Annals of Parasitology 2022, 68(4), 779–786
doi: 10.17420/ap6804.485

Original paper

Molecular identification of hookworm species infecting free-roaming and owned dogs from an urban area in inner São Paulo State, Brazil

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ABSTRACT. Dogs are the most popular pet animals worldwide, but on the other hand, they are main hosts of pathogens potentially transmissible to humans. The aim of this study was to assess the occurrence of intestinal parasites in free-roaming and owned dogs in an urban area in southeastern Brazil and to identify the hookworm species infecting them. Faecal samples (80 from free-roaming and 53 from owned dogs) were examined for intestinal parasites using concentration methods. DNA extracted from hookworm microscopy-positive samples were tested by PCR targeting the ITS1–5.8S–ITS2 region and the amplicons retrieved were sequenced. Intestinal parasites were detected in 43.60% (58/133) of the dogs and hookworm infection was found at the highest prevalence rate (38.30%), followed by *Toxocara canis* (10.50%), *Trichuris vulpis* (2.25%), *Giardia* spp. (0.75%) and *Cystoisospora* spp. (0.75%). Out of the 51 samples positive for hookworm eggs, 26 (50.90%) were successfully amplified and sequenced. Single infections with *Ancylostoma caninum* and *Ancylostoma braziliense* were recorded in 18 (69.20%) and two (7.70%) isolates, respectively, and mixed infections were found in the remaining six samples (23.10%). Both species were found infecting free-roaming and owned animals, but *A. caninum* was more common. These findings highlight the public health relevance of dogs as reservoirs of zoonotic parasites, with emphasis on hookworm species commonly implicated in cutaneous larva migrans (CLM) in poor and deprived areas.

Keywords: dogs, gastrointestinal parasites, zoonosis, hookworms, PCR, *Ancylostoma*

Introduction

The relationship between dogs and humans became established before the dawn of human civilization, but it can have positive or negative repercussions on the health of individuals and communities depending on how dogs are treated and managed [1,2]. A global phenomenon of increasing canine populations has been observed, mainly in developing regions, where overpopulation has become a public health threat.

The global population of domestic dogs is estimated at approximately 700 million, with around 75% classified as “free-roaming” [3]. In contrast to these high numbers, responsible pet ownership remains low and most of these animals are literally abandoned, living freely in the streets without care and/or without human supervision. Undoubtedly, these practices have an important impact on the dispersion of these companion animals as well as on the widespread dissemination of their pathogens, which are potentially transmissible to the human population [4]. In this context, infections caused by hookworms are of importance in terms of dispersion and risk to animal and human health [4].

The common hookworms of dogs are *Ancylostoma caninum*, *Ancylostoma braziliense*, *Ancylostoma ceylanicum* and *Uncinaria stenocephala* [5]. Among these species, *A. caninum*, *A. braziliense* and *A. ceylanicum* are ubiquitous hookworms found in dogs, especially in tropical climates, where conditions are conducive for hookworm survival [6]. All these hookworm species are blood-feeding intestinal parasites, but they differ in the severity of the disease that they cause to their hosts and in their roles as agents of zoonotic disease, particularly as causes of cutaneous larva migrans (CLM). This is a neglected parasitic skin disease commonly found in populations living in low-income settings in tropical and subtropical regions, and in travelers returning from these areas [5,7]. In this regard, *A. caninum* is the most pathogenic species and causes severe clinical signs and a high death rate, especially among puppies and immunosuppressed animals [4,5]. On the other hand, *A. braziliense* has been considered to be the major etiological agent responsible for human CLM [7]. In addition, *A. caninum* has also been implicated in CLM and is the leading cause of human eosinophilic enteritis [6].

Developing settings worldwide, including Brazil, are still home to large populations of dogs living as free-roaming animals in both rural and urban centers [8]. Despite programs to reduce overpopulation, stray dogs or even owned animals living in deprived areas impose a risk to human health relating to transmission of zoonotic pathogens, including hookworms.

Thus, given the public health significance of these animals that are the most popular pets worldwide, the aim of the present study was to verify occurrences of intestinal parasites infecting free-roaming and owned dogs in an urban area in southeastern Brazil, and to identify the hookworm species among these parasites.

