African Journal of Biotechnology Vol. 11(8), pp. 2099-2106, 26 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3065
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

PCR-based identification reveals unique Southern African internal transcribed spacer (ITS) haplotypes of hookworms (*Ancylostoma*) of dogs from the Durban metropole, South Africa

Jennifer Lamb, Melanie Napier and Samson Mukaratirwa*

School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville Campus, South Ridge Road, Westville, Durban 4001, South Africa.

Accepted 28 December, 2011

Previous studies on helminths of dogs have revealed a relatively high prevalence of *Ancylostoma* spp. in dogs in Southern Africa. *Ancylostoma caninum*, and to a lesser extent, *A. braziliense* have been reported as the predominant species in dogs based on either morphology of eggs or of adult parasites. The reliability of both methods is questionable and hence the aim of this study was to identify the species infecting dogs in the Durban metropole, South Africa, by comparing morphology of the eggs and results from a polymerase chain reaction (PCR)-based test applied to the third stage larvae of the parasites. Morphology of the egg (measurements of width and length of egg) revealed that the mean length and width of all specimens collected were compatible with *A. caninum.* However, phylogenetic analysis of the ITS-5.8S-ITS2 region of the DNA of third stage larvae of the same specimens revealed the presence of *A. caninum* and *A. braziliense*; the most common haplotype of each species had been previously reported, whilst the others were novel. *Ancylostoma* eggs were identified in 19 of the 63 dogs sampled (30%). Of these, 18 (95%) were infected with *A. caninum*, whereas 5 (26%) were infected with *A. braziliense*. Four (21%) of specimens had mixed infections of both *A. caninum* and *A. brasiliense*.

Key words: *Ancylostoma* spp., hookworms, dogs, Durban metropole, egg morphology, PCR, ITS sequencing.

INTRODUCTION

Hookworms are nematodes belonging to the family *Ancylostomidae*. The species that affect dogs in tropical regions are *Ancylostoma caninum*, *A. braziliense*, *A. ceylanicum* and *Uncinaria stenocephala* (Smyth, 1976), although, *U. stenocephala* is mainly found in temperate regions (Smith et al., 2003). Morphological differentiation of the three species from the genus *Ancylostoma* is difficult even for experienced parasitologists (Palmer et al., 2007). *A. caninum* causes iron-deficiency anaemia in dogs because of its haematophagus behaviour, the severity of which depends on the age and nourishment of the dog (Loukas and Prociv, 2001). Pups and undernourished dogs are more susceptible to severe anaemia and other hookworm-associated conditions, including eczema, bloody diarrhoea and even death (Smyth, 1976). *A. braziliense* is less pathogenic to dogs although it may cause hypo-proteinemia (Smyth, 1976).

Previous studies have revealed a relatively high prevalence of *Ancylostoma* spp. in dogs in Southern Africa (Mukaratirwa and Busayi, 1995; Minaar et al., 2002; Mukaratirwa and Singh, 2010). These species affect not only dogs, but also humans, and hence are of zoonotic importance (Traub et al., 2004). *A. braziliense* is the primary cause of cutaneous larva migrans, a condition caused by penetration and migration of the infective hookworm larvae in the epidermis of human skin, resulting in skin lesions (Kaewthamasorn et al.,

*Corresponding author. E-mail: mukaratirwa@ukzn.ac.za. Tel: +27 78 869 5542. Fax: +27 31 260 2029.

2006; Urquhart et al., 1987). *A. caninum* is known to cause eosinophilic enteritis and diffuse unilateral sub-acute neuroretinitis (Smyth, 1976).

Formerly, *Ancylostoma* spp were identified based on morphological characteristics of the adult worm as well as egg morphology (Palmer et al., 2007). Recently, however, techniques based on polymerase chain reaction (PCR)-amplification and sequencing of the nuclear ribosomal internal transcribed spacer (ITS) region have been used to identify *Ancylostoma* spp. with relative precision (Traub et al., 2004, 2007, 2008; Palmer et al., 2007). Molecular identification techniques have not previously been applied to the study of hookworms in the southern African region and there is a need to validate them for use in this context, particularly as evolutionary divergence among and between *Ancylostoma* spp across continents could result in unique South African forms and changes in expected amplification products.

