



SHORT COMMUNICATION

Occurrence and Molecular Identification of *Ancylostoma caninum* As the Predominant Hookworm of Dogs in Faisalabad, Pakistan

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ABSTRACT

Several canine zoonotic soil-transmitted helminths (cSTHs) represent important veterinary and public health concerns in tropical and subtropical regions. Most of the diseases caused by these helminths also classified as neglected tropical diseases (NTDs). Despite the endemic nature of these cSTHs, they are poorly studied in underdeveloped countries. This study investigated the occurrence of *Ancylostoma* (*A.*) spp. in owned and stray dogs in Faisalabad, Pakistan, using microscopy and multiplex TaqMan probe-based real-time PCR. A total of 150 faecal samples (75 owned, 75 stray) were analyzed. *Ancylostoma* eggs were detected in 52% (95% CI, 43.7–60.2%) of dogs, with a significantly higher occurrence in stray (77.3%) than owned dogs (26.7%). Molecular analysis confirmed *A. caninum* as the predominant hookworm species infecting dogs in the region. Lack of deworming, uncontrolled feeding, poor hygiene, outdoor housing and bad health status (based on BCS) were identified as significant risk factors. These findings highlight the need for molecular surveillance and integrated control strategies, including deworming, improved hygiene, and effective dog population management, to mitigate zoonotic risks.

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INTRODUCTION

Canine zoonotic soil-transmitted helminths (cSTHs), particularly different species of hookworms such as *Ancylostoma* (*A.*) *caninum*, *A. ceylanicum*, *A. braziliense* and *Uncinaria stenocephala* are known to impact the lives of dogs, cats and occasionally humans. The haematophagous hookworms, particularly *A. caninum*, cause significant damage to the intestinal mucosa, resulting in melaena and anaemia, and can be fatal, especially in untreated puppies (Raza *et al.*, 2018). Chronic hookworm infection can lead to iron deficiency anaemia and is typically observed in older dogs. In addition to their veterinary significance, these cSTHs are well-established zoonotic pathogens. The hookworms, *A. braziliense*, *A. caninum*, and *A. ceylanicum* are primarily distributed in the tropics and subtropics, whereas *Uncinaria stenocephala* is mostly restricted to temperate regions (Traub *et al.*, 2021).

All species of canine hookworms are zoonotic and capable of producing cutaneous larva migrans (CLM) in humans. *A. braziliense* is a cause of prolonged CLM or creeping eruptions, which requires medical intervention (White and Dove, 1928). *A. ceylanicum* and *A. caninum* are known to produce eosinophilic enteritis (Prociv and Croese, 1996), with the former also likely to result in chronic infections (Kladkempetch, Tangtrongsup, and Tiwananthagorn, 2020). *A. ceylanicum* is increasingly recognized as the second most common hookworm of humans in the Asia Pacific, after *Necator americanus* (Colella *et al.*, 2021). In neighbouring India, stray and domesticated dogs are documented key sources of environmental contamination with the zoonotic *Ancylostoma* (Traub *et al.*, 2014). Despite an estimated 18 million dogs in Pakistan, data on zoonotic STH prevalence are scarce, underscoring the importance of investigating their potential transmission to humans and animals

(GARC, 2022). Therefore, this study was designed to determine the occurrence and potential risk factors of zoonotic species of *Ancylostoma* in owned and stray dogs in metropolitan Faisalabad. In addition, we aimed to ascertain the species of hookworms infecting dogs to gain better insight into their expected zoonotic risks to the public.

MATERIALS AND METHODS

Sampling area: Faisalabad is the second largest city in Punjab Province and the third most populous city in Pakistan. It is located at 31.420°N, 73.080°E and at an elevation of 186m above sea level. The total area of Faisalabad district is about 5,856km², with a total population of 7.88 million. The climatic conditions are generally extreme, with mean minimum temperatures around 4.1°C in winter and mean maximum temperatures around 40.5°C in summer and classified as steppe and hot with dry winters (Sarfaraz *et al.*, 2014). The average rainfall in this city is approximately 542mm annually (Arshad *et al.*, 2019).