Materials and Methods

Study site and faecal samples collection

The study was conducted at the municipal animal shelter in Botucatu (22º53'09"S and 48º26'42"W), state of São Paulo, Brazil. Over a one-year period, fecal samples were collected in two different occasions: (1) from free-roaming dogs that had been rescued from the streets and were housed at the shelter; and (2) from owned dogs that had been enrolled in a spaying/neutering program at this shelter that was provided for low-income pet owners. A single individual stool sample was obtained from each animal, with no preference given for sex, breed, age, or clinical status. However, only animals that had not previously been dewormed (either at the shelter after capture or in the household by the owner) were included in the study. Data regarding age, sex and breed were recorded. The animal ages were mainly estimated by dentition analysis and were categorized as follows: pups less than six months, juveniles from six months to less than one year and adults more than one year old.

Samples were collected from free-roaming dogs within the first 24 hours after rescue and shelter admission. Each animal was kept alone overnight in an enclosure and fresh stool samples were collected in the early morning, before the enclosures were cleaned. Concerning owned dogs, faecal samples could be collected from the animals under anesthesia, by a veterinarian at the time of implementing sterilization. Faecal samples were taken from anesthetized animals after spontaneous defecation when they were handled or were taken directly from the rectum. All the faecal samples were collected in sterile flasks and were transported under refrigeration (in a cooler box with ice packs) to the Department of Parasitology of UNESP for analysis.

Microscopic examination of faecal samples

In the laboratory, each sample was filtered through gauze into a test tube washed three times with phosphate buffered saline (800×g for three minutes) to remove excess debris and to concentrate the material. An aliquot of the final sediment was examined using saline and Lugol-iodine stain by light microscopy under ×100 and ×400 magnifications to search for parasite eggs, cysts and oocysts. Next, an aliquot of faecal sediment was stored at –20°C for subsequent DNA extraction, and the remaining material was processed by using the zinc sulfate flotation technique and examined as previously mentioned.

DNA extraction and molecular analysis

For molecular characterization, DNA was extracted from all the faecal samples that were microscopically positive for hookworm eggs. To optimize disruption of eggs prior to DNA extraction, 200 mg of faecal sediments were suspended in 1.4 ml lysis buffer and then subjected to three cycles of freezing at liquid nitrogen temperatures followed by thawing at 96–98°C [9]. The resulting material was then used to obtain purified DNA using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purified DNA was eluted in 200 µl of the elution buffer and stored at –20°C until use. Amplification of the ITS1–5.8S–ITS2 region was carried out according to previously published protocol [9]. For species identification, the primers RTGHFI (5'-CGTGCT AGTCTTCAGGACTTTG-3') and RTABCR1 (5'-CGGGAATTGCTATAAGCAAGTGC-3') were employed to amplify a 545-bp DNA fragment of *A. caninum*, *A. ceylanicum* and *U. stenocephala* hookworms. A 673-bp fragment of *A. braziliense* was amplified using RTGHF1 and the specific reverse primer RTAYR1 (5'-CTGCTGAAAAGTC CTCAAGTCC-3'). All the PCR reactions were performed in 50 volumes and samples were heated to 94°C for 2 min, 64°C for 1 min, 72°C for 2 min followed by 50 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 30 s and 1 cycle of 72°C for 7 min. The amplicons were subsequently viewed on 1.5% agarose/ethidium bromide gels. In all PCR reactions, positive (DNA recovery from positive samples and confirmed via amplicon sequencing) and negative controls (ultrapure water) were included.

For determining the nucleotide sequences, PCR products were excised from the agarose gel, purified using the Ultrafree® DA kit (Millipore Corp., USA) and sequenced on both strands at a sequencing service (Biotechnology Institute, Sao Paulo State University), using the same set of primers as in the PCR assays. The sequences thus generated were edited manually using Chromas 2.6.6 (Technelysium Pty Ltd, South Brisbane, Australia) and then were aligned with each other and with reference sequences downloaded from GenBank, using Clustal X. Hookworm species were confirmed through identity search of the National Center for Biotechnology Information (NCBI) reference sequences, using the Basic Local Alignment Search Tool (BLAST).