We therefore sought to evaluate the utility of previously-published PCR primers for amplification of the ITS1-5.8S rRNA-ITS2 region of *Ancylostoma* species (Traub et al., 2004, 2008), and to carry out the first molecular identification of hookworm species of dogs in South Africa. We created a phylogenetic analysis including local and Genbank-derived *Ancylostoma* ITS sequences, and used this to identify haplotypes and species of *Ancylostoma* present in our samples. Thus, the aim of this study was to identify species of hookworms infecting dogs in the Durban metropole, South Africa, by comparing morphology of the eggs with identifications based on sequences of the nuclear ribosomal ITS region.

MATERIALS AND METHODS

Collection of faecal samples

Sixty-three faecal samples from stray dogs were collected from the Kloof Society for the Prevention of Cruelty to Animals (SPCA), which is a catchment of stray and impounded dogs in the Durban metropole, South Africa, during a six month period from March to September, 2010. The sampled dogs were of mixed sex, of various breeds and ranged from 8 weeks to 7 years of age.

Faecal egg counts, larva culture and isolation

The McMaster technique was used to detect hookworm eggs in faeces (Gibbons et al., 1996). The length and width of a minimum of 10 *Ancylostoma* spp. eggs per positive sample were measured using a calibrated stage micrometer on a compound light microscope (Gibbons et al., 1996). A portion of each faecal sample positive for hookworm eggs was cultured individually in semi-wet wood shavings (Vidhikar, 1927; Urquhart et al., 1987). The cultures were placed in an incubator at 27°C for 10 days. Third-stage larvae were recovered using the Baermann technique (Gibbons et al., 1996) and preserved in 70% ethanol for molecular analysis.

DNA isolation

Third-stage-larvae were used in the molecular identification of hookworm species. Adult *A. caninum* worms kindly donated from the University of Zimbabwe Veterinary Teaching Hospital, were used as a positive control. DNA was extracted from the larvae samples and the control adult sample with a DNeasy® Blood and Tissue Kit (Qiagen, Germany) according to manufacturer's instructions.

Molecular approach to sample identification

The ITS region of the DNA was PCR-amplified using three sets of primers, described by Traub et al. (2004). Amplifications were carried out using the same forward primer, RTGHF1 (5'-CGTGCTAGTCTTCAGGACTTTG-3') in combination with three different reverse primers; RTGHR1 (5'-CGTTGTCATACTA-GCCACTGC-3'), RTABCR1 (5'-CGGGAATTGCTATAAGCAAG-TGC-3') and RTAYR1 (5'-CTGCTGAAAAGTCCTCAAGTCC-3'). Primer pair RTGHF1 and RTGHR1 is reported to amplify a 679–690 bp region of the ITS region of *A. tubaeforme*, *A. caninum*, *A. duodenale* and *A. ceylanicum* (Traub et al., 2004). Primer pair RTGHF1 & RTABCR1 amplify 545 base pairs of the ITS region of *A. caninum*, *A. ceylanicum* and *Uncinaria stenocephala*, and primer pair RTGHF1 and RTAYR1 amplify 673 base pairs of the ITS region of *A. braziliense* (Traub et al., 2008).

PCR products were sequenced, after which Blast searches of the NCBI Genbank database were carried out in order to identify the closest matches for each sequence obtained. Experimental and Genbank-derived sequences were aligned, trimmed and analysed by the neighbour-joining and maximum parsimony methods. Samples were identified according to their cladal affiliation in the resulting tree, as well as their genetic distance from other samples and clades.

