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## Short Communication

## Filarial infections in domestic dogs in Lusaka, Zambia

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## ABSTRACT

Filariae are common parasites of dogs in many parts of the world, but little is known about the status of these infections in sub-Saharan Africa. A study was carried out to determine the occurrence and species of filariae among 272 dogs in Lusaka, Zambia. Giemsa stained blood smear and Knott's concentration methods revealed microfilariae in 16 (5.9%) of the dogs. PCR confirmed that most of these dogs had *Acanthocheilonema reconditum* infection. Ten (4.0%) of the examined dogs were positive for *Dirofilaria immitis* circulating antigen (by DiroCHEK<sup>®</sup> test), but *D. immitis* microfilariae were not identified in any of the dogs and the status of this infection remains unclear. Further studies are needed to explore the occurrence of filariae in Zambian dogs and the zoonotic potential for humans.

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## 1. Introduction

Filariae are vector-borne parasitic nematodes of which several species are of major public health importance, especially in warm climate countries (Simonsen et al., 2014). Dogs are also commonly infected with filarial parasites. Thus, infections in dogs with the mosquito-transmitted filariae *Dirofilaria immitis* and *Dirofilaria repens* are well known due to severe clinical manifestations elicited in the dogs, but also since some of the vectors are anthropophilic and may cause zoonotic transmission of the infections to humans (Genchi et al., 2007; McCall et al., 2008; Simon et al., 2009). *Acanthocheilonema reconditum*, *Acanthocheilonema dracunculoides*, *Cercopithifilaria grassi* and *Onchocerca lupi* are other widespread but less known filarial species of dogs (ESCCAP, 2012; Otranto et al., 2013a,b). Knowledge on the distribution, biology and veterinary and medical significance of these is limited, probably due to the

less distinct clinical picture seen during infection and to a general lack of diagnostic expertise.

The traditional method for diagnosing filarial parasites is by finding and identifying their larvae (microfilariae) in blood or skin samples, usually after application of various staining and concentration techniques (Genchi et al., 2007; McCall et al., 2008). *D. immitis* diagnosis in dogs has been simplified in recent years following development of serological tests for detection of specific circulating antigens, and several brands of these tests are now commercially available (Klotins et al., 2000; McCall et al., 2008). Of late, molecular PCR and DNA sequencing techniques applied on blood samples have moreover proved valuable as highly sensitive and specific tools for detecting and identifying filarial infections in dogs (Casiraghi et al., 2006; Rishniw et al., 2006; Latrofa et al., 2012).

Information on the occurrence of canine filariasis in dogs in sub-Saharan Africa is scarce, and mainly stems from case reports. From a review of available literature by Schwan (2009) it appeared that all the above mentioned species have been reported from various places on the continent. A large survey for filariae in dogs in northern Kenya

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has later been documented (Albrechtova et al., 2011). However, in general knowledge on the extent and distribution is limited. In Zambia, little attention has so far been given to the parasites of dogs, and no information is available on the occurrence of dog filarial infections in the country. As a starting point, the present study therefore aimed at determining the occurrence and species of filarial infections in dogs in Lusaka, Zambia.

## 2. Materials and methods

### 2.1. Study area, study design and sampling

The study was conducted in Lusaka, the capital of Zambia. The city is located in the central part of the country, at an altitude of about 1300 meters and with a human population of about 1.75 million. Lusaka experiences three main seasons: a cold and dry season from May to August (temperature range 6–25 °C), a hot and dry season from September to October (17–35 °C) and a wet season from November to April (14–30 °C). The study was conducted from April to December, 2013. Dogs aged six months and above according to owners information were routinely sampled when presented for medical consultation at the veterinary clinic at the University of Zambia School of Veterinary Medicine ( $n=125$ ) or other nearby veterinary clinics ( $n=76$ ) and during an anti-rabies vaccination campaign in one of the townships of Lusaka (Mtendere compound;  $n=71$ ).

