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Author: Rebecca J. Traub Riddhi P. Pednekar Leigh Cuttell Ronald B. Porter Puteri Azaziah Abd Megat Rani Mukulesh L. Gatne

PII: S0304-4017(14)00390-2
DOI: http://dx.doi.org/doi:10.1016/j.vetpar.2014.06.037
Reference: VETPAR 7319

To appear in: *Veterinary Parasitology*

Received date: 12-3-2014
Revised date: 24-6-2014
Accepted date: 28-6-2014

Please cite this article as: Traub, R.J., Pednekar, R.P., Cuttell, L., Porter, R.B., Rani, P.A.A.M., Gatne, M.L.,The prevalence and distribution of gastrointestinal parasites of stray and refuge dogs in four locations in India, *Veterinary Parasitology* (2014), http://dx.doi.org/10.1016/j.vetpar.2014.06.037

The prevalence and distribution of gastrointestinal parasites of stray and refuge dogs in four locations in India

Rebecca J. Traub[a*], Riddhi P. Pednekar[b], Leigh Cuttell[c], Ronald B. Porter[c], Puteri Azaziah Abd Megat Rani[d], Mukulesh L. Gatne[b]

[a] Faculty of Veterinary Science, The University of Melbourne, Parkville 3052, Australia

[b] Bombay Veterinary College, Maharashtra Animal and Fishery Sciences University, Parel, Mumbai 400012, Maharashtra, India

[c] School of Veterinary Science, The University of Queensland, Gatton, Queensland 4343, Australia.

[d] Faculty of Veterinary Medicine, University of Putra, Malaysia, 43400 Serdang, Selangor, Malaysia

* Corresponding author details –Email address: rtraub@unimelb.edu.au

Abstract

A gastrointestinal parasite survey of 411 stray and refuge dogs sampled from four geographical and climactically distinct locations in India revealed these animals to represent a significant source of environmental contamination for parasites that pose a zoonotic risk to the public. Hookworms were the most commonly identified parasite in dogs in Sikkim (71.3%), Mumbai (48.8%) and Delhi (39.1%). In Ladakh, which experiences harsh extremes in climate, a competitive advantage was observed for parasites such as *Sarcocystis* spp. (44.2%), *Taenia hydatigena* (30.3%) and *Echinococcus granulosus* (2.3%) that utilise intermediate hosts for the completion of their life cycle. PCR identified *Ancylostoma ceylanicum* and *A. caninum* to occur sympatrically, either as single or mixed infections in

Sikkim (Northeast) and Mumbai (West). In Delhi, *A. caninum* was the only species identified in dogs, probably owing to its ability to evade unfavourable climatic conditions by undergoing arrested development in host tissue. The expansion of the known distribution of *A. ceylanicum* to the west, as far as Mumbai, justifies the renewed interest in this emerging zoonosis and advocates for its surveillance in future human parasite surveys. Of interest was the absence of *Trichuris vulpis* in dogs, in support of previous canine surveys in India. This study advocates the continuation of birth control programs in stray dogs that will undoubtedly have spill-over effects on reducing the levels of environmental contamination with parasite stages. In particular, owners of pet animals exposed to these environments must be extra vigilant in ensuring their animals are regularly dewormed and maintaining strict standards of household and personal hygiene.

Keywords: Dogs, gastrointestinal parasites, zoonosis, *Ancylostoma ceylanicum*, India

1. Introduction

Canine gastrointestinal parasites can be divided into three broad categories; those of veterinary importance, for example *Spirocerca lupi*, those of public health importance, for example *Echinococcus granulosus* and those that produce morbidity in both canines and humans, namely hookworms and *Toxocara canis*. All three categories of gastrointestinal parasites are known to be endemic in India (Traub et al., 2005), especially among stray and semi-domesticated dogs. These parasites may be transmitted to humans either directly, through the ingestion of infective stages via close contact with a dog; or indirectly, through skin penetration or ingestion of infective stages in the environment, including those that may be food- or water-borne.

Although investigated, there appears to be a lack of widely accessible up-to-date information available on the prevalence and distribution of canine gastrointestinal parasites in India. The population of stray or community dogs in India is estimated as high as 20 million, despite efforts to curb numbers through sterilisation campaigns (Menezes, 2008). These uncared for animals not only pose an important source of parasites for the 5 million-odd 'owned' or 'pet' dogs, but also for the general public.

There is substantial evidence to show that canine intestinal parasites are a public health concern in India particularly in relation to hydatid disease, toxocarosis and zoonotic ancylostomosis (reviewed by Traub et al., 2005).

