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Characterization of aortic and brachiocephalic filariasis by *Filarioidea* sp (Nematoda:Spirurida:Filarioidea) in Mexican ramphastids

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ABSTRACT

The manuscript presented herein documents the findings of filaria nematodes in 5 keel-billed toucans, and one emerald toucanet, originated from 2 private aviaries in Mexico City during two years. The birds displayed ruffled feathers, depression, inability to perch, convulsions, and sudden death. Furthermore, thickened wall of the aortic and brachiocephalic arteries, with connective tissue proliferation and chondroid metaplasia were observed. Molecular characterization matched *Filarioidea* sp (Nematoda: Spirurida: Filarioidea). To the authors' knowledge, this is the first documented report of filariae *Filarioidea* sp. causing mortality in ramphastids in Mexico. This manuscript may contribute to expand current knowledge of filariasis and the health risks and livability of wild birds.

1. Introduction

Filariasis is a parasitic disease associated with nematodes of the *Filarioidea* family. These parasites may infect a variety of vertebrates including reptiles, amphibians, aves, and mammals, such as humans. It is known that approximately 160 filarial species from 16 genera are parasitic to birds. All these species are included in the *Onchocercidae* family (Bartlett, 2008). Nevertheless, it is likely that more *Filaria* species exist, and the infection may occur more frequently than is known or documented to date in scientific reports. Filariae are difficult to find at the necropsy. Some species are only viable for short periods of time and may disappear after breeding and yielding microfilariae. Furthermore, most filarial species are non-pathogenic, thus they are undetected (Bartlett, 2008). However, clinical outbreaks have been reported in wild species of the order Passeriformes, Piciformes, Psittaciformes, Gruiformes, and Falconiformes (Allen et al., 1985; Bartlett and Anderson, 1989; Oniki et al., 2002; Spratt, 2010; López et al., 2011; Madani and Dorrestein, 2012; Muñoz-García et al., 2018).

The genus *Pelicitus*, is the most widely spread filariasis in birds, and the only filarial species that may infect humans and hares. In South America, cases of intraocular filariasis associated with the genus *Pelicitus* (Bartlett and Greiner, 1986; Bain et al., 2011) have been detected in humans. Therefore, avian filariasis may have implications to human public health, and it is important to broaden our understanding of this disease and which avian species might be affected. The objective of the study presented herein is to characterize for the first time cases of aortic and brachiocephalic filariasis in ramphastids in Mexico.

2. Materials and methods

2.1. Case description

Between March 2015 and September 2017, five dead keel-billed toucans (*Ramphastos sulfuratus*) and one emerald toucanet (*Aulacorhynchus prasimus*) were submitted to the Research and Diagnostics Laboratory for Avian Diseases (College of Veterinary Medicine, UNAM). The birds were kept in captivity in two aviaries in Mexico City.

From the first aviary, two female toucans, introduced as adult birds, with two years in the aviary, presented ruffled feathers, depression,

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inability to perch, and convulsions. The birds were typically fed on a fruit-based diet (papaya, watermelon, banana), and were kept in a cage in a natural environment, the birds had no preventive medical care. From the second aviary, two male and one female toucans, and a male emerald toucanet were submitted to the laboratory. All the birds introduced as adult birds and commingled with six more toucans and a military macaw (*Ara militaris*). These birds were fed on a fruit-based diet (watermelon, cantaloupe, papaya), honey, and low-iron pellets. The preventive medical care consists in Vitamin A 2000 U/kg IM once (Vigantol ADE, Bayer de México S.A. de C.V.) and 2000 U/kg PO q 7 days (Vitafort-A, Parfarm México, S.A.) and topic Ivermectin (1 drop to skin 2 times per year). These birds exhibited depression prior to sudden dead.

Upon arrival to the laboratory, the dead birds were subjected to a necropsy (Bello et al., 2012). Tissue samples were collected and preserved in a 10%-formalin solution for 24 h, thereafter embedded in paraffin for histological analysis. The tissues were sliced with a standard microtome to a thickness of 3 μm per slice. Tissues were then stained with standard hematoxylin-eosin. This study was carried out in accordance with the recommendations of the Guidelines of the Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Zootechnics, UNAM.

