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Occurrence of filaria in domestic dogs of Samburu pastoralists in Northern Kenya and its associations with canine distemper

Kateřina Albrechtová[a,*], Kamil Sedlák[b], Klára J. Petrželková[c,d], Jan Hlaváč[a], Andrei D. Mihalca[e], Alison Lesingirian[f], Paul W.N. Kanyari[g], David Modrý[a,h]

[a] Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, 612 42 Brno, Czech Republic
[b] Department of Virology and Serology, State Veterinary Institute Prague, Sídlištní 136/24, Prague, Czech Republic
[c] Institute of Vertebrate Biology v.v.i., Academy of Sciences of the Czech Republic, Květná 8, 603 00 Brno, Czech Republic
[d] Liberec ZOO, Masarykova 1347/31, 460 01 Liberec, Czech Republic
[e] Department of Parasitology and Parasitic Disease, University of Agricultural Sciences and Veterinary Medicine, Calea Mănăstur 3-5, 400372 Cluj-Napoca, Romania
[f] Samburu Health Advancement, Diagnosis and Education, Tuum, Kenya
[g] Department of Parasitology, College of Agriculture and Veterinary Sciences, University of Nairobi, Kapenguria Road, P.O. Box 29053, 00625 Nairobi, Kenya
[h] Biology Center, Institute of Parasitology, Academy of Sciences of the Czech Republic, Branišovská 31, 37005 České Budějovice, Czech Republic

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ABSTRACT

Samples of blood (serum, smears and blood preserved with ethanol) were collected from dogs during a vaccination campaign in northern Kenya in the years 2006 and 2007. Blood was screened for filarial parasites using molecular and microscopy methods and sera were tested for antibodies against canine distemper virus (CDV). Parasitological examination revealed the presence of two species of canine filariae: *Acanthocheilonema dracunculoides* and *A. reconditum*. The DNA from the former species was detected in 58% dogs sampled in 2006 and 36% dogs sampled in 2007, whereas the latter was found only in 4.2% samples collected in 2007. Microfilariae were found in 33.8% blood smears collected in 2006 and 10.6% blood smears collected in 2007. The seroprevalence of CDV was 33.4% in 2006 and 11.2% in 2007. The effect of sex, age and CDV-seropositivity/seronegativity on the occurrence of *A. dracunculoides* was evaluated. Infection by *A. dracunculoides* was more common in males and in dogs with a positive antibody titer for canine distemper, but evenly distributed among different age groups. The difference in the prevalence of *A. dracunculoides* in two isolated mountain ranges was not statistically significant. Methodologies available for detection and determination of canine filariae are compared, underlining methodical pitfalls arising through the determination of less common filarial species. The role of single epidemiological factors and possible association between canine distemper and filariasis are discussed.

Abbreviations: ALRMP, Arid Land Resource Management Project; CNLI, Continental New Life International; CDV, canine distemper virus; pM, pmol; RFLP, restriction fragment length polymorphism; SHADE, Samburu Health Advancement, Diagnosis and Education; WHO, World Health Organization.

* Corresponding author. Current address: Department of Biology and Wildlife Diseases, University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, 61242 Brno, Czech Republic. Tel.: +420 776890848.
E-mail address: albkat@seznam.cz (K. Albrechtová).

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1. Introduction

There are several species from the family Onchocercidae occurring as vector-borne pathogens in carnivore populations worldwide. In recent years, considerable attention has been given to the genus *Dirofilaria*, especially the causative agent of canine heartworm disease, *D. immitis* (Leidy, 1856). The biology of the genera *Acanthocheilonema* (formerly *Dipetalonema*), *Onchocerca* and *Brugia* remains obscure despite their significant prevalence in certain regions (Snowden and Hammerberg, 1989; Genchi et al., 2005; Sréter and Széll, 2008).

