Journal of Helminthology

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Visceral infection by *Porocephalus* spp. (Pentastomida) in Neotropical wild mammals

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Short Communication

Cite this article: Gomez-Puerta LA, Basselly L, Lopez-Urbina MT, Gonzalez AE, Mayor P (2020). Visceral infection by *Porocephalus* spp. (Pentastomida) in Neotropical wild mammals. *Journal of Helminthology* **94**, e141, 1–5. https://doi.org/10.1017/S0022149X20000231

Received: 21 December 2019
Accepted: 2 March 2020

Key words:
Pentastomida; *Porocephalus*; pentastomiasis; Amazon; wildlife

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Abstract

Larval stages of pentastomids were collected from different organs of small mammals from the Peruvian Amazon. These parasitized mammals included: a western Amazonian oryzomys (*Hylaeamys perenensis*), an elegant oryzomys (*Euryoryzomys nitidus*), a lowland paca (*Cuniculus paca*), two kinkajous (*Potos flavus*), two silvery woolly monkeys (*Lagothrix poeppigii*) and a brown-mantled tamarin (*Leontocebus fuscicollis*). Pentastomids were found in the mesentery and parenchyma of the liver and lungs of these animals. All pentastomids were morphologically identified as nymphs of *Porocephalus* spp. Only the nymphs collected from select animals (the western Amazonian oryzomys, the elegant oryzomys and the brown-mantled tamarin) were analysed molecularly. Molecular analysis was performed amplifying the mitochondrial cytochrome *c* oxidase subunit I gene from select nymphs collected from the western Amazonian oryzomys, the elegant oryzomys and the brown-mantled tamarin. The nucleotide sequences exhibited 95.8–97.7% similarity between them. Also, these sequences showed an identity of 95.8–97.9% to *Porocephalus crotali* (GenBank accession numbers MG559647–MG559655). Molecular analysis indicated the presence of at least two *Porocephalus* species. These findings represent the first record of *Porocephalus* in these mammals, thus adding new intermediate hosts for this pentastomid genus. This work represents the first molecular data of *Porocephalus* in a Neotropical climate.

Introduction

Pentastomida is a group of obligate parasites genetically related to crustaceans and is represented by approximately 131 species (Christoffersen & De Asis, 2015). Reptiles, mainly snakes and crocodiles, are most frequently the definitive host for many of the species of pentastomids. These hosts are parasitized by species of the families Porocephalidae (snakes) and Sebekidae (crocodiles) (Christoffersen & De Asis, 2013). Members of Porocephalidae use small mammals as intermediate host, while those of Sebekidae use freshwater fishes (Riley, 1986).

In the Neotropical region, the genus *Porocephalus* (Porocephalidae) parasitizes the respiratory system of crotalid and boid snakes. Their immature stages have been found encysted in the lungs, liver and mesenteries of a variety of small mammals (Esslinger, 1962; Riley & Self, 1979; Christoffersen & De Asis, 2013). In Peru, three species of *Porocephalus* have been found in snakes (as definitive hosts): *Porocephalus clavatus* in *Boa constrictor* and *Epicrates cenchria*; *Porocephalus crotali* in *Bothrops atrox*; and *Porocephalus stilessi* in *Lachesis muta* (Tantaleán & Gozalo, 1985; Gárate *et al*., 2007; Gomez-Puerta *et al*., 2011). However, reports of the occurrence of *Porocephalus* in intermediate hosts are scarce. In the present report, nymphal stages of *Porocephalus* spp. were collected from small- and medium-sized Peruvian mammals and identified using morphological and molecular methods. The results of this survey add new intermediate hosts for the genus *Porocephalus*.

Material and methods

The research protocol was approved by the Research Ethics Committee for Experimentation in Wildlife at the Dirección General de Flora y Fauna Silvestre from Peru (0350-2012-AG-DGFFS-DGEFFS) and by a Resolution of the Head of the National Reserve of Pucacuro (no. 03-2012-SERNANP-RN Pucacuro–Jef). Some parasite specimens from the parasite collection of the School of Veterinary Medicine, Universidad Nacional Mayor de San Marcos (SERFOR RDG no. 023-201) were included in this study. Host data and collection date are summarized in table 1.

