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Short Communication

First molecular detection of *Haemoproteus* spp. and *Plasmodium* spp. in eared doves (*Zenaida auriculata*) in Brazil

Primeira detecção molecular de *Haemoproteus* spp. e *Plasmodium* spp. em pombos (*Zenaida auriculata*) no Brasil

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Abstract

The aim of this study was to verify the presence and identify the species of haemosporidian parasites in eared doves (*Zenaida auriculata*) in Brazil. Two hundred and eleven male and female eared doves were trap-captured in four different regions of Londrina city, in southern Brazil. Whole blood was collected in EDTA tubes through heart puncture after euthanasia in a CO_2 chamber. A nested PCR targeting the mitochondrial cytochrome b gene (cyt b) of *Haemoproteus* spp./*Plasmodium* spp. was performed, followed by an enzymatic digestion to identify the genus. Phylogenetic trees were constructed to determine the closely related species. Out of 211 eared doves, 209 (99.05%) were positive for *Haemoproteus* spp. and/or *Plasmodium* spp. RFLP analysis showed that 72.72% (152/209) of eared doves were positive only for *Haemoproteus* spp., 6.22% (13/209) were positive only for *Plasmodium* spp., and 21.05% (44/209) of eared doves had mixed infections. Genetic analysis found four samples that were homologous with *Haemoproteus multipigmentatus* and one that was homologous with *Plasmodium* sp. This is the first molecular study of hemoparasites from eared doves in Brazil, and it is also the first description of *H. multipigmentatus* and *Plasmodium* spp. infection in eared doves in Brazil.

Keywords: Haemosporidian, avian malaria, blood parasites, Columbidae, PCR.

Resumo

O objetivo deste estudo foi verificar a presença e a identificação espécies de parasitas hemosporídeos em pombos (*Zenaida auriculata*) no Brasil. Duzentos e onze pombos machos e fêmeas foram capturados em quatro regiões diferentes de Londrina, sul do Brasil. Amostra de sangue foi coletada em tubos contendo EDTA por meio de punção cardíaca, após eutanásia em câmara de CO_2. Uma nested PCR com alvo no gene mitocondrial citocromo b (cyt b) de *Haemoproteus* spp./*Plasmodium* spp. foi realizada, seguida de digestão enzimática para identificar o gênero. A árvore filogenética foi construída para determinar a relação com outras espécies. Das 211 pombas, 209 (99,05%) foram positivas para *Haemoproteus* spp./*Plasmodium* spp. A análise RFLP demonstrou que 72,72% (152/209) das pombas foram positivas somente para *Haemoproteus* spp.; 6,22% (13/209) foram positivas somente para *Plasmodium* e 21,05% (44/209) das pombas tiveram infecções mistas. A análise genética mostrou quatro amostras homólogas com *H. multipigmentatus* e uma com *Plasmodium* spp. Este é o primeiro estudo molecular de hemoparasitas em pombos no Brasil. E é também a primeira descrição da infecção por *H. multipigmentatus* e *Plasmodium* spp. em pombos *Z. auriculata* no Brasil.

Palavras-chave: Hemosporídeos, malária aviária, hemoparasitas, Columbidae, PCR.

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Introduction

Eared doves *Zenaida auriculata* (Des Murs, 1847) (Aves: Columbiformes), are native to Brazil, and can also be found in both rural and urban areas from the Caribbean Islands to southern Argentina (Shibatta et al., 2009). These birds are considered crop pests, and are also considered pests in cities (Adriano et al., 2003), as they have adapted easily to urbanization. Eared doves have become synanthropic, and often obtain their nest site and materials and food from human resources. In many cities in Brazil, large numbers of doves are a serious concern due to the transmission of diseases, agriculture losses caused by eared doves, and the problems caused by eared dove feces in urban areas (Shibatta et al., 2009).

Haemosporidian parasites occur worldwide in several birds. However, little is known about hemoparasites in doves (Valkiūnas, 2005). *Haemoproteus* spp. and *Plasmodium* spp are genetically closely related, however, they have different life cycles, and pathogenesis (Martinsen et al., 2008). Furthermore, although *Haemoproteus* spp. and *Plasmodium* spp. are known as avian malaria parasites, the World Health Organization defines only *Plasmodium* spp. as malaria parasites (Pérez-Tris & Bensch, 2005).