Acanthocheilonema dracunculoides (Cobbold, 1870) and *A. reconditum* (Grassi, 1890) are widespread in East Africa, in patterns corresponding to the occurrence of their vectors and favorable climatic conditions (Nelson et al., 1962; Lightner and Reardon, 1983). Adults of *A. dracunculoides* develop in the thoracic cavity and on the greater omentum of dogs. Ticks (family Ixodidae) or louse-flies (family Hippoboscidae) are believed to transmit microfilariae (Nelson, 1963; Olmeda-Garcia et al., 1994). *A. reconditum* can be found in the subcutis of hosts and is transmitted by fleas and chewing lice (Zajac and Conboy, 2006). Both *A. dracunculoides* and *A. reconditum* are generally considered non-pathogenic for dogs, yet are capable of causing skin lesions, body cavity effusions and even neurological symptoms, depending on the abundance and localization of adults (Bolio et al., 2002). Both species should be considered within differential diagnosis of microfilariaemia in dogs. *A. dracunculoides* is of special importance because of its close morphological similarity of its microfilariae to those of highly pathogenic *D. immitis* (Schrey and Trautvetter, 1998). Discrimination of microfilariae to the species level by molecular tools is considered far more specific than use of microscopy, especially when dealing with less-known filarial species (Rishniw et al., 2006; Furtado et al., 2009).

In recent years, many studies focused on the host immune mechanisms induced by filarial nematodes, since its understanding is assumed to be necessary for the elimination of human lymphatic filariasis (Ravindran et al., 2003; Duerr et al., 2008). Interactions between filaria and other infectious agents are also broadly discussed, especially concerning the co-infection of human lymphatic filariasis and HIV (Bundy et al., 2000; Brown et al., 2006; Nielsen et al., 2007). Since both diseases are immunosuppressive, it was suggested that acquisition of one can predispose the host for the other (Nielsen et al., 2007). Among dog populations, analogous interactions can be expected between filariasis and canine distemper, perhaps serving as an epidemiological model.

The canine distemper virus (CDV) (genus *Morbillivirus*, family Paramyxoviridae) occurs worldwide and is pathogenic for a range of carnivores. It replicates in macrophages and lymphocytes of the host, leading to lymphopenia, loss of the delayed-type hypersensitivity response and suppression of lymphoproliferation (Schobesberger et al., 2005). Long lasting immunologic abnormalities are present even after clearance of the virus from peripheral leukocytes in convalescent dogs (Beineke et al., 2009). There is no doubt about the substantial direct impingement of CDV on carnivore populations (Cleaveland et al., 2000). Yet the impact of CDV-induced immunosuppression on the epidemiology of other diseases remains an area to be explored (Munson et al., 2008).

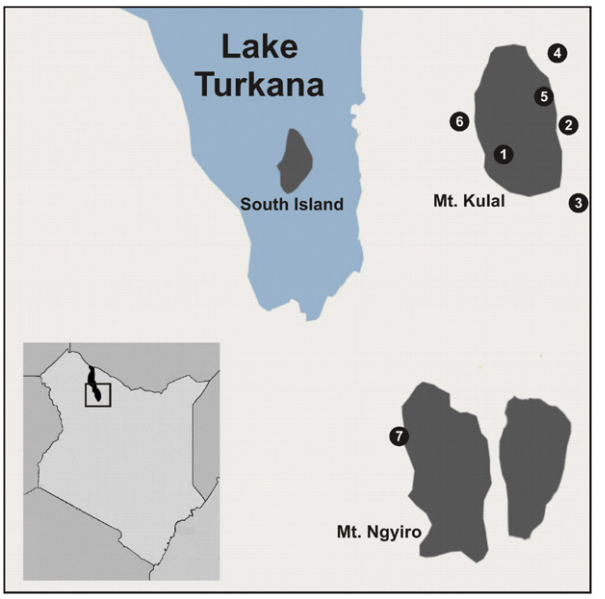

Fig. 1. Sampled localities: (1) Gatab, (2) Olturot, (3)Makutano, (4) Arapal, (5) Kororoi, (6) Larachi, and (7) Tuum.

The principal aim of this study was to describe the spectrum and prevalence of filarial nematodes in dogs living on two isolated mountain ranges in Northern Kenya. For its considerable prevalence on the study site, special emphasis was given to *A. dracunculoides*. We discuss the challenging features of determination of this species, far less known than its pathogenic counterpart, *Dirofilaria immitis*. We compare the prevalence of *A. dracunculoides* in males and females, different age groups and two sampled localities. Besides, we address whether the prevalence of this parasite is associated with canine distemper virus, also highly prevalent in the area.

