A survey on gram-negative bacteria in saffron finches (*Sicalis flaveola*) from illegal wildlife trade in Brazil

Pesquisa de bactérias gram-negativas em canários-da-terra (Sicalis flaveola) resgatados do tráfico ilegal de aves no Brasil

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Abstract

Passerines such as canaries or finches are the most unlawfully captured species that are sent to wildlife centers in São Paulo, Brazil. Captured birds may have infection by opportunistic bacteria in stressful situations. This fact becomes relevant when seized passerine are reintroduced. The aim of this study was to evaluate the health state of finches from illegal wildlife trade using microbiological approaches. Microbiological samples were collected by cloacal and tracheal swabs of 100 birds, captured during 2012 and 2013. The results indicate high frequency of gram-negative bacteria in feces and oropharynx, especially from the Enterobacteriaceae family (97.5%). The most frequent genera were *Escherichia coli* (46.5%) and *Klebsiella pneumoniae* (10.4%). *Enterobacter cloacae, Serratia liquefaciens, Serratia* spp. *Klebsiella oxytoca* and *Citrobacter freundii* were isolated with lower frequency from asymptomatic birds. The presence of enteropathogenic *Escherichia coli* (EPEC) and Shiga toxin-producing strain (STEC) confirm the zoonotic risks and public health concern.

Keywords: Birds. *Sicalis flaveola*. Microbiology. Enterobacteria. Public health.

Resumo

No Estado de São Paulo, Brasil, os pássaros como os canários-da-terra têm sido uma das espécies mais frequentemente resgatadas do tráfico ilegal e enviadas aos centros de vida selvagem. Em situações de estresse estas aves podem ser acometidas por infecções causadas por bactérias oportunistas. Este fato é de grande importância quando é planejada da reintrodução das aves na natureza. O presente trabalho foi delineado para avaliar o estado de saúde de canários-da-terra resgatados do tráfico ilegal. Foram colhidas soabes da traqueia e da cloaca de 100 aves resgatadas durante os anos de 2012 e 2013. Os resultados obtidos revelaram alta frequência de bactérias gram-negativas nas fezes e no orofaringe dos animais, com maior frequência para os membros da família Enterobacteriaceae (97,5%). Os gêneros mais frequentes foram *Escherichia coli* (46,55) e *Klebsiella pneumoniae* (10,4%). Outros microorganismos incluindo *Enterobacter cloacae*, *Serratia liquefaciens*, *Serratia* spp, *Klebsiella oxytoc*a e *Citrobacter freundii* também foram isolados em menor frequencia de aves assintomáticas. A presença de estirpes de *Escherichia coli* enteropagênicas (EPEC) e as produtoras da toxina de Shiga confirmam o risco de zoonose e a importância para saúde pública deste tipo de ave.

Palavras-chave: Aves. *Sicalis flaveola*. Microbiologia. Enterobactérias. Saúde pública.

DOI: 10.11606/issn.1678-4456.bjvras.2016.109042

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Received: 29/12/2015
Approved: 01/06/2016

Introduction

Illegal wildlife trade is the major cause of biodiversity reduction in Brazil, contributing to the annual removal of approximately 38 million specimens of nature. The mortality rate of such captured specimens reaches 90% due to the conditions of capture and transportation. It is estimated that this illegal activity moves approximately US $ 10 billion/ year worldwide (PAGANO et al., 2009).

The Passeriformes order is one of the main targets of the illegal wildlife trade in Brazil, representing 79% of the seized birds. Four species belong to *Sicalis* Genera, and among these stand out native subspecies (*Sicalis flaveola brasiliensis*) and exotic subspecies, such as Venezuelan canary (*Sicalis flaveola flaveola*) (SIQUEIRA et al., 2013). Some exotic subspecies present great phenotypic similarity to the native saffron finch and when introduced into a new environment, competes for territory and food, promoting environmental imbalance, especially in fragmented areas. This can decimate native species. It can also cause spread of diseases, exposing native birds to potential pathogens (SANCHES, 2008; PINTO et al., 2016).

The evaluation of the health status of canaries can provide relevant data to assist in determining zoo sanitary protocols and bird destination in wildlife centers (DUTRA et al., 2016). Passerines can carry several microorganisms that can become pathogenic in adverse situations, such as being under stress (SANDMEIER; COUTTEEL, 2006). A histopathological study by Godoy and Matushima (2010) revealed that 78.6% of the seized birds (283/360) analyzed died due to infectious processes. Sanches (2008) also points to bacterial disease as the leading cause of death in passerines seized from illegal wildlife trade, especially infections by gram-negative bacteria, mainly enterobacteria. Multiple gram-negative bacteria such as *Salmonella* spp., *Pasteurella* spp., *Klebsiella* spp., *Yersinia* spp., *Campylobacteriosis* spp., and *Escherichia coli* are also potential zoonotic pathogens identified in birds (EVANS, 2011). Therefore, a microbiological study of the sanitary status of confiscated animals that will be reintroduced is important in evaluating whether these animals act as carriers of pathogenic agents to other animals and humans (BRACONARO et al., 2015).

