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First Report of *Thelazia callipaeda* (Spirurida, Thelaziidae) in Wolves in Italy

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ABSTRACT: *Thelazia callipaeda* (Spirurida, Thelaziidae) infects the eyes of humans and rabbits as well of domestic and wild carnivores (i.e., dogs, cats, and foxes). The first three cases of infection by *T. callipaeda* eyeworms in wolves (*Canis lupus*) are here described with up to 96 nematodes collected from a single animal. The host competence of wolf was demonstrated by the retrieval of adult worms in the eyes of all examined animals. All nematodes collected were evaluated by morphologic and molecular techniques. Sequence analysis of the partial mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1) confirmed that only one haplotype of *T. callipaeda* was present. The competence of wolves as a definitive host for *T. callipaeda* is discussed in view of the relevance of wild fauna in maintaining and spreading eyeworm infection in humans and domestic animals.

Key words: *Canis lupus*, host-specificity, parasite, reservoir, *Thelazia callipaeda*, thelaziosis.

Thelazia (Spirurida, Thelaziidae) nematodes infect the orbital cavities and surrounding tissues of several species of mammals causing mild (e.g., conjunctivitis, epiphora, ocular discharge) to severe (e.g., keratitis, corneal ulcers) injuries (Anderson, 2000). *Thelazia callipaeda* has been referred to as "oriental eyeworm" due to the large distribution in humans (reviewed in Shen et al., 2006) and dogs in the former soviet republics and Asia (reviewed in Otranto et al., 2003). Recently, thelaziosis caused by *T. callipaeda* has been demonstrated in parts of Europe (Italy, France, Switzerland and Germany: Rossi and Bertaglia, 1989; Otranto et al., 2003; Chermette et al., 2004; Hermosilla et al., 2004; Deplazes pers. comm.), with the highest prevalence ever registered in dogs (60.14%) in some municipalities of Southern Italy (Otranto et al., 2003).

Differing from other species of *Thelazia* affecting livestock, *T. callipaeda* displays the broadest range of definitive hosts, including domestic and wild carnivores (e.g., dogs, cats, foxes), rabbits, and humans (Anderson, 2000). Nonetheless, there is no evidence of genetic variability in *T. callipaeda* worms collected from different definitive host species in Europe (Otranto et al., 2005c). In the past few years, studies have elucidated several aspects of the biology of *T. callipaeda*, both in the intermediate host (under experimental and natural conditions, see Otranto et al., 2005a, 2006b) and in the definitive host (Otranto et al., 2004a; Shen et al., 2006). This knowledge had constituted a foundation to further genetic epidemiological investigations at the population level and raised questions on the role played by wild carnivores in maintaining the life cycle of *T. callipaeda* in areas where the competent vector is present (Otranto et al., 2006a). The prevalence of infection in foxes (5.1%) in endemic areas for dog thelaziosis leads one to consider this species as the main sylvatic reservoir of *T. callipaeda* (Rossi et al., 2002; Otranto et al., 2003). No information is available on the role of other wild carnivores as hosts of *T. callipaeda*. In the present paper, the first three cases of thelaziosis in wolves caused by *T. callipaeda* are reported and the competence of this species as a definitive host is discussed.

In March and October 2006, two male adult grey wolves (*Canis lupus*) about 4 yr (wolf 1—W1) and 3 yr of age (wolf 2—W2), respectively, were retrieved dead in the National Park of Gallipoli Cognato (39–46°N, 6–7°E Basilicata region, south-

ern Italy). Wolf 1 was 110 cm long, 65 cm tall, and weighed 40 kg; W2 was 145 cm long, 70 cm tall, and weighed 50 kg.

In July 2006, a third male adult grey wolf (wolf 3—W3) about 2 yr of age (100 cm long, 50 cm tall, and 30 kg) was found dead in the National Park of Pollino (39–40°N, 15–16°E). Wolves were illegally killed and came from an area with a high prevalence of canine thelaziosis (Otranto et al., 2003).

Wolves were necropsied and adult nematodes were retrieved at the inspection of conjunctival sacs. All nematodes were collected by flushing with saline solution (0.9% NaCl), stored in 70% ethanol, and transported to the Parasitological Unit of the Faculty of Veterinary Medicine (University of Bari) for the morphologic and molecular identification.