2. Materials and methods

2.1. Study area

Blood samples of dogs were collected during two antirabies vaccination campaigns on Mt. Kulal and Mt. Ngyiro. Both mountain ranges are situated close to the southern tip of Lake Turkana in the Rift Valley and Eastern provinces of Kenya. The lowlands of the Turkana land region are semiarid, covered by a mosaic of *Acacia-Commiphora* bushland, while the peaks of the mountain ranges are covered with mountain mist forest dominated by *Juniperus procera* and *Olea africana*. The lake is 410 m above sea level, whereas the summit of Mt. Kulal reaches up to 2335 m and Mt. Ngyiro to 2752 m. The campaign was held in seven villages; six on Mt. Kulal and one on Mt. Ngyiro (Table 1 and Fig. 1). Both areas are inhabited by pastoralists, mainly of the tribes Samburu and Turkana. Dogs are kept to guard the herds and their population is characterized by an accelerated turnover rate and sex-ratio bias towards

Table 1
Characterisation of sampled localities.

Locality	Coordinates	Altitude	Characteristics	Human population[a]
Mt. Kulal				
Gatab	N 02°42′ E 37°00′	1800	Permanent village on the lower margin of montane forest	2655
Olturot	N 02°35′ E 37°06′	550	The driest locality in area, surrounded by semidesert flats with *Acacia* on temporary river beds	811
Makutano	N 02°31′ E 6°55′	880	Semi-permanent encampment on the slopes of Mt. Kulal in the area of dry thorny *Acacia* bush	Approx. 50
Arapal	N 02°47′ E 7°00′	850	Large village on the margin between dry Acacia bush and a semi-desert environs	915
Ngorori	N 02°41′ E 7°00′	800	Small settlement, environmentally similar to Arapal	Approx. 50
Larachi	N 02°42′ E 36°51′	750	Permanent village situated on the western slope of Mt. Kulal, environmentally similar to Arapal	312
Mt. Ngyiro				
Tuum	N 2°07′ E 6°47′	1500	Small montane town rich in vegetation, several mountain water sources	2890

[a] Based on September 2008 population census.

females (Macpherson et al., 2000). Dog-oriented veterinary medicine is virtually absent in this area, except for the very scarce use of locally produced antiparasitic medication. Before the campaign depicted in this study, only several dogs were vaccinated against rabies, let alone other infectious diseases. Thus it can be presumed, that all CDV-positive titers represent actually post-infection antibodies.

2.2. Sampling

This study includes samples of 235 dogs, 74 sampled in August and September 2006 and 161 in September and October 2007. From every dog, one sample of serum, one sample of blood preserved with ethanol and two blood smears were collected. Owners were inquired about basic anamnestic data of their dogs, including sex and age. Blood was taken from the cephalic vein into a Monovette syringe (S-Monovette, 7.5 ml, Sarstedt AG&Co, Germany). Blood smears were air-dried and preserved in absolute methanol. Serum was separated within the Monovette syringe and then transferred into 2.5 ml Eppendorf tubes, preserved with sodium azide (0.01% concentration in serum) and kept at refrigerator temperatures (4–10 °C). Approximately 0.5 ml of the blood clot remaining in the Monovette was preserved with 96% ethanol for the purpose of molecular typing.

2.3. Microscopy of blood smears

Blood smears were stained panoptically following the Pappenheim technique (WHO, 2006). Two blood smears per dog were examined under 400× magnification using an Olympus AX70 microscope. The referred prevalence determined by microscopy is cumulative prevalence, rating positive every dog in which microfilariae were found at least on one of the two blood smears examined.

2.4. DNA isolation and molecular typing

DNA was extracted by the phenol–chloroform method described in work by Maslov et al. (1996). In brief, approx. 300 μl of clotted blood was sliced, dried at 37 °C, than re-suspended in 1.5 ml lysis buffer (0.1 M NaCl, 0.05 M EDTA, 0.01 M Tris, 4.8% SDS; pH 8) and digested with 40 μl of Pronase E (12.5 g/l; Sigma–Aldrich, Germany) for 3 h on ice. After the protein lysis, the mixture was extracted three times with a 1:1 blend of phenol and chloroform, followed by two extractions with chloroform alone. Every extraction was performed by 10 min of shaking followed by a 10 min centrifugation step (18,500 × *g*). DNA was precipitated from the phenol-extracted samples by ethanol at −70 °C for 10 min and the DNA pellet was re-suspended in 50 μl of PCR water.

PCR was performed in the Biometra T personal thermocycler (Biometra bio.An. GmbH, Germany). Each PCR reaction consisted of 75 mM Tris–HCl (pH 8,8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 50 U/ml Taq Purple DNA polymerase, 19 nM anti-Taq monoclonal antibody, 10 pM of forward primer, 10 pM of reverse primer (Generi-Biotech, Czech Rep., see Table 2 for sequence) and 1.2 μl of DNA template in a total volume of 25 μl. To asses the adequacy of DNA extraction, all 74 samples from 2006 and the first 40 samples from 2007 were tested in PCR for eukaryotic 16S-like rRNA-coding regions, using primers Eurib-F and Eurib-R and reaction conditions as previously described (Medlin et al., 1988).