Data analysis

The prevalence of each enteric parasite was calculated based on microscopic examination. Regarding hookworms, the prevalence was determined taking into account positive result obtained by microscopy and/or PCR screening. The Chi-square test or Fisher's exact test was applied to compare parasite prevalence rates in the canine population, assessed according to sex, age and status as free-roaming or owned dog. Multiple regression analysis (Cox model) was used to evaluate possible risk factors associated with the prevalence of the most common parasites and the aforementioned variables. All statistical analyses were performed using SAS 9.3 and $P<0.05$ was taken as the level of significance.

Ethical considerations

All the procedures in this study were guided by the principles of animal welfare and were approved by the Ethics Committee on Animal Experimentation (CEEA) of the Bioscience Institute, IBB-UNESP (protocol number 601). All the dog owners voluntarily accepted that their animals could be included in the study, and all gave oral consent.

Results

Single faecal samples were obtained from 133 dogs, comprising 69 females (51.90%) and 64 males (48.10%), with predominance of mixed-breed animals (129/133; 97.00%). Regarding age, 45.90% were adults (61/133), while 20.30% (n=27) and 33.80% (n=45) were puppies and juveniles, respectively. A total of 60.15% (80/133) of these animals were free-roaming dogs that were housed in a shelter after they had been caught in the city, while 39.85% (53/133) were living with an owner and had been taken to this shelter for surgical sterilization. Based on information provided by the shelter administration, 67.50% (54/80) of the free-roaming dogs were caught in districts outside the city center, among which 50.00% (27/54) had been encountered on the outskirts of the urban area, mainly near to roads. Regarding owned dogs, most of them (75.50%) were living in households in poorly resourced communities in the city.

Microscopic examination revealed that at least one gastrointestinal parasite was detected in 43.60% (58/133) of the dogs surveyed, including 24.10% (32/133) and 19.50% (26/133) of the free-roaming and owned animals, respectively. Hookworms were the parasites most frequently detected, in 38.30% (51/133) of the dogs examined dogs, followed by *Toxocara canis* (10.50%), *Trichuris vulpis* (2.25%),

Table 1. Frequency of gastrointestinal parasites detected in faecal samples from free-roaming and owned dogs from an urban in inner São Paulo State, Brazil

Parasite	Free-roaming dogs (n=80)		Owned dogs (n=53)		Total (n=133)	%
	No. of positives	%	No. of positives	%		
Hookworms	26	32.50	25	47.20	51	38.30
Toxocara canis.	10	12.50	4	7.50	14	10.50
Trichuris vulpis	2	2.50	1	1.90	3	2.25
Giardia spp.	1	1.25	–	–	1	0.75
Cystoisospora spp.	1	1.25	–	–	1	0.75

Giardia spp. (0.75%) and *Cystoisospora* spp. (0.75%) (Tab. 1). The analysis on the prevalence of parasites in the dogs of the present study according to age, sex and status as free-roaming or owned did not show any statistically significant differences (*P*-values from 0.197 to 0.987). However, younger dogs (puppies and juveniles) were at higher risk of harboring *Toxocara canis* than were adults (prevalence ratio 4.66; 95% confidence interval 1.38–15.68; *P*=0.013). Although hookworms were found infecting both free-roaming and owned dogs without any significant difference in infection rates, they were more prevalent in owned dogs (25/53; 47.20%) than in free-roaming dogs (26/80; 32.50%) (Tab. 1).

Of 51 samples positive for hookworm eggs, 50.90% (26 samples) were amplified by PCR and successfully sequenced for species identification. PCR amplicons generated with ITS primers were sequenced and two hookworm species, namely *A. caninum* and *A. braziliense*, were identified in the present study. The DNA sequencing results revealed that out of the 26 PCR-positive samples, 18 samples (69.20%) and two samples (7.70%) matched with single infections with *A. caninum* or with *A. braziliense*, respectively (Tab. 2). The remaining six samples assessed (23.10%) were shown to have mixed *A. caninum* and *A. braziliense* infections (Tab. 2). In total, *A. caninum* and *A. braziliense* were detected in 80.80% (21/26) and 19.20% (5/26), respectively, and both species were found infecting free-roaming and owned animals (Tab. 2).

