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Morphological and molecular data on the dermal microfilariae of a species of *Cercopithifilaria* from a dog in Sicily

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ABSTRACT

Dermal microfilariae found in a dog from Sicily, Italy, were characterized morphologically and genetically and differentiated from those of all the other blood microfilariae commonly found in dogs. In particular, the microfilariae were short (mean length of 186.7 μm), presented a body flattened dorso-ventrally and a rounded head, bearing a tiny cephalic hook. The genetic identity of microfilariae herein studied was also assessed by molecular amplification, sequencing and analyzing of multiple ribosomal ITS-2 and mitochondrial (*cox*1 and 12S) target genes. Both morphologic and genetic characterization as well as the molecular phylogenetic history inferred using sequences of a barcoding dataset were concordant in supporting the identification of *Cercopithifilaria* at the genus level. Surprisingly, microfilariae here examined were well distinct from *Cercopithifilaria grassii* (Noè, 1907), from northern Italy, and resembled those of a species described in Brazil, *Cercopithifilaria bainae* Almeida & Vicente, 1984. This paper provides evidence for the existence of a *Cercopithifilaria* species infesting a dog from Sicily and also presents a PCR protocol on skin samples as a tool for further epidemiological studies, which could provide evidence on the aetiology and the natural history of this filarial species.

1. Introduction

Filarioids (Spirurida, Onchocercidae) parasitizing wild and domestic mammals can cause zoonosis in tropical and subtropical regions (Orihel and Eberhard, 1998; Otranto and Eberhard, 2011). Several of these species are parasites of dogs, and have either blood microfilariae such as of *Dirofilaria* spp. (Orihel and Ash, 1995; McCall et al., 2008; Pampiglione et al., 1995, 2009) and *Acanthocheilonema reconditum* (Huynh et al., 2001), or dermal microfilariae as *Onchocerca lupi* (Otranto et al., 2011). Canine filariae with dermal microfilariae are not restricted to *O. lupi* and two other species have been reported in the genus *Cercopithifilaria* (Eberhard, 1980), although they are little known and usually not searched for in dogs (Almeida and Vicente, 1984; Bain et al., 1982a).

The genus *Cercopithifilaria*, originally described as a subgenus of *Dipetalonema* by Eberhard (1980), is now well defined and comprises 28 species, either described in or reclassified to this genus (Bain et al., 2002). Adult worms are most often tiny, located in subcutaneous tissues and uneasy to detect. Microfilariae are always in the dermis instead of in the blood circulation (Bain et al., 2002). As far as it is known, species of *Cercopithifilaria* are primarily transmitted by hard ticks (Ixodida, Ixodidae), such as *Rhipicephalus* and *Ixodes* (Noè, 1908; Winkhardt, 1980; Bain

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et al., 1986; Spratt and Haycock, 1988; Petit et al., 1988). The host range of *Cercopithifilaria* is made of a diversity of ruminants, primates, carnivores, rodents, lagomorphs, marsupials and monotremes. However, species currently placed within this genus are well supported by traditional and molecular data (Bain et al., 2008).

In 1907, Noè found a filarial nematode with dermal microfilariae in samples collected from a dog in Rome (Italy). This species was originally described as *Filaria grassii* and later transferred to *Cercopithifilaria* by Bain et al. (1982b). This filaria presents characteristic "*gigantesche*" (from Italian, giant) microfilariae, as Noè stated in his original description for this species (Noè, 1907). These microfilariae developed in ticks (Noè, 1908). *C. grassii* species remained ignored until two interesting reports of this parasite, dated back to nearly 30 years ago, in Switzerland (Bain et al., 1982a) and in northern Italy (Pampiglione et al., 1983).

In south-eastern Brazil, the species was reported twice, by Costa and Freitas (1962) and Almeida and Vicente (1982). However, two years later, the last authors examined additional material of this rare filarioid and concluded that it represented a new species, *Cercopithifilaria bainae* Almeida & Vicente, 1984, distinguished for its much smaller microfilariae (Almeida and Vicente, 1984).

The primary aim of the present investigation was to reassess the occurrence of *C. grassii* in Italy. Dermal microfilariae were retrieved from skin samples of a dog from Sicily, southern Italy, and coupled morphological and molecular analyses confirmed that this filarial nematode belonged to the genus *Cercopithifilaria*. However, the morphological study indicated that this species was not *C. grassii*, but seemed related morphologically to the Brazilian filarioid described by Almeida and Vicente (1984).