Nematodes collected were identified according the keys of Skrjabin et al. (1967) and Otranto et al. (2004b). To confirm the morphologic identification, all specimens were processed for molecular evaluation as described previously (Otranto et al., 2005c). Briefly, genomic DNA from single nematodes was isolated using the QIAamp Tissue Kit (Qiagen GmbH, Hilden, Germany) and eluted in 50 µl H_2O. A partial sequence of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1; 689 base pairs [bp]) was amplified using primers NTF (5′-TGATTGGTGGTTTTGGTAA-3′) and NTR (5′-GATATTGATACTCGTACTTAT-3′) (Casiraghi et al., 2001). Genomic DNA (4 µl) was added to the PCR reaction mix (46 µl) containing 2.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3 and 50 mM KCl, 250 µM of each dNTP, 50 pmol of each primer and 1.25 U of Ampli Taq Gold (Applied Biosystems, Branchburg, New Jersey, USA). The PCR was performed in a Applied Biosystems 2700 thermal cycler using the following cycling protocol: 94 C for 12 min (polymerase activation), followed by 40 cycles at 95 C for 1 min (denaturation); 48 C for 1 min (annealing); 72 C for 1 min (extension), followed by 7 min at 72 C (final extension). Negative and positive control reactions were also carried out by substituting the *Thelazia* DNA template with sterile water and *T. callipaeda* DNA, respectively. Amplicons were purified using Ultrafree-DA columns (Amicon, Millipore; Bedford, Massachusetts, USA) and sequenced in an ABI-PRISM 377 using the *Taq* DyeDeoxyTerminator Cycle Sequencing Kit (Applied Biosystems). Sequences were determined in both directions (using the same primers individually as for the PCR) and the electropherograms verified by eye. Sequences were aligned using the ClustalX program (Thompson et al., 1997). The alignments were verified by eye and compared with the sequences available for the *cox*1 of *T. callipaeda* (GenBank accession nos. AM042549-556; Otranto et al., 2005c).

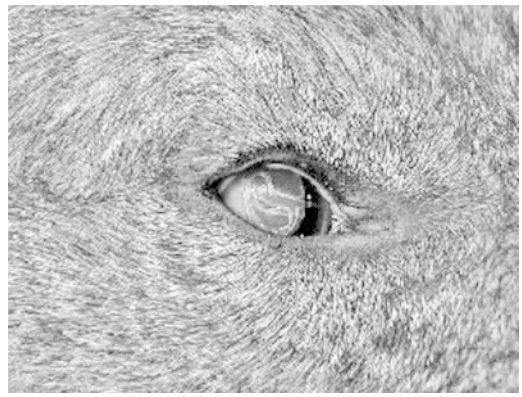

FIGURE 1. Adult specimens of *Thelazia callipaeda* in the right eye of wolf 3.

A total number of three adult female nematodes (one in the left and two in the right eye) were collected from W1; W2 was found infected by 96 adult nematodes (19 females and 21 males in the right eye; 21 females and 25 males in the left eye). The third wolf (W3) was infected by 15 adult nematodes (five males and ten females) in the right eye (Fig. 1). For W3 it was not possible to examine the left eye because it was extensively damaged.

All nematodes collected were morphologically identified as *T. callipaeda.*

Sequences obtained were identical to the sequence representing haplotype 1 (h1) of *T. callipaeda* (available in GenBank accession no. AM042549) (see Otranto et al., 2005c). This is the first report of thelaziosis in wolves caused by *T. callipaeda*. The host competence of wolf is demonstrated by the retrieval of adult *T. callipaeda* specimens in the eyes of animals, because it implies that third stage larvae, released by the insect intermediate host, developed to adult nematodes in the ocular cavity. This finding confirms that the spectrum of definitive hosts of *T. callipaeda* is much wider than for other species (e.g., *Thelazia gulosa* and *Thelazia rhodesi* in cattle; and *Thelazia lacrymalis*, which infects cattle and horses) (Anderson, 2000).

In a recent study, the genetic variability of *T. callipaeda cox*1 was investigated using a single strand conformation polymorphism (SSCP) analysis in worm specimens collected from different species of definitive hosts (i.e., dogs, cats, and foxes) from Europe and Asia (Otranto et al., 2005c). Our results confirm that h1 is the only haplotype present in southern Italy despite the species of animals from which they are collected, and they further support the hypothesis that although there is a high degree of specificity of *T. callipaeda* for their vectors (Otranto et al., 2005a, b, 2006b) there is not for their definitive host.

The existence of a sylvatic life cycle of *T. callipaeda* has been proved in foxes in areas with high prevalence of canine thelaziosis, thus indicating that foxes act as one of the main reservoirs of *T. callipaeda* where pet animals are under veterinary control (Rossi et al., 2002; Otranto et al., 2003). This could also account for the increasing number of reports of canine thelaziosis from different European areas over the past few years (Otranto et al., 2003; Chermette et al., 2004; Hermosilla et al., 2004). Nevertheless, the fact that wolves can act as competent definitive hosts for *T. callipaeda* eyeworms raises new questions on the role this carnivore plays in spreading the disease in endemic areas, considering that their habits fit with the ecological characteristics of the vector in the same environment (Mech, 1995; Otranto et al., 2006a). Although wolves are scantly present in the area under investigation as compared to foxes, they move greater distances (over 800 km, reviewed in Mech, 1995) than foxes (between 10 and 30 km; see Phillips et al., 1972); thus, wolves might play a crucial role in the spread of *T. callipaeda* nematodes for pet carnivores as well as humans.

Gabriella Testini deserves thanks for some lab work.

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