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Molecular identification of zoonotic hookworm species in dog faeces from Tanzania

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Research Paper

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Abstract

The presence and distribution of various species of canine hookworms in Africa are poorly known. The main objective of this study, therefore, was to identify the hookworm species present in canine faecal samples from Morogoro, Tanzania, using molecular techniques. Faecal samples from 160 local dogs were collected and hookworm positive samples processed to recover larvae for further molecular characterization. DNA was extracted from pools of larvae from individual samples (n = 66), which were analysed subsequently using two different molecular approaches, polymerase chain reaction-linked restriction fragment length polymorphism (PCR-RFLP) and species-specific PCR coupled with Sanger sequencing. The PCR-RFLP technique detected only the presence of the ubiquitous *Ancylostoma caninum* in the 66 samples. However, by species-specific PCR coupled with Sanger sequencing we identified ten samples with *A. braziliense*, two with *Uncinaria stenocephala* and five with *A. ceylanicum*. Thus, all four known species of canine hookworms were identified in Morogoro, Tanzania. To our knowledge this is the first report of the detection of the presence of *U. stenocephala* and *A. ceylanicum* in Africa using molecular techniques. In addition to their veterinary importance, canine hookworms have zoonotic potential and are of public health concern.

Introduction

Ancylostomatids (hookworms) together with ascarids (roundworms) are the most ubiquitous parasitic worms of companion animals (Traversa, 2012). Four species of ancylostomatids are considered as canine hookworms: *Ancylostoma caninum*, *A. braziliense*, *A. ceylanicum* and *Uncinaria stenocephala* (Bowman *et al.*, 2010). Hookworm infections may cause anaemia and hypoproteinemia (Loukas & Prociv, 2001); the severity depends on factors such as the age of the animal, intensity of infection and species of hookworm involved (Traversa, 2012; Ng-Nguyen *et al.*, 2015).

The geographical distribution of different ancylostomatids is not clear, mostly because the eggs are morphologically indistinguishable (Palmer *et al.*, 2007; Bowman *et al.*, 2010). Overall, *U. stenocephala* is adapted to cold, temperate and subarctic regions, whereas *Ancylostoma* spp. occur in warmer areas worldwide (Kalkofen, 1987; Traversa, 2012). *Ancylostoma caninum* is the most common of all species of canine hookworms, having a more global distribution. *Ancylostoma braziliense* is restricted to the southern hemisphere, and *A. ceylanicum* is endemic in Asia and the Pacific, and is widespread in this region (Bowman *et al.*, 2010; Traub, 2013; Traversa *et al.*, 2014).

Canine hookworms can infect or penetrate human skin, leading to hookworm infection, localized dermatological lesions (cutaneous larva migrans) and/or intestinal distress. However, the specific species responsible for clinical manifestations in humans are not always identified, and therefore it is not known whether all species of canine hookworms are of health concern for the human population. There is evidence for the zoonotic importance of *A. braziliense*, *A. caninum* and *A. ceylanicum*; however, the zoonotic potential of *U. stenocephala* remains unclear (Prociv & Croese, 1990, 1996; Croese *et al.*, 1994; Landmann & Prociv, 2003; Traub *et al.*, 2008; Bowman *et al.*, 2010; Traversa, 2012).

Ancylostoma ceylanicum is the only species of canine hookworm known to cause natural patent infections in human hosts. Previously, human infections with *A. ceylanicum* have been categorized as uncommon, but this assumption has been questioned following studies that have identified *A. ceylanicum* as the second most common hookworm infecting human populations in South-east Asia (Traub *et al.*, 2008; Sato *et al.*, 2010; Jiraanankul *et al.*, 2011; Conlan *et al.*, 2012; Ngui *et al.*, 2012a, c; Traub, 2013; Inpankaew *et al.*, 2014).

Traditionally, egg morphology, and especially egg size, has been used to differentiate between species. However, this is not a reliable method, given the similar morphology and

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egg sizes between hookworm species (Ehrenford, 1953; Lucio-Forster *et al.*, 2012). Species identification based on post-mortem recovery of adult worms or after anthelmintic expulsion is time-consuming and requires highly skilled staff, and there is a high possibility of misidentification (Traub *et al.*, 2004).