Three different PCRs were used in a stepwise manner to determine the species of filaria in the blood-DNA isolates. Firstly, all isolates were screened by pan-filarial primers (DIDR-F1 and DIDR-R1) as described by Rishniw et al. (2006). Samples producing 580–600 bp band in the pan-filarial PCR were considered positive for either *A. dracunculoides* or *A. reconditum* or both species concurrently. To distinguish between the two species pan-filarial PCR products were digested by MseI (3 h at 37 °C and 20 min at 65 °C) as suggested in the original publication (Rishniw et al., 2006). Samples in which the RFLP reaction resulted in two bands (approx. 250 and 330 bp) were considered as positive for *A. dracunculoides*, while those which remained undigested (580–600 bp) were taken as positive for *A. reconditum*. In cases where the RFLP resulted into three

Table 2
Primers used for determination of microfilaria.

Primer pair	Primer sequence	Product size	Reference
A.rec-F1 A.rec-R1	CAG GTG ATG GTT TGA TGT GC CAC TCG CAC TGC TTC ACT TC	384 bp	Mar et al. (2002)
D.rep-F1 D.rep-R1	TGT TTC GGC CTA GTG TTT CGA CCA ACG AGA TGT CGT GCT TTC AAC GTC	247 and 153 bp	Casiraghi et al. (2006)
DIDR-F1 DIDR-R1	AGT GCG AAT TGC AGA CGC ATT GAG AGC GGG TAA TCA CGA CTG AGT TGA	578 bp (*A. reconditum*) 584 bp (*A. dracunculoides*)	Rishniw et al. (2006)

bands (approx. 250, 330 and 580–600 bp) additional *A. reconditum*-specific PCR (primers A.rec-F1 and A.rec-R1) was used as described by Mar et al. (2002). This second PCR was done to distinguish whether both *A. reconditum* and *A. dracunculoides* DNAs are present in the sample or whether the restriction was insufficient and the 580 bp band represents an unrestricted DNA remnant. Third primer pair (D.rep-F1 and D.rep-R1) was used in PCR assay for DNA of *Dirofilaria repens* in samples where the pan-filarial primers produced bands of 580 bp and 480 bp (the 480 bp band was unexpected and due to its sequence might have corresponded to *D. repens*). The D.rep primers were used in PCR as described by Casiraghi et al. (2006).

Three products of *A. reconditum*-specific PCR and five products of pan-filarial PCR originating from different sampled localities were sequenced using the Single Extension Sequencing Service provided by Macrogen Inc., Seoul, Korea. All the sequenced PCR products were extracted from agarose gel using QuickClean DNA Gel Extraction Kit (GenScript Corp., USA). Obtained sequences were analysed using the Blastn version 2.2.18+ program (Altschul et al., 1997), with cut-off *E*-value 0.001. Samples identified by sequencing were employed as positive controls in PCR with pan-filarial primers and the *A. reconditum*-specific PCR reaction. Blood of 2-year-old healthy dog living in Czech Republic was used as negative control in all PCR reactions.

2.5. Serology

Serum samples were examined by an indirect immunofluorescent test (IFAT) for detection of antibodies against CDV. Sera were diluted in a twofold series starting at 1:100 as a basic dilution. Commercial CDV-positive serum (VMRD Inc., Pullman, USA) was used as positive control, serum of nonvaccinated laboratory dog was used as negative control. Diluted sera were placed on the 12-well substrate slides (VMRD Inc., Pullman, USA) and incubated in a humid chamber for 30 min at 37 °C. After incubation, slides were rinsed and soaked for 10 min in PBS. Anti-Canine IgG FITC conjugate (Sigma–Aldrich, Germany) was placed in the wells, incubated and rinsed again. Slides were viewed in fluorescent microscope, using immersion oil at 1000×. Titers were taken as CDV-positive if higher of equal to 1:100.

