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Molecular diagnosis of diarrheagenic *Escherichia coli* isolated from Psittaciformes of illegal wildlife trade[1]

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ABSTRACT.- Lopes E.S., Maciel W.C., Medeiros P.H.Q.S., Bona M.D., Bindá A.H., Lima S.V.G., Gaio F.C. & Teixeira R.S.C. 2018. **Molecular diagnosis of diarrheagenic *Escherichia coli* isolated from Psittaciformes of illegal wildlife trade.** *Pesquisa Veterinária Brasileira 38(4):762-766.* Laboratório de Estudos Ornitológicos, Faculdade de Veterinária, Universidade Estadual do Ceará, Campus do Itaperi, Av. Silas Munguba 1700, Fortaleza, CE 60714-903, Brazil. E-mail: elisangeladesouzalopes@hotmail.com

Diarrheagenic *Escherichia coli* (DEC) are considered one of the major causes of human diarrhea in developing countries. Some studies have pointed wild birds as important reservoirs for these pathogens. However, scarce species from the Psittaciformes order have been investigated. This study aimed to evaluate the presence of DEC strains in Psittaciformes from illegal wildlife trade. A total of 78 *E. coli* strains isolated from cloacal swab samples of 167 Psittaciformes in the Ceará State, Brazil, were evaluated regarding the presence of the following DEC virulence genes by polymerase chain reaction (PCR): *eae*A and *bfp*A genes (Enteropathogenic *E. coli* – EPEC); *stx*1 and *stx*2 (Shiga toxin-producing *E. coli* - STEC); *est*A and *elt*B (Enterotoxigenic *E. coli* - ETEC); *ipa*H (Enteroinvasive *E. coli* - EIEC); *aat*A and *aai*C (Enteroaggregative *E. coli* - EAEC). Positive strains for *eae*A and *bfp*A genes were considered typical EPEC, while strain positive exclusively for the *eae*A gene were classified as atypical EPEC. The *eae*A gene was identified in 20 *E. coli* strains and *bfpA* in 22 isolates. In addition, 11 and 9 belonged to tEPEC and aEPEC, respectively. No strain was positive for *stx1* or *stx2.* A total of 47 (60.3%) strains and a total of 136 birds (81.4%) were negative for the remaining DEC pathotypes investigated. In conclusion, psittacine from illegal wildlife trade in Ceará State, Brazil, presented a relevant prevalence of typical and atypical EPEC, potentially playing a role as reservoirs of DEC strains in the environment. Thus, proper control measures must be adopted to block the spread of these pathogens.

INDEX TERMS: EPEC, *Escherichia coli*, Psittaciformes, wildlife traffic, birds, bacterioses.

RESUMO.- [**Diagnóstico molecular de *Escherichia coli* diarreiogênicas isoladas de Psittaciformes do tráfico ilegal de animais silvestres.**] *Escherichia coli* diarreiogênicas (DEC) são consideradas uma das causas mais importantes de diarreia em países em desenvolvimento. Alguns estudos têm apontado aves silvestres como importantes reservatórios destes patógenos, entretanto, poucas espécies da ordem Psittaciformes têm sido investigada. O objetivo deste estudo foi analisar a presença de cepas de *E. coli* diarreiogênicas em Psittaciformes do tráfico de animais silvestres. Um total de 78 amostras de *E. coli* isoladas de suabes cloacais provenientes de 167 de Psittaciformes do Ceará, Brasil, foram avaliadas quanto a presença dos seguintes genes de virulência DEC por meio de reação em cadeia de polimerase (PCR): *eae*A e *bfp*A (*E. coli* Enteropatogênica - EPEC); *stx*1 e *stx*2 (*E. coli* produtora de Shiga - STEC); *est*A e *elt*B (*E.coli* Enterotoxigênica – ETEC); *ipa*H (*E.coli* Enteroinvasiva - EIEC); *aat*A e *aai*C (*E. coli* Enteroagregativa - EAEC). As cepas positivas para os genes *eae*A e *bfp*A foram consideradas EPEC típicas, enquanto que as positivas exclusivamente para o gene *eae*A foram classificadas como EPEC atípicas. O gene *eae*A foi

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identificado em 20 cepas de *E. coli* e o gene *bfp*A em 22 dos isolados. Adicionalmente, 11 e 9 cepas foram classificadas como EPEC típicas e atípicas, respectivamente. Nenhuma cepa foi positiva para os genes *stx*1 e *stx*2. Um total de 47 cepas (60,3%) e um total de 136 aves (81,4%) foram negativas para os demais patotipos DEC pesquisados. Em conclusão, psitacídeos provenientes do tráfico de aves silvestres do estado do Ceará, Brasil, apresentaram relevante prevalência de EPEC típicas e atípicas, potencialmente participando como reservatórios de cepas DEC no ambiente. Portanto, medidas de controle devem ser adotadas para inibir a disseminação destes patógenos.