To overcome this obstacle several studies in recent decades have used molecular tools to identify species of canine hookworms. Zhu & Gasser (1998) used polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP), and Gasser *et al.* (1996), Traub *et al.* (2004, 2007), e Silva *et al.* (2006), Palmer *et al.* (2007) and Liu *et al.* (2015) used PCR-linked restriction fragment length polymorphism (PCR-RFLP). Ngui *et al.* (2012b) used PCR-high resolution melting (PCR-HRM), and Conlan *et al.* (2012), Mahdy *et al.* (2012), Ngui *et al.* (2012c), Liu *et al.* (2014) and Oliveira-Arbex *et al.* (2017) used PCR and DNA sequencing. The objective of the present study was to identify the hookworm species present in canine faecal samples from Morogoro, Tanzania using PCR-RFLP and species-specific PCR coupled with Sanger sequencing.

Materials and methods

Study site and sampling

Non-randomized faecal samples were collected from local dogs within Morogoro municipality, approximately 195 km west of Dar es Salaam. In total, 160 samples were collected between September and December 2015 from four locations within Morogoro municipality: a dipping well at Sokoine University of Agriculture (SUA) main campus, a veterinary clinic at SUA main campus, private houses in Kilakala Ward, and a dipping well in Kihonda Ward. Most of the samples (78%) were from the two dipping facilities, where owners could have their dogs treated for ectoparasites. All samples were obtained *per rectum* except eight that were collected fresh from the floors of kennels. Samples were stored at 4°C for further analysis. To avoid duplicates, we recorded the name of the owner, the date and location of sampling, and distinctive features of each dog.

Parasitological procedures

Hookworm positive samples were identified using the McMaster technique (Roepstorff & Nansen, 1998). As electrical power cuts were frequent in the Morogoro laboratory, we were not able to preserve the faecal samples at −20°C and had to rely on ethanol preservation at room temperature. Therefore we decided to produce and recover hookworm larvae (L3) as we expected this approach to generate parasite DNA of a higher quality compared to DNA extracted from faeces stored in ethanol. This would also enable us to perform single larva PCR, if needed. Of the 160 samples, 92 were found to be positive for strongyle-type eggs by McMaster, but only 68 had enough faecal material to set up a coproculture (Roepstorff & Nansen, 1998). Larvae from those 68 samples were recovered after 10–15 days by the Baermann technique, stored in 70% ethanol and transported to the University of Copenhagen, Denmark for further molecular analysis.

Ethical considerations

A research associate permit was granted by the Directorate of Research and Postgraduate Studies, Sokoine University of Agriculture, Morogoro. At the sample setting and prior to any interaction with the dogs, the owners were informed in Swahili about the aim of the study and were asked for permission to take faecal samples from their dogs. All dogs were treated with ivermectin 1% (4 mg per 10–15 kg body weight) administered subcutaneously. Treatment was free of charge, with the exception of puppies younger than three months old and/or when interfering with the facility business (veterinarian care or deworming service). Toxicosis caused by macrocyclic lactones (ivermectin) might occur, especially in some collie-related breeds (Mealey, 2008). Based on phenotypic characteristics, there were no collie-type dogs among the dewormed animals.

Molecular procedures

Hookworm controls

Uncinaria stenocephala L3 larvae recovered from naturally infected Danish dogs were kindly supplied by Dr Helena Mejer (University of Copenhagen). A pool of *A. ceylanicum* L3 larvae was graciously provided by Professor Jennifer Keiser (Swiss Tropical and Public Health Institute), obtained after experimental infections in mice. Adult specimens of *A. caninum* and *A. braziliense* recovered from necropsied local dogs from Morogoro, Tanzania were generously provided by Mr Lufunyo Msalilwa (Sokoine University of Agriculture). The controls were confirmed by PCR-RFLP and Sanger sequencing (see below).

DNA extraction

Prior to DNA extraction, the larva pellet was incubated at 95°C to evaporate the ethanol used for storage. The DNeasy Blood & Tissue Kit spin column (QIAGEN, Germany) was used to extract DNA from the larvae and worms according to the manufacturer's instructions, except that the DNA was eluted with 50 µl of AE buffer.

