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Brief Report

First report of *Ancylostoma ceylanicum* in wild canids ☆

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ABSTRACT

The parasitic nematode *Ancylostoma ceylanicum* is common in dogs, cats and humans throughout Asia, inhabiting the small intestine and possibly leading to iron-deficient anaemia in those infected. It has previously been discovered in domestic dogs in Australia and this is the first report of *A. ceylanicum* in wild canids. Wild dogs (dingoes and dingo hybrids) killed in council control operations ($n = 26$) and wild dog scats ($n = 89$) were collected from the Wet Tropics region around Cairns, Far North Queensland. All of the carcasses (100%) were infected with *Ancylostoma caninum* and three (11.5%) had dual infections with *A. ceylanicum*. Scats, positively sequenced for hookworm, contained *A. ceylanicum, A. caninum* and *Ancylostoma braziliense*, with *A. ceylanicum* the dominant species in Mount Windsor National Park, with a prevalence of 100%, but decreasing to 68% and 30.8% in scats collected from northern and southern rural suburbs of Cairns, respectively. Due to the ability of *A. ceylanicum* to cause a patent infection in humans, the zoonotic risk arising from this wild dog reservoir to communities in the Wet Tropics should be determined.

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1. Introduction

Ancylostoma ceylanicum is a common hookworm of domestic dogs and cats in countries throughout Asia (Yoshida et al., 1968; Traub et al., 2007; Conlan et al., 2012). This parasite has also been reported in South America (Rep and Heinemann, 1976), Africa (Baker et al., 1989; Schuster et al., 2009), New Guinea (Anten and Zuidema, 1964), and more recently, Australia (Palmer et al., 2007).

Heavy infection can result in bloody diarrhoea and iron-deficient anaemia (Carroll and Grove, 1984). It was long thought that *A. ceylanicum* was a synonym of *Ancylostoma braziliense*, which has led to some confusion. Biocca (1951) demonstrated that the two are distinct species. Human infection with *A. ceylanicum* was previously considered to be abnormal and unimportant (Lane, 1913; Yoshida et al., 1968; Hotez et al., 2004). Subsequent studies, however, have revealed that this parasite can cause severe abdominal discomfort and diarrhoea (Carroll and Grove, 1986; Tu et al., 2008; Hsu and Lin, 2012) as well as cognitive impairment (Wijers and Smit, 1966) and should be considered to be of significant zoonotic importance (Traub et al., 2008; Thompson and Conlan, 2011; Conlan et al., 2012; Mahdy et al., 2012; Ngui et al., 2012).

In wild animals, *A. ceylanicum* has been identified in wild felids including the Asian golden cat (*Viverricula malaccensis*), the leopard cat (*Felis bengalensis*) and the civet (*Felis temminchii*) (Biocca, 1951; Chowdhury and Schad, 1972). Here, we look at the geographical distribution of hookworm species in the Cairns region of northern Australia and report for the first time *A. ceylanicum* in wild canids, specifically in the dingo (*Canis lupus dingo*).

2. Materials and methods

The wild dog of Australia, otherwise commonly known as the dingo (*C. lupus dingo*) is distributed widely in all states with the exception of Tasmania. European settlement has led to hybridisation of the wild dog with the domestic dog and this has resulted in fewer pure dingoes. The degree of hybridisation was not investigated in the animals used in this study and so we have chosen to use the term wild dogs, even though all of the animals examined resembled dingoes morphologically.

2.1. Study area and collection of specimens

The study area was restricted to localities within the Wet Tropics World Heritage Area in north-east Queensland, Australia. This region was further sub-divided into four localities, Mount Windsor National Park (NP), northern Cairns (rural areas around Cairns outer northern suburbs e.g. Barron), southern Cairns (rural areas around Cairns outer southern suburbs e.g. Walsh's Pyramid) and Atherton. Eighty-nine wild dog scats were collected over a

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12 month period during 2010/11 from Mt. Windsor NP, northern and southern Cairns locations. Twenty-six wild dog carcasses were supplied by Cairns Council animal control officers and local landholders following routine control measures from 2007 onwards around farmland and outer suburbs of northern and southern Cairns and Atherton. No wild dogs were killed specifically for this study. All of the specimens were bagged and frozen as soon as possible following collection and information regarding collection date and location were noted. All protocols were reviewed and approved by James Cook University Animal Ethics Committee (Approval no. A1546).