Molecular diagnostics based on PCR have been frequently used for the identification and differentiation of genera (Pérez-Tris & Bensch, 2005), and the mitochondrial cytochrome b gene has been used to identify haemosporidian, although this method does not differentiate between *Haemoproteus* and *Plasmodium* species (Valkiūnas et al., 2006, 2008). Studies of haemosporidian parasites showed a huge genetic diversity and the possibility to identify undescribed species (Beadell & Fleischer, 2006; Valkiūnas et al., 2010). However, sequencing is necessary to identify the lineages and phylogenetic relationships of haemosporidian parasites (Valkiūnas et al., 2006).

The aim of this study was to verify the presence and identify the species of haemosporidian parasites in eared doves in southern Brazil using molecular methods.

Material and Methods

Between January 2010 and December 2011, 211 male and female eared doves were trap-captured in four different locations in Londrina city (23°08'47" to 23°55'46" S, 50°52'23" to 51°19'11" W), in southern Brazil. The capture sites were as follows: State University of Londrina Campus (University: n = 53), Crop Cooperative I (Coop. I: n = 133), Dairy Farm (n = 16) and Crop Cooperative II (Coop. II: n = 9).

The eared doves were euthanized in a CO_2 chamber, and during the necropsy, blood was collected in EDTA tubes through heart puncture. The blood samples were used for blood smear and stored at -20 ^{0}C until DNA extraction.

All procedures involving the animals were 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 eared doves were euthanized according to the guidelines of the National Council for Animal Experimental Control (CONCEA/Brazil).

Blood smear was performed after blood collection and subsequently dried and fixed by methanol. Then, the smears were stained according to the Giemsa method for 1 hour and microscopic examination was performed under a light microscope (Nikon E200) at 1000x.

DNA extraction was performed using a commercial kit (BioPur Extraction Kit Mini Spin Plus®, Mobius Life Science, Brazil), following the manufacturer's instructions. The extracted DNA was eluted in 50 µL of elution buffer and samples were stored at -20 °C until PCR analysis.

A nested PCR (nPCR) followed by analysis of restriction fragment length polymorphism (RFLP) was performed based on the cytochrome b mitochondrial gene (cyt b). The external primers used were HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3'). These primers are specific for the genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*. The internal primers HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3'), previously described by Bensch et al. (2000), were used in the second reaction for the detection of the *Haemoproteus*/*Plasmodium* genus

The nPCR was performed as previously described by Hellgren et al. (2004), with minor modifications. The PCR reaction was performed in 25 µL, and was composed of 0.6 µM of each primer, 0.2 mM of each of the dNTPs, 2.5 mM $MgCl_2$, 1X PCR Buffer (Invitrogen®, USA), 1.25 U of Platinum Taq DNA polymerase (Invitrogen®, USA), and 5 µl of genomic DNA. The DNA amplification was done in a thermocycler (Veriti, Applied Biosystem, USA), under the following conditions: 95 °C for 3 minutes, 40 cycles at 95 °C for 45 seconds, 48 °C for 45 seconds and 72 °C for 45 seconds, with a final extension at 72 °C for 10 minutes. After the first reaction, 50 µL of ultrapure water was

added to each PCR tube. The second reaction was performed under the same conditions described, except for the primer concentration (0.4 µM of each primer), and using 2 µL of the first diluted amplicon. DNA from *Haemoproteus* spp. obtained from naturally infected pigeons, and *Plasmodium gallinaceum*, obtained from experimentally infected chickens, were used in all PCR reactions as positive controls, while ultrapure water was used as a negative control. Nested PCR products were visualized under UV light (Safer Imager, Thermo Fisher, USA), after electrophoresis on 2% agarose gel stained with Sybr Safe DNA Gel Stain (Thermo Fisher, USA). A 100 bp DNA (Thermo Fisher, USA) ladder was included in all agarose gels, and samples that showed a 525 bp band were considered positive.

For differentiation of *Haemoproteus* spp. and *Plasmodium* spp., an enzymatic digestion was performed in accordance with methodology previously described (Kistler et al., 2013). Enzymatic digestion reaction included 5 units of EcoRV enzyme (New England Biolabs, UK), 1X NEB Buffer, 0.2 µL of BSA, 2 µL of amplicons, and ultrapure water to create a 20 µL sample volume. This reaction was incubated at 37 °C for 60 min, and subsequently the enzyme was inactivated at 80 °C for 20 min. Digested PCR products were submitted to electrophoresis on 2.5% agarose stained gel with Sybr Safe (Thermo Fisher, USA), visualized, and photographed under UV light. A 100 bp DNA (Thermo Fisher, USA) ladder, and positive and negative controls were run in each agarose gel. Bands compatible with *Haemoproteus* spp. and *Plasmodium* spp. were expected to be seen as shown in Kistler et al. (2013).