The aim of this study was to evaluate the health status of confiscated canaries from Brazilian illegal wildlife trade and destined for reintroduction by assessment of gram-negative bacteria from cloacal and oropharyngeal microbiota, and assist in the development of more effective zoo sanitary protocols for the reintroduction of wild birds.

Materials and Methods

The development of this project was approved by the Ethics Committee on Animal Use of the College of Veterinary Medicine and Animal Science of the University of São Paulo (USP-FMVZ), protocol number 3134/2013, SISBIO license: 36470-1.

Animals

One hundred male and female saffron finches (*Sicalis flaveola*) of different ages were evaluated. Birds were seized by the Brazilian Institute of Environment and Renewable Resources (IBAMA), forest police and firefighters from September 2012 to September 2013. The birds were confiscated in cities of São Paulo: Itapecerica, Morungaba, Embu das Artes, Parque Cocáia, Parque Turquesa, Guaianazes, Vila Missionária, Jardim Angela, Jardim Marilu, Santo André, Osasco, São Bernardo do Campo, Barueri, Carapicuíba, Guarulhos, Cajamar and São Paulo. After seizure, the birds were housed at the Wild Animal

Recovery Tiete Ecological Park Center - Engineer Goulart Center (CRAS/PET), located at 23°25'S and 46°28'W, and administered by the Department of Water and São Paulo State Energy (DAEE). The birds were fed with seeds and grains and drinking water *ad libitum*, and were kept in collective enclosures after the quarantine period in CRAS/PET. The birds were physically restrained on the day of collection of samples for microbiological examination.

Microbiological and Molecular Evaluation

Stool samples, cloacal and oropharynx samples were collected from individual cages in which the canaries remained for 12 to 24 hours. The material was identified, packed and kept refrigerated until referral to the Laboratory of Avian Medicine at the Department of Pathology of FMVZ-USP for microbiological culture and isolation.

The individual samples were inoculated in BHI (brain heart infusion, Difco™) and Tetrathionate broth (Difco™), incubated at 37°C for 24 hours. The isolation was performed on MacConkey and XLT4 agar (Difco™), and incubated at 37°C for 24 hours, and 48 hours, respectively. The selected colonies were identified through specific biochemical tests (EPM, MiLi, and Citrate).

For MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) sample preparation, bacterial proteins were extracted using an ethanol/formic acid protocol (KUHNERT et al., 2012). The protein suspension (1 µl) was transferred to a polished steel MALDI target plate (Bruker Daltonik) and allowed to dry at room temperature. The sample was overlaid with 1 µL of matrix (10 mg α-cyano-4-hydroxy-cinnamic acid mL^{-1} in 50% acetonitrile/ 2.5% trifluoroacetic acid), and mass spectra in the 2–20 kDa range were acquired using a Microflex™ mass spectrometer (Bruker Daltonik). For the MALDI-TOF MS analysis, the spectra were loaded into MALDI BioTyper™ 3.0 and compared with the manufacturer's library, which resulted in the log (score) value. Standard Bruker interpretative criteria were applied; scores ≥ 2.0 were accepted for species assignment and scores ≥ 1.7 but ≤ 2.0 for genus identification.

In this study, the diarrheagenic *Escherichia coli* pathotypes (EPEC and STEC) were identified by molecular method using the polymerase chain reaction (PCR). The DNA extraction was performed according to the method described by Boom et al. (1990).

Table 1 presents detailed information about the virulence targeted genes, primers and expected amplicons, according to the methodology described by Costa et al. (2010). The strains were classified into pathotypes according to the combination of genes, including typical EPEC (*eae*+,*bfp*+ and *stx1*/ *stx2* negative), atypical EPEC (*eae*+, *bfp*- , *stx1* and *stx2* negative) and STEC (*eae*+, *stx1* and/or *stx2* positive).

The amplification mixture consisted of Tris-HCl buffer (pH 8.3) 10 mM, KCl 50 mM, $MgCl_2$, deoxynucleotide triphosphates 0.2 mM, pairs of primers, Taq DNA polymerase 0.5U, and ultrapure water autoclaved in a final volume of 25µl. The amplification products were separated by electrophoresis on a 1.5% agarose gel and examined after staining. The molecular weight marker used was the 100-bp DNA ladder.

