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Spreading of *Thelazia callipaeda* in Greece

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Short title: Thelaziosis in Greece

Summary

Thelazia callipaeda (Spirurida, Thelaziidae), the so-called oriental eyeworm, has been increasingly reported as an agent of infection in animals and humans from many European countries. Clinical signs range from subclinical to moderate or severe ocular disorders (e.g. epiphora, photophobia, conjunctivitis, keratitis, ulcers). The disease has been also diagnosed in animals from countries of the Balkan area (e.g., Croatia, Bosnia and Herzegovina and Serbia), but only a single case of canine thelaziosis, presumably autochthonous, was reported in Northern Greece. In this study we provide robust information of the occurrence of thelaziosis in Greece by reporting autochthonous cases of thelaziosis in dogs (n =46), cats (n =3) and in one rabbit (*Oryctolagus cuniculus*) living in Northern and Central regions of Greece. The occurrence of a single haplotype of the cytochrome oxidase subunit 1 gene confirms that the same zoonotic haplotype of the parasite circulating in Europe is also spreading in Greece. The increased awareness of this parasitosis is crucial in order to limit the risk of further infections in both humans and animals in European countries.

Keywords *Thelazia callipaeda*, eyeworm, dog, cat, rabbit, ocular, Greece

1. Introduction

Adult nematodes of *Thelazia callipaeda* (Spirurida, Thelaziidae) inhabit the ocular tissues of the eyes of animals and humans, mainly dwelling under the third eyelid of animals (Otranto and Traversa, 2005). In the infected animals, the mechanical damage and the load of adults and first-stage larvae (L1) are responsible for clinical signs ranging from subclinical to moderate or severe ocular disorders (e.g. epiphora, photophobia, conjunctivitis, keratitis, ulcers) (Shen et al, 2006; Otranto et al, 2013). Though dogs and foxes are the predominantly infected hosts, also cats, hares and wolves have been reported as suitable definitive hosts of *T. callipaeda* (Otranto et al, 2009). In Europe, the vector and intermediate host of this spirurid is the male drosophilid fly *Phortica variegata* (Drosophilidae, Steganinae), which displays a zoophilic behaviour feeding around the eyes of mammals (Otranto et al, 2006a, b). Over the last decade, *T. callipaeda* has been increasingly reported from domestic animals and wildlife in several European countries including Italy, France, Switzerland, Germany, Spain and Portugal (Otranto et al, 2015). Human infections are mainly documented from people living in East Asian countries (e.g. Japan, Korea, China, India) (Colwell et al, 2011), though reports of ocular infection from humans in Italy, France, Spain, Serbia and Croatia strengthen the medical importance of this parasitic infection also in European countries (Otranto and Dutto, 2008; Fuentes et al, 2012; Paradžik et al, 2016; Tasić-Otašević et al, 2016).

Recently, an increase in *T. callipaeda* infections has been reported in animals and humans living in Eastern European countries (Colella et al, 2016). In Greece information on *T. callipaeda* is limited to a single case report (presumably autochthonous) from an infected dog living close to the borders with the former Yugoslavian Republic of Macedonia (Diakou et al, 2015). In this study we provide information of the occurrence, the clinical presentation and the treatment of a large case series of thelaziosis in dogs, cats and a domestic rabbit (*Oryctolagus cuniculus*) living in Northern and Central regions of Greece.

2. Materials and methods

2.1 Clinical examination and parasite collection

From 2014 to 2016, a total of 50 client owned animals (46 dogs, 3 cats and 1 rabbit) were referred to different veterinary practitioners due to ocular problems or for routine veterinary examination. After administration of topical anaesthetic eye drops of

Proparacaine hydrochloride 0.5% (Alcaine®, ALCON, Belgium), animals were subjected to ocular examination and the parasites were collected from the conjunctival sac using fine forceps, sterile cotton swabs or flushing with saline solution. A brief history of each animal was recorded including data on breed, age, gender, location, lifestyle, clinical presentation and treatment.