PCR-RFLP

PCR. The set of primers used for amplifying the ITS-1, 5.8S and ITS-2 regions of the rDNA was a modification of the primers RTGHFI/RTABCR originally designed by Traub and co-workers (Traub *et al.*, 2004, 2007). Sequences from the following species and their GenBank accession numbers were used to identify suitable target regions for primers: *A. caninum* (KP844730, JQ812694, DQ438077, DQ438074, DQ438075, DQ438079), *A. ceylanicum* (LC036567, KF279132, KF279134, DQ381541), *A. braziliense* (JQ812692, JQ812693, DQ438062, DQ438061, Q438050), *U. stenocephala* (AF194145, HQ262052–HQ262055), as well as sequences of the human hookworms *Ancylostoma duodenale* (EU344797) and *Necator americanus* (LC036565) and the cat hookworm *Ancyclostoma tubaeforme* (JQ812691). The forward primers CDCTSF (5′-GTGCTAGTCTTCACGACTTTG-3′) and the reverse CDCTSR (5′-CATGTTGCAATATATTCTG ATCT-3′) amplifying a 570 bp region were designed using Primer3 (http://primer3.wi.mit.edu) (Rozen & Skaletsky, 1999) and synthesized by TAG Copenhagen A/S, Denmark. We conducted the PCR in a total volume of 30 µl containing 3 µl of PCR 10X ammonium buffer, 20 mM $MgCl_2$, 1.5 µl of deoxynucleotide triphosphate (dNTP) (4 mM), 1.2 pmol of each primer, 1.5 units of hot-start DNA polymerase and 1 µl of DNA as template. The PCR conditions were an initial denaturation of 95°C for 15 minutes followed by 40 cycles of 95°C for 30 s, 58°C for 40 s and 72°C for 60 s, and the final extension of 72°C for 10 minutes. The products were stained with GelRed (Biotium, USA) and

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visualized after gel electrophoresis (1.5% agarose) under UV light using Gel Doc (Bio-Rad, USA) and Quantity One software, version 4.3.1 (Bio-Rad, USA).

Restriction Fragment Length Polymorphism. The PCR products were subjected to digestion by three different restriction enzymes: *Hin*fI, *Rsa*I and *Psp*1406I (*Acl*I) (Thermo Fisher Scientific, USA). Palmer *et al.* (2007) found that *Hin*fI and *Rsa*I could be used to distinguish between *U. stenocephala*, *A. caninum*, *A. ceylanicum* and *A. tubaeforme* because of their distinct cutting profiles (table 1) (Gasser *et al.*, 1996; Palmer *et al.*, 2007). *Acl*I was included because *A. caninum* and *A. duodenale* are undistinguishable by *Hin*fI/*Rsa*I digestion. Only *A. duodenale* shows a cutting site for *Acl*I (George *et al.*, 2015). We used three units of endonuclease to digest 2 µl of the PCR product at 37°C for three hours; however, in case of weak PCR amplification up to 7 µl of product was digested. The band profiles were visualized by gel electrophoresis as described above. To confirm the species identity obtained by the PCR-RFLP procedure, PCR products from ten samples were bidirectional sequenced by Macrogen Inc. (Seoul, South Korea). The sequences were analysed and assembled using the software Vector NTI (Lu & Moriyama, 2004), aligned and trimmed with BioEdit version 7.2.5 (Hall, 1999) and compared to sequences in the NCBI database using BLAST (National Center for Biotechnology Information, Maryland, USA).

Species-specific PCR and sequencing

As the samples potentially contained DNA from larvae of different species, we aimed to develop species-specific primers to identify the canine hookworm species with lower infection intensities, which might be overlooked by the PCR-RFLP technique. As *A. caninum* was identified in all samples by sequencing the CDCTSF/CDCTSR PCR products, we only designed primers to target *A. braziliense*, *A. ceylanicum* and *U. stenocephala* (table 2). For primer design, we used Primer-Blast (Ye *et al.*, 2012) and the previously mentioned reference sequences in addition to the following sequences of the *cox*1 region: *A. caninum* (AJ407961, AJ407962, AJ407964, AJ407966, AJ407941), *A. ceylanicum* (KP072070-1, KP072079-0, KP072075-7, KM066109, AF225917, LC036568, KC247734, KC247737, KC247740, KC247743-5), *U. stenocephala* (AJ407939), *A. duodenale* (AJ407942, AJ407952, AJ407953, AJ407954, AJ407956, AJ407958, AJ407959, AJ407960, AJ407968), *N. americanus* (AB793562, AM980929, AM980900, AJ407963) and *A. tubaeforme* (FR846510.1, FR846503.1, FR846511.1, FR846505.1, AJ407940.1).

Table 1. Approximate fragment lengths (bp) generated for various hookworm species after enzymatic digestion of the 570 bp PCR product using three restriction enzymes: *Hin*fI, *Rsa*I and *Psp*1406I (*Acl*I).