PCR amplification and fragment isolation

The ITS-1 and ITS-2 regions of the *Ancylostoma* genome were amplified in 25 µL PCR reactions containing 0.8 µL water, 2.5 µL buffer (10X), 4 µL $MgCl_2$, 0.5 µL dNTPs, 4 µL forward primer and 4 µL reverse primer, 0.2 µL Taq polymerase (Promega) and 9 µL extracted DNA. Thermal cycling parameters were: 1 cycle of (94°C for 2 min, 64°C for 1 min and 72 °C for 2 min), followed by 50 cycles (94°C for 30 s, 64°C for 30 s, 72 °C for 30 s) and a finally at 72°C for 7 min. PCR products were electrophoresed in 2% agarose gels in 0.5 x TBE running buffer at 15 V overnight. The DNA bands were excised from the agarose gel and recovered by use of a Gel DNA Recovery Kit (Zymoclean, USA) according to manufacturer's instructions.

Sequencing and phylogenetic analysis

Purified amplicons were sent to the van Heerden laboratory, Stellenbosch University, Stellenbosch, Western Cape, South Africa, for sequencing. The sequences were edited and aligned together with Genbank-derived sequences of *A. caninum*, *A. braziliense*, *A. duodenale*, *A. ceylanicum*, *A. tubaeforme* and *U. stenocephala* (Table 1) using the BioEdit Sequence Alignment Editor (Hall, 1999). PAUP 4.0b10 (Swofford, 2002) was used to create neighbor-joining trees using p-distances and maximum parsimony trees based on haplotype data. For parsimony analysis, we used the random additions sequence option (n=100) for discrete, unordered characters. The shortest tree was searched for with the heuristic search option using the tree bisection-reconnection (TBR) branch swapping option. The degree of character support for each node of the resulting tree was estimated using bootstrap re-sampling analysis (1000 iterations) (Felsenstein, 1985). For genetic distance

Table 1. Haplotype status of Genbank-derived ITS sequences used in molecular identification of hookworms infecting dogs from the Durban metropole, South Africa.

Haplotype	Identification	Genbank accession number
G1	*A. caninum*	EU159415
G2	*A. caninum*	EU159416, DQ438070
G3	*A. caninum*	DQ438071, DQ438072, DQ438074, DQ438075, DQ438076, DQ438077, DQ438078, DQ438079
G4	*A. braziliense*	DQ359149, DQ438050, DQ438052, DQ438053, DQ438055, DQ438056, DQ438057, DQ438060, DQ438062, DQ438063, DQ438064
G5	*A. braziliense*	DQ438059
G6	*A. braziliense*	DQ438054
G7	*A. duodenale*	EU344797
G8	*A. tubaeforme*	EMBZ70741.1
G9	*A. ceylanicum*	DQ780009
G10	*Uncinaria stenocephala*	F194145

The samples were obtained by BLAST searches of the NCBI Genbank, and by searches of the nucleotide database for *Ancylostoma* and *Uncinaria* sequences.

analysis, samples were divided into the following groups according to the results of the neighbor-joining and maximum parsimony analyses: (1) Genbank *A. caninum*, (2) RTGHF1 - RTABCR1 product, (3) Genbank *A. braziliense*, (4) RTGHF1–RTAYR1 product, (5) Genbank *A. duodenale*, (6) Genbank *A. tubaeforme*, (7) Genbank *A. ceylanicum* and (8) Genbank *U. stenocephala* (outgroup) species. The net mean genetic p-distance between groups was calculated in Mega version 5 (Tamura et al., 2011).

RESULTS

Morphometric measurements

The mean egg length of ten eggs from each dog sample varied between 51.3 ± 10.8 and 65.6 ± 2.9 µM, whereas the mean egg width varied between 36.6 ± 4.2 and 42.8 ± 5.2 µM and these dimensions fell within the range described for *A. caninum* by Gibbons et al. (1996) (Figure 1). However, one sample with mean egg length of 51.3 ± 10.8 µM and width of 38.7 ± 2.3 was not classified as the dimensions did not match those of either *A. caninum* or *A. braziliense*. Meanwhile, no eggs fell within the combined length and width range described for *A. braziliense* as described by Gibbons et al. (1996).