Samples that tested positives in the nPCR were randomly selected for sequencing analyses. The amplicons were purified with a QIAquick Gel Extraction Kit (Qiagen, GER), and submitted to sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher, USA), using an ABI3500 Genetic Analyzer (Applied Biosystems, USA). Sequences were edited using Bioedit (version 7.2.5) and identified using the nucleotide BLAST application from NCBI. A maximum likelihood tree was constructed with MEGA6 software (Tamura et al., 2013), using the Kimura 2-parameter distance matrix (Kimura, 1980). Statistical analysis was conducted with the bootstrap method in 1,000 repetitions. DNA sequences from *Haemoproteus* spp. and *Plasmodium* spp. standards obtained from GenBank were included in the tree, and *Toxoplasma gondii* was used as the outgroup.

The chi-square ($\chi 2$) test with Yates correction was used to compare differences in infection rates between male and female eared doves, and between the sampling sites, using the software Epi Info (version 6.04). A p-value of ≤ 0.05 was considered significant.

Results

In total, 47.87% (101/211) of the captured eared doves were male and 52.13% (110/211) were female. According to microscopic examinations of the blood smears, 209 (99.05%) were positive. All positive samples in the blood smears was also positive in the nPCR analysis, indicating a high occurrence of *Haemoproteus* spp. and/or *Plasmodium* spp. in these birds. The RFLP analysis showed that 72.72% (152/209) of eared doves were infected with *Haemoproteus* spp., while 21.05% (44/209) had mixed infections, and 6.22% (13/209) of eared doves were infected only with *Plasmodium* spp.

There was no statistical difference ($p>0.05$) between infection rates in males and females at each capture site (University, Coop. I, Dairy Farm, and Coop II). Furthermore, no statistical significance ($p > 0.05$) was observed between the occurrence of *Haemoproteus* spp. and/or *Plasmodium* spp. infection at the different capture sites (Table 1).

Based on the sequencing analysis, four sample, named as HaemZaBr151, HaemZaBr152, HaemZaBr153, and HaemZaBr155 (GenBank accession numbers: MT374716 - MT374718, MT416577) showed similarity with *H. multipigmentatus* (JN788946.1 and JN788947.1) and one (PlasmZaBr154) (GenBank accession number: MT416112) *Plasmodium* sp. showed 99% of similarity with *Plasmodium* sp. (KU057967.1) (Table 2). The phylogenetic analyses (Figure 1) from the consensus sequences obtained in this study agreed with the results obtained with the BLAST tool.

Discussion

This study is the first molecular investigation of hemoparasites in eared doves in Brazil. According to the results, 99.05% of the eared doves were considered positive by microscopic examination and DNA amplification of cyt b, demonstrating that a high number of eared doves infected with *Haemoproteus* spp. and/or *Plasmodium* spp. Based on sequencing analysis, we found *H. multipigmentatus* in eared doves. A previous study found *H. multipigmentatus* in eared doves in Chile, demonstrating that this parasite is present in other countries in South America (Martínez et al., 2016). Adriano and Cordeiro (2001) reported the first description of haemosporidians in Giemsa stained blood smears from three different Columbiformes in Brazil, and found *H. columbae* in 100% of eared doves, in 51.6% of

Table 1. Results of nPCR-RFLP of *Zenaida auriculata* from Brazil.

	N	Positive samples (%)		X^2	p-value
***Haemoproteus* spp.**					
University Campus	53	19	(35.8)	3.64	0.30
Crop Coop. I	133	117	(88.0)		
Dairy Farm	16	10	(62.6)		
Crop Coop. II	9	6	(66.7)		
***Plasmodium* spp.**					
University Campus	53	11	(20.8)	nc	nc
Crop Coop. I	134	1	(0.8)		
Dairy Farm	16	1	(6.2)		
Crop Coop. II	9	0	0		
Mixed infection					
University Campus	53	23	(43.4)	0.64	0.89
Crop Coop. I	133	14	(10.6)		
Dairy Farm	16	4	(25.0)		
Crop Coop. II	9	3	(33.4)		

N: total number of samples in each local of capture; χ2: chi-square; nc: not calculated.

Table 2. Blast results of *Haemoproteus* and *Plasmodium* sequences obtained from eared doves from Brazil.