This study aimed to determine the prevalence and distribution of gastrointestinal parasites of veterinary and public health importance in stray dogs from four distinct geographical and climatic locations in India, the north-east (Sikkim), far north (Ladakh, Jammu and Kashmir), north (Delhi) and west (Mumbai).

2. Materials and methods

2.1 Study sites and sampling

The study was stratified to include four climatic zones, wet tropical (Mumbai), semi-arid (Delhi), arid mountainous (Leh, Ladakh) and humid temperate (Gangtok, Sikkim) based on information produced by The World Meteorological Organization.

Field work for this project was conducted between June to September 2008 with in-kind support provided by veterinary charity-based organisations conducting animal birth control programs in Ladakh, Sikkim, Delhi and Mumbai (Vets Beyond Borders, Jeevasharam, Krishanasharam and In Defence of Animals, India). The refuge centres provide shelter, de-sexing and veterinary care where appropriate, for dogs that are either rescued from the streets or abandoned by their owners. An estimate of each animal's age was made (based

on dentition and body size) and classified as puppy (less than 6 months old), juvenile (between 6 months to 1 year old), adult (between 1 to 7 year old) and geriatric (more than 7 year old). Each animal's sex, body condition score and source (stray or refuge) was noted. A single stool sample was collected per-rectum from 411 dogs from Ladakh (n=86), Sikkim (n=94), Delhi (n=110) and Mumbai (n=121) and preserved separately in 10% formalin and in 90% ethanol for future microscopic screening and molecular analysis, respectively. This project was approved by the University of Queensland Animal Ethics Committee.

2.2 Parasitological Techniques

Formalin preserved faecal samples were initially subjected to a sedimentation in water technique followed by faecal flotation using zinc sulphate ($ZnSO_4$) (S.G. 1.20). Faecal samples which were positive on microscopy for the presence of taeniid and hookworm eggs were further subjected to molecular analysis.

2.3 Extraction of the genomic DNA

Approximately 25 mg of faeces was washed once with 1 × TE buffer (40mM Tris HCl, 10mM EDTA), then boiled (100^{o}C) for 10 min to reduce the presence of inhibitors.

For taeniid egg-positive samples, DNA was extracted using QIAmp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol except that samples were subjected to an initial overnight incubation step (200 µl ASL buffer and 30 µl proteinase K) followed by 5 cycles of freeze-thawing and 3 cycles of freeze-fracturing prior to DNA extraction.

For hookworm egg-positive samples**,** Zirconia beads (Daintree Scientific, Australia) were added to faecal samples and the samples homogenised for 5 min at high speed using a

bench-top Beadbeater (Biospec Products, Bartlesville OK). Samples were then centrifuged at 11,700 × gfor 1 min before 100 µl of supernatant was transferred to a clean 1.5 mL tube.

2.4 PCR identification of taeniid eggs with multiplex PCR

A multiplex PCR using primers 'Cest1-5' was utilised for the detection and identification of taeniid eggs (Trachsel et al., 2007). The PCR amplicons were electrophoresed on a 2% agarose gel run in 1× TE buffer, stained using ethidium bromide and visualised using a GelDoc system (Bio-rad).

2.5 PCR-RFLP for hookworm egg species identification

Due to faecal sample exhaustion, 75-80% of hookworm positive samples could be subjected to species identification using PCR-RFLP (Palmer et al., 2007; Traub et al., 2004b). PCRs were carried out using an inhibitor-resistant DNA polymerase on each crude faecal lysate. Lysates were diluted 1/5 in 1× TE buffer and the 25 µl reactions carried out using 0.2U of Phusion Hotstart II High Fidelity DNA polymerase (Thermo Scientific, catalogue # F-5495), 12.5 pmol of each primer, 0.2 µl of 20mg/mL bovine serum albumin and 2 µl diluted DNA. Cycling conditions included an initial denaturation at 99^{o}C for 30 secs, then 50 cycles of 98^{o} C for 10 secs, 60^{o} C for 15 secs and 72^{o} C for 30 secs. The RFLP products were run on 1-2% agarose gels in 1× SB (Sodium Borate) buffer, stained using SYBR Safe (Invitrogen/Life Technologies) and visualised using a GelDoc system (Bio-rad).

