A broad insight onto the distribution pattern of Dirofilaria immitis in community dogs in Nepal

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SUBJECT AREAS
Parasitology

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Dirofilaria immitis; stray dogs; zoonosis; haematobiochemical; epidemiology; Nepal
Abstract
Background Nematodes of the genus Dirofilaria are widespread vector-borne helminths (VBH) of increasing relevance. Indeed, dirofilariosis is frequently diagnosed in domestic pets, often dogs, associated with a severe clinical condition known as heartworm disease caused by D. immitis. Assessing the distribution pattern of canine dirofilariosis is pivotal to undertake appropriate control measures and define the risk of infection in animals and humans. This study revealed the occurrence of D. immitis in naïve community dogs from Nepal. Methods An epidemiological study was performed in 2019 in Siddharthanagar (Lumbini region, Nepal). A total of 150 blood specimens were examined using direct microscopy, buffy coat centrifugation and modified Knott’s method aiming at isolating and identifying Dirofilaria microfilariae. In addition, hematobiochemical parameters, including packed cell volume (PCV), alanine aminotransferase (SGPT), aspartate aminotransferase (SGOT), alkaline phosphate (ALP), creatinine, blood urea nitrogen (BUN), urea and total protein were analyzed, along with a clinical scoring (i.e., body temperature and respiratory rate) of the animals enrolled in this study. Results Out of 150 dogs sampled, 29 (19.3%) had D. immitis microfilariae. The prevalence of the infection varied according to the technique used, ranging from 16.0% to 19.3% based on direct blood smear microscopy and modified Knott method, respectively. The infection rate was significantly (P <0.05) higher in dogs aged more than 3 years. A positive correlation was observed between SGPT and SGOT and the presence of microfilariae (P <0.05). Conclusion The current findings revealed the presence of D. immitis in dogs from Nepal, thus providing an explanation for the diagnosis of this VBH is human cases. Further investigations are warranted to accurately define the prevalence of the infection in other pets, instrumentally to reduce the potential burden on the infection in dogs and, accordingly, to control the spread of this parasite to humans.

Background
Canine heartworm disease is a vector-borne helminth (VBH) infection caused by *Dirofilaria immitis* [1], being particularly notorious for its implications in veterinary and human medicine [2–4]. Infected dogs might suffer from a potentially-fatal clinical condition featured by cardiorespiratory alterations, such as pulmonary hypertension, dyspnoea, ascites, caused by the localization of adult worms within
the pulmonary arteries and right heart chamber [5]. Conversely, humans are dead-end hosts for *D. immitis*, as the infection often results in a self-limited pulmonary condition. Nonetheless, the inflammatory response against the parasites induces so-called “coin lesions” potentially misdiagnosed as tumors [6]. Overall, dirofilariosis in dogs and humans is extremely relevant and requires attention by public health authorities and veterinary bodies, ultimately involving a constant need for up to date epidemiological information on the distribution of the parasite [2, 7, 8].

The life cycle of *D. immitis* includes a reservoir and a susceptible host, biologically connected by a competent arthropod vector [5]. While feeding on a native dog, infected mosquitoes of approximately 70 species included in the genera *Culex, Aedes, Ochlerotatus, Anopheles, Armigeres or Mansonia* [9] can transmit *D. immitis* infective third-stage larvae (L3) into the definitive host. Larvae migrate into the skin through the insect bite wound, developing in L4 in 3 days and reaching the pulmonary arteries in 47-67 days [2, 5]. Worms continue their development and growth for 4-5 months, finally reaching the adult stage. Over their lifespan, sexually-mature females release microfilariae into the bloodstream, which are eventually ingested by mosquitoes during their meal. Inside the vector, microfilariae moult into L3 in 10-14 days [5]. Totally, a period of 7 to 9 months is necessary for *D. immitis* to conclude its life cycle, persisting for around 4-5 years in the pulmonary arteries of the infected dog [10]. During this long period, microfilaraemic animals represent a continuous source of microfilariae for female mosquitoes, indirectly regulating the distribution of the parasite and the seasonality of the infection [5, 10].

*Dirofilaria immitis* has been diagnosed worldwide in dogs from different countries and climatic areas, including temperate, tropical, subtropical countries, such as Southeast Asia. In this continent, however, the literature on dirofilariosis is mainly limited to reports from Korea [11], India [12], Taiwan [13], or China [14], with scant information from Nepal. Although the presence of the parasite has been suspected following an isolated clinical record in a dog with chylothorax [15] and a human dirofilariosis case in an elderly woman [16], no epidemiological data is available from this country, which actually counts a large number of stray dogs, potentially serving as reservoir for the infection [17-18].
In the present study, the occurrence of *D. immitis* was investigated in naïve stray dogs and documented for the first time through the use of traditional parasitological and hematobiochemical assays, providing original data of the prevalence of canine dirofilariosis in Nepal.