	Restriction enzymes		
	*Hin*fI	*Rsa*I	*Psp*1406I (*Acl*I)
U. stenocephala	50, 219, 301	115, 455[a] 570	570
A. ceylanicum	49, 221, 301	267, 302	570
A. duodenale	34, 49, 84, 187, 216	266, 303	284, 286
A. caninum	33, 50, 84, 187, 216	267, 304	570
A. tubaeforme	33, 50, 84, 187, 216	40, 76, 151, 303[b] 40, 76, 454	570

[a]For *U. stenocephala* the *Rsa*I digestion region contains a polymorphic site, which may result in two distinct band profiles (Nadler *et al.*, 2013).
[b]For *A. tubaeforme* allelic polymorphism may result in two distinct band patterns (Palmer *et al.*, 2007).

The PCR volume was adjusted to 20 µl per reaction, containing 2 µl of PCR 10X ammonium buffer, 20 mM $MgCl_2$, 1 µl of dNTP (4 mM), 0.8 pmol forward and reverse primers, 1 unit of hot-start DNA polymerase and 1 µl of DNA as template. The PCR running conditions were the same as for the PCR-RFLP but using annealing temperatures of 61, 62 and 63°C for *A. braziliense*, *A. ceylanicum* and *U. stenocephala*, respectively. The PCR products were visualized by gel electrophoresis as described above.

To test the specificity of the species-specific PCR, ten *A. braziliense*, five *A. ceylanicum* and five *U. stenocephala* PCR-positive samples were sequenced in both directions (Macrogen Inc., Seoul, South Korea) and analysed as previously described.

Results

Copromicroscopy examination

Eggs of three species of gastrointestinal helminths were identified in 94 of 160 samples (59%). Hookworms were the most frequently found parasite, present in 92 samples (58%), followed by *Toxocara* spp. in nine samples (6%). *Dipylidium caninum* proglottids were detected macroscopically and confirmed microscopically in two samples (1%). Of the 92 hookworm positive samples we were able to recover larvae from only 68 samples because of the limited amount of faecal material collected from some dogs (fig. 1). The median and mean numbers of larvae stored were 137 and 1993, respectively (range 3–44,150). The number of larvae recovered varied greatly from sample to sample, mostly related to differences in number of eggs per gramme of faeces (epg) and weight of faecal material.

PCR-RFLP

We extracted DNA successfully from 66 of the 68 samples. For the PCR-RFLP all 66 samples showed the same band profile and were identified as *A. caninum*. Ten of the CDCTSF/CDCTSR PCR products were sequenced successfully and confirmed as *A. caninum* with 100% identity with previously published GenBank sequences (KP844730.1, JQ812694.1 and DQ438078.1). Sequences are deposited in GenBank with the accession numbers MG890369–MG890378.

Species-specific PCR and sequencing

For *A. braziliense*, 56 of the 66 samples were positive, and ten samples were randomly selected and all sequenced successfully and confirmed as such with a similarity of 98–100% with previously published sequences of this species (i.e. DQ438069.1, JQ083592.1 and DQ438054.1). Sequences are deposited in GenBank with the accession numbers MG890379–MG890388.

Five samples were PCR positive for *A. ceylanicum* and the five were sequenced successfully and identified as such with a similarity of 99–100% with *A. ceylanicum* sequences in GenBank (KF896598 and AF225917). Sequences are deposited in GenBank with the accession numbers MG914068–MG914072.

For *U. stenocephala* five samples were PCR positive but only two were sequenced successfully and deposited in GenBank,

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Table 2. The three species-specific primer sets used, with species targeted, nucleotide sequence, and the size and region of the product amplified.

Species targeted	Primer set sequence	Amplicon size	Target region
A. braziliense	Forward 5′-CTTGTGTTGGTGGTTGAGCG-3′ Reverse 5′-AGAACGGGAGTTGCTGAAGA-3′	373 bp	ITS (rDNA)
A. ceylanicum	Forward 5′-GTGTTTGGATCTTTGGGG-3′ Reversed 5′-TAAACTAGAATTTGATAAAACTACACC-3′	291 bp	*Cox*1 (mDNA)
U. stenocephala	Forward 5′-CATTAGGCGGCAACGTCTGG-3′ Reverse 5′-CAAGTGCCGTTCGACAAACA-3′	334 bp	ITS (rDNA)

with the accession numbers MG890389–MG890390. They showed a similarity of 99% with all five previously published *U. stenocephala* sequences (HQ262055.1, AF194145.1, HQ 262053.1, HQ262052.1 and HQ262054.1).

Discussion

We identified four canine hookworm species in dog faeces from Morogoro, Tanzania. To our knowledge this is the first report documenting the presence of *U. stenocephala* and *A. ceylanicum* in Africa using molecular techniques.