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Table 1. Hook measurements of nymphal stages of *Porocephalus* spp. isolated from different wild mammals from the Peruvian Amazon.

Host ($n = 1$)		*Hylaeamys perenensis*[a]			*Potos flavus*			*Potos flavus*			*Cuniculus paca*			*Lagothrix poeppigii*			*Lagothrix poeppigii*			*Euryoryzomys nitidus*[a]			*Leontocebus fuscicollis*[a]		
Infected organ		Liver and mesentery			Liver			Lung			Lung			Lung			Lung			Liver			Lung		
Sex		Male			Male			Female			Male			Male			Female			Male			Male		
Department		Madre de Dios			Loreto			Loreto			Loreto			Loreto			Loreto			Madre de Dios			Loreto		
Locality		El Carmen			Datém del Marañón			Datém del Marañón			Ramón Castillas			Ramón Castillas			Ramón Castillas			El Carmen			Maynas		
Collection date		11-Oct-2010			19-Jul-2014			7-Sep-2013			6-Jun-2015			17-Jan-2015			20-Mar-2014			19-Jul-2016			21-Mar-2017		
Measurements[b]		Range	Mean	SE	Range	Mean	SE	Range	Mean	SE	Range	Mean	SE	Range	Mean	SE	Range	Mean	SE	Range	Mean	SE	Range	Mean	SE
Total specimens collected		13			8			6			1			1			1			1			1		
Body (mm)																									
Length		8.8–11.2	9.7	0.4	7.3–10.2	8.7	0.4	6.5–8.4	7.7	0.4	8.1			12.2			12.9			10.1			12.5		
Maximum width		1.4–1.6	1.5	0.03	1.5–2.2	1.9	0.1	1.5–1.8	1.7	0.7	1.6			1.6			2.0			1.7			1.6		
Number of annuli		38–42	40	0.7	40–42	41	0.3	40–41	41	0.3	43			50			43			40			40		
Mouth (μm)																									
Length		237–336	300	20.6	275–368	316	13.8	228–245	238	7.8	237			278			267			242			303		
Width		191–279	222	15.9	154–181	168	3.4	178–196	190	1.4	160			173			212			163			193		
Lateral hooks (μm)																									
Hook length	AB	190–216	201	3.4	155–177	169	6.7	171–175	173	2.1	148–149	148	0.2	164–173	168	4.3	202–204	203	1.3	189–210	200	10.7	165–175	170	5.0
Base length	BC	230–257	242	3.6	227–242	233	4.6	241–242	241	0.7	156–177	166	10.1	172–180	176	4.1	205–220	212	7.6	233–237	235	2.4	239–259	249	10.0
Gap of blade	AC	88–124	103	4.9	132–140	137	2.4	128–133	130	2.2	90–101	95	5.6	99–108	104	4.1	111–125	118	6.7	104–107	106	1.9	139–151	145	6.1
Total length	AD	250–296	282	4.9	265–278	270	4.0	273–277	275	2.4	248–252	250	2.1	250–259	255	4.6	299–314	306	7.6	275–276	276	0.7	302–305	303	1.2
Basal length	CD	276–318	298	5.9	272–292	282	5.8	299–307	303	4.2	217–231	224	6.9	215–236	225	10.4	263–267	265	2.1	275–278	277	1.6	329–332	331	1.3
Blade curvature	E	107–132	119	3.0	85–98	92	3.7	96–100	98	1.8	74–84	79	5.2	86–101	93	7.5	81–91	86	4.7	110–111	110	0.8	99–117	108	9.0
Accessory shield length	G1–G2	113–134	123	2.1	114–120	117	1.6	112–121	116	4.4	136–141	139	2.4	114–120	117	3.1	129–131	130	1.3	109–112	111	1.3	136–137	136	0.2
Fulcrum length	F1–F2	347–395	378	5.1	358–397	376	9.6	355–368	361	6.8	314–338	326	11.8	269–276	273	3.5	368–383	375	7.8	314–316	315	0.9	366–373	369	3.3
Internal hooks (μm)																									
Hook length	AB	159–223	194	6.5	158–190	174	6.8	169–178	174	5.0	144–161	152	8.6	187–191	189	2.0	194–208	201	7.0	217–218	218	0.8	167–171	169	1.8
Base length	BC	225–258	245	3.8	221–230	225	2.1	215–225	220	5.0	171–191	181	10.0	182–190	186	3.8	199–200	200	0.6	196–204	200	4.4	200–203	202	1.7
Gap of blade	AC	100–148	125	5.0	115–142	132	6.0	101–135	118	17.2	107–113	110	2.9	101–109	105	4.0	108–116	112	3.8	112–116	114	1.7	119–127	123	4.0
Total length	AD	240–315	292	8.8	224–282	253	13.2	280–302	291	11.1	248–259	254	5.2	254–255	255	0.8	293–294	293	0.6	271–274	273	1.9	292–302	297	5.0
Basal length	CD	278–322	304	5.2	258–283	271	6.0	284–301	292	8.4	229–253	241	11.9	226–234	230	3.9	261–271	266	4.9	230–245	238	7.7	258–279	268	10.5
Blade curvature	E	104–129	115	2.4	86–124	108	8.1	88–101	95	6.4	72–82	77	5.0	91–96	94	2.9	94–96	95	0.8	96–107	101	5.3	104–110	107	3.4
Fulcrum	F1–F2	346–417	389	6.9	322–335	331	3.0	331–338	335	3.6	319–335	327	7.8	238–253	246	7.5	349–407	378	29.1	320–326	323	3.1	369–370	370	0.8