2.6 Presence of amplifiable DNA

Samples that were microscopy positive for hookworm but which failed to generate a result by PCR were tested for the presence of PCR inhibitors by using published primers (18SEUDIR and 18SEUINV) that amplify a 140 bp fragment of the 18SrRNA gene of

eukaryotes (Fajardo et al., 2008). PCR products were visualised on a 1 % agarose gel in 1× SB (Sodium Borate) buffer, stained using SYBR Safe (Invitrogen/Life Technologies) and visualised using a GelDoc system (Bio-rad)

2.7 DNA sequencing

PCR products were purified using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA). For samples with mixed infections, PCR amplification products were excised from the agarose gel and purified using QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer`s recommendation. DNA sequencing was performed in both directions using sequencing primers Cest 3 and Cest 5 for *Taenia* spp. and Cest 4 and Cest 5 for *E. granulosus*. Sequence chromatograms were read and analysed using the software program Finch TV v 1.4.0 (Geospira Inc.) The sequence were aligned and compared to previously published sequences using BLAST® (Altschul et al., 1990)..

2.8 Statistical analysis

Prevalence and 95% upper and lower confidence intervals were calculated for the gastrointestinal parasites using Epi Tools (Seargent, 2014). . SPSS Statistics 17.0 was utilised to determine the associations between parasitism and host factors. These were initially made using Chi squared or Fisher's exact test for independence. Animals with missing data were excluded from the analysis for that particular risk factor. The independent variables significant at $P \leq 0.20$ in the univariable analyses were selected for multivariable logistic regression. The backward elimination approach was used to determine which factors could be dropped from the multivariable model ($P < 0.05$) and adjusted odds ratios (OR) and 95% CI were reported for the factors retained in the final model (Hosmer and Lemeshow, 1989).

3. Results

Of 411 dogs sampled, 42% were entire female, 38% entire male, 13% sterilised female and 7% castrated male. The majority of dogs were adults (81.2%), followed by juveniles (11.1%), geriatrics (6.1%) and pups (1.2%). Most of the dogs sampled were classified as stray (89%).

3.1 Microscopy

The prevalence of gastrointestinal parasites in dogs from all four geographical locations in India is summarised in Table 1. Microscopic examination of the faecal samples from dogs revealed that 55% of dogs were parasitized with one or more gastrointestinal parasites, of which 82% were parasites of potential veterinary significance. Hookworms, followed by *S. lupi* were most commonly identified in dogs from Sikkim, Mumbai and Delhi, whereas *Sarcocystis* spp. and *Taenia/ Echinococcus* spp. were the most prevalent parasites identified in Ladakh.

Male dogs were 1.76 (p=0.016) and 2.12 (p= 0.014) times more likely to shed hookworm and *Spirocerca* eggs, respectively. *Cystoisospora* oocysts were 5.27 times (p=0.002) more likely to be shed by juvenile dogs less than one year of age.

3.2 Molecular identification of taeniid eggs

Thirty samples positive for taeniid eggs on microscopy originating from Ladakh and Mumbai were subjected to multiplex PCR (Trachsel et al., 2007). Of these, 26 (86.6%) samples produced amplicons corresponding to the expected sizes for *Taenia* spp. (26/26) and / or *Echinococcus* spp. (2/26). Clear and readable DNA sequences were obtained for 25 amplicons which showed 100% sequence identity to the *rrnl* gene segment of *Taenia hydatigena* and two samples, to *E. granulosus* on BLAST® (Altschul et al., 1990). .

3.3 Molecular identification of hookworm eggs

Table 2 summarises the results of the molecular identification of a portion of hookworm egg-positive samples for the three hookworm endemic regions. In Delhi, all canine hookworms were identified as *A. caninum*, whereas in Mumbai and Sikkim, both *A. ceylanicum* and *A. caninum* were identified in dogs, as either single or mixed infections. No *A. braziliense* was identified in this study.

4. Discussion

Stray and refuge dogs from all four locations were commonly infected with one or more gastrointestinal parasite. In addition to having implications on animal (including companion animal) and public health, transmission of canine parasites to livestock may also have economic impacts.

Hookworms and *S. lupi* were found to be the most common gastrointestinal parasites identified in dogs from Mumbai, Delhi and Sikkim. From a veterinary perspective, both parasites cause considerable morbidity and mortality in dogs. Infection with *S. lupi* in particular, may result in a multitude of clinical signs varying from dyspnoea, regurgitation, vomiting and wasting, to sudden death resulting from rupture of an aortic aneurism (Mazaki-Tovi et al., 2002; van der Merwe et al., 2008).

