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Short communication

Angiostrongylus species in wild carnivores in the Iberian Peninsula

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ABSTRACT

A survey of *Angiostrongylus* parasites was carried out between 2003 and 2006 in wild carnivore species in the Basque Country (Northern Spain). Parasitological examination consisted in the dissection of heart and lungs for the extraction of adult worms. Nematodes were identified using morphometrical features and also PCR amplification and sequencing analysis. The animal species included in this study were Eurasian badger (*Meles meles*), Weasel (*Mustela nivalis*), Beech marten (*Martes foina*), Pine marten (*Martes martes*), Polecat (*Mustela putorius*), American mink (*Mustela vison*), Red fox (*Vulpes vulpes*), Wolf (*Canis lupus*), Wild cat (*Felis silvestris*), and Small-spotted genet (*Genetta genetta*). *Angiostrongylus* parasites were only found in foxes and badgers at prevalences of 33.3% and 24%, respectively. Identification of the nematodes by morphometrical features revealed that foxes were infected with *A. vasorum* while badgers were infected by a different species of *Angiostrongylus* most likely *A. daskalovi*. Sequencing data of the second internal transcribed spacer region of ribosomal DNA (ITS2) of isolates from each species confirmed the species difference.

The high prevalence of *Angiostrongylus* found in the present survey, indicates that the wild cycle of two different species of *Angiostrongylus* is present in the Basque Country. To our knowledge this is the first report of *A. daskalovi* in the Iberian Peninsula.

1. Introduction

Angiostrongylus is a metastrongylid nematode genus that includes species found in insectivores, rodents and carnivores. Different species of *Angiostrongylus* have been described worldwide and those infecting carnivores are: *A. vasorum* in dogs, foxes, wolves, coyotes (*Canis latrans*), red pandas (*Ailurus fulgens fulgens*) and badgers in Europe, America and Africa; *A. gubernaculatus* in the badger (*Taxidea taxus*) and striped skunk (*Mephitis mephitis*) in North America; *A. chabaudi* in the wild cat in Italy and *A. daskalovi* in the badger, pine marten and beech marten in Bulgaria (Anderson, 2000; Bourque et al., 2005; Janchev and Genov, 1988; Patterson-Kane et al., 2009; Segovia et al., 2001; Torres et al., 2001; Ubelaker, 1986). Some species inhabit the pulmonary arteries and the right ventricle of the heart of the host, while others colonize the mesenteric veins (Anderson, 2000). The species *A. cantonensis* and *A. costaricensis* may infect humans, who act as a definitive host, producing eosinophilic meningitis with central nervous system symptoms and abdominal angiostrongyliasis, respectively. That means that these parasites may cause serious public health problems (Alicata, 1988; Eckert and Lammler, 1972; Morera and Céspeces, 1971). The definitive host of *A. cantonensis* and *A. costaricensis* are rodents, whereas *A. vasorum* is a canine parasite (Anderson, 2000) and human infections occur when paratenic hosts like fish and crustaceans are ingested (Alicata and Brown, 1962; Alto, 2001).

In the Iberian Peninsula, *A. vasorum* was first reported in 1974 in a fox in the province of Huesca by Tarazona (1974) and then in several cases of disease in dogs during the 80s published by Juste et al. (1993). Later this species was reported again in foxes (Miquel et al., 1993), wolves (Torres et al., 2000) and badgers (Miquel et al., 1993; Torres et al., 2001) and *A. dujardini* in rodents (Cordero

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del Campillo et al., 1994). The life cycle of *A. vasorum* is indirect with mollusks, such as slugs and snails, acting as intermediate hosts (Anderson, 2000). Experimental work with *A. vasorum* has demonstrated that amphibians can act as intermediate hosts as well as paratenic hosts (Bolt et al., 1994).

Since no published data exists of the prevalences of angiostrongylids in wild carnivores in the Basque Country, a survey was carried out to obtain this information.

2. Materials and methods

2.1. Sample collection

Between 2003 and 2006, 162 wild carnivores were collected in the provinces of Gipuzkoa, Bizkaia and Álava, in the Basque Country, Northern Spain. The animals had died of natural causes, were killed in road accidents or were shot for hunting purposes and their carcasses were transported to the laboratory for direct pathological examination within a few hours. Most of them were examined fresh, although a few ($n = 32$) had already been frozen.