The dogs were brought by their owners who were asked for permission to collect samples after explaining the purpose of the study. Dogs that were very ill or presented with parvovirus enteritis were excluded from the study. Serum and EDTA stabilized whole blood samples were collected from the cephalic vein of each dog. A structured questionnaire was used to obtain information about the dogs, including age, sex, breed, main function and main diet. Dogs were moreover examined systematically for ticks and fleas by visual inspection of the entire body (including ears and inguinal area) and palpation (to detect ticks that were not immediately visible). The body condition was recorded by using the Nestle Purina score system (Lafamme, 1997).

### 2.2. Examination of blood for microfilariae

Duplicate thick blood smears were prepared on glass slides from whole blood. Smears were air dried overnight, thereafter dehaemoglobinised (by soaking in tap water in vertical position for 1–2 min) and allowed to dry before being fixed with methanol for 30–60 s. Subsequently, the smears were stained for 30 min with a 1:14 Giemsa stock solution in distilled water, gently rinsed in a flow of tap water and allowed to dry. A compound microscope was used to identify and measure the microfilariae at 100–400× magnification.

Blood was furthermore examined for microfilariae with Knott's concentration technique (Melrose et al., 2000; Genchi et al., 2007). One ml of whole blood was mixed with 9 ml of 2% formalin in a conical centrifuge tube and then centrifuged for 5 min at 692 ×  $g$ . The supernatant was removed by inverting the tube. The deposit was mixed with

one drop of 1% methylene blue stain. Ten  $\mu$ l of the sample was transferred to a glass slide and examined under a compound microscope at 100× to identify the microfilariae. This was repeated until the entire sample in the tube had been examined.

### 2.3. Test for circulating *D. immitis* antigen

The DiroCHEK® Canine Heartworm Antigen test kit (Synbiotics Corporation, San Diego, USA), which is an enzyme linked immunosorbent assay, was used to detect circulating *D. immitis* antigen in the dog sera according to manufacturer's instructions. Briefly, 50  $\mu$ l of each test sera (plus positive and negative controls) were individually dispensed into the antibody-precoated microtitre wells, and one drop of conjugate (Reagent 1) was added. After 10 minutes of incubation the wells were emptied and washed 5 times with distilled water. Two drops of substrate (Reagent 2) were then added to each well. After 5 min of incubation the results were read as positive (distinctly blue) or negative (completely clear) against a white background.

### 2.4. Identification by sequencing

Fourteen of the 16 blood samples identified positive for microfilariae by microscopy were further analyzed by PCR for speciation of the microfilariae. The MasterPure™ DNA Purification Kit (Epicentre Biotechnologies) was used to extract DNA from 190  $\mu$ l of the whole blood samples according to the manufacturer's protocol except that samples were incubated with 200 mg proteinase K overnight at 56 °C.

The ITS2 region was PCR amplified in a total volume of 20  $\mu$ l using the primers DIDR-F1 and DIDR-R1 (Rishniw et al., 2006) under standard PCR conditions using 1  $\mu$ l DNA as template and an annealing temperature of 58 °C. Likewise, a part of the mitochondrial *cox1* gene was PCR amplified by primers COIntF and COIntR (Casiraghi et al., 2006) using annealing temperature of 48 °C. PCR products were stained using GelRed™ (Biotium) and visualized under UV light in 1.5% agarose gels. PCR products were cleaned up and sequenced in both directions using the PCR primers by Macrogen Inc. (Seoul, South Korea).

The sequences were manually checked using vector NTI (Lu and Moriyama, 2004) and aligned and trimmed in BioEdit (Hall, 1999). Sequences were compared to already published sequences in GenBank (BLAST search). For the *cox1* sequences the genetic relationship between the unknown dog filariae were compared to *A. reconditum* (JF461456), *D. immitis* (FN391553), *D. repens* (JF461458) and *Cercopithifilaria* sp. (JF461457) using the Neighbour-Joining clustering method and the Kimura-2-parameter for distance estimation as implemented in MEGA 6.1 (Tamura et al., 2013). The stability of the topology was assessed with 1000 bootstraps. Unfortunately no *cox1* sequences were available in GenBank for *A. dracunculoides*.

### 2.5. Data analysis

Parasitology, serology and dog data were analyzed using STATA version 13 (StataCorp, Texas, USA). Proportions

**Table 1**

Microfilaria and *Dirofilaria immitis* antigen status of domestic dogs in Lusaka, Zambia. Blood was examined for microfilariae both by examination of Giemsa stained blood slides and by Knott's technique whereas *D. immitis* antigen was detected by a commercial kit.