2.6. Statistical analysis

To explore the effect of sex, age and CDV status on the occurrence of *A. dracunculoides* in Samburu dogs, we fitted several general linear models (GLMs) with binomial distribution using R software (R Development Core Team, 2009). Dogs were classified into groups by age (group 1: up to 4 months; group 2: from 5 months to 18 months; group 3: 19 months to 7 years), sex (males, females), presence of antibodies against canine distemper virus (CDV-seropositive, CDV-seronegative) and locality of origin (Mt. Kulal, Mt. Ngyiro). Selected interactions to the second degree, namely year × CDV, age × locality, age × CDV, locality × CDV and sex × CDV, were included into the maximum model. Using model simplification, we gradually removed from the maximum model the least significant terms to obtain a minimal adequate model (Crawley, 2007).

3. Results

3.1. Identification of microfilariae in blood smears

Microfilariae were detected in 18% of 235 blood smears (33.8% in 2006 and 10.6% in 2007). The central body of the microfilaria was filled with dense nuclei, while the cephalic and caudal ends were nuclei-free. The excretory pore was localized in the second fifth of the microfilaria and the anal pore was visible in the last fifth. The caudal end of the microfilariae was straight (Fig. 2).

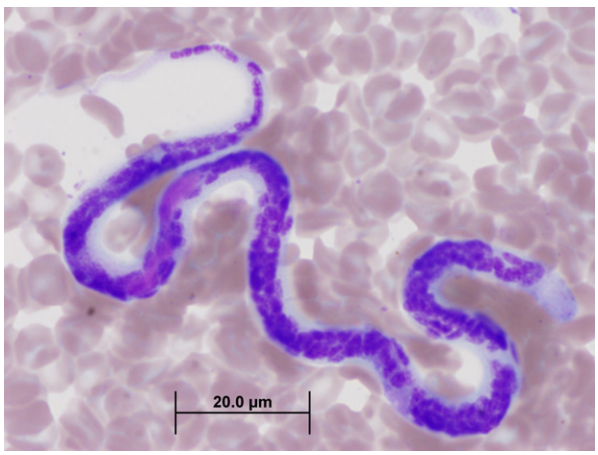

Fig. 2. Microfilaria of *A. dracunculoides*. Dog D 111 – peripheral blood smear, Gatab, Kenya, 2007. Light microscopy, May-Grünwald, Giemsa–Romanowski stain.

Table 3
Prevalence of *A. dracunculoides* and *A. reconditum* at different sampled localities.

	2006	2007	
	A. dracunculoides	*A. dracunculoides*	*A. reconditum*
Mt. Kulal			
Gatab	66.0% (31/47)	46.2% (18/39)	0
Olturot	Not sampled	58.8% (10/17)	41.2% (7/17)
Makutano	55.6% (5/9)	33.3% (4/12)	0
Arapal	Not sampled	25.0% (5/20)	0
Kororoi	Not sampled	83.3% (5/6)	0
Larachi	Not sampled	18.2% (2/11)	0
Mt. Ngyiro			
Tuum	38.9% (7/18)	25.0% (14/56)	0
Total per year	58.0% (43/74)	36% (58/161)	4.3 (7/161)

Table 4
Sequences of PCR products obtained by BLAST.

No. sample	PCR/primers used	Main BLAST hit	Similarity	*E*-value
D 111	Pan-filarial	*Acanthocheilonema dracunculoides*, ITS-2 complete sequence	97%	0
D 136	Pan-filarial	*Acanthocheilonema dracunculoides*, ITS-2 complete sequence	98%	0
D 249	Pan-filarial	*Acanthocheilonema dracunculoides*, ITS-2 complete sequence	97%	0
D 278	Pan-filarial	*Acanthocheilonema dracunculoides*, ITS-2 complete sequence	96%	0
D 120	Pan-filarial	*Acanthocheilonema reconditum*, ITS-2 complete sequence	80%	0
D 126	*A. rec.*-specific	*Acanthocheilonema reconditum*, COI complete sequence	70%	5e−132
D 130	*A. rec.*-specific	*Acanthocheilonema reconditum*, COI complete sequence	84%	8e−142
D 139	*A. rec.*-specific	*Acanthocheilonema reconditum*, COI complete sequence	75%	1e−145
D 111	Pan-filarial[a]	*Dirofilaria repens*, ITS-2 complete sequence	94%	2e−72

[a] Unexpected band of approx. 480 bp;Fig. 3.