2.2. Necropsy technique and parasite preservation

The stomach and intestines of 26 wild dogs (15 males and 11 females) ranging in age from four weeks to over five years of age (mean age approximately 22 months), were excised. The stomach, small intestine, and large intestine were each ligated at the junctions and examined separately. The intestinal lumen was exposed via an incision along its length and the contents washed into a 250-μm aperture sieve. Stomach washings were also examined for the presence of helminths. A representative sample of stomach contents was preserved in 10% formalin for use in a separate ecological study to determine the species of prey consumed. Intestines were then passed between the examiner's thumb and forefinger several times to scrape off any attached worms whilst a visual inspection was made of the mucosa. All contents were washed thoroughly and preserved in 70% ethanol for later microscopical examination. Faecal samples were also collected directly from the large bowel and preserved in 5% SAF for microscopy and 80% ethanol for molecular procedures.

2.3. Microscopic examination

All specimens were transported to the School of Veterinary and Biomedical Sciences, Murdoch university, Western Australia. Intestinal contents were examined under dissecting and compound microscopes. Positive identification of *A. ceylanicum* was established using those criteria documented in Biocca's (1951) paper on the morphological differentiation of *A. braziliense* and *A. ceylanicum*. Where present, at least fifty individual hookworms were identified before deciding on the species present. In samples where *A. ceylanicum* was detected, all hookworms were identified.

Faecal scats and necropsy-collected faeces were examined by simple smear technique, where faeces were mixed on a slide with a small volume of water, and those samples positive for strongyle eggs noted. Given the high number of positive samples detected it was decided to include all samples for molecular analysis.

2.4. Genomic DNA extraction

DNA was extracted directly from faeces using a Promega Maxwell® 16 research instrument system and tissue kit. The final DNA elution was prepared in 300 μl of elution solution and stored at −20 °C until required.

In order to confirm morphological identification, male *A. ceylanicum* specimens from two separate animals, and male *Ancylostoma caninum* specimens also underwent molecular identification. Worms were washed and DNA was extracted using an Epicentre MasterPure™ Complete DNA and RNA Purification Kit according to the manufacturer's instructions.

2.5. Molecular methods – PCR

A direct PCR assay modified from Traub et al. (2008) was used for the DNA amplification of hookworm species. A forward primer RTHWIF (5′-GATGAGCATTGCWTGAATGCCG-3′) and reverse primer RTHWIR (5′-GCAAGTRCCGTTCGACAAACAG-3′) were used to amplify an approximately 485 bp and 380 bp section of the internal transcribed spacer-1 (ITS-1), 5.8S and internal transcribed spacer-2 (ITS-2) regions of *Ancylostoma* spp. The PCR assay was prepared in a volume of 25 μl consisting of 1X PCR buffer, 25 mM $MgCl_2$, 0.4 mM of each dNTP, 10 pmol of each primer, 1.0 U *Taq* DNA polymerase (Biotech International, Perth, Australia) and 1 μL of template genomic DNA. Due to the presence of inhibitors, DNA template often needed to be diluted to 1:2 or 1:4 concentration. PCR cycling conditions consisted of a pre-heating step at 95 °C for 5 min. This was followed by 40 cycles of 95 °C for 30 s (denaturing), 60 °C for 30 s (annealing), 72 °C for 30 s (extension), a final extension of 72 °C for 7 min and a holding temperature of 14 °C. Cycling was performed on an Applied Biosystems 2720 Thermal Cycler. The verification of the PCR product was established on a 1.5% agarose gel dyed with SYBR®Safe DNA gel stain.

2.6. DNA sequencing of canine hookworm

DNA sequencing was conducted on all positive samples. PCR products were purified using an Agencout® AMPure® XP PCR purification kit. DNA was quantified using a spectrophotometer and sequenced using an ABI 3730XL 96 capillary DNA sequencer (Applied Biosystems using Big Dye version 3.1 dye terminators). All chromatograms were viewed using Finch TV Version 1.4.0 (Geospiza Inc.). Dual infections were characterised by the presence of overlapping nuclotide peaks at specific positons in the chromatograms which corresponded to the specific hookworm species. Sequences were compared to a variety of GenBank *Ancylostoma* spp. submissions for similarity.