2.2 Morphological and molecular identification

Nematodes collected from the eyes of infected animals were morphologically identified at species level based on previous descriptions (e.g., shape of the buccal capsule, presence of transversally striated cuticle and cloacal papillae, morphology of the spicules in males, and the position of the vulva in females) (Skrjabin et al, 1971; Otranto et al, 2003). The identification of the recovered specimens was further confirmed by molecular analysis. Briefly, genomic DNA was extracted using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen, GmbH, Hilden, Germany) and a partial sequence of the mitochondrial cytochrome oxidase subunit 1 (*cox*1) gene (689 bp) was amplified by PCR as previously described (Otranto et al, 2005). The amplicons were purified and sequenced using the Taq Dye Doxy Terminator Cycle Sequencing Kit (v.2, Applied Biosystems) in an automated sequencer (ABI-PRISM 377). Sequences were compared with those available in GenBank database by Basic Local Alignment Search Tool (BLASTn – http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3 Ethic Statement

All medical procedures were carried with the owner's approval. Nematodes were sent to the laboratory of Parasitology (Aristotle University of Thessaloniki and University of Bari) for morphological and molecular characterization.

3. Results

All infected animals (n=50) were located in 11 different areas of Northern and Central Greece (i.e., Thessaloniki, Drama, Serres, Grevena, Kilkis, Edessa/Giannitsa, Veroia, Chalkidiki, Kalampaka, Larisa and Athens - Figure 1). Animals were of both sexes, aging from 0.6 to 11 years old with a history of irregular administration of endo- and ecto-parasiticides (Table 1). None of them had travelled abroad or spent some period close to bordering countries. Ophthalmological examination revealed the presence of motile nematodes in the conjunctival sac and surrounding tissues of one or both eyes

(Figure 2). The number of removed nematodes ranged from 6 to 80 (mean= 29.4; sd= ±23.2), even though nematode counting was not possible in 10 out of 50 cases. All animals presented ocular signs of various severity, except for two dogs and one cat. Nematodes collected were morphologically identified as *T. callipaeda*. The *cox*1 sequences obtained from nematodes collected from all animal species were identical to the sequence of *T. callipaeda* haplotype-1 (h1) (GenBank accession no. AM042549).

Based on clinical presentation and the decision of the clinician, different drugs were administered (orally and/or locally) to reduce the gravity of ocular signs. Treatment consisted in antibiotics (e.g., tobramycin, doxycycline, chloramphenicol) and cortico-/steroids (dexamethasone, prednisolone; Table 1). In order to kill any remaining parasites, ivermectin was administered in some animals (off label use of VALANEQ injectable solution for ruminants, PNG Gerolymatos, Greece). The treatment, in some cases, was completed with the monthly administration of antiparasitic combinations of imidacloprid 10% and moxidectin 2.5% spot on (Advocate®, Bayer Animal Health, Germany) or milbemycin oxime/praziquantel tablets (Milbemax®, Novartis-Animal Health, France). Details on the lifestyle, sex, age, breed, main presenting reason, ocular findings and treatment of infected animals are presented in Table 1.

4. Discussion

The large case series of infection by *T. callipaeda* in dogs, cats and a rabbit from Northern and Central Greece, clearly indicate that the infection is rapidly spreading throughout the country. This is in line with the increase in the number of reports of thelaziosis in animals and humans living in Eastern European countries, including Romania (Mihalca et al, 2015), Bosnia and Herzegovina (Hodžić et al, 2014), Croatia (Hodžić et al, 2014; Paradžik et al, 2016), Serbia (Tasić-Otašević et al, 2016), Hungary and Bulgaria (Colella et al, 2016). Indeed, except for a supposedly autochthonous case report from a dog in 2015 (the parasite was not molecularly sequenced; Diakou et al, 2015), at the best of authors' knowledge, *T. callipaeda* has never been diagnosed before in Greece. The occurrence of 50 autochthonous cases of thelaziosis in dogs, cats and in a rabbit from several locations of Northern and Central Greece, indicates that *T. callipaeda* is present among animals living in both urban and rural areas. However, pathways associated with the introduction of *T. callipaeda* in Greece are not yet fully understood. For instance, hunting dogs travelling from endemic areas of Italy and France have been indicated as one of the possible route for the introduction of this parasite in Spain (Mirò et al, 2011). Alongside, the occurrence of the vector and intermediate host *P. variegata* has not yet been studied in Greece, though clinical cases were reported from animals living in rural forested and meadow areas with a low altitude (e.g. Kalambaka 244m, Kilkis 280m and Polygyros 426m above sea level), which represent suitable ecotopes for this drosophilid (Otranto et al, 2006b; Màca and Otranto, 2014). Nonetheless, three infected dogs (Figure 1) lived in Athens, which is farther to the south of Greece with altitude ranging from 70m to 338m above sea level. In this study, the occurrence of *T. callipaeda* reached its southernmost point in Europe (39°N/21°E and 37.9°N/23.7°E). The recent economic crisis in Greece may have favoured the establishment and spread of *T. callipaeda* in animal populations, due to the possible decrease in number of routine antiparasitic treatments of pets.

