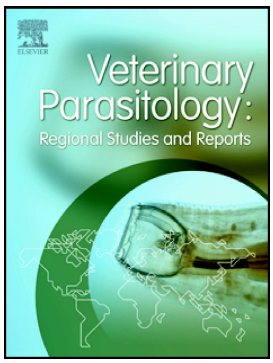

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Morphological and molecular evidence of *Oslerus osleri* (Nematoda: Filaroididae) in the Andean fox (*Lycalopex culpaeus*)

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Abstract

Oslerus osleri is a cosmopolitan filaroid nematode that parasitizes the respiratory system of domestic and wild canids. Natural infection by *O. osleri* is reported in the Andean fox (*Lycalopex culpaeus*) in this study. Nematodes, enclosed in small and compact fibrous nodules of 1 to 5 mm in diameter, were found on the surface of the trachea near the bronchial bifurcation on four Andean foxes during necropsy (one from Cuzco, Peru and three from Northwestern Patagonia in Argentina). The nematodes were identified as *O. osleri* by morphological and molecular methods. Ribosomal and mitochondrial DNA analyses were performed amplifying the second internal transcribed spacer region (ITS-2), the partial cytochrome c oxidase 1 (cox1), and the large subunit of nuclear ribosomal RNA (LSU rRNA) genes. Sequences of the ITS-2 and LSU rRNA had a genetic variation of 1.5% and 1.0%, respectively, with previous sequences of *O. osleri* registered in Genbank. This is the first amplification of the cox1 gene of *O. osleri* and demonstrated an identity of 92% to *Perostrongylus falciformis* (KY365437), and 90% to *Angiostrongylus cantonensis* (KY779735) and *Angiostrongylus costaricensis* (AP017675).

Keywords: Nematode; *Oslerus osleri*; Andean fox; *Lycalopex culpaeus*; Canidae.

Introduction

The Andean fox (*Lycalopex culpaeus*), also called Culpeo, is an endemic South American wild canid with distributions in the Andes, northern Ecuador down to southern Chile and Argentina, and extending to the Pacific coast (Novaro, 1997). The Andean foxes occupy a wide variety of habitats, including deserts, hills, puna grasslands, montane rainforests among others (Novaro, 1997). Some of these habitats overlap with farmland occupied by farmers and their livestock such as alpacas, llamas, sheep and cows (Moro et al., 1998). This close contact with humans and domestic animals possesses a disease transmission risk, as the Andean fox is a definitive host for many infectious diseases. Studies have shown that the Andean foxes act as definitive hosts for *Echinococcus granulosus*, *Taenia hydatigena*, *Taenia multiceps*, *Dipylidium caninum*, *Spirocerca lupi*, *Uncinaria stenocephala*, and *Toxascaris leonina* (Gomez-Puerta et al., 2018; Moleon et al., 2015; Moro et al., 1998; Novaro, 1997), which are common parasites of domestic and shepherd dogs (Zaldivar, 1991).

Oslerus osleri (Syn. *Filaroides osleri*) is an important nematode that parasitizes the trachea and bronchi of domestic and wild canids causing respiratory disease (Conboy, 2009; Yao et al., 2011). This parasite has a global distribution with very few research studies originating from South America (Kontrimavichus and Deliamure, 1985). Currently, *O. osleri* had been only reported in canids from Chile, Colombia, and Brazil (Avelar et al., 2013; Muñoz et al., 2007; Varela-Arias et al., 2014). This study demonstrates the occurrence of *O. osleri* in Andean foxes from Argentina and Peru, confirmed by morphological and molecular diagnosis, adding new geographical records for the parasite.