The identification of *A. caninum* and *A. braziliense* was to be expected, as they are ubiquitous species among dogs in the southern hemisphere, adapted to warm and humid climates. However, the presence of *U. stenocephala,* which is assumed to be distributed in colder areas, as well as *A. ceylanicum,* thought to be confined to Asia and the Pacific, was unexpected (Gibbs & Gibbs, 1959; Bowman *et al.*, 2010; Traub, 2013).

Nevertheless, there have been previous reports of *A. ceylanicum* in Africa, based on morphological identification (Yoshida, 1971), and *A. ceylanicum* has been morphologically identified in South African cats (Baker *et al.*, 1989). However, identification of *A. ceylanicum* from reports prior to 1951 may not be reliable, as it was in that year that Biocca resolved that *A. braziliense* and *A. ceylanicum* were different species (Biocca, 1951).

Our results support the idea that *A. ceylanicum* has been overlooked and that its distribution is more geographically widespread than formerly reported (Traub, 2013). This is important for both veterinary and public health. *Ancylostoma ceylanicum* has been considered to be a rare or non-relevant species regarding human hookworm infection (Ngui *et al.*, 2012a; Traub, 2013); however, recent studies in Asia using molecular techniques have shown that *A. ceylanicum* was present in between 5 and 51% of people infected with hookworms (Traub *et al.*, 2008; Sato *et al.*, 2010; Jiraanankul *et al.*, 2011; Conlan *et al.*, 2012; Ngui *et al.*, 2012c; Traub, 2013; Inpankaew *et al.*, 2014; George *et al.*, 2015; Bradbury *et al.*, 2017). Therefore, the presence of *A. ceylanicum* in dogs from Morogoro suggests that this hookworm species may also be present in people living in the area.

The presence of *U. stenocephala* in Morogoro could be explained by the close relationship of Sokoine University of Agriculture with Denmark. The Division of Veterinary Sciences was established at SUA in 1976, supported by Danida and followed up by professor exchange programmes, during which Danish professors visited for extended periods of time and may have travelled with their dogs infected with *U. stenocephala*.

Applying the PCR-RFLP method, we overlooked *A. ceylanicum* and *U. stenocephala*. This could be explained by the exponential amplification of DNA of the most abundant species during the PCR (*A. caninum* in this case). Consequently, the PCR products subjected to enzymatic digestion were either just *A. caninum* or were outnumbered by this species. This has important implications, as this method cannot be used reliably to estimate the prevalence of specific hookworm species in an area and may overlook the presence of certain species if few in numbers. Furthermore, for the PCR-RFLP it should be noted that intraspecific and intra-individual variation in the target region can lead to complex cutting profiles and therefore dubious species characterization (Chilton, 2004). This was most likely the case for our samples, as we consistently observed an undigested band (570 bp) after *Rsa*I digestion. This band remained intact despite the enzyme increment (3 times) and was also observed in the *A. caninum* control extracted from a single adult worm. Thus, this pattern may be related to intra-individual variation in the rDNA repeats, an observation that was reported previously by Gasser *et al.* (1996). Importantly, this polymorphism can lead to misidentifications with *U. stenocephala*, as this species' PCR product can also be left undigested by the *Rsa*I enzyme. However, this can be excluded in our case as we did not observe the *U. stenocephala* band profile by *Hin*fI digestion.

In addition, the *in silico* analysis showed that the cutting site for the *Psp*1406I (*Acl*I) enzyme is located in a polymorphic

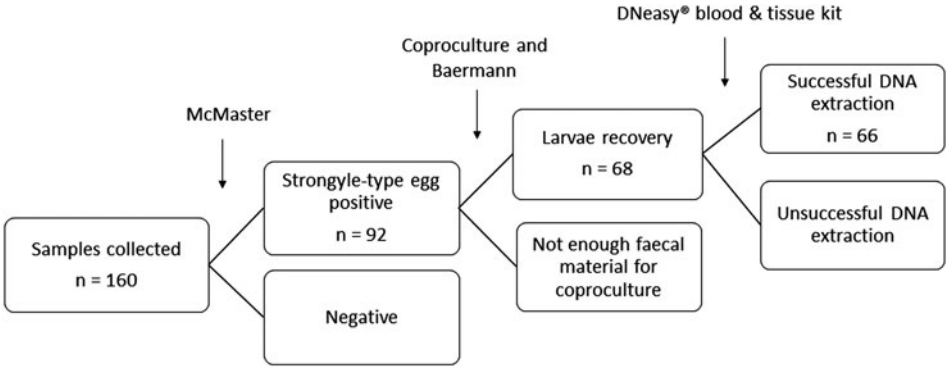

Fig. 1. Process used for analysis of faecal samples from 160 local dogs in Morogoro, Tanzania, to identify the hookworm species present.