Factor	Microfilariae		<i>D. immitis</i> antigen	
	No. examined	No. positive (%)	No. examined	No. positive (%)
Sex				
Male	154	11 <sup>a</sup> (7.1)	142	7 <sup>b</sup> (4.9)
Female	118	5 (4.2)	109	3 <sup>c</sup> (2.8)
Total	272	16 (5.9)	251	10 (4.0)
Age group				
0–3 years	190	8 (4.2)	178	8 <sup>c</sup> (4.5)
4–7 years	57	5 <sup>a</sup> (8.8)	49	1 <sup>c</sup> (2.0)
≥8 years	8	0 (0)	8	0 (0.0)
Unknown	17	3 (17.6)	16	1 <sup>c</sup> (6.2)
Total	272	16 (5.9)	251	10 (4.0)

<sup>a</sup> One dog was positive in Giemsa stained blood slide only and one was positive in Knott's technique only. All other microfilaria positive dogs were positive in both tests.

<sup>b</sup> 2 were positive for microfilariae.

<sup>c</sup> 1 was positive for microfilariae.

were compared by Chi-square test. *p*-values <0.05 were considered statistically significant.

### 3. Results

#### 3.1. Characteristics of dogs

A total of 272 dogs were sampled (118 females, 154 males). Most dogs were young, with 190 being 0–3 years of age (Table 1). Sixty-two were purebreds (32 German Shepherd, 9 Labrador retriever, 6 Boerboel, 5 Maltese, 5 Rottweiler, 2 Bull Mastiff, 2 Jack Russel, 1 Pomeranian) while the remaining were cross breeds. The majority were guard dogs (153/233) while 60 were pet dogs and 20 were both pets and guard dogs. Most were fed kitchen leftovers (130/233), while the others were fed either commercial pet food (54/233) or a combination of pet food and left overs (49/233). The body condition ranged from thin to obese, but with most dogs (195/233) being in the ideal category (score 4–6). A total of 39 dogs had incomplete questionnaire data.

#### 3.2. Microfilariae in blood samples

Venous blood was collected from all 272 dogs. Of these, 16 (5.9%) were found positive for microfilariae, namely 14 by both Giemsa stained slides and Knott's technique, one by Giemsa stained slide only and one by Knott's technique only (Table 1). There was no significant difference in microfilaria prevalence between male and female dogs (*p* = 0.31). All microfilaria positive dogs were less than 8 years old, and there was no significant difference in microfilaria prevalence between age groups 0–3 and 4–7 years (*p* = 0.18).

The microfilariae seen in the blood slides had fairly similar morphology. They were un-sheathed and many had a small hook-like protrusion from the anterior end. For 36 measured microfilariae from 11 of the dogs (all those seen in 9 of the dogs and 5 randomly selected from 2 dogs with high microfilaria counts), the average length was 236 μm (range 204–270 μm) and average width was 5.1 μm (range 4.5–6.0 μm). Many of the microfilariae thus showed morphological resemblance to *A. reconditum*.