TERMOS DE INDEXAÇÃO: EPEC, *Escherichia coli*, Psittaciformes, tráfico de animais silvestres, aves, bacterioses.

INTRODUCTION

Recent studies have observed the isolation of *Escherichia coli* from captive, traded and free-living psittacine (Saidenberg et al. 2012a, 2012b, Hidasi et al. 2013). However, there are scarce reports on virulence characterization of these strains. Currently, pathogenic *E. coli* is classified as intestinal pathogenic (or diarrheagenic *E. coli* - DEC) and extra-intestinal pathogenic *E. coli* (ExPEC) (Russo & Johnson 2000). DEC are further classified as enteropathogenic (EPEC), enterohemorragic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and diffusely adherent (DAEC) *E. coli* (Croxen et al. 2013).

EPEC are the most prevalent DEC in psittacine (Saidenberg et al. 2012a). These strains are characterized by an "attaching and effacing" lesion (A/E), marked by the presence of *eae*A gene (Ochoa & Contreras 2011). Moreover, EPEC are divided in atypical (aEPEC) and typical (tEPEC). Only tEPEC contains a virulence plasmid known as EPEC adherence factor (EAF), which harbors the bundle-forming pili (BFP) gene. This virulence factor provides adhesion of the bacterium to the host cell, which can be observed up to three hours after contact. However, aEPEC does not possess this plasmid (Trabulsi et al. 2002, Donnenberg & Finlay 2013).

tEPEC are classically responsible for most severe diarrhea in children less than five years of age (Croxen et al. 2013), while aEPEC are found more frequently, regardless of diarrhea (Beutin et al. 2003, Carvalho et al. 2003, Nakazato et al. 2004, Aidar-Ugrinovich et al. 2007). Although there are some reports of tEPEC identification in psittacine (Schremmer et al. 1999), this pathotype is known to be rarely isolated from animals (Trabulsi et al. 2002, Carvalho et al. 2003, Nakazato et al. 2004). On the other hand, farm, pet and wild animals are recognized as possible reservoirs and sources of aEPEC infection for humans (Carvalho et al. 2003, Nakazato et al. 2004, Krause et al. 2005, Ishii et al. 2007, Almeida et al. 2012).

Other DECs of human clinical importance have also been investigated in psittacine, such as EHEC and EAEC, which were identified in captive birds (Marietto-Gonçalves et al. 2011). EHEC are associated with hemorrhagic colitis and uremic hemolytic syndrome, while EAEC is highly prevalent in children and adults (Ferens & Hovde 2011), in which is responsible for acute diarrhea outbreaks in developed and developing countries (Steffen et al. 2015).

The demand for breeding psittacine has increased in recent years and this growth continues to be a tendency. This interest leads to the acquisition of these birds in legal and illegal wildlife trade. In this manner, the contact of man and psittacine, as occurs in domestic animals, is increasingly closer, generating the possibility of mutual transmission of microorganisms. Considering the health risk that pathogenic members of the Enterobacteriaceae family pose, researches have raised concern on the dissemination of these pathogens in the environment in which psittacine are maintained (Lopes et al. 2016). In addition, considering the importance of these pathogens for human health and the paucity of studies about these microorganisms in psittacine, the objective of this study was to evaluate the presence of DEC strains in Psittaciformes from illegal wildlife trade in Fortaleza, Brazil.

MATERIALS AND METHODS

Bacterial strains. A total of 78 *Escherichia coli* strains isolated by Lopes et al. (2015) from 167 clinically healthy psittacine, seized from illegal wildlife trade in the wildlife rehabilitation center located in the city of Fortaleza, Brazil, was collected from July to November 2013. Each bird was submitted to a single collection of individual cloacal swab and one representative colony was selected per bird. To avoid repetitions, all birds within the same pen were sampled in the same visit and different pens were collected in different periods. Strains maintained in nutrient agar were reactivated in BHI broth and plated in MacConkey for 24h at 37°C.

DNA extraction. From each plate, two to three colonies were collected and placed in tubes containing 1mL of 0.5% Triton X-100, which were vortexed for 15s and boiled for 20min at 94°C. Tubes were then centrifuged at 10.000rpm for 10min. Supernatant containing DNA was quantified and qualified by spectrophotometry using NanoDrop Spectrophotometer 2000 (Thermo Scientific, Wilmington, USA).

