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First molecular detection of *Eimeria* spp. in eared doves (*Zenaida auriculata*) from Brazil

Primeira detecção molecular de *Eimeria* spp. em pombos (*Zenaida auriculata*) do Brasil

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Highlights:
The epidemiology of *Eimeria* species in doves is scarce.
A nested-PCR for detection of *Eimeria* spp. in fecal samples has been developed.
Molecular detection of *Eimeria* spp. in *Zenaida auriculata* has been described worldwide for the first time.

Abstract

The aim of the present study was to detect *Eimeria* spp. in eared doves (*Zenaida auriculata*) from Brazil. Two hundred and fourteen birds were trap-capture in different regions of Londrina city, Paraná state, Southern Brazil. Fecal samples were collected, and DNA extraction was performed. A nested PCR based on the subunit I of the cytochrome c oxidase gene of the *Eimeria* mitochondrial genome was used to detect the DNA presence of this coccidian in eared dove feces. From 214 birds, 171 (79.9%) were positive for *Eimeria* spp. There was significantly difference of positivity between the site of capture. When analyzing the genders, the numbers of positive males were 84/103 (81.5%) and females 87/111 (78.4%). To the best of the authors' knowledge, this is the first study with molecular prevalence of *Eimeria* sp. in *Z. auriculata*. Further studies should be done to identify the species of *Eimeria* that infect eared doves *Z. auriculata*.
Key words: Eimeriidae. Coccidiosis. PCR. Columbiformes. Cytochrome c oxidase I gene.

Resumo

O objetivo do presente estudo foi detectar *Eimeria* spp. em pombos (*Zenaida auriculata*) do Brasil. Duzentos e quatorze pombos foram capturados em diferentes regiões da cidade de Londrina, estado do Paraná, Sul do Brasil. Amostras fecais foram coletadas e realizada a extração de DNA. Uma nested-PCR baseada na subunidade I do gene oxidase do citocromo c oxidase do genoma mitocondrial de *Eimeria* spp. foi utilizada para detectar a presença de DNA deste coccídeo nas amostras fecais dos pombos. Das 214 aves, 171 (79,9%) foram positivas para *Eimeria* spp. Houve uma diferença significativa de positividade entre o local de captura. Quando analisado o gênero, o número de machos positivos foi 84/103 (81,5%) e fêmeas 87/111 (78,4%). Para o conhecimento, este é o primeiro estudo com prevalência

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molecular de *Eimeria* spp. em *Z. auriculata*. Novos estudos devem ser conduzidos para identificar as espécies de *Eimeria* que infectam pombos *Z. auriculata.*
Palavras-chave: Eimeriidae. Coccidiose. PCR. Columbiformes. Citocromo c oxidase I gene.

Introduction

The eared dove (*Zenaida auriculata*) is found in most of Latin America countries in both urban and rural areas (Adriano & Cordeiro, 2001). It belongs to the family Columbidae, which comprises over than 300 avian species worldwide (Jamriška & Modrý, 2012). In Londrina region, it has been described 10 Columbidae species, including *Z. auriculata* (Shibatta et al., 2009).

Eimeria is the most diverse coccidia from Order Eucoccidiorida, causing damages in all classes of vertebrates (Berto, McIntosh, & Lopes, 2014) Eimeriosis has been causing losses especially in poultry industry for its high pathogenicity (Cardozo & Yamamura, 2004; Reid et al., 2014) There are many species of *Eimeria* described infecting pigeons such as: *E. labbeana, E. columbarum, E. columbae, E. tropicalis, E. kapotei, E. janovyi, E. livialis, E. sphenocerae, E choudari, E. turturi, E. waiganiensis, E. duculai, E. gourai, E. palumbi, E. curvata, E. zenaidae, E. mauritiensis* and *E. columbapalumbi* (Ball, Daszak, Swinnerton, Jones, & Snow, 2012; Yang, Brice, Elloit, & Ryan, 2016a).

Over the years, the methodologies to diagnose *Eimeria* in birds have been developed, not only in the traditional morphologic characterization, but also in molecular biology (Carvalho et al., 2011). Furthermore, the identification of Eimeria species can be based on morphology, geographic distribution, host species and pathology (Yang, Brice, Elloit, Lee, & Ryan, 2014). In this way, molecular tools are used for diagnostics and to describe the genetic variability of *Eimeria* oocysts (Carvalho et al., 2011)

The sequences of the subunit I of the cytochrome c oxidase gene (COI) have been used to identify *Eimeria* and *Isospora* species in birds; however, there are still few studies of coccidia in birds worldwide (Berto et al., 2014; Hafeez et al., 2015; Ogedengbe, Hanner, & Barta, 2011; Rathinam, Gadde, & Chapman, 2015; Yang, Brice, & Ryan, 2016b). Thus, the aim of this study was to verify the presence of *Eimeria* sp. in *Z. auriculata* from Southern Brazil.