PCR amplification products

Primers RTGHF1 (forward) and RTGHR1 (reverse) produced an amplification product of ~680 bp. Sequence electropherograms contained either single traces, consistent with *A. caninum* or mixed traces in which the major component (tallest peaks) was identifiable as *A. caninum*, and the minor component as *A. braziliense*, consistent with mixed infections with these two species.

Amplification with primers RTGHF1 and RTABCR1 produced a single amplification product of approximately 545 bp, which gave a clear, easily-readable single-trace sequence electropherogram. Amplification with primers RTGHF1 and RTAYR1 resulted in a single amplification product of approximately 673 bp, and a clear, single-trace sequence electropherogram.

Phylogenetic analysis

We analysed a trimmed alignment (327 nucleotides) of ITS1-5.8S-ITS2 haplotypes of samples derived from the NCBI Genbank (*A. caninum*, *A. braziliense*, *A. duodenale*, *A. ceylanicum*, *A. tubaeforme* and *U. stenocephala*) (Table 1) and experimental samples (Tables 2 and 3). Three characters were variable and parsimony-uninformative, whereas 32 were parsimony-informative. Both maximum parsimony and neighbor-joining analyses provided strong support (bootstrap value (BV) 100 %) for the monophyly of *Ancylostoma* samples (group A) with respect to the outgroup *U. stenocephala* (Figure 2). The mean genetic distance (p-distance) between *U. stenocephala* and all *Ancylostoma* samples was 6.0% (Table 4).

Within the *Ancylostoma* group, *A. ceylanicum* occupies an unsupported position consistent to an unsupported group (B) comprising of *A. caninum*, *A. duodenale*, *A. braziliense*, *A. ceylanicum* and *A. tubaeforme* haplotypes. *A. tubaeforme* (G8) and Genbank *A. caninum* haplotype 1 form an unsupported group (B1), similar to an un-supported group B2 comprising Genbank-derived haplotypes of *A. caninum*, *A. braziliense*, *A. duodenale* and experimental samples.