Results

Table 2 shows the distribution of gram-negative bacteria identified. Three hundred sixteen colonies were selected and identified. Of these isolates, 308 (97.5%) were from *Enterobacteriaceae* family, and 8/316 were from the families *Moraxellaceae* (*Acinetobacter calcoaceticus*), *Aeromonadaceae* (*Aeromonas caviae*), *Alcaligenaceae* (*Bordetella avium*) and *Pseudomonadaceae* (*Pseudomonas* spp.). Among the *Enterobacteriaceae*, the species most often isolated were *Escherichia coli* (46.5%), of which 129 strains were isolated from feces and cloaca, and 18 strains from oropharynx. Other genera of bacteria less frequently isolated were *Klebsiella pneumoniae* (10.4%), *Enterobacter cloacae* (8%), *Serratia liquefaciens* (6.4%),

Table 1 – Target genes, primers sequences and amplicon sizes used to identify *E. coli* pathotypes in oropharynx, cloaca and feces of saffron finches from illegal wildlife trade

Patothypes of *E. coli*	Gene	Pairs of *primers* (5`-3`)	*Amplicon* (pb)
EPEC	*eae*	AAACAGGTGAAACTGTTGCC CTCTGCAGATTAACCTCTGC	454
	bfb	CAATGGTGCTTGCGCTTGCT GCCGCTTTATCCAACCTGGT	550
STEC	*stx1*	CAACACTGGATGATCTCAG CCCCCTCAACTGCTAATA	349
	stx2	ATCAGTCGTCACTCACTGGT CTGCTGTCACAGTGACAAA	110

Table 2 – Frequency of gram-negative bacteria in fecal and oropharynx samples from saffron finches from illegal wildlife trade in São Paulo, Brazil. Samples obtained from 2012 to 2013

Bacteria	Feces/Cloaca (n)	Percentage (%)	Oropharynx (n)	Percentage (%)	Total n (%)
Acinetobacter calcoaceticus	1	0,4	-	-	1 (0,3)
Aeromonas caviae	1	0,4	1	1,6	2 (0,6)
Bordetella avium	-	-	1	1,6	1 (0,3)
Escherichia coli	129	51,0	18	28,5	147 (46,5)
Escherichia fergusonii	1	0,4	-	-	1 (0,3)
Enterobacter aerogenes	-	-	1	1,6	1 (0,3)
Enterobacter asburiae	1	0,4	-	-	1 (0,3)
Enterobacter cloacae	16	6,3	9	14,2	25 (8,0)
Enterobacter cowanii	2	0,8	-	-	2 (0,6)
Enterobacter kobei	1	0,4	-	-	1 (0,3)
Pantoea aglomerans	1	0,4	1	1,6	2 (0,6)
Pantoea septica	-	-	1	1,6	1 (0,3)
Pantoea aglomerans	1	0,4	-	-	1 (0,3)
Serratia spp.	12	4,7	3	4,8	15 (4,8)
Serratia odorifera	1	0,4	1	1,6	2 (0,6)
Serratia liquefaciens	16	6,3	4	6,3	20 (6,4)
Serratia marcescens	3	1,2	1	1,6	4 (1,2)
Serratia rubidaea	2	0,8	1	1,6	3 (0,9)
Hafnia alvei	2	0,8	4	6,3	6 (1,8)
Klebsiella pneumoniae	28	11,0	5	7,9	33 (10,4)
Klebsiella oxytoca	9	3,5	2	3,2	11 (3,6)
Morganella morganii	1	0,4	-	-	1 (0,3)
Proteus mirabilis	4	1,6	3	4,8	7 (2,3)
Proteus vulgaris	4	1,6	3	4,8	7 (2,3)
Citrobacter diversus	4	1,6	1	1,6	5 (1,7)
Citrobacter farmei	1	0,4	-	-	1 (0,3)
Citrobacter freundii	8	3,2	1	1,6	9 (2,9)
Citrobacter youngae	1	0,4	-	-	1 (0,3)
Edwardsiella tarda	1	0,4	-	-	1 (0,3)
Pseudomonas koreensis	-	-	1	1,6	1 (0,3)
Pseudomonas montilii	-	-	1	1,6	1 (0,3)
Pseudomonas oryzihabitans	1	0,4	-	-	1 (0,3)
Pseudomonas putida	1	0,4	-	-	1 (0,3)
Total	**253**	100	**63**	100	**316** (100)

Serratia spp. (4.8%) *Klebsiella oxytoca* (3.6%) and *Citrobacter freundii* (2.9%). There was no isolation of *Salmonella* spp.