3.2. Identification and prevalence of filariae by molecular techniques

A total of 105 out of 235 samples were positive in PCR with pan-filarial primers (Fig. 3). Subsequent RFLP by MseI and *A. reconditum*-specific PCR revealed that DNA only from *A. dracunculoides* was present in 42.9% samples (101 out of 235) and DNA only from *A. reconditum* was in 3.0% samples (7 out of 235). Four samples contained DNA from both species. The prevalence of *A. dracunculoides* and *A. reconditum* by localities is outlined in Table 3. Seven of the pan-filarial positive samples displayed, besides the expected 580 bp band, an additional band of lower molecular weight (around 480 bp, Fig. 3). This band was sequenced and showed 70–84% similarity with the ITS-2 sequence of *Dirofilaria repens*. In subsequent *D. repens*-specific PCR, all of those 7 samples yielded negative results. The results of sequencing are outlined in Table 4.

3.3. Serology test for Canine distemper virus

Seroprevalence of CDV was 33.4% in 2006 and 11.2% in 2007. Taken by localities, the highest seroprevalence (42.8%; 24 out of 56 dogs) was on Mt. Kulal in 2006, which decreased to 13.3% (14 out of 105 dogs) in 2007. The prevalence on Mt. Ngyiro was 5.8% (1 out of 18 dogs) in 2006 and 7.1% (4 out of 56 dogs) in 2007.3.4 (Fig. 4).

3.4. Statistical analysis

The minimal adequate model that fit for the occurrence of *A. dracunculoides* included the significant parameters

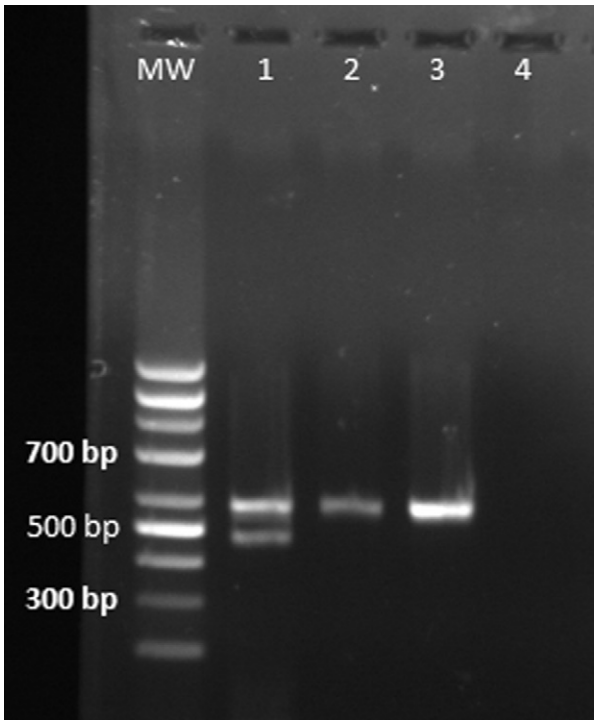

Fig. 3. Gel electrophoresis of pan-filarial amplicons (1% EtBr–agarose gel). Line MW – molecular weight marker; line 1 – DNA of *A. dracunculoides* showing unexpected band of approx. 480 bp (sample D 111); line 2 – DNA of *A. dracunculoides* (sample D 136); line 3 – DNA of *A. reconditum* (sample D 139); line 4 – negative sample (D 137).

Table 5
Occurrence of *A. dracunculoides* in groups by age, sex, CDV status, year and locality.

Parameter assessed	No. positives/total in group	Prevalence in group	Significance
CDV			$p = 0.004$
Seronegative	70/193	36.3%	
Seropositive	31/42	73.8%	
Sex			$p = 0.023$
Female	19/59	32.2%	
Male	82/176	46.6%	
Age			$p = 0.153$
<4 months	16/38	42.1%	
5–18 months	48/122	39.3%	
>18 months	37/75	49.3%	
Locality			$p = 0.143$
Mt. Kulal	80/161	49.7%	
Mt. Ngyiro	21/74	28.4%	
Year			$p = 0.179$
2006	43/74	58.1%	
2007	58/161	36.0%	

sex ($z = -2.272$, $p = 0.023$) and CDV ($z = 2.916$, $p = 0.004$), the non-significant parameters year ($z = -1.342$, $p = 0.179$) and locality ($z = 1.080$, $p = 0.143$) and the significant interactions year × CDV ($z = -2.105$, $p = 0.036$) and age × locality ($z = -2.133$, $p = 0.033$). *A. dracunculoides* was more prevalent in males and CDV-seropositive dogs (Table 5).