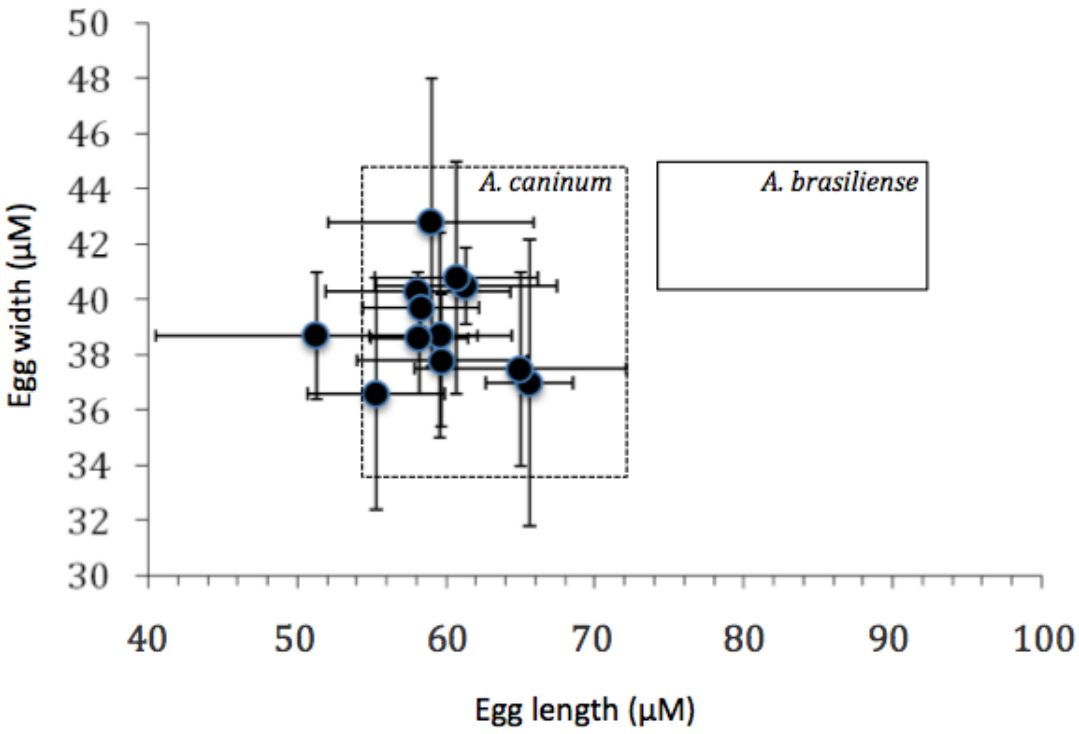

Figure 1. Plot of dimensions of *Ancylostoma* eggs recovered from faeces of dogs sampled in the Durban metropole, South Africa. Solid rectangle; expected position of *A. braziliense* eggs (74 to 95 µM long and 41 to 45 µM wide) according to Gibbons et al. (1996). Dotted rectangle; expected position of *Ancylostoma caninum* eggs (55 to 72 µM long and 34 to 45 µM wide). Measurements are means of 10 eggs per sample; error bars indicate the standard deviation.

Table 2. Haplotype status of experimental samples used in molecular identification of hookworms infecting dogs from the Durban metropole, South Africa.

Haplotype	Primer	Product size (nt)	ID	Genbank accession number	Sample
E1	RTABCR1	673	*A. caninum*	JQ083587	1 - 15, control
E2	RTABCR1	673	*A. caninum*	JQ083588	16
E3	RTABCR1	673	*A. caninum*	JQ083589	17
E4	RTABCR1	673	*A. caninum*	JQ083590	18
E5	RTABCR1	673	*A. caninum*	JQ083591	19
E6	RTAYR1	545	*A. brasiliense*	JQ083592	18, 19
E7	RTAYR1	545	*A. brasiliense*	JQ083593	14
E8	RTAYR1	545	*A. brasiliense*	JQ083595	15
E9	RTAYR1	545	*A. brasiliense*	JQ083596	13

The nuclear ribosomal ITS region was amplified and sequenced using forward primer RTGHF1 in combination with reverse primer RTABCR1 and, separately, with reverse primer RTAYR1. Sample identifications are based on a phylogenetic analysis of haplotype data (Figure 2). ITS – internal transcribed spacer; nt – nucleotide.

Primer RTAYR1

Within B2, there is strong support from both analyses (neighbor-joining BV 87%, maximum parsimony BV 83%) for a monophyletic group (C1) containing all samples primed with RTGHF1 - RTAYR1, and all Genbank-derived *A. braziliense* samples. Members of Group C1 are also unified by the presence of a one nucleotide insertion at position 3 in the alignment. The net mean genetic distance between *A. braziliense* (Genbank) haplotypes G4 - G6 and experimental samples primed with RTGHF1- RTAYR1 (haplotypes E6 – E9) is 0.1% (Table 4). Experimental haplotypes tend to be basal to more derived Genbank *A. braziliense* haplotypes. Experimental RTAYR1-primed haplotype E6 is identical to Genbank *A. braziliense* haplotype G6, whereas haplo-

Table 3. Variable positions in an alignment of 327 nucleotides of the nuclear ribosomal ITS region of experimental (E1-E9) (Table 2) and Genbank-derived (G1-G9) (Table 1) haplotypes of *Ancylostoma* species.