Material and methods

This study was included in protocols N° 023-2018-MINAGRI-SERFOR-DGGSPFFS approved by the Forest and Wildlife Service of Peru (SERFOR) and N° IF-2020-07979976-APN-DRPN#APNAC of the National Park Administration (Administración de Parques Nacionales) of Argentina. The foxes were found as roadkill in different regions of Northwestern Patagonia in Argentina and Cuzco, Peru (Table 1), during the period 2012-2019. The lungs of 10 Andean foxes (*L. culpaeus*) were macroscopically examined to assess the presence of parasites. Upon inspection of the lungs, several nematodes were found in the trachea and bronchi. The nematodes were collected from the nodules, using fine needles under a stereomicroscope in the fresh trachea, and then preserved in 70% ethanol. The morphological diagnosis was based on 15 male and 15 female nematodes. They were cleared

using an ethanol-phenol solution (50% ethanol, phenol 1V: 2W) to facilitate the observation of internal organs. Photographs and dimensions were taken with the aid of an optical microscope. Measurements are given in range followed by means and standard deviation (SD) values in parentheses. Measurements are expressed in millimeters (mm) and micrometers (µm). The identification was based on the taxonomic keys according to Avelar et al. (2013) and Kontrimavichus and Deliamure (1985).

Total DNA was extracted from some of the specimens for molecular diagnosis using Chelex (Gomez-Puerta et al., 2016). The partial cytochrome c oxidase 1 (cox1) gene, the second internal transcribed spacer region (ITS-2) and the partial large subunit of nuclear ribosomal RNA (LSU rRNA) gene were amplified using previously published primers (JB4.5 – JB3 for cox1, 391 – 501 for LSU rRNA, and NC1 – NC2 for ITS-2) and polymerase chain reaction (PCR) protocols (Bowles and McManus, 1994; Gasser et al., 1993; Nadler et al., 2000). All amplicons were sequenced in an ABI 3100 automated sequencer (Applied Biosystems). Sequences were edited and analyzed using the Chromas Pro software (https://technelysium.com.au/wp/chromaspro). Nucleotide sequences were compared with sequences recorded in the GenBank database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Unique nucleotide sequences of the ITS-2 region, partial cox1 and LSU rRNA genes from this study were deposited into the GenBank database under accession numbers ITS-2 (Accession numbers: MN238776 – MN238785, MT232876, and MT232878), cox1 (Accession numbers: MN231005 – MN231014, MT229983, and MT229994), and LSU rRNA (Accession number: MN227204 – MN227207, MT232877, and MT232879).

Results

Four (three from Argentina and one from Peru) out of 10 foxes were infected with small nematodes in the trachea. The parasitic load was lower in foxes found in Argentina compared to the one found in Peru; they were infected with only 12 to 14 worms each. The fox from Peru had a severe infection. Between eight to 16 nematodes were enclosed in 13 small and compact fibrous nodules (1 – 5 mm in diameter) located on the surface of the trachea in the caudal one third portions near to the bronchial bifurcation (Fig. 1). The nematodes had a short and muscular esophagus. Their buccal cavities were a simple mouth without labia. Male nematodes had small and equal spicules and a gubernaculum. The vulva, in the female nematodes, was located near the anus. These feature characteristics identified the nematodes

as part of the *Oslerus* genus. All measurements were similar between nematodes from Argentina and Peru. The only notable difference was the length of the body. The nematodes from Argentina were longer than those from Peru (Table 2).

Male:

Measurements were taken from five nematodes from Argentina and ten nematodes from Peru. The nematode total body length ranged from 4.43 – 12.40 (7.18; SD = 3.33) mm. The maximum body width ranged from 150 – 168 (161; SD = 6.20) µm. The esophagus was 210 – 263 (232; SD = 14.99) µm long and 48 – 74 (67; SD = 6.38) µm wide. The nerve ring was located 62 – 75 (69; SD = 3.70) µm from the anterior end. The posterior end had two pairs of preanal papillae and three pairs of postanal papillae located ventrally with a curved posterior end. Spicules, uniform in size, measured 101 – 114 (107; SD = 3.89) µm long. The gubernaculum was 22 – 25 (23; SD = 0.86) µm long. The cloacal opening measured 29 – 43 (37; SD = 4.35) µm from the posterior end (Fig. 2).