The survey of virulence factors showed two strains of *E. coli* (2/147) positive for the *eae* gene (*attaching and effacing*) and negative for *bfp* (*bundle forming pili*). One of these strains was also positive for $stx2_f$ gene (*Shiga-like* toxin) and showed cytotoxic effect in cell cultures (VERO cells). Therefore, these two strains were classified as atypical EPEC (*eae*+/ *bfp*-) and STEC (*eae*+/*stx2f*+).

Discussion

This is the first study to describe the occurrence of atypical EPEC and STEC in saffron finches seized from the illegal wildlife trade, and one of the few studies to evaluate the gram-negative intestinal microbiota of passerines. In this study, 87% of the passerines presented colonization by gram-negative bacteria. In asymptomatic birds, gram-negative bacteria should not be considered alarming pathogens, but rather an indication of need for improvement of health management, thereby minimizing the risk of developing opportunistic infections (BOSERET et al., 2013). The main issue is not only related to the pathogenicity of certain strains, but the possibility of transmission to other animals and humans (SAIDENBERG et al., 2012b).

Normal fecal bacteria cultured from healthy birds include gram-positive bacteria (*Lactobacillus* spp., *Staphylococcus* spp. and *Streptococcus* spp.). Gram-negative bacteria occasionally recovered in clinically normal birds include *Pseudomonas* spp., *Escherichia coli, Enterobacter* spp. and *Klebsiella* spp. (BRACONARO et al., 2015), but the same bacteria isolated from sick birds (*Serinus canaria*) are considered clinically significant (GIACOPELLO et al., 2015).

In this study 2.5% of the isolates (8/316) were gram-negative bacteria belonging to the Moraxellaceae (*Acinetobacter calcoaceticus*), Aeromonadaceae (*Aeromonas caviae*), Alcaligenaceae (*Bordetella avium*) and Pseudomonadaceae (*Pseudomonas* spp.) families.

Acinetobacter spp. are widely distributed in the environment (soil) and constitutes one of the predominant organisms in water. It is commonly found in hospitals for humans and has evolved as one of the most important nosocomial pathogens in the past decade, particularly in immunosuppressed patients, and can affect different organs causing pneumonia, meningitis, septicemia, urinary tract and skin infections (ÖZVATAN et al., 2016). It is not commonly isolated from birds, but there are reports of infection by *Acinetobacter* spp. associated with mycobacteriosis in falcons that were probably infected by feces of wild birds (MULLER et al., 2010).

Aeromonas spp. is ubiquitous in aquatic environments and can be a primary pathogen causing septicemia in reptiles, fish and amphibians. Predisposing factors such as immunosuppression and noninfectious and infectious diseases are usually associated with gastroenteritis and septicemia in human beings. It has been described in ostrich (*Struthio camelus*) toucan (*Ramphastos toco*), budgerigar (*Melopsittacus undulatus*) and domestic canaries (*Serinus canaria*), as a facultative pathogen that requires infection by other bacteria, environmental stress, or injury (FRANÇA et al., 2009).

Bordetella avium is among the several causative agents of respiratory tract diseases leading to severe economic losses in the poultry industry worldwide. Is most frequently diagnosed in 2- to 6-week-old turkeys as a sudden onset of sneezing and clear nasal discharge, but it has been described in other avian species as well (SZABÓ et al., 2015). Farrington and Jorgenson (1976) described the first occurrence of *Bordetella bronchiseptica* in wild birds, but it is related to the proximity to domestic animals and humans. Szabó et al. (2015) also isolated *Bordetella avium* from wild ducks, geese and partridge that were asymptomatic. It is associated with rhinitis, sinusitis and temporomandibular joint rigidity (Lockjaw Syndrome) in psittacines birds (GRESPAN et al., 2012; MORENO et al., 2015), but has never been described in passerines.

Pseudomonas spp. is ubiquitous in the environment and is an opportunistic pathogen in animals and humans associated with high morbidity and mortality due to its ability to develop antibiotic resistance and expression of multiple virulence factors. In birds, it may cause respiratory infections, sinusitis, keratoconjunctivitis, osteomyelitis, septicemia and mortality in embryos or newly hatched birds. Canaries can become carriers of resistant strains, with the risk of a possible dissemination and transmission of these pathogens to humans through direct contact (GIACOPELLO et al., 2015), but this pathogen has also

been described in cloacal swabs of asymptomatic wild birds (BRITTINGHAM et al., 1988).