Both *A. caninum* and *A. ceylanicum* may cause sufficient blood loss in the acute phase to produce severe haemorrhagic diarrhoea, anaemia and hypoproteinaemia in pups (Areekul et al., 1975; Miller, 1968) and an chronic microcytic hypochromic anaemia in adult dogs (Carroll and Grove, 1984).

In addition to being pathogenic in dogs, both species of canine hookworms may also produce a temporary pruritic papular rash known as 'ground itch' in humans (Maplestone, 1933). Although there have been no reports of *A. caninum*-induced eosinophilic enteritis in

India (Prociv and Croese, 1996), the widespread nature of *A. caninum* and the obscure clinical presentation, makes it plausible that the condition may be more common than reported due to a lack of investigation.

This study represents the fourth known report of *A. ceylanicum* in India. The hookworm was first reported in humans in Calcutta (Kolkata) by Lane in 1913 (Lane, 1913), shortly after it discovered by Looss (Looss, 1911) in civet cats in Ceylon (Sri Lanka). The parasite was frequently recovered in civet and domestic cats in Kolkata in the 1920s (Chandler, 1925) and again in the 1970s (Chowdhury and Schad, 1972). In 2004, *A. ceylanicum* was detected in 62% of community dogs in northeast Assam, half of which were present as mixed infections with *A. caninum* (Traub et al., 2007; Traub et al., 2004b). The discovery of the expanding distribution of *A. ceylanicum* to other parts of India, most notably Mumbai, does justify the renewed interest in this emerging zoonosis. Recent molecular-based surveys in Southeast Asia have demonstrated *A. ceylanicum* as the second most common hookworm species infecting humans (Conlan et al., 2012; Inpankaew et al., 2014; Ngui et al., 2012). Natural infections with *A. ceylanicum* in humans have been reported in almost all geographical areas in which the hookworm is known to be endemic in dogs and cats (Traub, 2013) and it is likely that this hookworm may be present, but overlooked in human parasite surveys in wet tropical and temperate regions of India.

The factors influencing the distribution of hookworms are likely climactic. *A.ceylanicum* was found to be endemic in Sikkim and Mumbai but not in Delhi, possibly owing to the dry winters that are detrimental to the survival of *Ancylostoma* larvae. In contrast, *A. caninum* can undergo 'arrested development' within the host tissue and evade unfavourable climactic conditions. The parasite stage can re-activate once conditions are favourable for its survival (Schad and Page, 1982), providing it with a significant competitive advantage over other hookworm species (Schad et al., 1973).

In Ladakh, which experiences harsh extremes in climate that range from -28°C in winter to 33°C in summer with low relative humidity, a competitive advantage was observed for parasites such as *Sarcocystis spp., Taenia spp.* and *E. granulosus* that utilise intermediate hosts for the completion of its life cycle. Notably, the high prevalence of *T. hydatigena* and *Sarcocystis* spp. and the presence of *E. granulosus* reflect the observed opportunity stray dogs have to meat trimmings and offal at locally run abattoirs/ butchers (personal observation, Rebecca Traub). Although the species of *Sarcocystis* remains unascertained, both *T. hydatigena* and *Sarcocystis* spp. may cause economic loss in the form of extra trimming and possibly carcase condemnation at slaughter. The lack of age-related resistance to infection with *Toxascaris leonina*, the ability of its eggs to tolerate temperatures of -15°C and to rapidly develop into the infective stage in as little as 3 days to one week at temperatures over 27ºC (Okulewicz et al., 2012) may explain the high prevalence of this roundworm in Ladakh.

The absence of *E. granulosus* in stray dogs within urban centres of India is consistent with the declining trend of hydatid disease observed in livestock over the past few decades, owing to economic development and improved government regulation of abattoirs (Pednekar et al., 2009). Nevertheless we acknowledge that the true prevalence of *E. granulosus* is likely to be underestimated due to the intermittent nature of egg shedding. Despite human reports of alveolar hydatid disease (Aikat et al., 1978; Sharma et al., 2003) in India's north, we did not encounter *E. multilocularis* (or *E. shiquicus*) infection in dogs. Nevertheless, the possibility of the parasites' presence, in Jammu and Kashmir cannot be excluded given the similarity between the geographical location, climatic, socio-economic conditions and sylvatic fauna of India's far north and neighbouring Tibet and Western China, where both species of tapeworm are known to be endemic (Xiao et al., 2006).