Molecular diagnosis of DEC. DNA samples were submitted to uniplex polymerase chain reactions (PCR) for the diagnosis of DEC from *E. coli* strains isolated from cloacal swabs. The presence of eight genes of virulence genes from five pathotypes was evaluated as follows: genes *stx1* (348pb) and *stx2* (584pb) for the identification of Shiga-Toxin producing *E. coli* (STEC); *elt*B (508pb) and *est*A (147pb) for enterotoxigenic *E. coli* (ETEC); *eae*A (881 pb) and *bfp*A (300 pb) for enteropathogenic *E. coli* (EPEC); *ipa*H (483 pb) for enteroinvasive *E. coli* (EIEC); *aat*A (630 pb) and *aai*C (215pb) for enteroaggregative *E. coli* (EAEC) (Taniuchi et al. 2012)(Table1). EPEC strains are classified as typical when possessing *eae*A and *bfp*A genes, without *stx*; and as atypical if only the *eae*A gene is found (Donnenberg & Finlay 2013). Strains EAEC 042, EHEC O157:H7, EIEC O124, EPEC 2348/69 and ETEC H10407 were used as positive controls for the reactions. PCR was performed using GoTaqGreen kit (Promega) and primers at 0.2uM in MyCycler Thermal Cycler (Biorad, CA, USA), with the following protocol: 95°C for 15min; 40 cycles of 95°C for 30s, 57°C for 30s and 72°C for 1min; followed by 72°C for 10min. Amplified products were submitted to 2% ethidium bromide stained agarose gel electrophoresis and photo-documented by the transilluminator ChemicDoc XRS System (Biorad, CA, USA).

RESULTS

From the total of 78 *Escherichia coli* strains isolated from psittacine of illegal wildlife trade, only *eae*A and *bfp*A genes were detected. The prevalence rates of these genes in the investigated psittacine were 12.0% (20/167) and 13.2 (22/167) for *eae*A and *bfp*A respectively. Considering

*eae*A and *bfp*A genes, 6.6% (11/167) of birds were positive for strains as tEPEC, while 5.4% (9/167) were classified as aEPEC. In addition, 60.3% (47/78) of isolated bacteria and 81.4% (136/167) of evaluated psittacine did not harbor any of the virulence genes used to diagnose EPEC, EIEC, EHEC, EAEC or ETEC. Furthermore, tEPEC prevalence within species was: 4/66 *Amazona aestiva* (6.1%), 3/13 *Amazona amazonica* (23.1%) and 4/67 *Eupsitulla cactorum* (6.0%) (Table 2).

Table 1. The primers used for detection of the various genes by PCR, amplicon size

Genes	Description of the target genes	Oligonucleotide primer pairs (5'-3')	Amplicon (pb)
*Stx*1	STEC	ACTTCTCGACTGCAAAGACGTATG ACAAATTATCCCCTGAGCCACTATC	348
*Stx*2	STEC	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	584
*elt*B	ETEC	TTCCCACCGGATCACCAA CAACCTTGTGGTGCATGATGA	508
*est*A	ETEC	TTCACCTTTCGCTCAGGATG AGCACCCGGTACAAGCAG	147
*eae*A	EPEC	GTAAAGTCCGTTACCCCAACCTG CAAAGCGCACAAGACTACCA	881
*bfp*A	EPEC	GGAAGTCAAATTCATGGGGG GGAATCAGACGCAGACTGGT	300
*ipa*H	EIEC	CCTTTTCCGCGTTCCTTGA CGGAATCCGGAGGTATTGC	483
*aat*A	EAEC	CTGGCGAAAGACTGTATCAT TTTTGCTTCATAAGCCGATAGA	630
*aai*C	EAEC	ATTGTCCTCAGGCATTTCAC ACGACACCCCTGATAAACAA	215

Adapted from Taniuchi et al. (2012).

DISCUSSION

This study identified important DEC virulence genes (*eae*A and *bfp*A) in *Escherichia coli* strains isolated from psittacine of illegal wildlife trade, which are important to diagnose the EPEC pathotype. Although these bacteria are recognized as important human pathogens (Peréz et al. 2010, Gomes et al. 2016), aEPEC have also been associated with increasing morbidity and mortality of Budgerigars (Seeley et al. 2014).

The detected percentages of tEPEC and aEPEC in this research were higher than results found in most reports involving psittacine. Studies about psittacine in free-life, zoos, rescue centers and commercial breeders present prevalence rates of up to 6.5% for tEPEC and 2.3% for aEPEC in strains isolated from cloacal swabs (Schremmer et al. 1999, Saidenberg et al. 2012a, 2012b, 2015, Marietto-Gonçalves et al. 2011).

The majority of *E. coli* strains isolated in this study (60.3%) did not harbor any of the genes analyzed and, therefore are not identified as any of the DEC investigated in this study harmful for human health (EPEC, EIEC, EHEC, EAEC, ETEC). Considering that reports of EPEC isolated from psittacine are scarce, the other DEC pathotypes appear to be even harder to identify. Marietto-Gonçalves et al. (2011) surveying for DEC in 86 captive psittacine observed strains of STEC and EAEC, in addition to EPEC. Koochakzadeh et al. (2015) investigated *stx*1, *stx*2 and *eae*A genes in 30 *E. coli* strains from psittacine of pet shops in Iran. However, none of the strains presented these genes. EPEC (*eae*) and STEC (*stx2*) genes have been isolated in a study performed by Gioia-Di Chiacchio et al. (2016) with cockatiels and budgerigars, which are common pet psittacine.