[a]Specimens from the parasite collection of the School of Veterinary Medicine, Universidad Nacional Mayor de San Marcos (SERFOR RDG no. 023-201).
[b]The measurements are expressed in millimetres (mm) and micrometres (μm). SE, standard error.

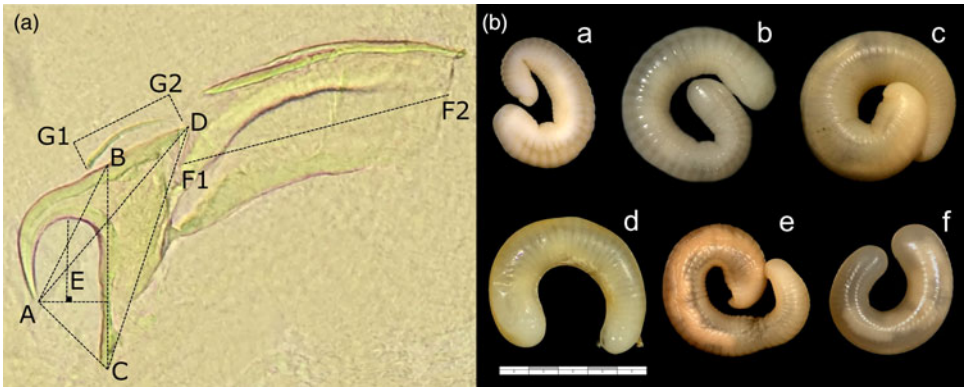

Fig. 1. (a) Lateral hook of *Porocephalus* nymph from *Hylaeamys perenensis*. Hook measurements used in this study: AD, total length of the hook; AB, length of the hook measured in a straight line from point A to point B; AC, distance between the tip of the hook (point A) and the most prominent point of the base (point C); BC, length of the base, from point B to point C; CD, distance in a straight line between points C and D; E, hook curvature; F1–F2, fulcrum length; G1–G2, accessory shield length. (b) *Porocephalus* nymphs collected from *H. perenensis* (1), *Leontocebus fuscicollis* (2), *Lagothrix poeppigii* (3), *Euryoryzomys nitidus* (4), *Lagothrix poeppigii* (5) and *Cuniculus paca* (6). Scale bar: 5.0 mm.