Material and Methods

Study location and sampling

Londrina city is located at Parana State, Southern Brazil (23°08'47" to 23°55'46" S, 50°52'23" to 51°19'11" W). Between January 2010 and December 2011, 214 male and female eared doves (*Z. auriculata*) were trap-captured in four different locations in Londrina: State University of Londrina Campus (University: n=54), Crop Cooperative I (Coop. I: n=136), Dairy Farm (n=16) and Crop Cooperative II (Coop. II: n=9).

The birds were euthanized in a CO_2 chamber, and during the autopsy, the intestines were collected and opened. The fecal samples were scraped from the guts, put into plastic microtubes and maintained at -20 ºC until DNA extractions.

All procedures involving the animals was approved by the National Institute for the Environment and Renewable Natural Resources (IBAMA - SISBIO N. 16428-1) and by the Ethics Committee of Animal Experiments of the State University of Londrina (n. 70/2008). The birds were euthanized according to the guidelines of the National Council for Animal Experimental Control (CONCEA/Brazil).

DNA extraction

Before the DNA extraction procedures, fecal samples were submitted to three cycles of freeze

and thaw (freezer -80 °C and water bath 55 °C). For the DNA extraction, 1 mL of Tris-EDTA (TE) was added for each 50 µL of fecal sample in a 1.5 mL microtube. After centrifugation (4000g for 15 min), the supernatant was discarded, and the pellet was used for extraction with Nucleospin Tissue® (Macherey-Nagel, Germany) according with manufacturer's instructions. DNA sample was eluted in 20 µl of elution and stored at -20 °C until further analysis.

PCR

A nested PCR based on detecting sequences in the subunit I of the cytochrome c oxidase gene (COI) of the *Eimeria* mitochondrial genome was performed according with methodology previously described (Dolnik, Palinauskas, & Bensch, 2009) molecular studies of wild birds' Coccidia are still in their infancy and are mostly based on DNA extracted from the blood stages of these parasites. Linking microscopic and molecular data requires a method that reliably extracts DNA from single oocysts with parallel detailed morphological examination of the same cell. We offer a thorough manual of isolating, photographing, and trapping single oocysts from avian feces, followed by extraction of parasite DNA and amplification of mitochondrial DNA from the same cells. In 39 single oocysts from 6 wild blackcaps, we combined microscopic studies of individual cells with studies on their mitochondrial haplotype. In 72% of the single oocysts sampled, we detected unambiguous sequences. From feces and blood of investigated birds, we obtained 6 different haplotypes of Isospora sp. (iSAT1-iSAT 6.

The first PCR was performed using the external primers COX tenella F4 (5'G(AT) TCATTAGTATGGGCACATCA3') and COX tenella R (5'CCAAGAGATAATAC(AG)AA(AG) TGGAA3'). The second round was performed with the internal primers COX tenella F2 (5'GGGCACATCATATGATGAC3') and COX tenella R2 (5'ATAGTATGTATCATGTA(AG)(AT)GCAA3').

For the DNA amplification, 0.8 µM of each primer, PCR mixture (0,2 mM of each dNTPs, 2.5 mM MgCl2, 1X PCR Buffer (Invitrogen, USA) and 1.25 U of Platinum Taq DNA polymerase (Invitrogen, USA) were added to 2 µL of isolated DNA. The first PCR was carried out in an Applied System Termocycle® (Applied Biosystems, USA) under the following conditions: 94 °C for 3 min, 12 cycles at 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 30 sec, 15 cycles at 94 °C for 30 sec, 48 °C for 30 sec and 72 °C for 30 sec, and a final extension at 72°C for 10 min. Two microliters of the first PCR product were used in the second amplification. This second PCR was carried out under the following conditions: 94 °C for 3 min, 35 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 10 min. Samples with 250bp length bands were considered positive.

A positive control (genomic DNA from *Eimeria acervulina* obtained from experimentally infected chicken) and negative control (ultrapure water) were included in each PCR reactions. The amplified fragments from the second PCR were visualized under ultraviolet light after the electrophoresis in 1.5% agarose gel stained with SYBR® Safe (Invitrogen, USA) and photodocumented by LPix Imagem ST Software (Loccus Biotecnologia). A 100 bp DNA ladder (Invitrogen, USA) was used in each agarose gel.