Searches in BLAST revealed that the sequences obtained in the present study were highly similar (96.50–100% identical) to previously published sequences available in GenBank. Comparison of the sequences classified as *A. caninum* revealed that they were 97.90–100% identical to sequences from Australia (KP844730), China (KC755026), USA (JQ812694), Kenya (MG271920), Tanzania (MG890369, MG890370, MG890373, MG890375, MG890369 and MG890375), and Brazil (DQ438070, DQ438071, DQ438072, DQ438073, DQ438075 and DQ438078). Regarding *A. braziliense*, the isolates that were successfully sequenced and confirmed as such showed similarity of 96.50–97.0% with previously published sequences of this species in USA (JQ812693) and Brazil (DQ438059-DQ438064).

Discussion

Over the years, dogs have assumed an important role as pets that provide positive psychological and

Table 2. Molecular determination of hookworm species infecting free-roaming and owned dogs from an urban area in inner São Paulo State, Brazil

	Samples tested	Positive samples		Hookworm species		
		Microscopy	PCR*	*A. caninum* n (%)	*A. braziliense* n (%)	Mixed infection n (%)
Free-roaming	80	26	16	11 (61.10)	02 (100.0)	03 (50.00)
Owned	53	24	10	07 (38.90)	_	03 (50.00)
Total	133	50	26	18 (69.20)	02 (7.70)	06 (23.10)

*Samples successfully amplified and sequenced physical benefits for their owners [10]. On the other hand, despite this close relationship, many animals are left free on the streets due to indiscriminate breeding of unowned dogs, along with abandonment by irresponsible owners [11]. In the present survey, among 133 dogs enrolled in the survey, 60.15% were free-roaming animals housed in a shelter, while 39.85% (53/133) were living with an owner and attending in the shelter for surgical sterilization. In urban centers, many dogs captured in the streets by the authorities are housed in public shelters, are sterilised and directed to adoption. Although shelters are not always environments that guarantee animal welfare, these places can provide a temporary home for stray, lost or owner-surrendered animals, until they can be reclaimed by the owner or adopted into another family's household [12]. In addition to providing a safe environment, food and veterinary care for stray dogs removed from public areas, the shelter enrolled in the present study was running a spaying-neutering program for pet owners who otherwise would not be able to afford it. Another point to be commented is that, interestingly, most of the owned dogs inhabit households in poorly resourced communities in the city, which often coincided with areas on the town's outskirts where free-roaming animals were frequently caught. High abundance of free-roaming dogs in urban areas, especially in regions under poorer socioeconomic conditions and with higher population densities, has been reported in other studies [13,14].

Close contact with dogs favors zoonotic transmission of parasitic infections to humans and thus is a public health concern. It is well known that some pathogens that infect dogs, especially gastrointestinal parasites, can affect humans either through close contact or through contamination of the environment [8,12]. In the present study, a considerable overall prevalence (43.60%) of canine gastrointestinal parasites was observed, highlighting infections by zoonotic parasites such as hookworms (38.30%) and *Toxocara canis* (10.50%) in both free-roaming and owned animals. The occurrence of these parasites, infecting free-roaming and owned dogs, was consistent with what was found in other recent investigations [15–20]. In general, these zoonotic helminths are commonly detected infecting dogs in different regions worldwide, regardless of whether they are free-roaming, kept in shelters or owned. However, prevalence rates may vary widely depending on factors such as age, climate, environment and socioeconomic conditions of settings [12,21].

The predominance of hookworms and *T. canis* should be considered a relevant threat to public health, since they are the causative agents of cutaneous and visceral larva migrans, respectively. Investigations have demonstrated that the frequencies of these gastrointestinal parasites in stray and shelter dogs are higher than in owned pet populations. Stray and shelter dogs can commonly become parasitized in developing countries due to the lack of regular prophylactic treatment [12]. In this regard, it is important to mention that none of the free-roaming or owned animals enrolled in the present study had previously undergone deworming, which thus reinforces the latter statement.

No statistically significant differences were observed between the prevalence of parasites and the variables age, sex and status as free-roaming or owned animal, however some aspects deserve comments. First, younger dogs were at higher risk of harboring *T. canis* than were adults. This species is more prevalent in puppies and young dogs, but it is also found in adult animals, however the infection frequently impacts young dogs from birth to 1 year old, determining respiratory symptoms [12,22]. Moreover, transplacental and transmammary transmission are important infection routes [22]. Regarding hookworms, these parasites were found infecting both free-roaming and owned dogs, however they were more prevalent in the latter. Hookworms are regarded as the most constantly present intestinal parasites of dogs, whether they are owned or stray [4,23]. This is particularly so in developing countries, where regular prophylactic treatment is lacking.