#### 3.3. *D. immitis* antigen

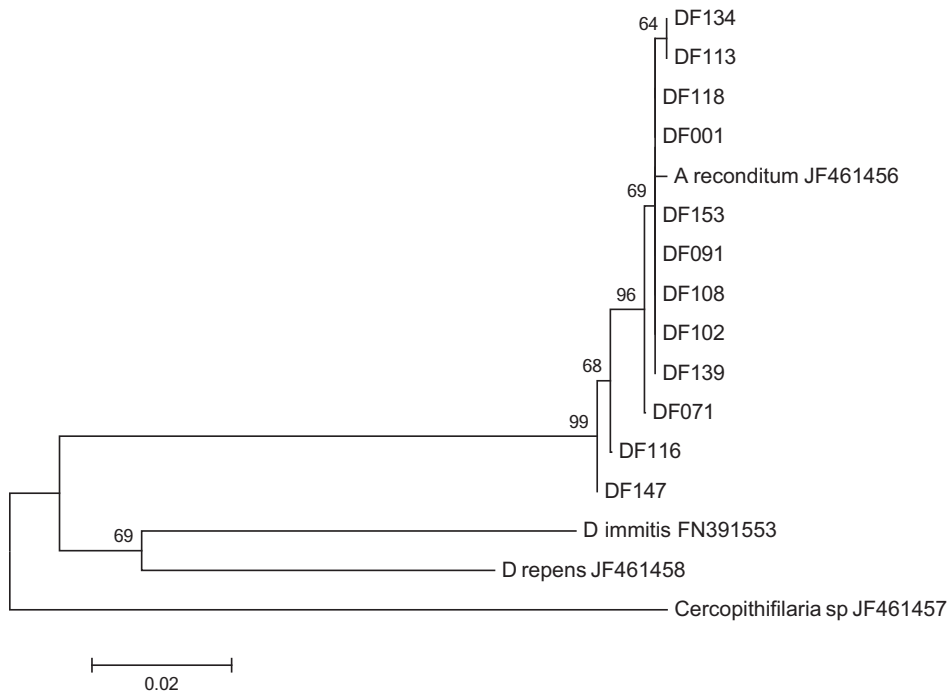
Sera from 251 of the dogs were tested for *D. immitis* antigen (Table 1) and 10 of these were positive (4.0%). There was no statistical difference in antigen prevalence between male and female dogs (*p* = 0.52). All antigen positive dogs were below 8 years old, and there was no significant difference in antigen prevalence between young (0–3 years) and medium-aged (4–7 years) dogs (*p* = 0.43). Three dogs that were positive for *D. immitis* antigen were also positive for microfilariae, whereas the remaining 13 dogs with microfilariae were negative for this antigen.

#### 3.4. Identification of microfilariae by PCR

The ITS2 region was amplified by PCR for all 14 of the microfilaria positive dogs. Only 107 bases of the first part of the ITS2 region could be obtained by direct sequencing which probably relates to intra-individual length variation. For 13 of the dogs, this part of the ITS2 region showed 100% homology to *A. reconditum* (AF217801.2) and 91% to *A. dracunculoides* (DQ018785.1). However, for dog DF096 only 91 bp could be sequenced and 56 bases showed 100% homology to *Onchocerca volvulus*, *O. ochengi*, *O. linealis*, *O. gutturosa* and *O. gibsoni* (the exact identity therefore remains unknown). The *cox1* gene was amplified and sequenced for all except dogs DF096 and DF200. All the sequenced microfilariae from the dogs clustered with *A. reconditum* (Fig. 1). The *p*-distances within this group were between 0% and 1.0% whereas the *p*-distances to *D. immitis* and *D. repens* are 11.7–13.2%. From the sequence chromatograms there was evidence that dogs FD108, DF116, DF147 and DF153 were infected with at least two haplotypes of *A. reconditum* as there were ambiguous peaks for one to three of the nucleotides for these samples.

#### 3.5. Relationship between dog characteristics and filarial infections

None of purebred dogs were positive for microfilariae, thus giving a prevalence of 7.6% among the cross breeds.



**Fig. 1.** Genetic relationship between sequenced samples based on 608 bp of *cox1* gene using Kimura-2-parameter distances and NJ clustering. DF refers to sequences obtained from individual dogs ( $n = 12$ ) and sequences for comparison are obtained from GenBank with accession number given after each species. The stability of the topology was assessed with 1000 bootstraps and values  $>50$  reported. Scale bar: number of base substitutions per site.

However, of the 10 *D. immitis* antigen positive dogs, two were purebred (German Shepherd). The microfilaria prevalence was slightly but not significantly higher in guard dogs than in pet dogs (8.5% vs. 3.3%;  $p = 0.19$ ), whereas the *D. immitis* antigen prevalence was significantly higher in pet dogs than in guard dogs (7.5% vs. 1.4%;  $p = 0.03$ ). The microfilaria prevalence was higher in dogs fed leftovers than in those fed commercial food (19.0% vs 3.7%) whereas the opposite trend was seen for antigen prevalence (3.3% vs. 6.7%) but these differences were not significant ( $p = 0.16$  and  $p = 0.33$ , respectively).