Pentastomids were fixed and preserved in 10% formaldehyde or 70% ethanol. All pentastomids collected were analysed morphologically, measured and, finally, body rings or annuli were enumerated. Hooks were dissected using fine needles and were mounted directly onto glass slides in Berlese's medium to facilitate examination of their morphology. Morphometric examination of hooks included eight different dimensions (fig. 1a). Photographs and measurements were taken using a Carl Zeiss microscope, Axioskop 40 (Göttingen, Germany), and the software Leica IM50 version, 4.0 R117 (Leica Microsystems, Wetzlar, Germany).

Molecular analysis involved total DNA extraction from internal tissue samples of select specimens using the Chelex method (Gomez-Puerta *et al.*, 2016) and a salting-out method (Kelehear *et al.*, 2014). A total of three pentastomids were studied molecularly – one from the western Amazonian oryzomys, one from the elegant oryzomys and one from the brown-mantled tamarin. A polymerase chain reaction was performed to partially amplify the mitochondrial cytochrome *c* oxidase subunit I gene (*cox*1) using a previously published protocol (Kelehear *et al.*, 2011) and primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). Amplicons were sequenced using an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). The MEGA-X software (http://www.megasoftware.net/) was used to create a phylogenetic tree via the neighbour-joining method with Kimura two-parameter distance. Nucleotide sequences from this study were registered in the GenBank database.

Results

A total of 31 pentastomids collected from eight hosts were studied morphologically. These pentastomids corresponded to the immature stage (nymphs) of *Porocephalus* spp., and they were found encysted mostly in the hosts' liver (13 nymphs), followed by the lung (ten nymphs) and then the mesentery (nine nymphs) (fig. 1b). All specimens had numerous annuli in the body (38 to 43) and the cephalic region contained two pairs of oral hooks (lateral and internal). Morphological analysis showed the occurrence of at least five *Porocephalus* morphospecies: type 1 were collected from one western Amazonian oryzomys (*Hylaeamys perenensis*) and one elegant oryzomys or elegant rice rat (*Euryoryzomys nitidus*); type 2 from two kinkajous (*Potos flavus*) and one brown-mantled tamarin (*Leontocebus fuscicollis*); type 3 from one lowland paca (*Cuniculus paca*); type 4 from one silvery woolly monkey (*Lagothrix poeppigii*); and, finally, morphospecies type 5 were collected from the second silvery woolly monkey.

Three Amazonian rodents were infected by more than one pentastomid. One western Amazonian oryzomys was infected with 13 *Porocephalus* nymphs found in the mesentery and the liver. One elegant oryzomys or elegant rice rat was infected with one *Porocephalus* nymph in the liver. One *Porocephalus* nymph was collected from the lung of a lowland paca (*C. paca*). The morphological parameters of these nymphs are tabulated in table 1.

Two kinkajous (*P. flavus*), a carnivorous mammal of the family Procyonidae, were examined post-mortem and both were parasitized with pentastomids. One kinkajou was infected in the hepatic parenchyma and the other in the pulmonary parenchyma. A total of eight and six *Porocephalus* nymphs were collected from the liver and lungs, respectively. The morphological dimensions are mentioned in table 1.

The pulmonary parenchyma of a brown-mantled tamarin (*L. fuscicollis*) and two silvery woolly monkeys (*L. poeppigii*) was each infected with one *Porocephalus* sp. nymph. The nymph from the brown-mantled tamarin was 12.5 mm long and 1.6 mm wide, with 40 annuli over the entire body length. *Porocephalus* sp. nymphs from each silvery woolly monkey varied morphologically. The morphological measurements are shown in table 1.

A total of 673 base pairs of the *cox*1 gene were amplified in three *Porocephalus* nymphs from the western Amazonian oryzomys, one from the elegant oryzomys and one from the brown-mantled tamarin (GenBank accession numbers MK903613–MK903617). Analysis of nucleotide sequences of the *cox*1 gene showed three distinct sequences with differences ranging from 2.3 to 4.2%. Differences occurred at 29 alignment positions, 27 transitions (19 C–T and eight A–G) and two transversions (one A–T and one A–C). In addition, the nucleotides G + C content of these sequences ranged from 37.44 to 38.34%.