Collection of samples: The sample size was calculated using Shiny Server Professional v1.5.18 (<https://shiny.vet.unimelb.edu.au>), assuming an average expected prevalence of hookworms in stray dogs of 10% and owned or community dogs of 5% (Farooqi *et al.*, 2014), using a 5% desired absolute precision and a 95% confidence. A total of 75 pet/owned and 138 stray dogs were targeted for sampling, but due to logistic limitations, we managed to collect 75 faecal samples from the stray dogs from July–November 2017, covering the summer, monsoon, and early winter seasons.

A single faecal sample per animal was collected either freshly from the ground or directly per rectum into a labelled wide-mouth sterilized disposable plastic jars. Samples were immediately put on ice and then transported to the University of Agriculture, Faisalabad for further processing. Stray or community dogs were sampled from five different locations (Madina town, Iqbal town, Chak Jhumra town, Samundri and Jinnah town) across the city of Faisalabad (Fig. 1), whereas owned dogs were sampled at the outdoor Veterinary Clinic, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan.

Body condition scores (BCS) were assessed on a 7-point algorithm score (German *et al.*, 2006) and correlation was made with health status. Low BCS denotes bad health status and noted together with age (< 6 months or > 6 months) and sex (male or female) of each animal. A brief questionnaire designed to collect information was provided to the owners requesting information on deworming status (treated with anthelmintic in the last 30 days or not), feeding pattern (controlled or uncontrolled), housing (indoors or outdoors) and their environmental hygiene practices (disposal or non-disposal of pet faeces). A controlled feeding pattern represented the use of commercial feed or homemade feed, whereas uncontrolled feeding represented allowing dogs to freely roam outdoors with potential access to food scraps, including raw meat and rodents.

Microscopic identification of helminth eggs: Faecal samples (~1.5g) were processed by formol-ether

sedimentation technique for copro-microscopic examination as previously performed by Tun *et al.*, (2015). The eggs of *Ancylostoma* were observed using 10x, 20x and 40x objective lenses under a light microscope and identified to a genus level for hookworms (Fig. 2).

DNA extraction: Genomic DNA was extracted from 26 (13 each from owned and stray dogs) randomly selected faecal samples positive for hookworm eggs. Genomic DNA was extracted directly from the faecal sample using E.Z.N.A.[®] Stool DNA Kit (OMEGA, Bio-tek) (Georgia, United States) according to the manufacturer's instructions. The extracted DNA was stored at -40°C for further molecular analysis.

Molecular identification of hookworm species: gDNA was transported by courier to the Melbourne Veterinary School, the University of Melbourne in Australia, and subjected to two previously optimized and validated TaqMan Probe-based multiplex qPCR assays for canine hookworm species, following the protocol of Massetti *et al.* (2020).

The qPCR multiplex assays targeted the ITS-1 rRNA region of *A. braziliense*, *A. ceylanicum*, *A. caninum* and *U. stenocephala* (Massetti *et al.*, 2021) and used two sets of primers to amplify a 103bp region of *A. caninum* and *A. ceylanicum* ITS-1 rRNA (AcanceyF and AcanceyR9, and a 119 and 118bp region of the ITS-1 rRNA of *A. braziliense* and *U. stenocephala* (UncbrazF and UncbrazR). Species-level identification of microscopy-positive faecal samples were achieved using specific probes as reported previously by Massetti *et al.* (2020).

Each sample was run in duplicate using Synthetic double-stranded DNA fragments (gBlocks[®] Gene Fragments, IDT[®] Technologies, Skokie, Illinois, USA) containing individual sequence targets of each hookworm species and the genomic DNA of *U. stenocephala*, *A. braziliense*, *A. caninum*, and *A. ceylanicum* as positive controls. Mammalian primers (Mam F and Mam R) and the MAM probe, designed to target a 92bp region of the mammalian 16S mitochondrial gene, were used as the DNA extraction control. Additionally, equine herpesvirus (EHV4) primers (EHVF and EHVR), along with the EHV probe, were used, with genomic DNA serving as the internal control for the reaction (Massetti *et al.*, 2020). The reactions and cycling conditions were performed as described by Massetti *et al.* (2020), using a 20µL reaction volume containing 10µL of GoTaq Probe qPCR Mastermix (Promega, Madison, WI), 1µL of known quantity of EHV4 gDNA, and 2µL of template DNA. Nuclease-free-water was added to reach the final reaction volume. The qPCR multiplex assays were performed using a four-channel Magnetic Induction Cycler (BioMolecular Systems, Sydney, Australia) which facilitated amplification, detection, and data analysis through the micPCR software.