Of the 233 dogs examined for ectoparasites, 90 had ticks (*Rhipicephalus* spp.) and/or fleas (*Ctenocephalides* spp.). The microfilaria prevalence was significantly higher in dogs with these ectoparasites than in those without (11.1% vs. 4.2%,  $p = 0.04$ ). No differences in *D. immitis* antigen prevalence was observed between dogs with or without ectoparasites (3.9% vs. 5.2%,  $p = 0.62$ ).

#### 4. Discussion

The dogs in Lusaka can roughly be divided into pets, guard dogs and stray dogs. Keeping dogs as pets is a relatively new but increasingly common habit. The majority of dogs are kept as guards to provide security for their owners, a practice which is also becoming more common. There are many stray dogs in the city, but these were not included in the study.

Microfilariae were identified in 16 of the examined dogs (5.9%). The microfilariae were sheath-less and relatively small when compared to those of *D. immitis*, *D. repens*

and *C. grassi*, and morphological assessment indicated a close resemblance to *A. reconditum* (Genchi et al., 2007; Brianti et al., 2012; Magnis et al., 2013). Subsequent PCR analysis on whole blood samples from the microfilaraemic dogs confirmed the presence of *A. reconditum* in 13 of these. *A. reconditum* is a common filarial parasite of dogs in many geographical areas. In Eastern and Southern Africa it has previously been reported from Kenya (Nelson, 1962; Albrectova et al., 2011), Uganda (Bwangamoi and Isyagi, 1973), Mozambique (Schwan and Durand, 2002) and South Africa (Schwan, 2009). The adult parasites are located in the subcutaneous tissues and show a preference for the limbs and back (Nelson, 1962). The infection is generally considered to be of little clinical importance (Brianti et al., 2012). The main vectors of *A. reconditum* are fleas, especially dog and cat fleas (*Ctenocephalides* spp.). In this respect, it is noteworthy that many of the examined dogs had fleas and ticks, and that the microfilaria prevalence was significantly higher in dogs with these ectoparasites than in those without. It is not clear if the infective larvae are transmitted through the flea bite or the dogs acquire the infection by ingesting the fleas, but a recent study suggests the later of these routes to be the most likely (Napolii et al., 2014). Guard dogs were found to have higher microfilaria prevalence than pet dogs, probably because guard dogs generally are allowed to roam more freely in the outside environment compared to pet dogs (personal observation).

Sera from 10 of the dogs (4%) were positive for *D. immitis* antigen. Surprisingly, none of these or any of the other examined dogs were found positive for *D. immitis* microfilariae. Three of the antigen positive dogs had microfilariae



but were PCR positive only for *A. reconditum* and not for *D. immitis*. The positive antigen tests showed a distinct blue color-response against a white background, although the color was less intense than seen with the positive control. Preventive treatments against *D. immitis* are generally not used, as these infections have so far not been recognized as a health problem in dogs in Zambia. There may be a couple of plausible explanations for this ambiguous result. First, as the circulating antigens are released by adult female *D. immitis*, the antigen positive dogs may have had adult worm infection without microfilaraemia, for example due to unisexual infections, immune-mediated clearance of microfilariae or previous ivermectin treatment (Bowman and Mannella, 2011). It is surprising, though, that not a single dog was found positive for *D. immitis* microfilariae, as these are essential for maintaining transmission. Second, it might be speculated that the positive tests relate to cross reactions. Although the *D. immitis* antigen test is considered to be very specific, cross reactions have recently been demonstrated in dogs infected with the French heartworm – *Angiostrongylus vasorum* (Schnyder and Deplazes, 2012). In this respect it is interesting to note that circulating *D. immitis* antigen was also found in dogs from several study sites in South Africa that were negative for microfilariae of this species (Schwan, 2009).

This study demonstrated, for the first time, the occurrence of filarial infections in dogs in Zambia, and identified *A. reconditum* infections to be relatively common in Lusaka. Regular control of ectoparasites should be encouraged among dog owners, e.g. by dog dipping, as a strategy for reducing transmission of this filarial parasite.

### Conflict of interest statement

The authors have no conflicts of interest to report.

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