Parasite	Isolate	Identity (%)	Query coverage (%)	Sequence with maximum % identity	GenBank accession
Haemoproteus multipigmentatus	HaemZaBr151	98.76%	100.00%	JN788947.1 - *Haemoproteus multipigmentatus* isolate SocH16	MT374716
	HaemZaBr152	98.66%	99.00%	JN788946.1 - *Haemoproteus multipigmentatus* isolate SocH15	MT374717
	HaemZaBr153	99.12%	100.00%		MT374718
	HaemZaBr155	98.87%	100.00%		MT416577
Plasmodium sp.	PlasZaBr154	98.66%	100.00%	KU057967.1 - *Plasmodium* sp. NYCNYC01	MT416112

ruddy ground doves (*Columbina talpacoti*) and in 19.3% of scaled doves (*Scardafella squammata*). In other countries, the prevalence of *Haemoproteus* spp., based on microscopy detection, ranges from 15.06% to 45.05% (Radfar et al., 2011), indicating a worldwide distribution of this parasite.

The number of described lineages from avian Haemosporida has increased in the last few years, as recent studies have described *H. multipigmentatus* in *Z. galapagoensis*, *H. multivolutinus* in *Turtur tympanistria* and *H. paramultipigmentatus* n. sp. in *Columbina passerina socorroensis* (Valkiūnas et al., 2010, 2013). Other species of *Haemoproteus* have been detected by PCR in different bird species, such as *Z. macroura* and *Z. graysoni*, demonstrating that *Haemoproteus* spp. are able to infect different species of birds (Carlson et al., 2013; Križanauskienė et al., 2013). Recently, Cepeda et al. (2019) developed a host-parasite system to study haemosporidian in avian hosts and insect vectors, allowing to experimentally characterize the complete life cycle of *H. columbae* (cytb lineage HAECOL1) in *Columba livia.*

In the present study, no statistical significance was observed regarding infection status and gender of doves, corroborating previous results on *Haemoproteus* spp. and *Plasmodium* spp. in rock pigeon (*C. livia*) and captive