2.7. Data analysis

Prevalence was calculated by dividing the number of samples positive for each hookworm species by the total number of samples positive for hookworm in each location (Table 1). The significance ($p < 0.05$) of the difference between hookworm species and location was determined using the two-sided Fisher's Exact test (Kirkman, 1996).

3. Results and Discussion

Sixty-three of the 89 scats (70.7%) were PCR positive for hookworm. Of these, 49 samples returned clear and readable sequences. Chromatograms showed low signal strength on repeatedly unreadable samples, this often corresponded with low spectrophotometer DNA readings.

BLAST results showed a 99% or greater homology to previously published sequences with GenBank accession nos. DQ780009 for *A. ceylanicum* and JQ812694 for *A. caninum*. One sample, from northern Cairns, was 100% homologous with Genbank accession no. DQ438069.1 for *A. braziliense*.

From the 26 wild dog intestines examined, only the hookworms *A. ceylanicum* and *A. caninum* were found. BLAST results from sequences of positively identified *A. ceylanicum* samples NSD25 (Fig. 1) and NSD26 (both from northern Cairns) were 100% homologous with GenBank accession no. DQ780009 for *A. ceylanicum*. Positively identified *A. caninum* from sample NSD20 (from southern Cairns) was 100% homologous with DQ438073 for *A. caninum* confirming that morphological identification of samples was correct. Other gastrointestinal helminths detected during necropsies were *Toxocara canis* (46%), *Trichuris vulpis* (0.04%) and the tapeworms, *Spirometra erinacei* (46%) and *Dipylidium caninum* (0.04%).

Table 1
Location and prevalence of hookworm species from wild dog scats and wild dog necropsies.

Parasite	Location	Number of positive samples			
		Scats*/*n* (% prevalence)		Necropsy†/*n* (% prevalence)	
Ancylostoma ceylanicum	Mt. Windsor NP	11/11	(100)	–	
	Northern Cairns	17/25	(68)	2/2	(100)
	Southern Cairns	4/13	(30.8)	1/17	(5.9)
	Atherton	–		0/7	(0)
Ancylostoma caninum	Mt. Windsor NP	5/11	(45.5)	–	
	Northern Cairns	19/25	(76)	2/2	(100)
	Southern Cairns	11/13	(84.6)	17/17	(100)
	Atherton	–		7/7	(100)
Ancylostoma braziliense	Mt. Windsor NP	0/11	(0)	–	
	Northern Cairns	1/25	(4)	0/2	(0)
	Southern Cairns	0/13	(0)	0/17	(0)
	Atherton	–		0/7	(0)
Dual infections	Mt. Windsor NP	5/11	(45.5)	–	
(*A. ceylanicum* and *A. caninum*)	Northern Cairns	12/25	(48)	2/2	(100)
	Southern Cairns	2/13	(15.4)	1/17	(5.9)
	Atherton	–		0/7	(0)

* PCR.
† Morphological identification.

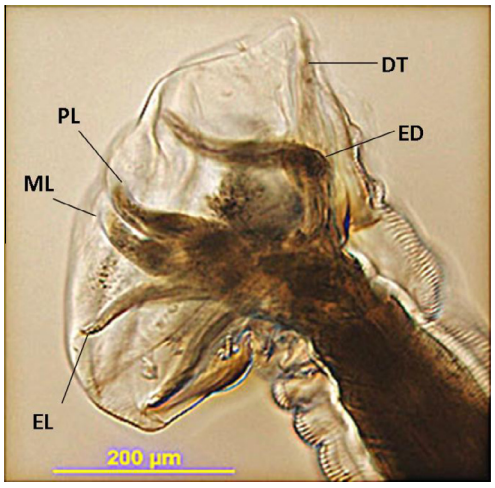

Fig. 1. Lateral view of male bursa of *A. ceylanicum* clearly showing divergent externolateral ray (EL) and closely associated mediolateral (ML) and posteriolateral (PL) rays. The externodorsal (ED) ray is indicated at the attachment point to the dorsal trunk (DT).