Statistical Analysis: The proportion of positive samples and corresponding 95% confidence intervals (95% CI) for *Ancylostoma* were calculated separately for owned and stray dogs using IBM SPSS Statistics 20. Associations between parasitism, host and management factors were made using Pearson's Chi-square test ($P < 0.05$) with Minitab 17.



Fig. 1: Geographical location of Faisalabad, Pakistan, and sampling sites for the study. The map on the left highlights Faisalabad within Pakistan (red outline). The enlarged map on the right provides a detailed view of sampling locations, including Iqbal Town, Chak Jhumra, Samundri Road, Madina Town, and Jinnah Colony. The latitude and longitude coordinates for each site are provided in the legend.

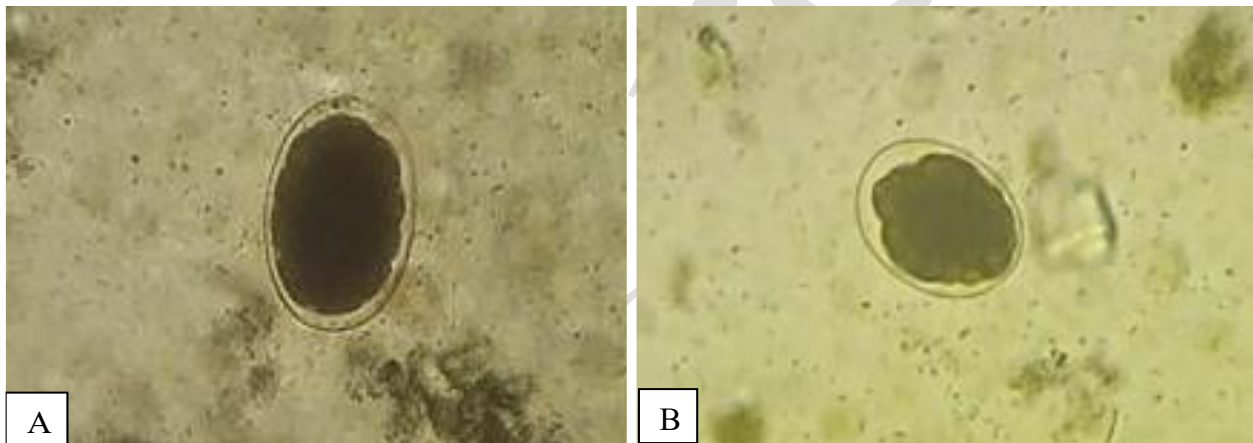


Fig. 2: (A) and (B) Microscopic image of hookworm eggs recovered from canine faecal samples using the formol-ether sedimentation technique. The image was captured under a light microscope at 20× objective magnification. The eggs exhibit the characteristic oval shape, thin smooth shell and internal morula typical of hookworm ova.

RESULTS

Occurrence of parasites: In total, single faecal samples with accompanying metadata were collected from 75 owned dogs and 75 stray dogs. Microscopic analysis showed that 78 of 150 dogs were positive for *Ancylostoma* eggs with an overall occurrence of 52% (CI:43.7-60.2%). The occurrence of hookworms in stray dogs (77.3%, CI:68.2-85.8%) was significantly higher ($P < 0.0001$) than that of owned dogs (26.7%, CI:17.4-36.9%).

The TaqMan probe-based multiplex qPCR assay for canine hookworm species allowed the characterization of a subset of microscopy-positive hookworm samples ($n=26$). The microscopy-positive hookworm samples subjected to the multiplex qPCR only yielded positive for the canine

hookworm *A. caninum*. None of the other targeted hookworm species (*A. ceylanicum*, *A. braziliense*, or *U. stenocephala*) were detected in the analysed samples. These results strongly indicate *A. caninum* as the predominant and potentially sole hookworm species infecting dogs in Faisalabad. The reaction internal control (EHV) and external control (Canine DNA) were successfully amplified in all 26 samples subjected to qPCR.