3. Results

3.1. Necropsy and histopathology

Gross lesions associated with filariasis and their location are described in Table 1. The truncus of the aorta and brachiocephalic arteries were thickened, hardened, and the lumen reduced in three out of six birds. Hypertrophy of the left cardiac ventricle (3/6), moderate hepatomegaly (3/6), *Sarcocystis* cysts in striated muscle (2/6), and renal uratosis (3/6). Body condition was between good and average, and one toucan appeared cachectic.

In the histological analysis, the arterial walls had areas of proliferation of connective tissue and chondroid metaplasia (3/6). In most cases, these areas surrounded parasitic structures visible through a transversal section, and identified as filariae. The parasites presented a thin striated cuticle, with coelomic muscular structures, small digestive tube, and a uterus containing eggs and microfilariae. In the lumen of blood vessels of lungs, heart, encephalon, liver, spleen, and kidney, unsheathed microfilariae were observed. Parasite dimensions were 2-3.5 x 40 - 94 μm; their anterior end was round, their posterior end was sharpened, and their body contained abundant somatic nuclei. Other histological findings were hemosiderosis in liver and spleen (4/6), and multifocal necrotic hepatitis (3/6) (Fig. 1).

3.2. Parasitology

Upon necropsy, filariae specimens were collected from the aorta artery, observed under the microscope, fixed in 70% ethanol, and cleared with lactophenol (Doster and Goater, 1997). Most specimens collected were microfilariae, and only a few segments of adult phases were observed (Fig. 2). Pictures were taken with a Nikon Coolpix S51 digital camera attached directly to the microscope.

3.3. Molecular biology

Genomic DNA was isolated from 4 samples of paraffin embedded tissue from sections of the aortic artery with intralesional filariae of both aviaries using a DNeasy blood and tissue kit (Qiagen, Ventura CA, USA) according to the manufacturer's instructions. A preliminary removal of paraffin by extraction with xylene protocol was used (Sepp et al., 1994). For polymerase chain reaction (PCR) amplification, 18S nuclear and 12S mitochondrial partial ribosomal sequence were used, the set of primers were 18SF 5′- GAC GGG CAG CTT CCG GAA ACG -3′ and 18SR 5′- CCG CTT TTC TCG AAA CGG CTC A -3′ and 12SF 5′-ATT GAC GGA TGR TTT GTA CC-3′ and 12SR 5′-GTT CCA GAA TAA TCG GCT A-3′ amplifying an expected size of 542 bp and 439 bp, respectively (Muñoz-Garcia et al., 2018). PCR conditions were stablished in a final volume of 25 μL, containing 200 ng of DNA as template, 20 pmol of each primer, 1X PCR buffer (8 mM Tris-HCl, pH 8, 20 mM KCl), 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 1 μL of BSA (1%) and 2 U of *Taq DNA Polymerase* (Invitrogen, USA). Amplification conditions were one cycle at 94 °C for 5 min, 35 cycles including denaturation, annealing and extension steps at 94°C-30 s, 60 °C 1 min to amplify 18S or 56 °C to amplify 12S and 72°C-30 s and a final extension step at 72 °C for 7 min. The presence of amplicons were detected by electrophoresis in 1.5% agarose gel with 0.5 mg/mL ethidium bromide and visualized under ultraviolet (UV) light.

The amplicons were purified and the nucleotide sequence was determined in both directions with Taq FS Dye Terminator Cycle Sequencing Fluorescence-Based Sequencing and analyzed on an Applied Biosystems 3730 xl DNA sequencing system.

Sequences of 18S and 12S genes obtained in this study were subjected to BLAST searches in GenBank database to confirm homology with the filarial sequences and multiple alignment were stablished using the Clustal W and Muscle algorithms included in MEGA software version 7.0.26 (Thompson et al., 1994; Edgar, 2004; Kumar et al., 2016). Phylogenetic reconstruction was conducted using a Bayesian approach with MrBayes version 3.2 (Ronquist et al., 2012). The analysis was performed for 10,000,000 generations with sampling trees every 100 generations. Trees with scores lower than those at the stationary phase ('burn-in') were discarded, and trees that reached the stationary phase were collected and used to build majority consensus trees.