Nucleotide sequences from this study were specific to each host, which formed three clades on the neighbour-joining tree (fig. 2). Also, these sequences were compared with sequence references from GenBank and showed an identity of 95.8–97.9% to *P. crotali* collected from the Burmese pythons (*Python bivittatus*)

https://doi.org/10.1017/S0022149X20000231 Published online by Cambridge University Press

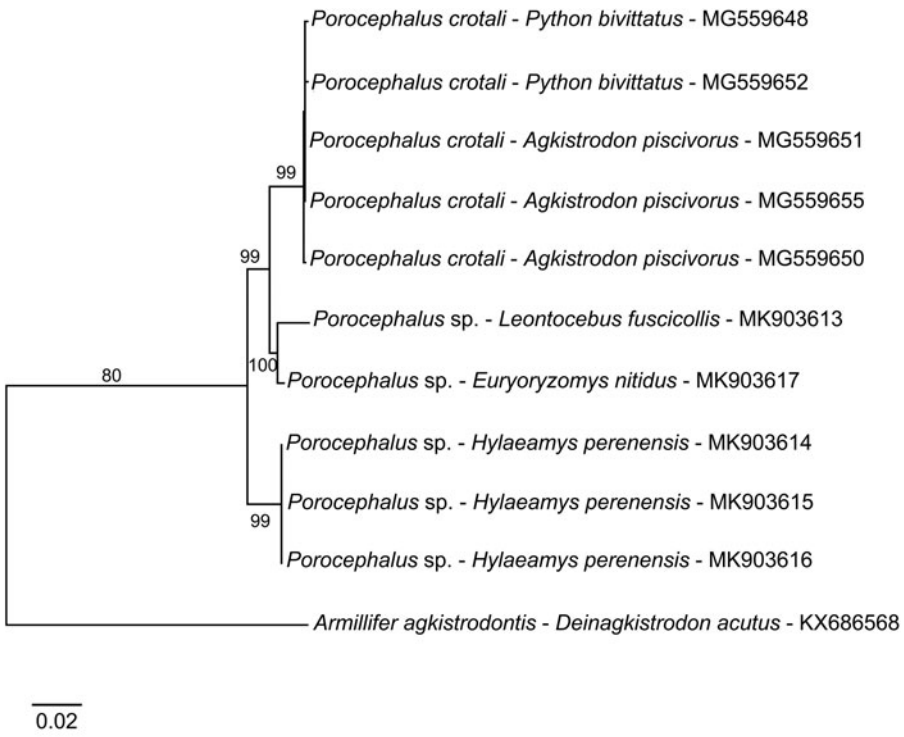

Fig. 2. Phylogenetic tree of nucleotide sequences of *cox*1 gene of *Porocephalus* spp. from wild mammals from the Peruvian Amazon and other pentastomids used as references from GenBank (accession numbers KX686568, MG559648, MG559650-2 and MG559655). The scale bar indicates 2 substitutions per 100 nucleotide positions.

and the cottonmouth snakes (*Agkistrodon piscivorus*) (GenBank accession numbers MG559647–MG559655).

Discussion

All pentastomids collected and studied were identified as nymphal stages of the genus *Porocephalus*, based on the morphology of mouth, hooks and the number of annuli (Esslinger, 1962; Vargas, 1970). This was confirmed by molecular analysis, the *cox*1 gene had more than 95% similarity to *P. crotali* collected from the Burmese pythons and the cottonmouth snakes (GenBank accession numbers MG559647–MG559655) (Miller *et al.*, 2018).