Hap	ID	Position in alignment																			
		1	2	3	25	26	40	41	46	93	94	95	110	137	139	141	152	247	295	311	322
G1	*A. caninum*	-	A	C	A	T	G	A	T	G	T	A	T	T	G	T	-	G	T	T	G
G2	*A. caninum*	-	A	C	A	T	G	A	G	G	T	A	T	T	C	C	-	G	T	T	G
G3	*A. caninum*	-	A	C	A	T	G	A	G	G	T	A	T	T	T	C	-	G	T	T	G
E1	*A. caninum*	-	A	C	A	T	G	A	G	G	T	A	T	T	T	C	-	G	T	T	G
E2	*A. caninum*	-	A	C	A	T	G	R	G	G	T	A	T	T	T	C	-	G	T	T	G
E3	*A. caninum*	-	A	C	A	T	G	R	G	G	Y	A	T	T	T	C	-	G	T	T	G
E4	*A. caninum*	-	A	C	A	T	G	R	G	G	Y	A	T	T	T	Y	-	G	T	T	-
E5	*A. caninum*	-	A	C	G	T	G	R	G	G	Y	A	T	T	T	Y	-	G	T	T	G
G4	*A. brasiliense*	A	C	C	G	C	G	G	G	G	C	A	A	G	T	T	-	G	T	C	G
G5	*A. brasiliense*	A	C	S	G	C	G	G	G	G	C	A	A	G	T	T	-	G	C	C	G
G6	*A. brasiliense*	A	C	C	G	C	G	G	G	G	C	A	A	G	T	T	-	A	T	C	G
E6	*A. brasiliense*	A	C	C	G	C	G	G	G	G	C	A	A	G	T	T		A	T	C	G
E7	*A. brasiliense*	A	C	C	G	C	G	G	G	G	C	A	W	G	T	T	-	R	T	C	G
E8	*A. brasiliense*	A	C	C	R	Y	G	R	G	G	C	A	W	G	T	T	-	R	T	C	G
E9	*A. brasiliense*	A	C	C	R	Y	G	R	G	G	C	A	W	G	T	Y	-	R	T	C	G
G7	*A. duodenale*	-	A	C	A	T	G	A	G	G	T	A	A	T	T	T	-	G	T	T	G
G8	*A. tubaeforme*	-	-	C	A	T	G	A	G	R	T	R	T	T	A	T	G	G	T	T	G
G9	*A. ceylanicum*	-	A	C	A	T	A	G	G	G	T	A	T	T	T	T	-	G	T	T	G

Hap – Haplotype; ID – identification.

types E7, E8 and E9 contain polymorphic sites.

Primer RTABCR1

With one exception (experimental haplotype E5, Table 2), all experimental samples primed with RTGHF1 and RTABCR1 (haplotypes E1 – E4) form a monophyletic group (C2) which includes the adult *A. caninum* positive control (haplotype E1) and Genbank *A. caninum* haplotypes G2 and G3 (Figure 2). This group is at best weakly-supported, however, the net mean genetic distance between *A. caninum* (Genbank) samples and experimental samples primed with RTGHF1 and RTABCR1 is 0.0%. Experimental haplotype E1 is identical to Genbank *A. caninum* haplotype G3, whereas haplotypes E2 – E5 contain substitutions, deletions and or polymorphisms (Tables 2 and 3). *A. duodenale* (haplotype G7) and experimental RTABCR1-primed haplotype E5 (from sample 18) occupy an unsupported intermediate position within group B2. Genbank *A. caninum* haplotype G1 also falls outside the main *A. caninum* clade (C2) and occurs in an unsupported clade with *A. tubaeforme*.

Ancylostoma samples were identified by cladal affiliation according to the phylogenetic species concept (Figure 2) and genetic distance from other clades (genetic species concept) (Table 4). Our molecular analyses identified the presence of *A. caninum* in 95% (18/19) of the infected samples. Although, derived from a 545 bp product primed with RTGHF1 – RTABCR1, the identification of haplotype E5 was uncertain, owing to its equivocal position in the phylogenetic analysis