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region. Some of the reference sequences of *A. duodenale* deposited in GenBank do not present the specific restriction site (AA^CGTT) in the amplified ITS region (accession numbers KC896800, AB504714-15, AB501348-51). Hence, it may not be a reliable method for discerning between *A. caninum* and *A. duodenale*. However, despite the ambiguity when analysing reference sequences, experimental studies have distinguished between these two species by the use of *Psp*1406I (*Acl*I), which were also confirmed by DNA sequencing (George *et al.*, 2015).

It is important to emphasize that this study aimed to identify which canine hookworm species were present, and not their prevalences. To achieve that, we developed a species-specific PCR, which cannot currently be used as a diagnostic method as it was not validated with other hookworm species (*A. duodenale, N. americanus* and *A. tubaeforme*) nor other species of helminths. Therefore, any positive sample should be sequenced to verify the expected identity of the PCR product. Considering that we could confirm the presence of *A. caninum* by sequencing the CDCTSF/CDCTSR PCR product, we did not develop *A. caninum*-specific primers. Nevertheless, we designed specific primers for *A. ceylanicum*, *A. braziliense* and *U. stenocephala* for PCR followed by sequencing to reliably confirm their identity. As 56 samples were positive for *A. braziliense* we sequenced only ten randomly selected samples, as we regarded this as sufficient to prove their presence among our samples.

Further studies on species prevalence and distribution are needed for improved control of hookworms in the future. To achieve this, it is of paramount importance to develop sensitive and specific diagnostic tools that are able to distinguish between human, feline and canine hookworm species. A cross-sectoral 'one health' approach is not only essential when addressing diagnoses but also regarding epidemiology. Current control of hookworms in the human population is based on treatment of the individual (pregnant women and children under five years of age) or as an added advantage in control programmes for schistosomiasis and lymphatic filariasis using mass drug administration (WHO, 2002, 2006). However, if zoonotic transmission is ignored, control programmes are unlikely to be fully successful with regards to reduction of reinfection among humans (Thompson & Conlan, 2011; Traub, 2013).

In conclusion, local dogs from Morogoro are reservoirs of zoonotic hookworm species. Of special interest is the finding of *A. ceylanicum*, previously thought to be confined to Asia, where it causes hookworm infection in high numbers of people. Nevertheless, we were able to identify this species (along with *U. stenocephala*) only by the use of species-specific primers coupled with Sanger sequencing, and not by the PCR-RFLP method, which may question its reliability as a diagnostic tool. Our results dispute the assumed geographical distribution or confinement of the various hookworm species and call for further research.

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References

Baker MK, Lange L, Verster A and van der Plaat S (1989) A survey of helminths in domestic cats in the Pretoria area of Transvaal, Republic of South Africa. Part 1: the prevalence and comparison of burdens of helminths in adult and juvenile cats. *Journal of the South African Veterinary Association* **60**, 139–142.

Biocca E (1951) On *Ancylostoma braziliense* (de Faria, 1910) and its morphological differentiation from *A. ceylanicum*. *Journal of Helminthology* **25**, 1–10.

Bowman DD, Montgomery SP, Zajac AM, Eberhard ML and Kazacos KR (2010) Hookworms of dogs and cats as agents of cutaneous larva migrans. *Trends in Parasitology* **26**, 162–167.

Bradbury RS, Hii SF, Harrington H, Speare R and Traub R (2017) *Ancylostoma ceylanicum* hookworm in the Solomon Islands. *Emerging Infectious Diseases* **23**, 252–257.

Chilton NB (2004) The use of nuclear ribosomal DNA markers for the identification of bursate nematodes (order Strongylida) and for the diagnosis of infections. *Animal Health Research Reviews* **5**, 173–187.

Conlan JV, Khamlome B, Vongxay K, Elliot A, Pallant L, Sripa B, Blacksell SD, Fenwick S and Thompson RC (2012) Soil-transmitted helminthiasis in Laos: a community-wide cross-sectional study of humans and dogs in a mass drug administration environment. *The American Journal of Tropical Medicine and Hygiene* **86**, 624–634.

Croese J, Loukas A, Opdebeeck J, Fairley S and Prociv P (1994) Human enteric infection with canine hookworms. *Annals of Internal Medicine* **120**, 369–374.

e Silva LMC, Miranda RRC, Santos HA and Rabelo E. ML (2006) Differential diagnosis of dog hookworms based on PCR-RFLP from the ITS region of their rDNA. *Veterinary Parasitology* **140**, 373–377.