Prevalence results for both scats and necropsies are illustrated in Table 1 and Fig. 2. From the 26 wild dog carcasses examined, all were infected with *A. caninum* (100%) and three (11.5%) contained *A. ceylanicum* and *A. caninum* together as dual infections from northern and southern regions of Cairns whilst Atherton wild dogs were solely infected with *A. caninum* (100%). The prevalence of *A. ceylanicum* in wild dog scats was highest at Mt. Windsor NP (100%) followed by northern Cairns (68%) and finally scats from southern Cairns (30.8%). A single infection of *A. braziliense* (4%) was identified in a scat collected from northern Cairns. Fisher's Exact test on frequency analysis showed that prevalence of hookworm species in wild dog scats varied significantly with location ($p = 0.0004$).

This study reports, for the first time, the presence of the hookworm *A. ceylanicum* in wild canids and the first occurrence of this parasite in Far North Queensland, Australia. It also demonstrates that *A. ceylanicum* is the dominant hookworm species of wild canids in Mt. Windsor National Park. This area has a history of logging (Crome et al., 1992), and over the past several decades there is evidence of rainforest recovery and expansion (Tng et al., 2012). A study by Palmer et al. (2007) found *A. ceylanicum* in other regions of Australia and although the survey did include Cairns, Far North Queensland, it looked only at domestic dogs. Previous reports of *A. ceylanicum* in Australia (Stewart, 1994; Gasser et al., 1996; Adams, 2003) were a result of misidentification of *A. braziliense* as *A. ceylanicum* (e Silva et al., 2006; Traub et al., 2007).

Previous studies on pound dogs in Cairns (Setasuban and Waddell, 1973) and wild dogs near Townsville (Brown and Copeman, 2003), approximately 350km south of the current study area, reported the prevalence of *A. caninum* at 100% and 90% respectively. These prevalences are similar to the findings in this study for the wild dogs necropsied (Table 1).

Further gastrointestinal helminths, found during necropsies, were *Spirometra erinacei* at a prevalence of 46%. This is consistent with previous findings in North Queensland (Brown and Copeman, 2003). Only one animal (0.04%) was found to be infected with *D. caninum,* this figure is substantially less than the previous report of 59% by Brown and Copeman (2003). A small number of fleas, *Ctenocephalides felis* and *C. canis*, the intermediate hosts of *D. caninum*, were seen on some of the wild dogs. As many of these animals were shot by farmers during pest control operations, they may have been left exposed for a period of time after death, whereupon fleas would leave the carcass as it cooled.

Interestingly, the zoonotic tapeworm, *Echinococcus granulosus* was not found in animals in this study. Banks et al. (2006), previously identified a pocket of high cystic echinococcosis infection in cattle on the Atherton Tableland, therefore, the parasite is present in the region. As the main definitive host of *E. granulosus,* dingoes have often been described with high worm burdens (Baldock et al., 1985). Wallabies, abundant in the region, have previously proven to be the most susceptible macropod to hydatid cyst infection (Durie and Riek, 1952), and can form a major dietary constituent of wild dogs. As the majority of our study animals were under two years of age, it is possible that they generally targeted smaller prey such as rats (*Rattus rattus*), bandicoots (*Perameles nasuta*) and in one pup's unfortunate case, an echidna (*Tachyglossus aculeatus*), all of which are not thought to carry hydatid infection.

The detection of *T. canis* at a prevalence of 46% is of some concern due to the zoonotic potential of this parasite. *T. canis* is predominantly a parasite of young dogs (Kelly, 1977), therefore, an increased prevalence in our study animals may be expected. The single finding of the canine whipworm, *T. vulpis,* is believed to be the first report of this parasite in a wild dog in Far North Queensland.