The sequence analyzed with 18S gene showed a scarce genetic variation and 100% of identity was observed with others filarial species, in particular *Onchocerca* sp., *Breinlia* sp., *Dirofilaria* sp. and *Dipetalonema* sp. In contrast, the 12S gene showed high genetic variation, with 91% of identity with *Filarioidea* sp. and with the rest of the

Table 1
Gross lesions and presence of parasites in various organs of ramphastid birds from two aviaries in Mexico City, Mexico.

Bird	Arterial lesions[a]	Filariae	Microfilariae						
			Lung	Liver	Heart	Encephalon	Kidney	Spleen	Size
Aviary 1									
Toucan	+ +	+ +	+	+	+	NI	–	+	40–70 μm
Toucan	–	–	+	+	–	NI	–	–	50–60 μm
Aviary 2									
Toucan	–	+	+ + +	+ + +	+ +	+ +	+ + +	+	44–94 μm
Toucan	+ + +	+ + +	NI	+ +	–	+	+	–	45–80 μm
Toucanet	+	+ +	+	–	+	+	+	NI	48–52 μm
Toucan	–	–	+	+	NI	NI	NI	NI	40–90 μm

Severity of the lesión/amount of parasites, mild or scarce (+), moderate (+ +), severe or abundant (+ + +), not present (−). Not included for evaluation (NI).
[a] Lesions in aorta and brachiocephalic trunk.