Univariate risk factors associated with the occurrence of *Ancylostoma*: The univariable risk factors associated with the occurrence of *Ancylostoma* sp., in stray and owned dogs are outlined in Tables 1 and 2. Briefly, sex and age were not found as significant risk factors for dogs with hookworm infection. In owned dogs, lack of anthelmintic

treatment was associated with a significantly higher occurrence of *Ancylostoma* sp., (OR=0.0, CI:0.0-0.09, $P=0.001$). In addition, other factors, including uncontrolled feeding (OR=0.01, CI:0.00-0.08, $P=0.01$ and environmental hygiene (OR=0.05, CI:0.01-0.25, $P=0.001$), were significantly associated with the prevalence of parasitism in owned dogs. Owned dogs that had bad health status based on body condition scores (BCS) of ≤ 4 were more likely to be infected with *Ancylostoma* than those with good health status (higher BCSs) (OR=0.01, CI:0.002-0.06, $P=0.001$).

Table 1: Risk factors associated with *Ancylostoma* infection in owned dogs (n=75)

Factor	Category	n=75			P-value	χ^2	OR	95% CI
		Positive	Negative	Positive rate				
Hookworm	Sex							
	Male	16	40	28.6%	0.62	0.25	1.5	0.43-5.21
	Female	4	15	21.1%			0	
	Age				0.80	0.06	1.1	0.40-3.19
	<6 months	9	23	28.1%			3	
	>6 months	11	32	25.6%	5		0	
	Deworming				0.00	43.2	0	0.0-0.09
	Yes (last 30 days)	0	46	0	1	6	0	
Feeding	Controlled	2	48	4%			0.0	0.00-0.08
	Uncontrolled	18	7	72%	0.01	5.57	1	
Housing	Inside	14	42	25%	0.67	0.17	0.7	0.23-2.26
	Outside	6	13	31.6%			2	
Owned hygiene	Good	2	37	5.1%	0.00	11.3	0.0	0.01-0.25
	Bad	18	18	50.0%	1	5	5	
Health status	Good	2	50	3.8%	0.00	22.6	0.0	0.002-0.06
	Bad	18	5	78.3%	1	5	1	

Table 2: Risk factors associated with *Ancylostoma* infection in stray dogs (n=75)

Factor	Category	n=75			P-value	χ^2	OR	95% CI
		Positive	Negative	Positive rate				
Hookworm	Sex							
	Male	31	12	72.1%	0.65	0.200	0.47	0.14-1.53
	Female	27	5	84.4%			0.59	0.19-1.81
	Age				0.74	0.111	0.59	
	<6 months	17	7	70.8%				
	>6 months	41	10	80.4%				

DISCUSSION

The current study demonstrates that zoonotic cSTH infections are highly endemic, in both stray and owned dog populations in Faisalabad, Pakistan, representing a public health concern. In this study, more than half of the dogs sampled were infected with *A. caninum*, with stray dogs being almost three times more likely to be infected (77%) than owned dogs (26%). In Pakistan, available studies reporting the prevalence of canine hookworms were geographically restricted to Lahore (Rehman *et al.*, 2017; Ashraf *et al.*, 2019). Previously, hookworm eggs in canine faeces were frequently referred to as *A. caninum* (Khan *et al.*, 2019); however, this was largely presumptive as morphological identification of the eggs alone is insufficient to characterize hookworms at the species level (Masseti *et al.*, 2021). Rehman *et al.* (2017) previously subjected a subset of faecal samples (26%) sourced from owned and street dogs positive for hookworm eggs to an *A. caninum*-specific conventional PCR and DNA sequencing, confirming the presence of this zoonotic hookworm for the first time in Lahore, Pakistan. The definitive characterization of the species of canine hookworms in this study from different locations in the Faisalabad metropolitan area suggests the wider geographical

distribution of cSTHs in the country. Multiplex real-time screening of a subset of hookworm egg-positive samples revealed that *A. caninum* is the predominant hookworm species infecting dogs in Pakistan. *A. caninum* induces mostly non-patent infection with accompanying eosinophilic enteritis in humans. Although *A. caninum*-induced eosinophilic enteritis has not been reported in humans in Pakistan, its subclinical presentation and diagnostic challenges suggest potential cases may have been overlooked (Traub *et al.*, 2021).