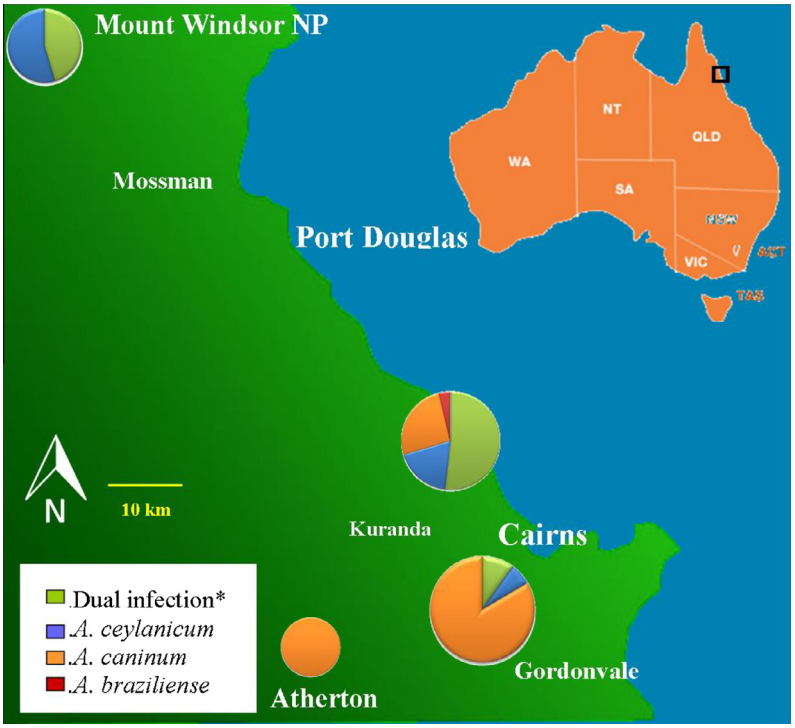

Fig. 2. Hookworm populations in wild dogs and scats collected in north-east Queensland, Australia. (Circle size reflects the number of positive samples collected in the surrounding area; the segments indicate the composition of the population according to species, as represented in Table 1.) *Dual infection indicates infection with both *A. ceylanicum* and *A. caninum*.

A. braziliense is known to occur in North Queensland (Stevenson and Hughes, 1980; Beveridge, 2002) and the finding of a single infection from a scat is consistent with previous reports of low prevalences in the region (Heydon, 1929; Setasuban and Waddell, 1973).

A recent paper by Conlan et al. (2011) described *A. ceylanicum* as the most neglected of all human hookworm species. Some studies have reported insignificant findings in several clinical studies involving *A. ceylanicum* infection in healthy well-fed adults (Wijers and Smit, 1966; Carroll and Grove, 1986). However, these findings should be balanced by reports where significant clinical disease has been seen such as that reported by Anten and Zuidema (1964) suggesting that severity is dependent on host and environmental factors as occurs with most diseases.

Wild dogs used in this study were generally 'problem' animals killed in council control operations. They were frequently found to be utilising areas close to people and farms and therefore may have been in close contact with domestic dogs in which *A. caninum* is the dominant hookworm infection. In contrast, the majority of wild dog scats collected were found further afield in National Parks and rural areas, away from human habitats and in areas dominated by rainforest or green corridors (areas of habitat designated as wildlife corridors between regions). This sampling bias may be one of the reasons for the higher prevalence of *A. ceylanicum* found in scats. Also, although some care was taken to collect scat samples following rainy periods to ensure they were recently deposited, the collection of multiple scats from the same individuals cannot be ruled out. Therefore, some differences may be due to sampling, and infection incidences in various locations, may not be as high as those stated.

The results showed a significant difference between species of hookworm present in scats and location. These differences cannot be attributed to reasons such as season or altitude as *A. ceylanicum* was found in scats collected year round and at elevations ranging from 7 m above sea level (Barron) up to approximately 1000 m (Mt. Windsor NP). Again, higher frequencies of *A. ceylanicum* were present in scats collected from more remote locations (100% from Mt. Windsor NP). It is possible, therefore, that *A. ceylanicum* is the more abundant hookworm in areas of rainforest vegetation and that in locations where domestic dogs, the reservoir host of *A. caninum*, are present there may be a spill-over of infection from domestic dogs that influences the hookworm species present in the wild dog population.

Given that *A. ceylanicum* has previously been found in cats and wild felids, further investigation is necessary to evaluate the hookworm population of domestic and feral cats in the region. Future studies should also concentrate on animals in potentially high risk Indigenous communities to determine the extent of *A. ceylanicum* infection in Far North Queensland and to assess the risk of zoonotic transmission and disease.

The zoonotic potential of this parasite should not be underestimated. Indigenous communities are at particular risk because of the limited management of domestic dog health and the presence of free-roaming community dogs that can be exposed to parasite eggs and larvae in soil contaminated by wild dogs. Together with the warm, moist conditions of the tropics this provides an ideal scenario for the success of soil-transmitted helminth infections.

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