The genus *Porocephalus* includes nine species, of which six are exclusive to the Americas (Christoffersen & De Asis, 2013), and three have been recorded in Peru: *P. crotali*, *P. clavatus* and *P. stilesi* (Gomez-Puerta *et al.*, 2011). For many years, *Porocephalus* nymphs have been reported in various species of wild mammals and the great majority of them were identified as *P. crotali* due to the morphological parameters of the hooks (Vargas, 1970; Rego, 1980; Martinez, 1982). However, these parameters have not been consistent in scientific works (Vargas, 1970; Riley & Self, 1979; Rego, 1980). For example, Vargas (1970) used measurements of total length (AD), gap of blade (AC), basal length (CD), blade curvature (E) and fulcrum length (F1–F2), while Rego (1980) used measurements of hook length (AB), base length (BC) and fulcrum length (F1–F2). For this reason, in our study, we included all the parameters used in these studies in order to have a standard for the morphological description of adult stage of *Porocephalus* hooks, which will serve for future studies.

Adult stages of *Porocephalus* show a dimorphism among species. This dimorphism occurs mainly in the hook parameters and the number of annuli (Riley & Self, 1979). However, this has not been demonstrated in larval stages (Vargas, 1970; Miller *et al.*, 2018). In our study, all nymphs studied corresponded to the sixth stage of *Porocephalus* according to annuli and hook morphology (Esslinger, 1962). Some morphological parameters of these *Porocephalus* nymphs were very similar to one another (table 1). However, morphological analysis indicated the occurrence of at least five species. Only the *Porocephalus* nymph collected from a silvery woolly monkey had a greater morphological difference compared with the others. This would indicate that larval stages of some *Porocephalus* species share similar morphological characteristics. However, molecular analysis indicated that at least two *Porocephalus* species were identified (fig. 2).

Procyonids such as raccoons and coatis (Christoffersen & De Asis, 2013) and other mammals may act as intermediate hosts for *Porocephalus* species. This study is the first record of *Porocephalus* nymphs in kinkajous.

Porocephalus nymphs also occur in several species of cricetid rodents (Christoffersen & De Asis, 2013). However, this is the first report of *Porocephalus* nymphs found in the western Amazonian oryzomys, the elegant oryzomys and the lowland paca. Likewise, some reports mentioned infection of immature stages of *Porocephalus* in non-human primates such as monkeys from the families Cebiidae and Callitrichidae (Christoffersen & De Asis, 2013). In the current study, a brown-mantled tamarin (Callitrichidae) and a silvery woolly monkey (Atelidae) were infected with nymphal *Porocephalus* in the lung. Pulmonary infection by *Porocephalus* had been previously reported in a Rio Tapajós saki (*Pithecia irrorata*) from Brazil that experienced sudden death (Pereira *et al.*, 2010). Of the few studies about pentastomids in New World monkeys, there are two reports that mention cerebral infection by *Porocephalus* nymphs in the common squirrel monkey (*Saimiri sciureus*, Cebiidae) (Fox *et al.*, 1972; Hall *et al.*, 1985). For this reason, it is necessary to be thorough and consider the brain in the inspection of mammals, mainly monkeys, at the time of necropsy.

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The mitochondrial *cox*1 gene has been used in many studies regarding the phylogeny and taxonomy of invertebrates (Hu *et al*., 2002; Zhang *et al*., 2014). The *cox*1 gene has proven to be a good marker to differentiate closely related species (Hu *et al*., 2002; Zhang *et al*., 2014). For example, the *cox*1 gene is able to distinguish species of the genus *Taenia*. Nucleotide sequences showed a difference of up to 18% in one study (Zhang *et al*., 2014). In our report, the nucleotide sequences from pentastomids had a difference of up to 5%. The morphological analysis supported by the molecular analysis indicates that the specimens studied are members of the genus *Porocephalus*.

Sequence analysis demonstrated two clades for the nymphs of this study (fig. 2), one for the western Amazonian oryzomys, and the other for the elegant oryzomys and the brown-mantled tamarin. Apparently, the elegant oryzomys and the brown-mantled tamarin were parasitized by the same species of *Porocephalus* (1.7% nucleotide difference). However, futures studies will be necessary to develop molecular analysis on existing *Porocephalus* species; this will help in molecular epidemiological studies regarding diagnosis and taxonomical investigations on *Porocephalus* nymphs.

Financial support. This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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