The findings of this study highlight the importance of molecular diagnostics in identifying *Ancylostoma* spp. While traditional microscopy provides initial prevalence estimates, it cannot differentiate *Ancylostoma* spp. due to morphological similarities in ova. The multiplex TaqMan probe-based qPCR assay used in this study offers a reliable method for species-specific identification with high sensitivity and specificity, as evidenced by consistent amplification results for *A. caninum*. The absence of *A. ceylanicum* and other zoonotic hookworm species in our samples is noteworthy, as *A. ceylanicum* has been reported in neighbouring countries like India, raising questions about its absence, low prevalence or wide-scale molecular analysis in different regions of Pakistan. Recent reports indicate that in rare instances, *A. caninum* may also complete its life cycle in humans (Ngcamphalala *et al.*, 2019; Furtado *et al.*, 2020). Nevertheless, the detection of *A. caninum* in dogs in Pakistan was expected owing to its wide geographical distribution. *A. caninum*'s ability to undergo arrested development in the tissue of the host, only to re-activate and undergo tracheal migration to enter the gut once climatic conditions are favourable for its survival (Marsh and Lakritz, 2023), thus providing it with a significant competitive advantage over other species of canine hookworm.

In this study, the occurrence of *A. caninum* was significantly ($P<0.05$) higher in stray compared with owned dog populations, likely owing to the absence of veterinary care and broad exposure to infective stages. In owned dogs, a lack of frequent deworming, allowing pets to scavenge and poor environmental hygiene were identified as significant risk factors associated with *Ancylostoma*. All faecal samples from the dogs that were dewormed in the past 30 days were negative for *Ancylostoma*, reflecting a high degree of anthelmintic efficacy. Feeding plays a significant role in controlling *Ancylostoma* infection. Dog feeding in the form of scavenging and hunting by dogs, compared to proper home feeding with commercial products, increases the chances of infection with gastrointestinal helminths (GIHs) in dogs (Dantas-Torres *et al.*, 2020). This study's findings corroborate previous research demonstrating that controlled feeding regimes significantly reduce the prevalence of gastrointestinal helminth (GIH) infections among dogs ($P<0.05$). This association clearly highlights the importance of dietary management as a practical intervention to limit parasitic burden.

Dog hygiene also plays a significant role in controlling hookworm infection. Regular removal and proper disposal of faeces from the shelter/kennel of dogs helps prevent GIHs infection (Raza *et al.*, 2018). Our results reinforce the importance of hygiene conditions in reducing infection risk ($P<0.05$). Dogs with poor health status (based on body-coat condition, body weight, alertness and clinical signs), were more likely to be infected with *Ancylostoma*. Dogs

with bad health status /malnourished may have a poor immune system to combat prevailing GIHs and ultimately compromised immune response to infection. A single adult *A. caninum* worm can suck up to 0.1ml of blood per day, which can directly be correlated with weight loss and increased susceptibility to infection (Saravanan *et al.*, 2011). In Pakistan, uncontrolled stray dogs, combined with poor environmental hygiene conditions in overcrowded urban areas, make the transmission of these zoonoses to humans more conducive. For dog owners and their families, factors including a lack of owner awareness about the importance of deworming and prompt removal of dog faeces following defecation in household backyards also add to the zoonotic risk.

A smaller sample size for the stray dog cohort and submission of the subset of microscopic positive faecal samples due to financial constraints were the main limitations of this study. It is common for studies to face challenges in achieving the target sample size, especially when working with hard-to-reach or underserved populations, such as stray dogs. In such cases, it may be necessary to use the term "occurrence" rather than "prevalence" to describe the study's findings.

Conclusions: Our findings highlight the need for incorporating molecular diagnostics in routine parasitological surveys to accurately assess zoonotic risks and design targeted control strategies. As both uncontrolled stray dog populations and owned dogs contribute to the environmental contamination of urban areas with zoonotic hookworms and roundworms, there is an urgency to advocate for humane dog birth control, public education and deworming programs to accompany current anti-rabies vaccination campaigns in the area. Veterinarians should be encouraged to educate pet owners about the importance of deworming their dogs (and cats) and regularly removing feces from common places and kennels to benefit their pets and their family's health. A limitation of this study is the relatively small number of samples subjected to molecular analysis, which may not fully represent the diversity of hookworm species in the region. However, the consistent identification of *A. caninum* across all analysed samples provides strong evidence for its predominance. Future studies should include a larger sample size and explore additional regions in Pakistan to confirm these findings and assess the potential presence of other zoonotic hookworm species.

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