Statistical analysis

The Chi-square ($\chi 2$) test corrected by Yates was used to compare the locations of capture and gender using the Epi Info software, version 6 (Dean et al., 1994). A p-value of ≤ 0.05 was considered significant.

Results and Discussion

Out of 214 birds, 171 (79.9%) were positive for *Eimeria* sp. by nPCR. There was a significantly difference of positivity between the places of

capture (p=0.0008), since there were more positive birds from Coop. I (119/136, 87.5%) than in the University (33/54, 61.1%), the Dairy Farm (12/15, 80%) and the Coop. II (7/9, 77.8%) as shown in Table 1.

Table 1
Association between the number of positive *Zenaida auriculata* for *Eimeria* detection by PCR in different site capture from Southern Brazil

Capture site	N	Positive		Negative			
		n	%	n	%	X^2	P
University [a]	54	33	61.1	21	38.9	16.79	0.0008
Crop Coop. I [b]	136	119	87.5	17	12.5		
Dairy Farm [a,b,c]	15	12	80.0	3	20.0		
Crop Coop. II [a,b,c]	9	7	77.8	2	22.2		
TOTAL	214	171	79.9	43	20.1		

N = total number of samples, $\chi 2$ = qui-square, p-value, different letters: a, b, c and d indicate significant differences ($p < 0.05$) in prevalence for the different places.

When analyzing the genders, 103/214 (48.13%) were male and 111/214 (51.87%) were female. The number of positive males were 84/103 (81,5%) and females 87/111 (78,4%), which showed no statistical differences between the genders ($p > 0.05$) and no differences when comparing the genders regarding the capture sites ($p > 0.05$).

To the author's knowledge, this is the first study with molecular prevalence of *Eimeria* sp. in *Z. auriculata* in Brazil. Yang et al. (2016b) studying Eimeria from *Columba livia domestica* in Australia, using the COI gene, described a new species named *Eimeria labbeana*-like. Most of the molecular characterization of *Eimeria* oocysts were done in chickens and turkeys (Hafeez et al., 2015; Ogedengbe et al., 2011; Rathinam et al., 2015)Springer-Verlag Berlin Heidelberg. Species-specific PCR primers targeting the mitochondrial cytochrome c oxidase subunit I (mtCOI, and despite over than 1700 species have been described in different hosts (Yang et al., 2014), there is still a lack of information about *Eimeria* in other birds, including Columbiformes.

A significantly difference was observed between the positivity on samples and the capture sites, especially when analyzing the results from the University and those from Coop. I. This might be because there were more eared doves captured from Coop I, giving a higher prevalence of *Eimeria* in this place, or because the *Z. auriculata* population from there site was much bigger than the one found at the University. The bigger the population, the higher amount of feces to be in contact with, the most birds can be affected. As these birds live in flocks (Adriano & Cordeiro, 2001; Shibatta et al., 2009), they can get infected rapidly from themselves and keep the *Eimeria* in the population.

There was no statistical importance between the genders, demonstrating that both male and female get infected equally. This can be due to their life in flocks and all of them feed on the ground even during the breeding. It is also important as the young population can get infected, and although most of free-living birds present asymptomatic infections, even if they shed oocysts, eimeriosis is reported to damage strongly the young birds,

which can shed more oocysts in the environment, depending on inoculation dose, stress, previous infection, coinfection with other diseases and immunocompetence (Atkinson, Thomas, & Hunter, 2009; Cole & Friend, 1999).

Most of studies with *Eimeria* in pigeons around the world were done with the prevalence of oocyst shedding and morphometric descriptions: in 1991, McQuistion (1991) found a new coccidian in *Zenaida galapagoensis*, named as *Eimeria palumbi*, at the Galapagos Island. Conti & Forrester (1981) studied two populations of *Zenaida macroura* and one population of *Zenaida asiatica* in Florida, USA and they found Eimeria sp. in 33.3%, 49.1% and 6% doves, respectively. In Brazil, Adriano, Thyssen, and Cordeiro (2000) described *Eimeria curvata* n. sp. in 8/46 *Columbina talpacoti* and 5/39 *Scardafella squamatta*. A few years later, Adriano, Thyssen and Cordeiro (2003), based on morphological characteristics of oocysts, first described a new species of *Eimeria* in *Z. auriculata*, named *Eimeria zenaidae* n. sp. González et al. (2004) studied *Z. auriculata* in Chile and found 5.5% (13/235) of then shedding oocysts of *E. labbeana*. These studies show a diversity of species that can be found in doves and the importance to research *Eimeria* worldwide. As *Eimeria* shows a specificity for hosts, it must be given relevancy to the knowledge of species infecting each kind of bird.