4. Discussion

African domestic dogs can host at least eight different species of filaria. Among them, *Dirofilaria immitis* and *D. repens* are well known because of their clinical importance and frequent occurrence all over the world. *A. dracunculoides* and *A. reconditum* have so far attracted somewhat less attention and some aspects of their biology remain unknown. Four other species could be characterized as mysterious, especially in respect to their occurrence in canines: *Acanthocheilonema grassii* (NOE, 1907), *Brugia patei* (Buckley, Nelson and Heisch, 1958), *B. malai* (Brug, 1927) and *B. pahangi* (Buckley and Edeson, 1956) (Nelson et al., 1962; Rinaldi and Cringoli, 2007; McCall et al., 2008).

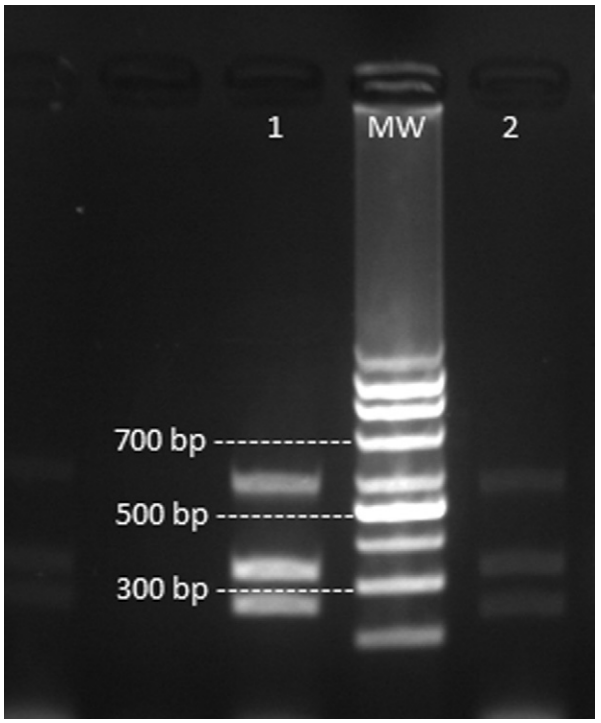

Fig. 4. Gel electrophoresis of pan-filarial amplicons, digested by MseI (1.5% EtBr-agarose gel). Line MW – molecular weight marker; line 1 – DNA of *A. dracunculoides*, showing remnant of original 584 bp band (sample D 136); line 2 – bands resulting from co-infection of *A. dracunculoides* and *A. reconditum* (sample D 126).

Among several methodical approaches available for detection and species-identification of filarial nematodes, molecular methods are assumed to be the most sensitive and specific (Rishniw et al., 2006; Furtado et al., 2009). Since microscopic examination of peripheral blood smear or the buffy coat lacks both of these qualities (Courtney and Zeng, 2001; Rinaldi and Cringoli, 2007), detection and determination of filaria in the "pre-molecular era" was based upon blood concentration methods followed by histochemical staining and eventual confirmation by serological tests. As there is certain ambiguity concerning descriptions of histochemical patterns of less common species of filaria such as *Acanthocheilonema* or *Brugia* (Ortega-Mora et al., 1989; Schrey and Trautvetter, 1998; Peribáñez et al., 2001), this method of diagnosis could have been misleading and was not employed in this study. Similarly, the value of serological tests, which are available only of *D. immitis*, would be limited. Finally the Knott's technique, classical method for filarial nematodes detection and discrimination, would have presumed formalin preservation of the blood samples. As the sampling was designed to be simple and suitable for molecular detection of various blood parasites, ethanol was preferred before formalin to preserve the blood cells. As the morphology of microfilariae found on blood smears was consistent with the key characteristics of the genera *Acanthocheilonema* and *Dirofilaria* (Schrey and Trautvetter, 1998; Rinaldi and Cringoli, 2007), we decided to pursue the RFLP-PCR reaction designed by Rishniw et al. (2006).

The biggest asset of the reaction used is its universality; pan-filarial primers are able to amplify DNA fragments of six different species of canine microfilariae, distinguishable by size of the band. Only the two species of *Acanthocheilonema* produce bands of very similar size and

have to be distinguished by MseI RFLP, where *A. dracunculoides* produces two bands while *A. reconditum* remains undigested. However, in cases where the RFLP results in three bands (two shorter and one original) it is unclear whether the original band is an undigested remnant of *A. dracunculoides*' DNA or whether co-infection is in place (Fig. 4). This was overcomed by a re-check by additional *A. reconditum*-specific PCR. Additionally in several samples positive for *A. dracunculoides* PCR with panfilarial primers resulted in two bands; approximately 580 and 480 bp. The sequence of the unexpected 480 bp band showed high similarity with ITS-2 sequence of *Dirofilaria repens* and led to the employment of *D. repens*-specific PCR. As this PCR was negative in all tested samples, the 480 bp band was considered as an unspecific amplicon of *A. dracunculoides* and its high similarity to *D. repens*-ITS-2 sequence was attributed to the close genetic relationship between the two species.