Ehrenford FA (1953) Differentiation of the ova of *Ancylostoma caninum* and *Undnaria stenocephala* in dogs. *American Journal of Veterinary Research* **14**, 578–580.

Gasser RB, Stewart LE and Speare R (1996) Genetic markers in ribosomal DNA for hookworm identification. *Acta Tropica* **62**, 15–21.

George S, Kaliappan SP, Kattula D, Roy S, Geldhof P, Kang G, Vercruysse J and Levecke B (2015) Identification of *Ancylostoma ceylanicum* in children from a tribal community in Tamil Nadu, India using a semi-nested PCR-RFLP tool. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **109**, 283–285.

Gibbs HC and Gibbs KE (1959) The effects of temperature on the development of the free-living stages of *Dochmoides stenocephala* (Railliet, 1884) (Ancylostomidae: Nematoda). *Canadian Journal of Zoology* **37**, 247–257.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Inpankaew T, Schär F, Dalsgaard A *et al.* (2014) High prevalence of *Ancylostoma ceylanicum* hookworm infections in humans, Cambodia, 2012. *Emerging Infectious Diseases* **20**, 976–982.

Jiraanankul V, Aphijirawat W, Mungthin M *et al.* (2011) Incidence and risk factors of hookworm infection in a rural community of central Thailand. *The American Journal of Tropical Medicine and Hygiene* **84**, 594–598.

Kalkofen UP (1987) Hookworms of dogs and cats. *Veterinary Clinics of North America: Small Animal Practice* **17**, 1341–1354.

Landmann JK and Prociv P (2003) Experimental human infection with the dog hookworm, *Ancylostoma caninum*. *Medical Journal of Australia* **178**, 69–71.

Liu Y, Zheng G, Alsarakibi M *et al.* (2014) The zoonotic risk of *Ancylostoma ceylanicum* isolated from stray dogs and cats in Guangzhou, South China. *BioMed Research International* **2014**, 208759. doi: 10.1155/2014/208759.

Liu YJ, Zheng GC, Zhang P *et al.* (2015) Molecular identification of hookworms in stray and shelter dogs from Guangzhou city, China using ITS sequences. *Journal of Helminthology* **89**, 196–202.

Loukas A and Prociv P (2001) Immune responses in hookworm infections. *Clinical Microbiology Reviews* **14**, 689–703.

Lu G and Moriyama EN (2004) Vector NTI, a balanced all-in-one sequence analysis suite. *Briefings in Bioinformatics* **5**, 378–388.

https://doi.org/10.1017/S0022149X18000263 Published online by Cambridge University Press

Lucio-Forster A, Liotta JL, Yaros JP, Briggs KR, Mohammed HO and Bowman DD (2012) Morphological differentiation of eggs of *Ancylostoma caninum*, *Ancylostoma tubaeforme*, and *Ancylostoma braziliense* from dogs and cats in the United States. *Journal of Parasitology* **98**, 1041–1044.

Mahdy MAK, Lim YAL, Ngui R, Fatimah MRS, Choy SH, Yap NJ, Al-Mekhlafi HM, Ibrahim J and Surin J (2012) Prevalence and zoonotic potential of canine hookworms in Malaysia. *Parasites & Vectors* **5**, 88.

Mealey KL (2008) Canine ABCB1 and macrocyclic lactones: heartworm prevention and pharmacogenetics. *Veterinary Parasitology* **158**, 215–222.

Nadler SA, Lyons ET, Pagan C *et al.* (2013) Molecular systematics of pinniped hookworms (Nematoda: *Uncinaria*): species delimitation, host associations and host-induced morphometric variation. *International Journal for Parasitology* **43**, 1119–1132.

Ng-Nguyen D, Hii SF, Nguyen V-AT, Van Nguyen T, Van Nguyen D and Traub RJ (2015) Re-evaluation of the species of hookworms infecting dogs in Central Vietnam. *Parasites & Vectors* **8**, 401.

Ngui R, Ching LS, Kai TT, Roslan MA and Lim YA (2012a) Molecular identification of human hookworm infections in economically disadvantaged communities in Peninsular Malaysia. *The American Journal of Tropical Medicine and Hygiene* **86**, 837–842.

Ngui R, Lim YA and Chua KH (2012b) Rapid detection and identification of human hookworm infections through high resolution melting (HRM) analysis. *PLoS One* **7**(7), e41996. doi: 10.1371/journal.pone.0041996.