This study supports the hypothesis indicating the lack of *Trichuris vulpis* in Indian dogs (Traub et al., 2002). The absence of *T. vulpis* in India is unexplained as other host-specific species within the genus *Trichuris* occur endemically throughout the country in humans (Naish et al., 2004; Traub et al., 2004a), rodents (Sharma et al., 2013) and livestock (Tariq et al., 2010). Reports of *T. vulpis* eggs in children from urban slum areas in New Delhi (Singh et al., 1998) and in human tribal populations of the Andaman and Nicobar Islands (Singh et al., 1993) are likely erroneous.

The prevalence of *T. canis* in Sikkim was comparable to that reported for a general population of stray dogs in Madhya Pradesh of 2.7% (Sahasrabudhe et al., 1969). The negligible presence or complete absence of *T. canis* in the other cities however, is likely due to the low proportion of puppies, pregnant and lactating females sampled. Intact males were significantly more likely to harbour hookworms and *S. lupi*, which is likely related to the compromised innate resistance to parasites produced by higher levels of testosterone (Hughes and Randolph, 2001). Age-related immunity was only found to be a significant factor for coccidia, in which dogs less than one year of age were more likely to be shedding oocysts. The lack of *Giardia duodenalis* and *Cryptosporidium* spp. in these dogs is likely due to the poor sensitivity of zinc sulphate flotation and microscopy compared to coproantigen- and/ or PCR-based tests (Traub et al., 2009; Helmy et al., 2014) for their detection. Future employment of the latter would allow better assessment of risk these stray animals pose as sources of zoonotic protozoa.

5. Conclusions

Stray dogs in India's cities continue to represent a source of environmental contamination with infective stages of gastrointestinal parasites that pose a zoonotic risk to the public and a source of parasites for well-cared for pets. In particular, owners of pet

273 animals exposed to these environments must be extra vigilant about deworming their animals
274 and maintaining strict standards of household and personal hygiene.

275 Vaccination and neutering programs implemented towards the control of rabies will
276 undoubtedly co-contribute to reducing populations of animal reservoirs of helminth zoonoses.

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393 Table 1. The prevalence (%) [95% CI lower, upper confidence intervals] of gastrointestinal
394 parasites in dogs from four different locations in India

Parasite	Delhi (n=110)	Mumbai (n=121)	Ladakh (n=86)	Sikkim (n=94)
Ancylostoma spp.	39.1 (30.5, 48.4)	45.5 (36.9, 54.3)	4.7 (0.2, 9.2)	70.2 (60.3, 78.5)
Taenia hydatigena	0 (0, 0.03)	4.1 (0.57, 7.63)	32.6 (22.7, 42.5)	0 (0, 0.04)
Echinococcus granulosus	0 (0, 0.03)	0 (0, 0.03)	2.3 (0.0, 5.5)	0 (0, 0.04)
Cystoisospora spp.	0.9 (0, 2.7)	1.7 (0, 4.0)	11.6 (4.8, 18.4)	0 (0, 0.04)
Toxocara canis	0 (0, 0.03)	0.8 (0, 2.4)	0 (0, 0.04)	3.2 (0, 6.8)
Trichuris vulpis	0 (0, 0.03)	0 (0, 0.03)	0 (0, 0.04)	0 (0, 0.04)
Spirocerca lupi	4.5 (0.6, 8.4)	5.8 (1.6, 9.9)	0 (0, 0.04)	26.6 (17.7, 35.5)
Sarcocystis spp.	0 (0, 0.03)	0 (0, 0.03)	44.2 (33.7-54.7)	0 (0, 0.04)
Toxascaris leonina	0 (0, 0.03)	0 (0, 0.03)	15.1 (7.6, 22.7)	0 (0, 0.04)
Dipylidium caninum	1.8 (0, 4.3)	0 (0, 0.03)	0 (0, 0.08)	1.1 (0, 3.2)

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396 Table 2. Molecular identification of hookworm positive eggs in canine stool from three
397 geographical locations in India

Region	Microscopy positive	PCR-RFLP positives	Proportion of (%)			
			Microscopy positives tested	*Ancylostoma caninum* only	*Ancylostoma ceylanicum* only	Mixed infections
Mumbai	55/121	33/42	60.0	60.6	24.2	18.2
Delhi	43/110	28/34	65.1	100	0	0
Sikkim	66/94	43/53	65.2	14.2	62.8	23.3

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Highlights

- A survey of enteric parasites of stray and refuge dogs was conducted in India
- Canine parasites of veterinary, public health and economic importance were endemic
- Hookworms were the most common parasite of dogs in Delhi, Mumbai and Sikkim
- In Ladakh, dogs were commonly infected with meat-borne parasites
- Canine neutering programs will reduce risks of parasitic zoonoses