In summary, *A. dracunculoides* was the predominant filaria parasitizing dogs in the studied areas, while *A. reconditum* was diagnosed only in few cases at a single locality. While the former species has been already reported from the Lake Turkana region (Nelson, 1963; Lightner and Reardon, 1983), the latter was supposed to be confined to humid montane and coastal areas (Nelson et al., 1962). Interestingly, we detected *A. reconditum* only in Olturot, the driest among sampled localities.

Consistently with other studies (Courtney and Zeng, 2001; Mishra et al., 2007; Furtado et al., 2009), the number of samples with microfilaria detectable on blood smear microscopy was considerably lower than the prevalence of filaria obtained by molecular methods. These findings correspond with the presumed ability of PCR to capture positive even individuals sampled within amicrofilariaemic stage of infection. Specifically, DNA released from adults, migrating L3/L4 stage larvae or remnants of dead microfilaria present in the blood may potentially lead to a PCR-positive diagnosis even before or after the stage of microfilariaemia (Lizotte et al., 1994; Touré et al., 1997; Fischer et al., 2000).

Our study showed that the prevalence of *A. dracunculoides* is significantly higher in males. This has been shown also in other studies of canine dirofilariasis and attributed to males' higher outdoor activity, resulting in higher exposure to vectors (Selby et al., 1980; Montoya et al., 1998; Aranda et al., 1998). We can support this hypothesis by our observation that male dogs usually follow livestock herds while females are more settled and thus less exposed to vectors. No significant differences in the prevalence of *A. dracunculoides* in different age groups could be attributed to high dynamics of the studied dog population and short average lifespan. Previous studies, which revealed the cumulative effect of *Dirofilaria* and *Acanthocheilonema* in dogs older than five years (Theis et al., 1995; Aranda et al., 1998; Cringoli et al., 2001; Bolio et al., 2004), investigated populations with considerably wider age-range than that of the dog population on Mt. Kulal, and thus should not be compared with our study. The factors "year" and "locality" did not cause significant variability in the distribution of *A. dracunculoides*, despite the geographical isolation of the two sampled areas and higher humidity in the first sampling year (ALRMP, 2007).

The titer of antibodies against CDV was the most significant among the evaluated factors, showing an association between infection with *A. dracunculoides* and seropositivity for CDV. Moreover, a significant interaction between the factors "year" and "CDV" in the occurrence of this nematode was revealed. Although the prevalence of *A. dracunculoides* was higher among CDV-seropositive dogs in both years, the influence of CDV was statistically significant only in year 2006. As indicated by the serological data, an epidemic of canine distemper occurred at Mt. Kulal in 2006. Correspondingly, the association between CDV-seropositivity and infection by *A. dracunculoides* was strongest within this year and locality.

We considered two hypotheses that could explain the association of these two diseases. First, immunosuppression caused by CDV can facilitate the acquisition and establishment of filariasis. This scenario has already been observed in cases of protozoan infections that were perpetuated by CDV co-infection, resulting in epidemics with fatal outcomes (Moller and Nielsen, 1964; Reed and Turek, 1985; Frank, 2001; Munson et al., 2008). However, immune processes occurring in filariasis are different from those produced by protozoa and their character varies depending on developmental stages of filaria. The first defense against infective L3 larvae is based on an acquired antibody response and is possibly reduced by immunosuppression (Duerr et al., 2008). In contrast, the persistence of adults is conditioned by a state of immune hyporesponsiveness of the host (Maizels and Lawrence, 1991). Thus even reverse interaction of the two pathogens is possible, rendering our second hypothesis; the presence of adult filaria in a host leads to immunosuppression, including down-regulation of Th1 cytokines and perpetuating development of viral infection.

Our data can hardly elucidate which of these hypotheses are correct, if they indeed are the only possibilities, but they are certainly not mutually exclusive. However, the results presented here are very suggestive that interaction between CDV and filariasis may represent a significant epidemiological phenomenon and is certainly worth further investigation.

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