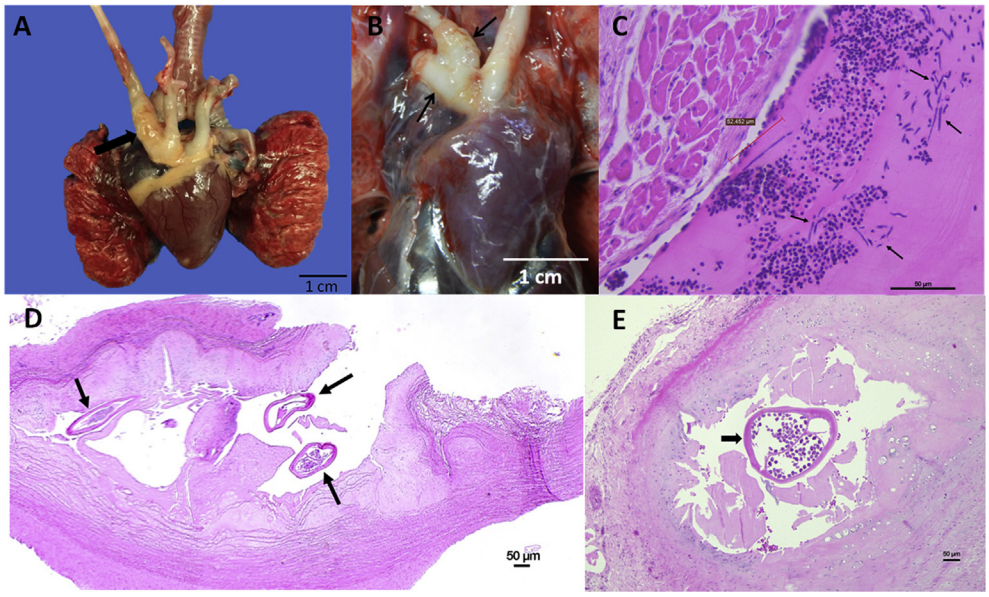

Fig. 1. Lesions associated with filariasis in ramphastid birds submitted to the Research and Diagnostic Laboratory for Avian Diseases, College of Veterinary Medicine-UNAM. (A) Cardiopulmonary system with severe thickening of the aortic trunk (arrow), and moderate hypertrophy of the left ventricle. (B) Heart with severe thickening of the aortic and brachiocephalic trunk (arrows), and left cardiac ventricle hypertrophy. (C) Photomicrography of the heart, in the lumen of the left auricle, there are numerous microfilariae, erythrocytes, and thrombocytes. Hematoxylin-eosin (H&E) stain, bar: 50 µm. (D) Photomicrography of a longitudinal section of the aorta artery. The wall is severely enlarged due to abundant presence of connective tissue, chondroid metaplasia, and adult filariae in a cross section (arrows). In the filarial section, cuticle, coelomic musculature, and a gravid uterus are observed. H&E stain, bar: 500 µm. (E) Photomicrography of the aortic wall (arrow); cross section of an adult, female filaria, surrounded by extensive areas of chondroid metaplasia and connective tissue. H&E stain, bar: 200 µm.

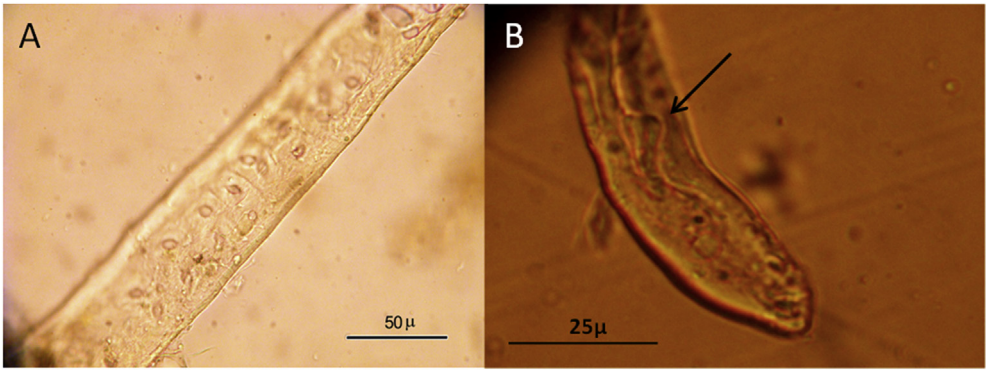

Fig. 2. (A) Mid-section of a filarial specimen. (B) Lateral view of the distal end of a male filaria. Primordial spicules in the copulatory bursa, distinctive of the gender, are observed (arrow).

available filarial species in the GenBank showed values lower than 86%.

The phylogenetic analyzes using the 18S partial sequence showed homoplasy among all filarial species without formation of particular clades. The 12S sequence could clearly separate in clades each of the filarial species and showed a particularly clade that grouped with high values of posterior probability (0.99) to *Filarioidea* sp (JX870434) with the sequence obtained in this work (Fig. 3).

4. Discussion

Gross and microscopic lesions, and morphologic characteristics of the parasites, are compatible with filariasis in the aorta and brachiocephalic arteries. This is described for the first time in keel-billed toucans (*Ramphastos sulfuratus)* and an emerald toucanet (*Aulacorhynchus prasimus*) in Mexico. Most filariae are considered non-pathogenic to avian species, and infections are generally regarded as subclinical. However, filariae detected in this report are deemed pathogenic due to the metaplastic chondroid and connective tissue proliferation observed surrounding the adult filariae. This reaction of the arterial wall tissue leads to reduced lumen, higher resistance to blood flow, and left cardiac ventricle hypertrophy observed in 3 out of 6 birds examined. These findings could have caused the mortality in the birds. Furthermore, the numerous hemosiderophages in liver and spleen might be due to phagocytosis of erythrocytes produced from intravascular hemolysis associated with microfilariae.

Filariosis is endemic in tropical regions, and the development of clinical disease is thought to occur only in those individuals exposed to prolonged contact with the infected fly vector–those living from months to years in an endemic region (Anderson, 2000). According to Lemine et al. (2017), the infection is transmitted in the form of larvae or microfilariae in the blood to vertebrates by an arthropod, are usually mosquitoes of the Culicidae or Phlebotomidae families or flies of the Tabanidae family. However, the pathogenicity of filarias is closely linked to various factors such as the tissue location of the parasite, and the disease is associated with episodes of acute and chronic inflammation (Anderson, 2000). It is likely that chronic inflammation, fibrosis, and aortic artery chondroid metaplasia caused obstruction to blood flow and arterial hypertension, hypertrophy of the left ventricle of the heart, congestion and pulmonary edema, and finally the death of the birds.