**Methods**

**Study area**

The study was conducted from November 2018 to May 2019 in Siddharthanagar, Nepal (Indo-Nepal border, 28°10′N, 84°15′E), a municipality located 107 m above sea level. The area is featured by a semi-tropical climate with annual temperature of 24.8°C (range 18.7°C - 30.9°C), and rainfall means 143.8 mm (range 8.2-545.6 mm).

**Animals and sampling procedures**

Blood specimens (approximately 10 ml) containing ethylene diamine tetraacetic acid (EDTA) were randomly collected from stray dogs and sent to the Veterinary Teaching Hospital (VTH, Institute of Agriculture and Animal Science, Tribhuvan University, Siddharthanagar). Animals were categorized into four age classes (i.e. <1, 1–3, 3–5 and >5 years) and subjected to an accurate anatomopathological examination at VTH if found dead during the study period. In particular, dogs were examined for cardiopulmonary parasites, which were identified at species level based on their morphological features [5].

**Parasitological examination**

Samples were examined by direct microscopy,uffy coat centrifugation and modified Knott's method. In the former, one drop of blood (approximately 20 µl) was placed onto a clean microscope slide with a cover slide and microscopically examined, with samples showing slight undulating movements of larvae considered as positive. In addition, micro-hematocrit tubes were filled with 20 µl blood, centrifuged at 12000 rpm for 5 min and observed for alive microfilariae on theuffy coat. Finally, a third subsample was evaluated according to the modified Knott's method. One ml of EDTA blood was mixed with 9 ml 2% formalin solution and centrifuged at 1500 rpm for 5 min. After discarding the
supernatant, 0.1% methylene blue was added to the sediment, which was transferred onto a microscope slide using a plastic pipette. The sample was examined and microfilariae were identified at species level based on their morphometry and morphology of the anterior and posterior extremities [19–22].

Hematobiochemical and biochemical evaluations

The following parameters were analyzed in microfilaria-positive and the -negative dogs: packed cell volume (PCV), alanine aminotransferase (SGPT), aspartate aminotransferase (SGOT), alkaline phosphate (ALP), creatinine, blood urea nitrogen (BUN), urea and total protein. All data were determined by using an advanced microprocessor-based colorimeter and biochemical reagents (Accurex Biomedical Pvt. Ltd., India).

Statistical analysis

Statistical analysis was conducted using Statistical package on social science (SPSS) v.25 and MS Excel were used for data analysis. The analysis was performed using Chi-square test and one sample T-test with comparison of means. The significance level was set at $P < 0.05$.

Results

A total of 150 dogs (81 males and 69 females) were enrolled. Of these animals, 25 (16.7 %) were less than 1 year, 34 (22.7%) aged 1-3 years, 44 (29.3%) aged 3-5 years and 47 (31.3%) over 5 years. Soon after the blood collection, 19 dogs (12.7%) were found dead and showed chylothorax and ascites upon necropsy. Nine (47.4%) of them were infected with *D. immitis* heartworms (range 2-14 adult nematodes) (Fig. 1). Overall, only 41 dogs (27.6%) suffered from elevated respiratory rate (more than 30 acts per minute) whereas the majority (129 animals, 86.2%) had elevated body temperature, ranging from 37.5 to 39.0°C, but not statistical correlation was observed ($P= 0.611$). Upon parasitological analysis of blood samples, 29 (19.3%) animals were positive for microfilariae. Larvae measured 305 µm in length and showed a conical front end a thin and straight rear end, suggestive for *D. immitis* (Fig. 2). The sensitivity of the diagnosis was higher in samples analyzed by
the modified Knott method (i.e., 19.3%) than those by direct smear microscopy on blood or buffy coat (i.e., both 16.0%). The prevalence of the infection was higher in female dogs than males (i.e., 21.7% vs 17.3%) but not statically significant ($P=0.49$), and in dogs aged 3-5 years (51.7%) than those younger than one year (3.5%) (Fig. 3).

PCV ranged 35%-54% in 79.3% of infected dogs, while it was less than 35% in 13.8% of infected dogs and more than 54% in 6.9% of infected dogs. A significant correlation between SGPT and SGOT was recorded in infected animals ($P<0.05$) (Table 1).

**Discussion**

Results of the present study documented the occurrence of *D. immitis* infection in community dogs from Nepal demonstrating that the parasite is circulating among native dogs living in the Indo-Nepal border. With the exception of previous reports from mainland and northeast India [12, 23–25] or Bangladesh [26], no epidemiological data was available from the study area. Interestingly, the prevalence of the infection herein reported is relatively higher than that reported from the eastern Indian boundaries [12, 23–26], and whether this is due to the different climatic conditions, activity of arthropod vectors or availability of a larger population of stray dogs is yet to define. Although it is scientifically proved that mosquitoes are the main intermediate hosts of *Dirofilaria* spp. and are crucial for the larval development of microfilariae into L3 and their transmission [27–30], no information is reported on the species involved into the circulation of these parasites within the Indo-Nepal region. Nevertheless, mosquitoes are active in this area, as it can be inferred from the efforts spent on the control and epidemiological surveillance of other vector-borne diseases, such as Dengue, Zika or other human filarial worms [30–32].