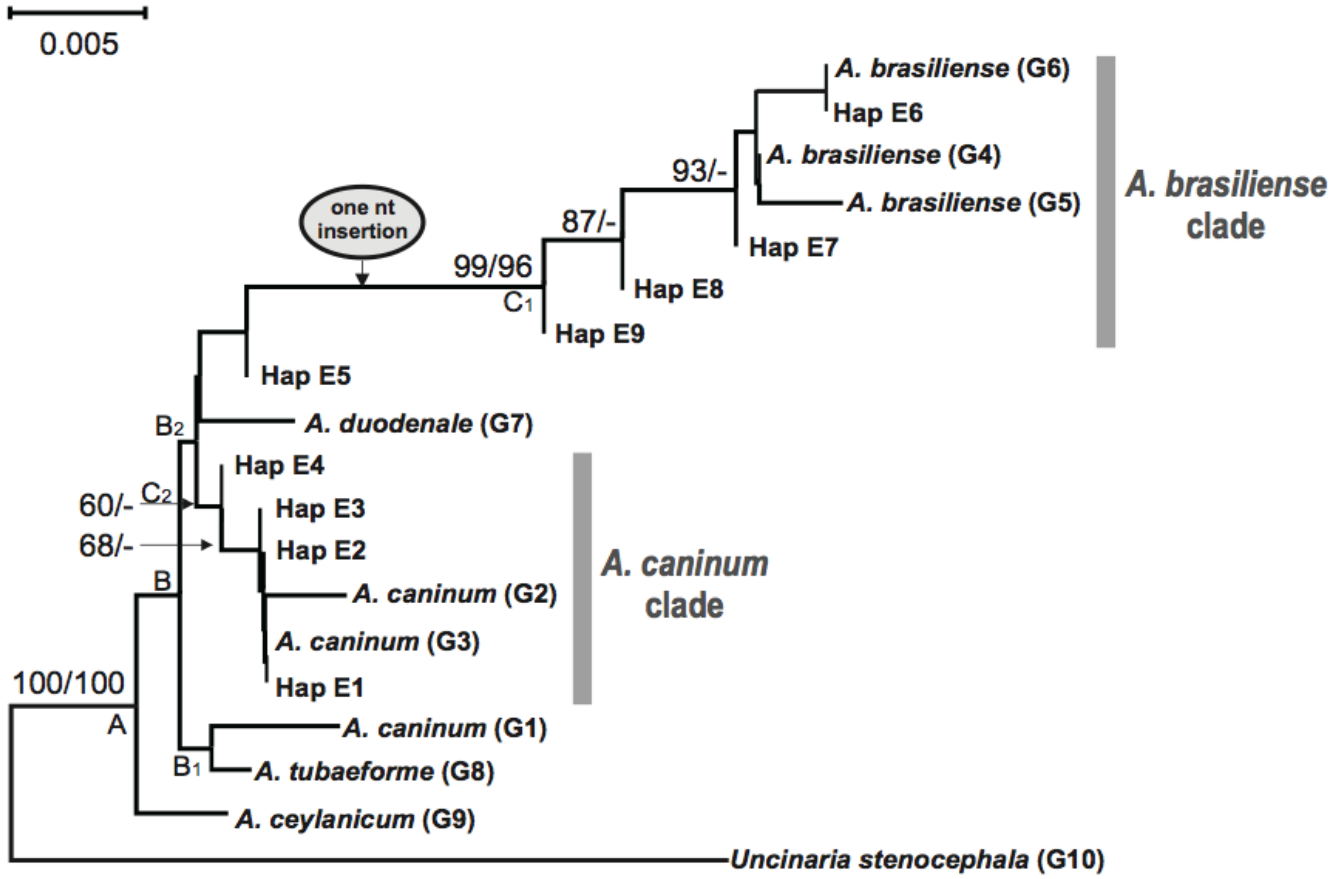

Figure 2. Neighbor-joining tree illustrating evolutionary relationships between experimental- and Genbank-derived *Ancylostoma* haplotypes and the outgroup, *Uncinaria stenocephala*, based on analysis of 327 nucleotides of the nuclear ribosomal ITS region. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (p-distances) used to infer the phylogenetic tree. The distance units are base substitutions per site. Bootstrap values (BV) from a congruent maximum parsimony (MP) analysis are included adjacent to nodes with support values of 60% or greater, in the format (NJ BV %/ MP BV %). Haplotypes derived by BLAST searches of the NCBI Genbank are abbreviated as G1 – G10, whereas experimental haplotypes are abbreviated as E1 – E9.