Fontenelle et al. (2008) described arterial lesions in green-billed toucans (*Ramphastos dicolorus*) and channel-billed toucans (*Ramphastos vitellinus*) in Brazil. The lesions observed were parasitic nodules on the tunica adventitia of the aortic arch and the brachiocephalic artery, associated with *Dessetfilaria braziliensis* (formerly known as *Dessetfilaria*

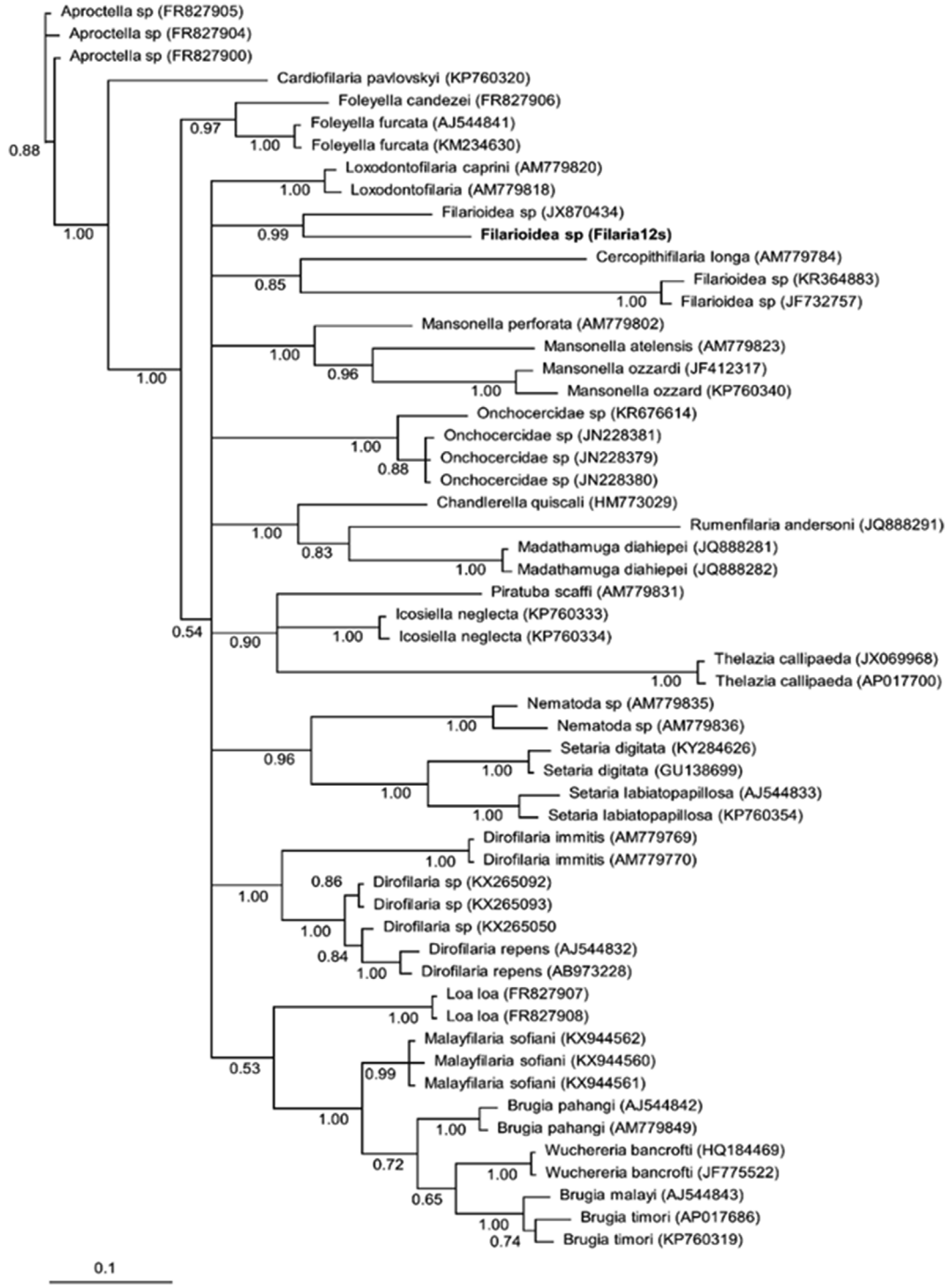

Fig. 3. Bayesian phylogenetic tree using the 12S mitochondrial sequences for different species of filariae. The number of the nodes indicate the values of support or posterior probability.