Studies with other avian species also showed that the DEC genes analyzed here may be detected elsewhere. The study of Chandran & Mazumder (2014) reported an elevated rate of positive samples for DEC diagnosing genes. From a total of 412 samples collected from captivity-bred birds of 15 different species, 63 isolates harboring the *stx* gene were detected.

Table 2. Absolute (n) and relative (%) frequencies of *Escherichia coli* pathotypes isolated from psittacine of illegal wildlife trade in the state of Ceará, Brazil

Sample source	Number of investigated birds	Absolute and relative frequencies of *E. coli* per avian species	EPEC		*E. coli* containing only *bfp*A	Birds negative for the investigated genes
			Typical	Atypical		
			n(%)	n(%)	n(%)	n (%)
Amazona aestiva	66	41 (62.1)	4 (6.1)	2 (3.0)	6 (9.1)	54(81.8)
Amazona amazonica	13	8 (61.5)	3 (23.1)	2 (15.4)	1 (7.7)	7 (53.8)
Eupsitulla cactorum	67	19 (28.4)	4 (6.0)	4 (6.0)	4 (6.0)	55 (82.1)
Brotogeris chiriri	2	2 (100.0)	-	1 (50.0)	-	1 (50.0)
Eupsittula aurea	3	2 (66.7)	-	-	-	3 (100.0)
Ara ararauna	7	4 (57.1)	-	-	-	7 (100.0)
Anodorhynchus hyacinthinus	3	1 (33.3)	-	-	-	3(100.0)
Ara severus	1	1 (100.0)	-	-	-	1(100.0)
Amazonas farinosa	1	-	-	-	-	1(100.0)
Ara macao	2	-	-	-	-	2 (100.0)
Psittacara leucophthalmus	1	-	-	-	-	1 (100.0)
Ara chloropterus	1	-	-	-	-	1(100.0)
TOTAL	167	78 (100.0)	11 (6.6)	9 (5.4)	11 (6.6)	136 (81.4)

The following genes were negative in all samples: *aai*C, s*tx*1, s*tx*2, *elt*B, *est*A, *ipa*H, *aat*A.

However, ETEC and EIEC related genes were not identified. In addition, Saviolli et al. (2016) looked for the genes *eae*A, *stx*1 e *stx*2 in free-living fragatas (*Fregata magnificens*) and did not detect EPEC or STEC genes.

The detection of *eae*A and *bfp*A genes in some of the bacterial strains isolated from psittacine suggests that these birds might have a contamination source for these pathogenic strains. Studies with psittacine in captivity or from illegal wildlife trade, in which tEPEC were identified, show that the likely source of contamination may have anthropozoonotic character, considering the poor environmental conditions of these places (Saidenberg et al. 2012b).

Illegal wildlife trade might have influenced the findings here described, as it contributes to a closer contact between wild and domestic species, in addition to humans (Kruse et al. 2004). Trafficked wild birds are potential reservoirs of important agents of human health (Matias et al. 2016). Thus, measures, such as quarantine, are essential for avoiding the spread of strains with zoonotic potential. Marietto-Gonçalves et al. (2010) explained that the monitoring of Gram-negative bacteria in the enteric microbiota of Psittaciformes is a procedure that must be included in the routine of private breeders, zoos, veterinary hospitals and main programs that aim to reintroduce captive birds back to the wild. Such sanitary care measures are important not only because *E. coli* is not a member of the normal microbiota of these birds, but also due to the risk of dissemination of these pathogens to the wild environment, contributing for the epidemiologic chain of a variety of enteric diseases for humans and other animals.

CONCLUSIONS

The findings suggest that psittacine from illegal wildlife trade in Ceará, Brazil, present important prevalence of typical and atypical EPEC. Thus we suggest that *Escherichia coli* isolates from these birds must be further investigated, considering that clinically healthy wild birds might harbor bacterial strains that present virulence factors relevant for human and animal health.

A possible explanation for the isolation of pathogens in this study is the close contact between wild birds and domestic species, or even directly with humans, that occurs in illegal wildlife trade or captivity. Therefore the importance of adequate sanitary conditions in wildlife rehabilitation centers is reinforced, considering that the absence of effective prophylactic measures and disease control may favor dissemination.

In addition, these birds may transmit pathogens to other animals in nature, following reintroduction into the wild, or even into private environment of legal breeders, maintaining the cycle of transmission of these microorganisms.

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