

First report of *Cercopithifilaria* spp. in dogs from Eastern Europe with an overview of their geographic distribution in Europe

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Abstract Dogs in Europe may be parasitized by a variety of species of filarioids. While some species have been studied for decades, others have been only recently reported, and their range of distribution is still unknown. The present study was aimed to investigate the occurrence of *Cercopithifilaria* spp., whose microfilariae reside in the dermis and are transmitted by *Rhipicephalus sanguineus* s.l. ticks, in a dog population from Romania and to present the current knowledge of their distribution in Europe. Sampling was performed in a rural locality from Danube Delta region of Romania, location chosen due to the presence of the only known vector for these species. Skin samples were collected from 39 dogs. The samples were tested for the presence of *Cercopithifilaria* spp. by PCR methods. One animal (2.56 %) was positive, and the amplified sequence showed a 100 % similarity to *Cercopithifilaria bainae*. This study reports *C. bainae* for

the first time in Eastern Europe, extending the known range of the genus in Europe.

Keywords Filarial nematodes · Dogs · Microfilariae · Romania · *Cercopithifilaria bainae*

Introduction

Among filarial nematodes (Spirurida, Onchocercidae) that may parasitize dogs, the greatest attention has been given to *Dirofilaria immitis* (Leidy, 1856) and *Dirofilaria repens* (Railliet & Henry, 1911) which are both regarded as zoonotic agents (Orihel and Eberhard 1998), and their abundance in dog populations is directly related to risks of human infection (Montoya-Alonso et al. 2010). Other species which have microfilariae residing either in the blood stream (e.g., *Acanthocheilonema reconditum* Grassi, 1889 and *Acanthocheilonema dracunculoides* Cobbold, 1870) or in the subcutaneous tissues (e.g., *Onchocerca lupi* Rodonaja, 1967 and *Cercopithifilaria* spp.) of dogs have also been reported in Europe. However, limited information regarding their distribution is available. There are very few reports of these filarioids in humans (Huynh et al. 2001; Otranto et al. 2011a, 2012a), but their zoonotic potential cannot be disregarded.

Filarioids of the genus *Cercopithifilaria* Eberhard, 1980 have a large host spectrum and geographical range, and they are transmitted by hard ticks (Bain et al. 2008). The adult stages of all species inhabit the subcutaneous tissues, while microfilariae are found in the dermis and are absent from the main blood stream (Bain et al. 2008). So far, in Europe, two species belonging to genus *Cercopithifilaria* have been reported in dogs (Otranto et al. 2012b). In particular, *Cercopithifilaria grassii*, initially described as *Filaria grassii* by Noè in 1907 in Italy, has exceptionally large microfilariae (567–660 µm in length and 12.5–15.5 µm in width) with bifid

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caudal end (Noè 1907, 1908, 1911). The length of adults varies between 17 and 21 mm in females and 7 and 8 mm in males (Noè 1908). This species was neglected for a long time, until its presence was reported in *Rhipicephalus sanguineus* s.l. ticks in Switzerland (Bain et al. 1982) and Northern Italy (Pampiglione et al. 1983). It has also been recently reported in dogs from Spain and Sicily (Otranto et al. 2012b). *Cercopithifilaria bainaie* was described in Brazil by Almeida and Vicente (1984). This species was not reported again until 2010 in a dog from Sicily (Otranto et al. 2011b) and then was redescribed (Otranto et al. 2013). Microfilariae are small (170–197 μm in length and 6.1–9.4 μm in width) with a blunt caudal extremity. The length of adults is 14–19 mm in females and 9–12 mm in males (Otranto et al. 2013). Microfilariae develop in *R. sanguineus* s.l. (Brianti et al. 2012; Ramos et al. 2013), but not in *Ixodes ricinus* (Ramos et al. 2013). In Europe, *C. bainaie* is known to be present in Italy, Spain, and Greece (Otranto et al. 2012b, c; Solinas et al. 2013). Along these two well-defined species, an additional *Cercopithifilaria* species (i.e., *Cercopithifilaria* sp. II sensu Otranto et al. 2012b) has been recently reported from dogs and *R. sanguineus* s.l. ticks from Italy and Spain (Otranto et al. 2012b; Solinas et al. 2013). Microfilariae measure 273–305 μm in length and 12–15 μm in width; the adults are not known (Otranto et al. 2013).

Three species of filarioids with blood-dwelling microfilariae have been reported so far in Romanian dogs: *D. immitis*, *D. repens*, and *A. reconditum* (Hamel et al. 2012). However, studies regarding the presence of species with dermal microfilariae have never been performed. Given the presence and distribution of *R. sanguineus* s.l. in Romania (Mihalca et al. 2012), the present study aims to determine through molecular methods whether *Cercopithifilaria* spp. occur in dogs from Danube Delta region of Romania, where dogs are known to be heavily infested by ticks.

Methods

Study area and sampling

The study was conducted in Chilia Veche (45° 25' 19" N, 29° 17' 23" E), a rural locality in the Danube Delta region of Romania.

The samples were collected from a small number ($n=39$) of randomly selected owned dogs of both sexes, with ages ranging from 1.5 months to 12 years. Most of them had never received antiparasitic drugs and had a history of tick infestation. Upon receiving the owners' consent, skin samples were collected using 3-mm disposable biopsy punches (Kai Medical, USA) from the interscapular region, where the frequency of *Cercopithifilaria* microfilariae is known to be higher (Otranto et al. 2012d). All samples were properly labelled, preserved in absolute ethanol, and kept at -20°C until further processing.