Interestingly, this predominance of infection in owned dogs in the present study is concordant with findings previously reported [24]. However, some points need to be highlighted in this regard. Firstly, most of the owned dogs surveyed consisted of puppies and young animals (71.70%), which are more susceptible to hookworm infections. Although dogs are susceptible to infection with hookworms throughout life, the level of infection tend to be higher in young animals since, in addition to the immaturity of these animals immune system, transmammary transmission is considered to be an important route through which puppies become infected [12]. Secondly, most of the owned dogs surveyed were living in poorly resourced communities on the outskirts of the urban area, where many streets are still unpaved and these animals are frequently in contact with the soil, thus increasing the risk of infection and contamination of the environment. Lastly, all these animals belonged to low-income owners and many of them were unable to afford the costs of veterinary care, which is a limitation to provision of regular anthelmintic treatment for their pets.

In both developed and developing regions, hookworms are responsible for a common gastrointestinal infection in the canine population. Despite some epidemiological particularities, the canine hookworm species generally include *A. caninum*, *A. braziliense*, *A. ceylanicum* and *U. stenocephala.* Given that the eggs of these species are morphologically indistinguishable, identification and differentiation of species have usually been based on post-mortem examination of the adult worms [5,25]. Advances in molecular techniques have made it possible for high-specificity assays to distinguish hookworm species without the need to recover the worms at necropsy [5,23,25].

Based on PCR, the analyses demonstrated that of 51 microscopy-positive hookworm samples, 50.17% were successfully amplified. Amplification was not observed for all samples; however, this is not an uncommon situation, and it has been reported and discussed in other investigations [9,15,26,30]. As highlighted in these previous studies, one reason for this failure may be poor concentration of DNA, especially in low-density infections, thus resulting in low numbers of eggs in stools. This is an important condition that can lead to no PCR product or can yield poor amplification.

Out of 26 samples that were both amplified and sequenced, *A. caninum* and *A. braziliense* were detected in 80.80% and 19.20%, respectively, whether in single (76.90%) or mixed (23.10%) infections, infecting both free-roaming and owned animals. Identification of *A. caninum* and *A. braziliense* was to be expected, since these species have been documented infecting canine populations worldwide, particularly in warm humid climatic zones [9,12]. *A. caninum* has often been the most common species reported in recent molecular-based surveys [15,20,27–31] or even in parasitological whether in simple or mixed infections findings from necropsies performed on stray dogs that were euthanized because of zoonosis control programs [16]. To a lesser extent, *A. braziliense* was also observed, thus corroborating the findings reported in other investigations [15,29–31]. Within a public health context, both species have the potential to cause cutaneous larva migrans, but the evidence has more often supported involvement of *A. braziliense* [5]. On the other hand, not only does *A. caninum* account for the majority of canine hookworm infections worldwide, but also this species has been related to eosinophilic enteritis in humans [5]. Apart from this, in a molecular-based study conducted recently in Brazil to identify single hookworm eggs in human faeces, the presence of *A. caninum* eggs was reported. This finding led the authors to raise the idea that this may have a direct implication for the epidemiology of human parasitism by this species, since the source of infection might not be restricted to infected animals only [32].

In brief, although the canine population surveyed and the number of isolates sequenced were relatively small, the current findings emphasize the role of free-roaming dogs and owned pets living in low-income communities as reservoirs of zoonotic gastrointestinal parasites, particularly the hookworm species *A. caninum* and *A. braziliense*. In view of this, the epidemiological information assembled here lead us to stress the need for improvements in urban infrastructure in order to avoid soil contamination by dog faeces in poor areas. Moreover, there is a need for educational actions focusing on responsible pet ownership, so as to prevent animal abandonment and guide owners regarding the public health relevance of canine zoonotic parasites and prophylactic measures for controlling infections.

Acknowledgements

The authors would like to thank all the professionals of the shelter and owners who accepted to collaborate in this study.

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Received 15 May 2022
Accepted 30 July 2022