guianensis) (Bartlett and Bain, 1987). In contrast with the report presented herein, no indication of parasitic damage to the arterial tunica intima or media was mentioned previously. Moreover, the distribution and morphological description of the microfilariae differed as well, thus it is possible that other filarial species were involved in the case. Nevertheless, the lack of whole specimens did not allow a full morphological description of the filariae nor a comparison with published scientific reports. Therefore, molecular biology testing was developed to further identify the parasites.

Although the molecular variation identified in the sequences of the 18S did not aid to identify the genus and species of the analyzed specimen, this sequence corroborated the taxonomic classification of family, in particular this marker is insufficient to establish a molecular diagnosis as other researchers have seen (Huang et al., 2017; Muñoz-García et al., 2018), for this reason it is necessary to evaluate other markers, such as the 12S that showed high genetic variation among all the filarial species available in the GenBank and grouped our specimen together with a new species of filaria (*Filarioidea* sp JX870434) identified in an Owl from North America with an identity of 91%, suggesting a different species. Nevertheless, there are no available 18S and

12S sequences of *Dessetfilaria braziliensis* to compare the sequences obtained in this study and more morphological and molecular studies are necessary to corroborate this finding.

Other genera of filariae within the *Ramphastidae* family are *Pelecitus*, *Chandlerella*, and *Eulimdana*. The genus *Pelecitus* has been detected in the coxofemoral, femoro-tibiotarsal, tibiotarsal-metatarsal, and phalangeal joints. Occasionally, also found the subcutaneous neck tissue (Bartlett, 2008). Nonetheless, there is only one description of *Pelicitus* sp. in a channel-billed toucan (*Ramphastos vitellinus*) associated with filarial tenosynovitis in the hock joint (Madani and Dorrestein, 2012). No parasites were found in any of the joints examined in the birds evaluated in the current study.

Chandlerella braziliensis has been reported in cervical air sacs of a green-billed toucan (*Ramphastos dicolurus)* (Liang-Sheng, 1957) and *Eulimdana micropenis* has been observed in a spot-billed toucanet (*Selenidera maculirostris*). However, parasite morphology or location were not documented (Pinto et al., 1996). Microfilariae have also been reported in ramphastids in Central and South America, although identification of adult filariae was not possible in that report (Sousa and Herman, 1982; Bennett et al., 1991; Young et al., 1993).

Splendidofilaria caperata causes lesions in pulmonary arteries in crows (*Corvus brachyrhynchos*) similar to those found in the birds reported herein. Microfilariae may be trapped in the arterial wall and not found in the blood stream. This condition is known as occult filariasis in crows (Bartlett and Anderson, 1981), which differs from our findings in ramphastids. Microfilariae were found in the majority of the examined organs and tissues, which suggests that the parasites find a way out of the arterial wall into the bloodstream. This migration pattern characteristic may serve as an antemortem diagnostic tool by microscopic examination of blood samples in ramphastid birds.

This type of study needs continuous attention, because species such as toucans and other birds are highly transported worldwide as part of zoos or by illegal traffic, not only causing the mobility of the bird, but also of its parasites to a new niche. This may increase the likelihood of zoonotic transmission and the incidence of atypical diseases as observed by other filarial species in humans (Otranto and Eberhard, 2011). It is possible that these ramphastid birds are aberrant hosts of *Filarioidea* sp because these birds were in captivity and lived with other species. Some authors proposed that the presence of these bird nematodes in mammals is a good example of the parasite's ability to overcome the species barrier (Bartlett and Greiner, 1986).

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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