Most of the isolates found in this study 97.5% (308/316) were Enterobacteriaceae. This data contrasts to the Glünder (1981) report that examined the stools of 98 asymptomatic granivorous passerines, demonstrating the absence of *Enterobacteriaceae* in 82.6% of the birds. On this occasion, the author suggested that enterobacteria are not part of the intestinal microbiota of these birds. However, in subsequent years, other researchers identified different enterobacteria in the digestive system in studies involving various species of symptomatic and asymptomatic birds (LOIKO et al., 2007; XENOULIS et al., 2010; BRACONARO et al., 2015). It is likely that maintaining captive birds can modify the intestinal microbiota of passerines, favoring the colonization by gram-negative bacteria (MATTES et al., 2005; BOSERET et al., 2013). The average life span of birds seen in our study was 313 days and perhaps this long period in captivity contributed to the horizontal spread of these microorganisms from the feces of carriers.

The data obtained in this study showed a predominance of some species of enterobacteria: *Escherichia coli* (46.5%) was the most frequently isolated, while *Klebsiella pneumoniae* (10.4%), *Enterobacter cloacae* (8%), *Serratia liquefaciens* (6.4%), *Serratia* spp. (4.8%) *Klebsiella oxytoca* (3.6%) and *Citrobacter freundii* (2.9%) were less frequent (Table 2). The identification of other genera such as *Hafnia* spp., *Proteus* spp. and *Edwardsiella tarda* was less frequent, with percentages of 1.8%, 4.6% and 0.3%, respectively. These data are consistent with the results obtained by Gibbs et al. (2007) and Braconaro et al. (2015), who identified *Klebsiella pneumoniae*, *E. coli* and *Serratia liquefaciens* in stool samples from several species of clinically healthy passerines. On the other hand, in a parrot study, Dorrestein et al. (1985) reported the isolation of *Serratia liquefaciens*, *Serratia marcescens* and *Serratia odoriferous* from asymptomatic birds, while the presence of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii* and *E. coli* was associated with clinical manifestations and macroscopic changes in necropsy.

In addition to the differences in the literature on the presence of gram-negative bacteria in the microbiota of clinically healthy passerines, one of the main current difficulties is to establish a correlation between the presence of certain pathogens in the microbiota, the occurrence of diseases and health risks involved in the transmission. A more accurate assessment of the risk can be obtained with the aid of molecular techniques to search for genes related to bacterial virulence factors, such as the production of adhesins and toxins. In this study, we chose the evaluation of virulence factors of *Escherichia coli*, since this was the most frequent agent. Due to the opportunistic nature of the agent, the isolation only of feces, cloaca or oropharynx is not enough to associate the presence of bacteria with the occurrence of disease (HIDASI et al., 2013).

Clinical disease is related to the presence of pathotype-specific virulence factors. One of the most common is EPEC (Enteropathogenic *E. coli*), which causes diarrhea by the intimate adhesion between the bacteria and the host cells, and STEC (Shiga-like toxin *E. coli*) with diarrhea caused by the production of cytotoxins Stx1 and Stx2 (COSTA et al., 2010). In this study, we found a strain characterized as atypical EPEC, and another classified as STEC. This fact deserves special attention because it indicates the zoonotic potential of these agents, since the animals act as reservoirs of atypical EPEC and STEC, both considered emerging pathogens (SAIDENBERG et al., 2012a; GIOIA-DI CHIACCHIO et al., 2016).

Some bird species have been described as possible asymptomatic carriers of atypical EPEC and STEC; among them are columbiformes and galliformes (FAROOQ et al., 2009); passeriformes, piciformes and anseriformes (OH et al., 2011); and psittaciformes (SAIDENBERG et al., 2012a; GIOIA-DI CHIACCHIO et al., 2016). In a study by Saidenberg et al. (2012b), typical EPEC strains were detected in asymptomatic parrots from illegal wildlife trade in Brazil. The authors note the existence of three birds positive for EPEC that

were kept together with others of the same species that remained negative, indicating an intermittent elimination of the pathogen or a transient carrier state. These findings reinforce the need for tests to check for the presence of *E. coli* with pathogenic potential and ensure the health of birds to be reintroduced into the wild.

Conclusion

Although the canaries analyzed were apparently in healthy clinical condition, the frequency of isolation of gram-negative bacteria, especially enterobacteria, was high, with a predominance of *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Serratia liquefaciens*. All these agents are potentially pathogenic for passerines and can cause systemic infections.

The study of *E. coli* virulence factors revealed the presence of pathogenic strains of atypical EPEC and STEC. The investigation of these issues is important to evaluate the pathogenicity, the zoonotic potential and the risks involved for animal health, public health and biodiversity conservation.

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