are docile companions and have colorful, exuberant plumage. These birds are frequently bred in urban areas, and the proximity to human beings and other domestic animals can facilitate environmental contamination and infection by Shiga toxin-producing *E. coli* (STEC) (Nielsen et al., 2004).

Infection with STEC can cause mild to bloody diarrhea (Clements et al., 2012). The first step of colonization is mediated by fimbriae, with some strains causing lesions in the intestinal epithelium through the intimate bacterial attachment to enterocyte microvilli. The microvilli lesions are classified as attaching–effacing (A/E lesion) and result from the expression of intimin, an external membrane protein encoded by the *eae* gene, which is present in a pathogenicity island named locus LEE (Clements et al., 2012). The primary STEC virulence factors are encoded by the bacteriophage genes *stx*1 and *stx*2 and are involved in the production of Shiga toxin. The *stx*2 seems to be more toxic than *stx*1, and is often associated with hemorrhagic colitis and hemolytic uremic syndrome (Chandran and Mazumder, 2014).

Shiga toxin-producing *E. coli* (STEC) has a well-defined zoonotic origin (Croxen et al., 2013). Cattle and poultry are its main reservoir of virulent strains, particularly EHEC O157:H7 (Caprioli et al., 2005; Foster et al., 2006). Knowledge about the routes of transmission has increased over the last 20 years and the origin of human infections occurs mainly through the ingestion of contaminated food. Currently, infection by STEC is not only a food-borne-disease; the agent can also be transmitted by contact with infected companion animals, such as dogs, cats and birds (Persad and Lejeune, 2014). Due to the zoonotic potential of these animals and the lack of reports regarding the prevalence of STEC in captive

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birds, this study investigated the presence of STEC strains in small psittacine birds kept as pets in domestic environments.

2. Materials and methods

2.1. Bacterial strains

This project was approved by the Ethics Committee of São Paulo University and authorized for scientific purposes (CEUA7423290414/2014). The study was conducted with fecal samples from 171 psittacine birds, housed as pet in São Paulo, Brazil: 67 cockatiels (*N. hollandicus*), 59 budgerigars (*M. undulatus*) and 45 agapornis (*Agapornis* spp.). All of the birds were considered clinically healthy upon physical examination at the time of collection, and the age and sex of the birds varied. The swabs were collected from cloacae and transported to the laboratory under refrigeration. Standard bacteriological methods were employed for *E. coli* isolation and identification. The swabs were cultured in brain–heart infusion broth—BHI (Difco—BBL, Detroit, MI, USA) and incubated at 37° C for 24 h. After enrichment, the samples were plated on MacConkey agar and incubated at 37° C for 24 h. Conventional biochemical tests (EPM, Mili and Citrate of Simmons-Probac®) were used to identify the bacterial genera. All of the isolates were stored in Luria Bertani broth containing 15% glycerol at −70° C.

2.2. Polymerase chain reaction (PCR)

The PCR was performed in 25 μL reaction mixtures containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide, 10 pmol of each oligonucleotide primer, 0.5 U of Taq DNA polymerase, autoclaved ultra pure water, and 3 μL of DNA template, as described by Yu and Kaper (1992) and Pal et al. (1999). The primers used in the amplification of the EPEC and STEC virulence factor genes, the amplicon size and references are described in Table 1. The amplified products were separated with Syber Safe® (Invitrogen Corporation, Carlsbad, CA, USA). A 100 bp DNA ladder (LGC Biotecnologia, São Paulo, Brazil) was used as a molecular size marker.

2.3. Hemolysin production and O157:H7 agglutination

The *stx*+ strains were grown in 3 mL of Tryptic Soy Broth (Difco—BBL, Detroit, MI, USA) at 37 °C for 18 h. Two microliters of each inoculum was seeded on blood agar plates containing 5% defibrinated sheep erythrocytes and supplemented with 10 mM calcium chloride. The plates were washed three times with PBS (pH 7.4) and incubated at 37 °C. The results were determined by visual inspection of the lysis zones around areas of bacterial growth after 3, 18, 24 and 48 h. of incubation (Beutin et al., 1989). One hemolytic UPEC, O6 serogroup, was used as a positive control.