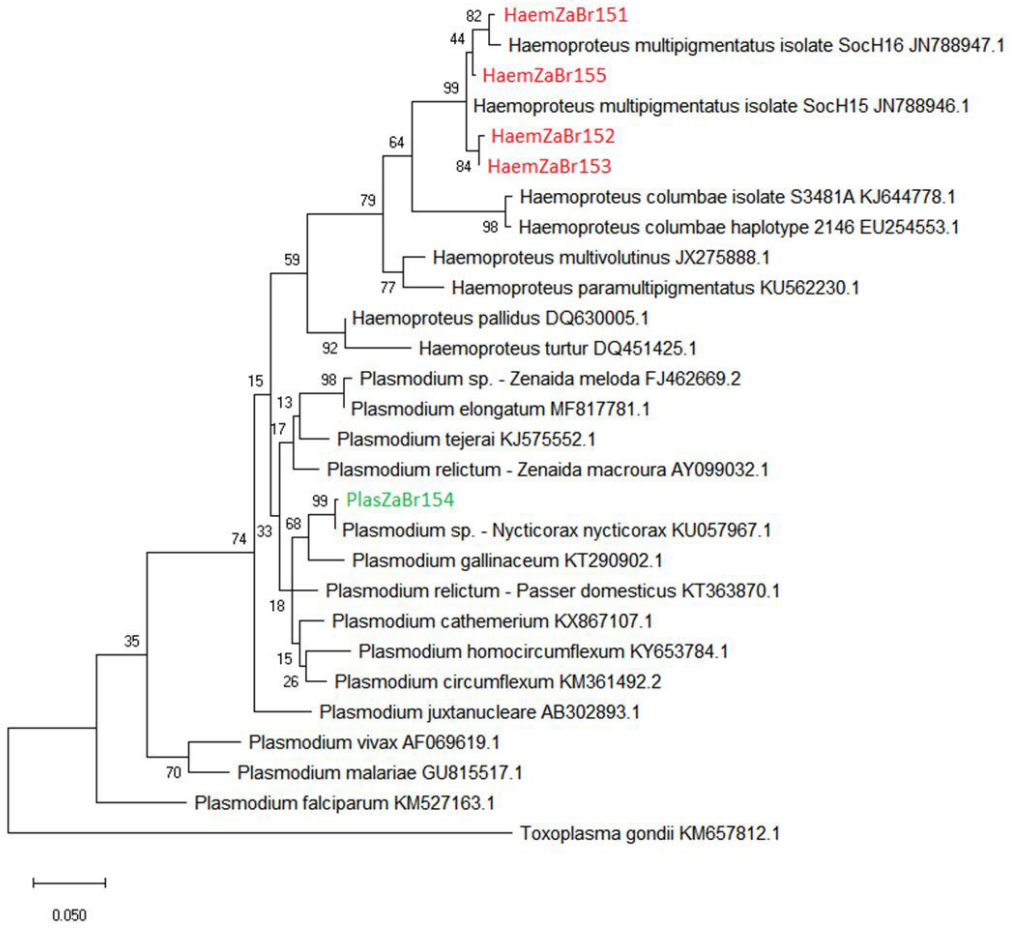

Figure 1. Phylogenetic tree based on the mitochondrial cytochrome b gene sequences of *Haemoproteus* and *Plasmodium* species from *Zenaida auriculata* from Brazil.

birds (Chagas et al., 2016, 2017). However, the rate of coinfection was very significant ($p < 0.01$), showing that the presence of a hemoparasite could predispose birds to another haemosporidian infection (Scaglione et al., 2015).

We observed a high percentage of eared doves that tested positive for haemosporidian infectious by nPCR. A previous genetic study in Brazil with non-Columbiformes also found a high prevalence of *Plasmodium* spp. and *Haemoproteus* spp. (Tostes et al., 2015). Passeriformes were also described as being infected with haemosporidian parasites, with many different lineages, revealing a huge genetic diversity in Brazil, and the importance of more investigations in haemosporidian parasites in the country. A survey of free-living birds from São Paulo Zoo showed that 100% of rock pigeons were positive for haemosporidian parasites in stained blood smears, similar to our molecular findings. However, four *Haemoproteus* lineages were obtained, including *H. columbae*, *H. iwa* and *H. multipigmentatus* (Chagas et al., 2016). *Zenaida auriculata* can cohabit with other animals and humans, what can cause a huge concern regarding transmission of infectious diseases (Barros et al., 2014). In wild birds, the disease could spread into the bird flocks causing decrease of the population and even the extinction of them, if the parasites are not endemic, while in the poultry farms, the presence of this parasite could cause a big economic loss because of loss of appetite, decrease of weight gain and death (Valkiūnas, 2005). Moreover, in the future, this exchange of hosts species could turn into phenotypic and genotypic disorders causing mutation, culminating with the raise of new and more resistant and aggressive strains and species.

Because *Plasmodium* and *Haemoproteus* are genetically closely related, restriction enzyme-based assays has been developed to differentiate between parasites in these to genera (Beadell & Fleischer, 2006; Kistler et al., 2013). Using RFLP, we were able to detect both *Haemoproteus* spp. and *Plasmodium* spp. infections in eared doves, providing accurate information about coinfection in these birds.

According to sequencing analysis, the *Plasmodium* spp. detected in this study showed 99% of similarity of a lineage NYCNYC01, which has been previously described in 16 bird species, including the order Accipitriformes,

Anseriformes, Galliformes, Pelecaniformes, Piciformes, Psittaciformes, and Struthioniformes from the São Paulo Zoo in Brazil. This lineage, considered a generalist parasite, has high degree of similarity of the lineage pPESA01, being both of them described only in birds from South and North America (Chagas et al., 2017; Durrant et al., 2006; Lacorte et al., 2013), however, studies evaluating the genetic diversity of *Plasmodium* spp. in other regions of the world are still scarce.

An ectoparasite was recovered from one dove (0.47%) from Coop. I. The identification was performed based on morphological characteristics under stereomicroscopy according to Graciolli & Carvalho (2003). The parasite was described as pigeon louse fly *Pseudolynchia canariensis*, a known vector of hemoparasites in pigeons (Graciolli & Carvalho, 2003; Valkiūnas, 2005). In Brazil, pigeon louse flies have been reported in eared doves, ruddy ground doves, and scaled doves, suggesting that the rate of transmission of vectors among birds may be high, as they live in large flocks (Adriano & Cordeiro, 2001).

Conclusion

In conclusion, this study found that a large proportion of eared doves were infected with haemosporidian parasites. Additionally, to the best of our knowledge, this is the first molecular characterization of *H. multipigmentatus* and *Plasmodium* spp. in eared doves in Brazil. Further studies should be performed to identify mixed infections and the presence of other blood parasites in this species.

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