Some studies with other species of pigeons also found *Eimeria* oocysts in feces by flotation methods. Sari, Karatepe, Karatepe and Kara (2008) studied 136 domestic pigeons (*Columba livia*) and 115 wild pigeons in Turkey, by Sheather's method. They found 81/136 (59.6%) positive domestic pigeons and 35/115 (30.4%) wild pigeons with coccidian oocysts. They identified *E. labbeana*, *E. columbarum*, *E. columbae* and *Isospora* sp. through morphology, which reveals that the birds can be infected by more than one species of coccidia spread in the environment.

Natala et al. (2009) also using Sheather's method, found 49.2% of positive birds for *Eimeria* sp., while Radfar, Fathi, Asl, Dehaghi and Seghinsara (2011) identified 41 (40.19%) of pigeons with *Eimeria* sp. in Iran. In Brazil, Marques, Cuadros, Silva and Baldo (2007) found 37/43 (86.05%) of domestic pigeons with *Eimeria* sp. oocysts. Ball et al. (2012) described for the first time the *Eimeria mauritiensis* in *Nesoenas mayeri* in Mauritius Island. In the same year, Jamriška and Modrý (2012) described *Eimeria columbapalumbi* n. sp. for the first time, from *Columba palumbus* in Czech and Slovak Republics. Aleksandra and Pilarczyk (2014)the degree of coccidia infection and the effect of the coccidiostat used in the course of the disease in two pigeon lofts located in the West Pomerania province. The material for the study came from 180 birds. A total of 330 faecal samples were investigated with two methods: Willis-Schlaafs (qualitative studied 180 *Columba livia* from two different lofts in a husbandry in West Pomerania Province. They had respectively in loft I and loft II: 17/19 (89%) and 62/67 (93%) of young pigeons, 27/43 (63%) and 28/51 (55%) of adult pigeons with *Eimeria* sp. Morphologically, the oocysts found were described as *E. labbeana*, *E. columbarum* and *E. columbae*. Yang et al. (2016b) first described an *Eimeria labbeana*-like from a *Columba livia domestica* attended in a rehabilitation center in Australia. All this data determine that Columbiformes are infected by different *Eimeria* and although some pigeons show specific species of *Eimeria*, the same species of pigeons show the same range of *Eimeria*. As *Z. auriculata* is spread over the Latin America, we can deduce that we can find the same *Eimeria* in different countries as the ones found in Brazil.

Most of these studies showed higher presence of oocysts in fecal samples, but it does not seem to be related to the total number of samples. The high prevalence found in *Z. auriculata* in this study (79.91%) by PCR can be due to the scraped samples, which could have brought not only DNA from oocyst free on stool, but also DNA from other intracellular stages of *Eimeria* (e.g. sporozoites, meronts).

Molecular methods are needed to complement morphological data, as it may not be enough to give full information about species identity and host specificity. Furthermore, molecular characterization might show the genetic diversity, the phylogenetic relationships and provide information about the geographic distribution. However, PCR methods can underestimate mixed infections, that can sometimes be observed as double peaks on sequence electopherogram, as the sequencing selects the amplification of only one species of parasite (Pérez-Tris & Bensch, 2005; Valkiūnas et al., 2006, 2008) and resolving such multiple infections is important for our understanding of host–parasite relationships. We propose a simple and reasonably accurate method for detecting and resolving multiple infections, based on the analysis of parasite cytochrome b DNA sequences: genetically mixed infections are first identified by double nucleotide peaks on sequence electropherograms, and later retrieved by TA-cloning. We applied this method to wild birds, and to experimentally created mixes with varying proportion of two parasites (Plasmodium spp. and Haemoproteus spp..

PCR studies with Eimeria in pigeons are still limited, so little is known about genetic characterization of this parasites in Columbiformes. As nuclear genomics has shown a lack of reliability on speciation, researchers employed the mitochondrial COI sequence as an alternative marker for molecular identification. Although the usage of COI gene has been increasing in the last few years, there is still not many information in public databases about coccidian COI sequences (Berto et al., 2014) which is why molecular studies about COI genes are important to understand the coccidia, such as the present research. Moreover, the sequencing is needed to describe the genetic characterizations of the species.

Conclusions

The evaluation of Eimeriidae coccidia species is very important not only to contribute to taxonomy and phylogeny, but to estimate the risk of infection of other bird species that could be living at the same environment of *Z. auriculata*. *Eimeria* spp. in *Z. auriculata* was first molecularly described in the present study. Further studies should be done in order identify the *Eimeria* species that infect *Z. auriculata*.

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