2. Materials and methods

2.1. Dermal microfilariae collection

On July 2010, one mongrel dog was found positive for *A. reconditum* microfilariae in blood during a survey carried out in the municipal dog shelter in Messina (38°11′N; 15°33′E), Sicily, Italy (data not shown). These larvae were identified on the basis of their length (260 μm), body width (4 μm), caudal filament, and characteristic prominent cephalic hook. In the same shelter, other dogs were found to be positive for *A. reconditum* (data not shown). None of the animals had received any anthelmintic or ectoparasiticide treatment during the months before and almost all of them were heavily infested by fleas (*Ctenocephalides* spp.) and by *R. sanguineus* ticks (Brianti et al., 2010). At the clinical examination a subcutaneous nodule was retrieved on the dog's right thigh (Fig. 1) and thus a biopsy of 3 mm was taken from skin (sample 1; s1). Skin samples were collected using a disposable scalpel after shaving the hair over an area of about 0.5 cm × 0.5 cm × 0.6 cm. Dermal sample was soaked in saline solution for 10 min at 37 °C and thereafter removed and stored at −20 °C. The sediment was observed under light microscopy after adding a drop of methylene blue (1%). Following the retrieval of motile microfilariae (see Section 3), five other skin biopsies (s2–s5) were performed (i.e., from the left thigh, s2; from both right and left temporal areas, s3 and s4; and from armpits, s5 and s6).

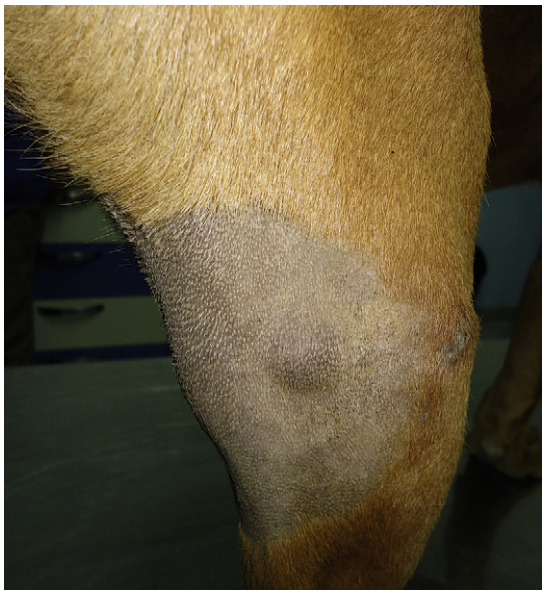

Fig. 1. Subcutaneous nodule on the animal's right thigh.

2.2. Morphological analysis

Morphological analysis was done with fixed microfilariae cleared in lactophenol. The cover-slide was unsealed in order to orient the microfilariae in dorso-ventral or lateral views, as previously described (Bain et al., 1988; Uni et al., 2001). Drawings were made with an optic microscope equipped with a camera lucida and measurements were made on drawings. Microscopic images were acquired using a digital camera (Zeiss Axiocam MRc, Carl Zeiss, Germany) mounted directly on the microscope (Zeiss Axioscop 2 plus, Carl Zeiss, Germany). The software AxioVision rel. 4.8 (Carl Zeiss, Germany) was used for the image analysis process including measuring of larvae, which are provided in micrometers. Slide-mounted microfilariae were deposited in the collection of the Muséum National d'Histoire Naturelle, Paris, France (MNHM), under the accession numbers 194 YU and 284 YU.

2.3. Molecular amplification and phylogenetic analyses

The molecular identification was performed by extracting genomic DNA from microfilariae isolated by the larval sediment of s1, using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen, GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. In addition, the remaining skin saline-soaked sample (s1) was extracted with the remaining samples (s2–s6) as above.

A *cox*1 (~689 bp) and 12S (~330 bp) gene fragments, which are usually employed for barcoding of filarioids (Ferri et al., 2009) were amplified. In particular, *cox*1 was amplified by using filarioid-generic primers (Casiraghi et al., 2004, 2006) whereas 12S was amplified by a set of primers (Fila12SF: 5′-CGGGAGTAAAGTTTTGTTTAAACCG-