Molecular amplification and sequencing

The extraction of genomic DNA from samples was performed using a commercial kit (Isolate II Genomic DNA Kit; Bionline, UK) according to the manufacturer's instructions.

Amplification of a partial *cytochrome c oxidase* subunit 1 (*cox1*) gene of *Cercopithifilaria* spp. (304 bp) was performed using the CbCox1F/NTR primer pair, following reaction procedures and protocols described in the literature (Otranto et al. 2011b). A positive control and a sample with no DNA were included in order to test the specificity of the reaction and to assess the presence of contaminants. Amplicons were visualized by electrophoresis in a 2 % agarose gel stained with ethidium bromide (Gellyphor, EuroClone, Italy), and their molecular weight was assessed by comparison to a molecular



Fig. 1 Distribution of *Cercopithifilaria* spp. in Europe

Table 1 Proportion of the species of *Cercopithifilaria* in dogs from Mediterranean countries evaluated by microscopy

Location (country)	<i>C. bainae</i> (%)	<i>C. grassii</i> (%)	<i>Cercopithifilaria</i> sp. II ^a (%)	Reference
La Vera (Spain)	52.9	2.9	44.1	Otranto et al. (2012b)
Basilicata (Italy)	75	0	25	Otranto et al. (2012b)
Apulia (Italy)	100	0	0	Otranto et al. (2012b)
Sicily (Italy)	90.6	9.4	0	Otranto et al. (2012b)
Sardinia (Italy)	94.2	0	5.8	Solinas et al. (2013)

^a Sensu Otranto et al. (2012b)

marker (Gene Ruler™ 100-bp DNA Ladder; MBI Fermentas, Vilnius, Lithuania).

Amplicons were purified using Ultrafree-DA columns (Amicon, Millipore; Bedford, USA) and then sequenced directly using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (v.2; Applied Biosystems, Inc.) in an automated sequencer (ABI PRISM 377; Applied Biosystems, Inc.). Sequences were compared to those available in GenBank™ dataset by Basic Local Alignment Search Tool (BLAST) analysis.

Results

A total of 39 skin samples were tested for the presence of *Cercopithifilaria* spp. by PCR. Out of these, a single one (2.56 %), from a 10-year-old male, was positive. The BLAST analysis of the sequence showed a 100 % similarity to a sequence of *C. bainae* available in GenBank™ (accession number JF461457).

Discussion

Prior to this report, the known range of *Cercopithifilaria* spp. in dogs from Europe was limited to the Mediterranean region (Fig. 1), but the occurrence of *Cercopithifilaria* spp. in dogs

from Romania was plausible, considering the occurrence of *R. sanguineus* s.l. (Mihalca et al. 2012), the only proven vector for this nematode (Brianti et al. 2012).

The geographic distribution seems to be slightly different in *Cercopithifilaria* spp. affecting dogs, but the limited number of studies is not enough for a more clear conclusion. Based on current knowledge, the most widely spread seems to be *C. bainae* (Table 1). Even if based on a small number of samples, this new report from Romania broadens current knowledge on the distribution of *C. bainae* in dogs.

While for other canine filarial species (i.e., *Dirofilaria*), the range is influenced by the distribution of the mosquito vector and by the local climatic conditions, mainly temperature (Genchi et al. 2009), in the case of tick-borne filariae, the temperature-dependent development has not been evaluated so far. The hypothesis that *Cercopithifilaria* spp. might be more common in warmer areas has not been explored but may be supported by the low positivity rate for *C. bainae* infection in dogs included in our study (1/39), compared to other reports (Table 2). Nevertheless, the prevalence rates seem to vary significantly depending on the detection method employed (Table 2). Moreover, if, in the case of mosquito-borne canine filariae, the microfilarial periodicity in the blood has been documented (Di Cesare et al. 2013), this aspect is unknown for the tick-borne *Cercopithifilaria*.

Table 2 Prevalence of *Cercopithifilaria bainae* infection in dogs from Europe

Location (country)	Microscopical prevalence (%)	Molecular prevalence (%)	Reference
Xanthi (Greece)	4.3	0	Otranto et al. (2012c)
La Vera (Spain)	21.6	45.4	Otranto et al. (2012c)
Bari (Italy)	ND	13.6	Otranto et al. (2012c)
Basilicata (Italy)	12	0	Otranto et al. (2012c)
Ginosa (Italy)	ND	5.3	Otranto et al. (2012c)
Putignano (Italy)	ND	12.5	Otranto et al. (2012c)
Sicily (Italy)	13.3	19.5	Otranto et al. (2012c)
Sardinia (Italy)	7.7	1.3	Solinas et al. (2013)
Chilia Veche (Romania)	ND	2.56	Present study

ND no data

Further investigations regarding species diversity and their geographical boundaries should be carried out at least in the areas where *R. sanguineus* s.l. ticks are present. Climatic correlations should be made in order to evaluate the possible impact of temperature on the distribution and development on *Cercopithifilaria* spp. in ticks.

Conclusion

Our results report for the first time, in Eastern Europe, the presence of *Cercopithifilaria* spp. in dogs, extending the known range of this genus, which raises the importance of more extensive studies in the areas where the main vectors are distributed.

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Conflict of Interest The authors declare that they have no conflict of interest.

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