Female:

Measurements were taken from five nematodes from Argentina and ten nematodes from Peru. The total body length measured 6.84 – 27.40 (13.70; SD = 8.11) mm. The nematodes had a maximum body width of 210 – 400 (294; SD = 66.28) mm. The esophagus measured 231 – 288 (248; SD = 16.05) µm long and 62 – 74 (69; SD = 4.60) µm maximum wide. The nerve ring was located 68 – 84 (73; SD = 5.28) µm from the anterior end. The anus opening was 19 – 38 (31; SD = 5.29) µm from the posterior end. The vulva opening was located 31 – 58 (48; SD = 8.39) µm from the posterior end. The uterus was saturated with eggs of45 – 49 (47; SD = 0.40) µm long and 76 – 81 (77; SD = 0.64) µm wide. The eggs from the terminal uterus contained spiraled larvae of 217 – 266 (245; SD = 13.53) µm long (Fig. 2).

Molecular analysis

Ribosomal and mitochondrial DNA analyses were performed on 10 nematode specimens from Peru and two from Argentina. A total of 520 base pairs (bp) of the ITS-2 region, 400 bp of the cox1 gene, and 933 bp of the LSU rRNA gene were amplified from these 12 nematodes. Sequences of the ITS-2 and adjacent regions (Accession numbers: MN238776 – MN238785, MT232876, and MT232878) were compared with a sequence of *O. osleri* registered in GenBank (Accession number: JQ730005) and they had a genetic variation of

1.5%. This difference corresponded to 8 nucleotide positions represented by 7 transitions (6 G – A, 1 C – T), 1 transversion (A – C), and 1 nucleotide deletion (gap) (Fig. 3).

Partial LSU rRNA sequences (Accession numbers: MN227204 – MN227207, and MT232877 and MT232879) were compared with previous *O. osleri* sequences registered in GenBank. They had an identity of 99.8% with *O. osleri* collected from *Canis latrans* in the USA (Accession number: AY292800) and 99.2% with *O. osleri* collected from *Canis lupus familiaris* in the Czech Republic (Accession number: JX185314). Differences in these sequences were in 5 transitions (3 C – T, 2 A – G) and 3 transversions (2 A – T, 1 G – T), respectively.

Sequences of the cox1 gene (Accession numbers: MN231005 - MN231014, MT229983, and MT229994) showed an identity of 98 to 100%. All sequences from Peruvian nematodes were identical and the sequences from Argentinian specimens were different by only three nucleotide positions (Figure 3). The nucleotide sequences were compared with sequences registered in GenBank and showed an identity of 92% to *Perostrongylus falciformis* (Accession number: KY365437), and 90% to *Angiostrongylus cantonensis* (Accession number: KY779735) and *Angiostrongylus costaricensis* (Accession number: AP017675), all members of the Metastrongylidae family.

Discussion

Natural infections by *O. osleri* in Andean foxes are reported in this work. All morphological parameters of specimens from Peru and some parameters of specimens from Argentina were very similar to previous studies (Avelar et al., 2013; Kontrimavichus and Deliamure, 1985). *Oslerus osleri* from Argentina had a marked difference in body size; they were very large compared to specimens from Peru (see Table 2). However, *O. osleri* diagnosis was confirmed by molecular analysis. The nucleotide sequences of ribosomal and mitochondrial genes from this study were more than 98% similar to sequences referenced from GenBank (Accession numbers: JQ730005, JX185314, and AY292800) (Carreno and Nadler, 2003; Husnik et al., 2016; Verocai et al., 2013). Studies have demonstrated that nematode body size can change by the action of their nutrients and microbial food parameters (So et al., 2012). Likewise, nematode body size and parasite load are negatively correlated (Poulin, 1999; Poulin and Morand, 2000). This fact could explain our finding, since the fox from Peru had a higher parasite load than the foxes from Argentina.

Oslerus osleri is a ubiquitous parasite recorded in many geographical parts of Europa, Asia, Africa, America, and Australia (Kontrimavichus and Deliamure, 1985; Yao et al., 2011). This nematode has been found parasitizing the trachea and bronchi of many canid species, mainly coyotes and wolves (Dias et al., 2012; Kontrimavichus and Deliamure, 1985; Yao et al., 2011), causing tracheobronchial and granulomatous nodule formation (Conboy, 2009) like in this report (Fig. 1). This verminous tracheobronchitis in domestic dogs can be asymptomatic in mild infections, but the nematodes can cause tissue damage in serious parasitosis, which could cause dyspnea, cough, nasal discharge, and even pneumonia (Conboy, 2009). However, the majority of studies in wild canids were discovered by incidental findings; none of them evaluated the effect of *O. osleri* in the host. Therefore, the consequences of *O. osleri* on the health and ecology of wild canids are not clear yet.