Ngui R, Lim YA, Traub R, Mahmud R and Mistam MS (2012c) Epidemiological and genetic data supporting the transmission of *Ancylostoma ceylanicum* among human and domestic animals. *PLoS Neglected Tropical Diseases* **6**(2), e1522. doi: 10.1371/journal.pntd.0001522.

Oliveira-Arbex AP, David EB, Oliveira-Sequeira TCG, Katagiri S, Coradi ST and Guimarães S (2017) Molecular identification of *Ancylostoma* species from dogs and an assessment of zoonotic risk in low-income households, São Paulo State, Brazil. *Journal of Helminthology* **91**, 14–19.

Palmer CS, Traub RJ, Robertson ID, Hobbs RP, Elliot A, While L, Rees R and Thompson RCA (2007) The veterinary and public health significance of hookworm in dogs and cats in Australia and the status of *A. ceylanicum*. *Veterinary Parasitology* **145**, 304–313.

Prociv P and Croese J (1990) Human eosinophilic enteritis caused by dog hookworm *Ancylostoma caninum*. *The Lancet* **335**, 1299–1302.

Prociv P and Croese J (1996) Human enteric infection with *Ancylostoma caninum*: hookworms reappraised in the light of a "new" zoonosis. *Acta Tropica* **62**, 23–44.

Roepstorff A and Nansen P (1998) *Epidemiology, diagnosis and control of helminth parasites of swine*, Rome, Food and Agriculture Organization of the United Nations.

Rozen S and Skaletsky H (1999) Primer3 on the www for general users and for biologist programmers. pp. 365–386 *in* Misener S and Krawetz SA (*Eds*) *Bioinformatics methods and protocols*. Totowa, Humana Press Inc.

Sato M, Sanguankiat S, Yoonuan T, Pongvongsa T, Keomoungkhoun M, Phimmayoi I, Boupa B, Moji K and Waikagul J (2010) Copro-molecular identification of infections with hookworm eggs in rural Lao PDR. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **104**, 617–622.

Thompson RC and Conlan JV (2011) Emerging issues and parasite zoonoses in the SE Asian and Australasian region. *Veterinary Parasitology* **181**, 69–73.

Traub RJ (2013) *Ancylostoma ceylanicum*, a re-emerging but neglected parasitic zoonosis. *International Journal for Parasitology* **43**, 1009–1015.

Traub RJ, Hobbs RP, Adams PJ, Behnke JM, Harris PD and Thompson RC (2007) A case of mistaken identity – reappraisal of the species of canid and felid hookworms (*Ancylostoma*) present in Australia and India. *Parasitology* **134**, 113–119.

Traub RJ, Inpankaew T, Sutthikornchai C, Sukthana Y and Thompson RC (2008) PCR-based coprodiagnostic tools reveal dogs as reservoirs of zoonotic ancylostomiasis caused by *Ancylostoma ceylanicum* in temple communities in Bangkok. *Veterinary Parasitology* **155**, 67–73.

Traub RJ, Robertson ID, Irwin P, Mencke N and Thompson RC (2004) Application of a species-specific PCR-RFLP to identify *Ancylostoma* eggs directly from canine faeces. *Veterinary Parasitology* **123**, 245–255.

Traversa D (2012) Pet roundworms and hookworms: a continuing need for global worming. *Parasites & Vectors* **5**, 91. doi: 10.1186/1756-3305-5-91.

Traversa D, di Regalbono AF, Di Cesare A, La Torre F, Drake J and Pietrobelli M (2014) Environmental contamination by canine geohelminths. *Parasites & Vectors* **7**, 67. doi: 10.1186/1756-3305-7-67.

WHO (World Health Organization) (2002) *Prevention and control of schistosomiasis and soil-transmitted helminthiasis: report of a WHO expert committee*. WHO Technical Report Series 912.

WHO (World Health Organization) (2006) *Coordinated use of anthelminthic drugs in control interventions: a manual for health professionals and programme managers*. Geneva, WHO.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S and Madden TL (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134. doi: 10.1186/1471-2105-13-134.

Yoshida Y (1971) Comparative studies on *Ancylostoma braziliense* and *Ancylostoma ceylanicum*. I. The adult stage. *The Journal of Parasitology* **57**, 983–989.

Zhu XQ and Gasser RB (1998) Single-strand conformation polymorphism (SSCP)-based mutation scanning approaches to fingerprint sequence variation in ribosomal DNA of ascaridoid nematodes. *Electrophoresis* **19**, 1366–1373.