2.2. Parasitological examination

Hearts and lungs were dissected and extraction of adult worms of *Angiostrongylus* sp. was prioritised over that of other species. Nematodes were preserved in 70% ethanol, cleared in lactophenol and studied using light microscopy and specialized keys for identification (Guilhon and Cens, 1973; Janchev and Genov, 1988; Soulsby, 1982).

2.3. PCR amplification: DNA extraction and rDNA ITS2 amplification

Total DNA was extracted from preserved adult worms. Briefly, worms were individually chopped with a sterile scalpel blade and resuspended in 100 μl of TE (10 mM Tris and 1 mM EDTA, pH 8). The whole homogenate was digested with proteinase K (20 mg/ml, Invitrogen) and DNA extraction was performed with Qiamp® DNA Blood mini kit (Qiagen) according to the manufacturer's instructions.

The internal transcribed spacer 2 (ITS2) of ribosomal DNA was amplified using primers NC1 (5′-ACGTCTGGTTCAGGGTTGTT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) as described by Gasser et al. (1993). PCR amplification was carried out as described by Caldeira et al. (2003) in a total volume of 25 μl consisting of: 1–10 ng of template DNA, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 of each primer and 0.8 Taq DNA Polymerase (Invitrogen). The conditions in thermocycler were: 90 s at 94 °C and then 39 cycles for: 58 °C for 1 min, 72 °C for 90 s, 94 °C for 50 s and a final extension step at 72 °C for 10 min. 10 μl of the amplified products was visualized on a 3% agarose gel.

2.4. Sequencing

The PCR amplicons (600–650 bp) were purified using an Exo SAP-IT kit (Amersham Biosciences) and subjected to BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Both strands were sequenced with specific primers in ABI 3130 (Applied Biosystems) sequencer. Sequences were compared with those already available in GenBank database by nucleotide sequence homology searches made at the network server of the National Center for Biotechnology Information (NCBI) using BLAST. Multiple sequence alignments were performed using the program AlignX (Vector NTI 8.0 suite, InforMax, North Bethesda, MD, USA) with an engine based on the CLUSTALW algorithm (Thompson et al., 1994). Similarity matrices were then constructed from aligned sequence data by the Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were constructed by the neighbour joining method (Saitou and Nei, 1987) as implemented in the Mega 4 package (Tamura et al., 2007). Stability or accuracy of inferred topologies was assessed via bootstrap analysis of 1000 replicates.

3. Results

Of the 162 animals studied, *Angiostrongylus* sp. was found in 33.3% of foxes and in 24% of badgers (Table 1). None of the other carnivores were infected. Parasitized foxes (75%) and badgers (33%) showed macroscopical lesions of verminous pneumonia. Nematodes found in foxes were identified by morphometrical features as *A. vasorum* (Guilhon and Cens, 1973; Janchev and Genov, 1988) and those found in the badger as *A. daskalovi* (Janchev and Genov, 1988) (Table 2).

The ITS2 region sequences obtained from 3 out of 27 of the parasites recovered from 16 foxes showed 100% homology with the sequences of *A. vasorum* deposited in

Table 1
Presence of *Angiostrongylus* species in wild carnivores from the Basque Country.

Host species	Scientific name	No. of animals	*Angiostrongylus* positive (%)	Species of *Angiostrongylus*
Eurasian badger	*Meles meles*	50	12 (24%)	*Angiostrongylus daskalovi*
Weasel	*Mustela nivalis*	5	0	
Beech marten	*Martes foina*	23	0	
Pine marten	*Martes martes*	11	0	
Polecat	*Mustela putorius*	5	0	
American mink	*Mustela vison*	2	0	
Red fox	*Vulpes vulpes*	48	16 (33.3%)	*Angiostrongylus vasorum*
Wolf	*Canis lupus*	3	0	
Wild cat	*Felis silvestris*	5	0	
Small-spotted genet	*Genetta genetta*	10	0	
Total		162	28 (17.3%)	