The lack of an overt clinical presentation in *D. immitis* infected dogs is related to the life cycle of this nematode and to the pathological alterations it may cause, which are often detected during the chronic phase of the infection. As confirmed in this study, a significant correlation between dirofilariosis and increased respiratory rate might be observed, although this clinical sign might be associated with other clinical conditions [33–34]. However, when observed during canine heartworm disease, dyspnoea is mainly attributed to the presence of adult nematodes in the arteries interfering
with normal cardiopulmonary function, ultimately inducing hemolysis, liver or kidney dysfunction, and respiratory failure [5, 10, 35]. The persistence of *D. immitis* for up to 6 years and long life cycle of the parasite (i.e., 7 to 9 months) also explains the significantly-higher and -lower prevalence of the infection is in dogs older than 5 years and younger than one year, respectively. In the latter, however, a false negative diagnosis cannot be excluded based on the sensitivity of the detection method tested in the present study, only allowing to confirm the occurrence of *D. immitis* when the infection is patent [5, 10]. Comparing the techniques herein tested, the modified Knott’s method was more sensitive than the direct detection of larvae either in blood or buffy coat, based on the principle that microfilariae were concentrated into a small volume before being analyzed [36]. The use of indirect diagnostic methods, like serology, might overcome this limitation. Although the direct parasitological methods are straightforward [10, 37], they are less sensitive and a potentially-low specific compared to antigen or antibodies detection methods, thus reducing the potential for misidentification of microfilariae species [20, 38]. Currently, the identification of *D. immitis* microfilariae has been corroborated by the detection of adult heartworms upon necropsy. Nonetheless, other zoonotic filarioid species might be present in this region, including *Dirofilaria repens* or *Acanthocheilonema* spp. [25].

Finally, the lack of significance between alteration in PCV and positivity for *D. immitis* was not surprising, as previously reported [5, 10]. Oppositely, the increase in SGPT and SGOT might be associated with predominant intracellular activity following the inflammation caused by the parasite, as previously reported [39]. Accordingly, the cellular damage increases alongside the progression of the disease [5, 10]. Although this epidemiological study was limited by the relatively small number of dogs enrolled, the present findings provided new data on the occurrence of *D. immitis* in a remote region of Nepal where no data was available so far, despite the report of human and animal cases [15, 16]. Further investigations are warrant to learn about the distribution of other canine VBD in Nepal.

**Conclusion**

To the best of our knowledge, this is the first report documenting the occurrence of *D. immitis* in
community dogs from the Indo-Nepal region and demonstrating an active circulation of the parasite. The veterinary care in the study area is considered inadequate and this results into free movement of untreated domestic animals and wildlife potentially acting as a source of the infection for large populations of stray dogs potentially serving as a reservoir for the infection [17–18] for humans living within the Nepal/India urban districts. Strict hygienic measures, control programs of stray dogs and adequate control of mosquitoes are urgently demanded in order to reduce the potential threat represented by D. immitis and other zoonotic parasites that might be present in this region.

Declarations

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Ethical approval

Ethical clearance and publication approval was given by the ethical review committee of the Nepal Veterinary Council.

Conflict of interest

The authors declare that there was no conflict of interest.

Consent of publication

No need the consent of publication

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Table 1
Table 1 Biochemical parameters evaluated in sera of examined community dogs in Siddharthnagar sub-metropolitan city, Nepal

| Parameter                          | Infected dogs     | Non-infected dogs |
|------------------------------------|-------------------|-------------------|
| Total protein (mg/dl)              | 7.37 ± 0.36       | 6.56 ± 0.29       |
| Aspartate Aminotransferase (SGOT) (IU/L) | 26.73 ± 3.54      | 17.98 ± 2.52      |
| Alkaline phosphatase (ALP) (IU/L)  | 376.11 ± 39.60    | 337.35 ± 53.71    |
| Alanine Aminotransferase (SGPT) (IU/L) | 27.25 ± 2.65      | 19.91 ± 2.47      |
| Urea (mg/dl)                       | 22.19.12 ± 1.91   | 25.24 ± 2.90      |
| Blood Urea Nitrogen (BUN) (mg/dl)  | 11.39 ± 0.101     | 12.21 ± 1.49      |
| Creatinine (mg/dl)                 | 1.63 ± 0.132      | 1.46 ± 0.12       |

Figures
Figure 1

Adult heartworm (Dirofilaria immitis) revealed from Right Ventricle and Pulmonary artery in necropsy finding
Microfilariae of the recovered Dirofilaria immitis. a. The whole microfilaria in direct blood smears with x 10. b. Magnified power of the microfilaria with x 20. c. The whole microfilaria of D. immitis. Scale bar = 50 µm. d. The posterior end of D. immitis microfilaria, Note the characteristic thin and straight end.
Figure 3

The prevalence of Dirofilaria in community dogs of different aged group of Siddharthanagar Sub-metropolitan city, Nepal using various traditional techniques

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