Furthermore, the role of wildlife, particularly foxes and wolves, as spreader of the infection (Hodžić et al, 2014; Otranto et al, 2015) would deserve investigations. For example, wolves have been recovered recently in Greece, which represents the south-eastern edge of their distribution in Europe (Karamanlidis et al, 2016). In addition, genetic studies on a population of wolves from Greece revealed the existence of a high genetic variability (Karamanlidis et al, 2016) similar to those found in the Balkan area (Moura et al, 2014). This data suggests that wolves roam and breed among bordering countries, and may favour the introduction of pathogens, including *T. callipaeda*, in suitable areas as suggested for Italy (Otranto et al, 2007). The participation of rabbits in the maintenance of thelaziosis should be further assessed as reported in highly endemic areas of Portugal (Gama et al, 2016).

Treatments of eyeworm infection include milbemycin oxime/praziquantel tablets (Motta et al, 2012; Otranto et al, 2016) and imidacloprid 10% and moxidectin 2.5% spot-on formulation (Lechat et al, 2015; Otranto et al, 2016), which showed to be effective against *T. callipaeda* in naturally infected animals. In this study, treatment of thelaziosis included macrocyclic lactones (in some cases off label use of ivermectin) and symptomatic therapy, which was successful in clearing the infection and in a fully recovering of animals from the associated clinical signs. Therefore, the treatment of domestic animals with proper and registered products together with an increased medical and veterinary communities' awareness of thelaziosis is central to limit further infections in both animals and humans in Europe.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure 1: Geographical location (dots) and number (within brackets) of infected animals in Greece (D=dog, C=cat, R=rabbit).

Figure 2: *Thelazia callipaeda* eyeworms in the eye of an infected dog from Greece (animal anesthetized).

Table 1: Details on the cases of animals infected with *Thelazia callipaeda* from different areas of Greece.

Table 1: Details on the cases of animals infected with Thelazia callipaeda from different areas of Greece.

Animal Species	Lifestyle	Sex	Age (years)	Breed	Main presenting reason	Ocular findings (Mean number of nematodes ±sd)	Treatment
Dog (n=46)	I/O/O+I	Male (n=26) Female (n=20)	0.6-11	Mongrel and breeds (Beagle, Boxer, German Shepherd, Greek hound, Griffon, Labrador retriever, Setter)	Ocular discharge with or without irritation, Conjunctival congestion, Incidental finding during physical examination or Routine vaccination	Conjunctivitis, mucoid or mucopurulent ocular discharge, follicular conjunctivitis, indolent corneal ulcer, corneal abrasions, blepharitis, anterior uveitis (37.02 ±21.48)	Tobramycin 0.1%+diclophenac 0.3% coll, TID x 10 D, Tobramycin 0.3%+Dexamethasone 0.1% coll, TID x 10D, Doxycycline 10 mg/kg po SID 28 D, chloramphenicol 0.5%+dexamethasone 0.1% coll, TID x 10 D, milbemycin oxime 0.9 mg/kg + praziquantel 9 mg/kg p.o. monthly, milbemycin oxime 1mg/kg + praziquantel 10 mg/kg p.o. monthly, 10% imidacloprid + 2.5% moxidectin spot on monthly,

							Ivermectin (400μg/kg, sc), Physical removal of nematodes
Cat (n=3)	I/O/O+I	Male (n=2) Female (n=1)	1.5-5	Greek shorthair	Ocular discharge- irritation, Routine vaccination	Conjunctivitis, No clinical signs (8.33 ±5.5)	Tobramycin 0.3%+Dexamethasone 0.1% coll, TID x 10D, Tobramycin 0.3% coll, Physical removal of nematodes
Rabbit (n=1)	O	Male	8.5		Ocular discharge	Conjunctivitis, mucoid ocular discharge (n=6)	Physical removal of nematodes

Lifestyle: O=outdoor, I=indoor

p.o.= per os, sc=subcutaneously, BID= bis in die, TID= ter in die (i.e. three times a day), D=day

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