Evaluation of DNA-barcoding is an efficient method for the rapid identification of some nematode parasites (Jex et al., 2015). However, one limitation of this method is the absence of DNA sequence profiles in a database like GenBank, which can be used as references. Currently, there are few molecular studies performed in *O. osleri*, which have analyzed various genetic markers that include ribosomal genes (large and small subunit) and the ITS-2 region (Carreno and Nadler, 2003; Husnik et al., 2016; Verocai et al., 2013). In this study, we amplified partial cox1, partial LSU rRNA, and the full ITS-2 region. Analysis of the LSU rRNA gene and the ITS-2 region showed an identity of 99.2% and 98.5%, respectively to previous *O. osleri* nucleotide sequences from GenBank (Accession numbers: JX185314, AY292800, and JQ730005) (Carreno and Nadler, 2003; Husnik et al., 2016; Verocai et al., 2013). Also, this is the first partial amplification of the cox1 gene for *O. osleri* and its sequence analysis showed an identity of 98% and 100% among 12 specimens analyzed.

In South America, *O. osleri* has been found in wild canids from Brazil, Chile, and Colombia, including the Andean (*L. culpaeus*) and Chilla foxes (*Lycalopex griseus*) (Oyarzún-Ruiz et al., 2020). However, many of these studies are ambiguous because they lack an adequate morphological diagnosis (Dias et al., 2012; Varela-Arias et al., 2014). Our study performed a brief morphological description and a molecular analysis for *O. osleri*, confirming the presence of the parasite in South America. This finding registers new geographical areas as well as a new host for the parasite.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Table 1. Data from the Andean foxes included in this study.

ID	Age	Host sex	Collection date	Country	Province	Locality	Coordinates	Infection status
1	Adult	NR*	August, 2012	Argentina	Neuquén	Los Lagos, Anfiteatro Route 237	40°59´S - 70°05´W	Negative
2	Adult	Female	January, 2014	Argentina	Neuquén	Los Lagos, Route 40 North	40°48´S - 71°35´W	Negative
3	Adult	Female	January, 2014	Argentina	Neuquén	Seccion Limay, Route 2	41°03´S - 71°08´W	Negative
4	Adult	Male	October, 2015	Argentina	Neuquén	Quin[illegible]queo	40°57´S - 71°19´W	Negative
5	Adult	Male	February, 2016	Argentina	Neuquén	Co[illegible]encia Traful	40°43´S - 71°05´W	Negative
6	Adult	Male	March, 2018	Argentina	Río Negro	Las Chacras	41°06´S - 71°12´W	Positive
7	Adult	Female	April, 2018	Argentina	Río Negro	Catedral Mountain	41°10´S - 71°26´W	Positive
8	Juvenile	NR*	May, 2018	Argentina	Río Negro	Cerro Frey Mountain Path	41°10´S - 71°26´W	Negative
9	Adult	Male	May, 2018	Argentina	Río Negro	Virgen de las Nieves Route 82	41°07´S - 71°24´W	Positive
10	Adult	Female	October, 2016	Peru	Cusco	Canchis, Sicuani-Condorsenca Road Km 18.	14˚15'S - 71˚09'W	Positive

* Not recorded

Table 2. Measurements of *Oslerus osleri* (in µm) isolated from the Andean fox (*Lycalopex culpaeus*) from Argentina and Peru