The STEC strains were submitted to the Reference Laboratory of the Oswaldo Cruz Institute, Rio de Janeiro, Brazil for agglutination tests with O157 and H7 antisera.

2.4. Antibiotic resistance

The bacterial resistance profile was determined using the agar diffusion technique with disks impregnated with antibiotics, following the protocol of the Clinical Laboratory Standard Institute (CLSI, 2008; CLSI, 2013). Ampicillin, chloranfenicol, amikacin, ceftriaxone, gentamicin, nitrofurantoin, cefepime, ceftiofur, cefoxitin, cefotaxime, polymyxin B, tetracycline, florfenicol, sulfamethazine, sulfamethoxazole–trimethoprim, fosfomycin, enrofloxacin, marbofloxacin, ciprofloxacin, nalidixic acid, streptomycin, neomycin and tobramycin were tested. A strain was considered multidrug resistant (MDR) when demonstrating resistance to three or more classes of antibiotic (Schwarz et al., 2010).

2.5. Phylogenetic analysis

The strains were assigned to phylogenetic groups using the Clermont method (2013). This method assigns strains to one of the seven phylogenetic groups (A, B1, B2, C, D, E and F) based on the presence of three genes (*arp*A, *chu*A and *yja*A) and a specific DNA sequence (TSPE4.C2). The amplicon sizes and references are described in Table 1.

2.6. Single-enzyme amplified fragment length polymorphism (SE-AFLP)

Restriction endonuclease digestion and ligation was performed as described by McLauchlin et al. (2000). Electrophoresis was performed on a 1.5% agarose gel at 22 V for 24 h. The amplified products were stained with GelRed™ (Uniscience) and compared to a 100 bp DNA ladder (Invitrogen, Inc.). The levels of relatedness of the isolates were determined by through the comprehensive pairwise comparison of restriction fragment sizes using the Dice coefficient. The mean values obtained from the Dice coefficients were employed in UPGMA, using Bionumerics 7.5 software (Applied Maths NV, Saint-Martens-Latem, Belgium) to generate the dendogram.

3. Results

The bacteriological results indicated that 74/171 (45%) of the birds were colonized by Gram negative Enterobacteria. *E. coli* was isolated from 42 of the 171 psittacine birds. A total of 22 *E. coli* strains were isolated from cockatiels (22/67), 10 *E. coli* were isolated from agapornis (10/45) and 10 *E. coli* were isolated from budgerigars (10/59).

The PCR results classified 19.4% (8/42) of the strains as STEC (positive for the *eae* and *stx*2 genes), with three of the strains being isolated from cockatiels and five isolated from budgerigars. None of the strains were positive for the O157:H7 serotype, and no hemolytic activity was observed. Most of the strains were classified as sensitive to the 18 tested antibiotics, which belonged to eight different classes. Only one (1/3) sulfamethoxazole–trimethoprim-resistant strain was isolated from the cockatiels. In the budgerigar, one strain was resistant to sulfamethazine (1/5) and one strain was resistant to streptomycin (1/5). None of the strains exhibited a multiresistance profile.

The Clermont phylogenetic analysis of the eight STEC classified three strains as B2, two as F, and one as Clade I. Two strains were classified as non-typeable. The AFLP results are shown in Fig. 1. Seven of the strains (3 from cockatiels and 4 from budgerigars) were clonal, presenting 90–100% similarity. Only one strain isolated from a budgerigar showed a different band pattern and was classified in a single cluster, with similarity below 70%.

4. Discussion

E. coli are part of the microbiota of mammals and some bird species; however, the presence of this microbe in the intestinal tract of psittacines is considered a sanitary risk and is mainly associated with sepsis and the death of birds (Saidenberg et al., 2012). Strains producing Shiga toxin (STEC), a potent cytotoxin that inhibits protein synthesis in eukaryotic cells, can also be considered a risk to public health (Croxen et al., 2013).