Country	Argentina			Peru			Consensus*		
	n	Range	Mean (SD)	n	Range	Mean (SD)	n	Range	Mean (SD)
Adult male									
Total body length (mm)	5	10.20 - 12.40	11.66 (1.0)	10	4.43 - 5.77	4.94 (0.4)	15	4.43 12.40	7.18 (3.33)
Maximum body width	5	150 - 167	157 (9.1)	10	159 - 168	163 (2.9)	15	150 - 168	161 (6.20)
Esophagus length	5	221 - 252	236 (12.6)	10	210 - 263	230 (16.40)	15	210 - 263	232 (14.99)
Esophagus width	5	48 - 74	62 (10.1)	10	68 - 71	69 (1.0)	15	48 - 74	67 (6.38)
Nerve ring	5	62 - 75	71 (5.4)	10	65 - 70	67 (1.5)	15	62 - 75	69 (3.70)
Spicules	5	103 - 114	109 (5.5)	10	101 - 111	107 (2.9)	15	101 - 114	107 (3.89)
Gubernaculum	5	23 - 25	24 (1.1)	10	22 - 24	26 (0.6)	15	22 - 25	23 (0.86)
Cloacal opening from the posterior end	5	38 - 43	41 (2.0)	10	29 - 40	35 (3.9)	15	29 - 43	37 (4.35)
Adult female									
Total body length (mm)	5	24.50 - 27.40	25.80 (1.38)	10	6.84 - 11.50	8.87 (1.84)	15	6.84 - 27.40	13.70 (8.11)
Maximum body width	5	365 - 400	383 (14.31)	10	210 - 302	259 (36.83)	15	210 - 400	294 (66.28)
Esophagus length	5	259 - 288	269 (13.67)	10	231 - 252	239 (6.25)	15	231 - 288	248 (16.05)
Esophagus width	5	60 - 62	62 (1.00)	10	68 - 74	71 (1.90)	15	62 - 74	69 (4.60)
Nerve ring	5	77 - 84	81 (3.11)	10	68 - 73	70 (1.88)	15	68 - 84	73 (5.28)
Anus	5	19 - 29	24 (4.08)	10	32 - 38	34 (1.98)	15	19 - 38	31 (5.29)
Vulva	5	31 - 48	38 (7.23)	10	47 - 58	53 (3.61)	15	31 - 58	48 (8.39)
Larvae length	8	240 - 261	251 (8.40)	10	217 - 266	241 (15.63)	18	217 - 266	245 (13.53)

* Consensus indicates measurements of the *O. osleri* collected from all infected foxes from Argentina and Peru.

Figure 1. Macroscopic features of the trachea of the Andean fox (*L. culpaeus*). (a) Dissected trachea and bronchi to show compact fibrous nodules (indicate by arrow). (b) Numerous worms are observed inside the nodules (indicate by arrow).

Figure 2. (a) Posterior region of an adult male stage of *O. osleri* from the Andean fox. Note the spicules (indicate by arrow) (scale bar = 100 μm). (b) and (c) Adult female stage of *O. osleri* from the Andean fox with details of the anterior and posterior extremities. The cephalic region in lateral view. Note the esophagus short and muscular (indicate by arrow). At the caudal end, note the presence of larval eggs (scale bar = 100 μm). (d) Presence of larvae in the uterus of *O. osleri* (scale bar = 50 μm).

Figure 3. Genetic diversity of *O. osleri* throughout analysis of the partial cox1 gene, the ITS-2 region and the partial LSU rRNA gene. A dot (.) indicates identical nucleotides and dash (-) indicates gaps with the sequence used as control from GenBank. (*) and (**) sequences used in the cox 1 gene are of *O. osleri* from Peru and Argentina, respectively. (*) The sequence used in the ITS-2 region is a consensus of all *O. osleri* sequences from the Andean fox (N° MN238776 – MN238785, MT232876 and MT232878). (*) The sequence used in the LSU rRNA gene is a consensus of all *O. osleri* sequences from the Andean fox (N° MN227204 – MN227207, MT232877, and MT232879).

- The first finding of *Oslerus osleri* (Nematoda: Filaroididae) in the Andean fox (*Lycalopex culpaeus*)
- Original data supporting the first molecular characterization of the *O. osleri* collected in South America
- We give a brief morphological description of the *O. osleri* recovered at necropsy from Andean foxes