(Figure 2). Also,26% (5/19) of samples were infected with *A. braziliense* and 21% (4/19) had mixed infections of both *A. caninum* and *A. braziliense*.

DISCUSSION

Morphometric studies of the *Ancylostoma* eggs from faecal samples of the dogs in this study revealed that they were all likely to be those of *A. caninum*, as, with one exception (length 51.3 ± 10.8 µM), they all fell within the size range recorded by Gibbons et al. (1996) for this species (55 to 72 µM long and 34 to 45 µM wide). No eggs fell within the combination length/width range described for *A. braziliense* (74 to 95 µM long and 41 to 45 µM wide), although some egg widths were consistent with *A. braziliense*. Identification based on such measurements could be regarded as unreliable, as the width range for *A. brasiliense* eggs lies within that described for *A. caninum* eggs (Figure 1) (Palmer et al., 2007).

Molecular identification of *Ancylostoma* spp. based on phylogenetic analyses of the nuclear ribosomal ITS region indicates that *A. braziliense* was present in 26% of the samples studied. In cases of mixed infection (21% of samples), *A. braziliense* appears to be present at much lower levels than *A. caninum.* For example, in double-trace sequence electropherograms obtained with the more general primer pair, RTGHF1 and RTGHR1, the set of peaks identifiable as originating from *A. caninum* were considerably taller than those from *A. braziliense*. Furthermore, the use of primer pair RTGHF1 and RTABCR1, which amplifies the ITS region of *A. caninum* DNA, produced heavy amplification, whilst the use of RTGHF1 and RTAYR1, specific for *A. braziliense*, resulted in at least 10-fold lower levels of amplification as indicated by thickness of bands on the gel. Considerably lower levels of *A. braziliense* relative to *A. caninum* in mixed infections may also account for the failure to identify *A. braziliense* eggs in faecal samples.

The primer pair RTGHF1–RTABCR1 produced a ~545

Table 4. Net between-groups mean genetic p-distances (percent) between experimental- and Genbank-derived *Ancylostoma* samples and the outgroup, *Uncinaria stenocephala*, based on 327 nucleotides of the nuclear ribosomal ITS region.

S/N	ITS Region	1	2	3	4	5	6	7	8
1	*A. caninum*	-							
2	RTABCR1 product	0.0	-						
3	*A. braziliense*	2.5	2.2	-					
4	RTAYR1 product	1.8	1.6	0.1	-				
5	*A. duodenale*	1.0	0.9	2.1	1.6	-			
6	*A. tubaeforme*	0.6	0.6	2.8	1.2	0.6			
7	*A. ceylanicum*	0.7	0.6	1.8	1.4	0.9	0.9	-	
8	*Uncinaria stenocephala*	5.7	5.3	7.3	6.8	6.5	5.2	5.2	-

bp ITS PCR amplification product, identifiable from phylogenetic analyses of ITS sequences as originating from *A. caninum*, consistent with the results of Traub et al. (2004) in the hookworms of dogs in India, who microscopically identified the larvae as *A. caninum*. Contrary to the findings of Traub et al. (2004), and in agreement with Traub et al. (2008) this primer pair did not amplify a product identifiable as *A. braziliense*. Our phylogenetic analyses showed that with the exception of sample 19, all experimental sequences derived from this band and the positive control obtained from Zimbabwe, formed a monophyletic group (C2) with Genbank-derived *A. caninum* haplotypes G2 and G3 (Table 1 and Figure 2). Although support levels were at best moderate, we identified these samples isolated from dogs as *A. caninum*, since the mean genetic distance between the experimental and Genbank-derived samples was 0%. The most common experimental *A. caninum* haplotype (E1) was found in 15 of the 19 experimental samples, and the positive *A. caninum* control. Haplotype E1 is identical to Genbank *A. caninum* haplotype G3, represented by samples DQ438071, DQ438072, DQ438074, DQ438075, DQ438076, DQ438077, DQ438078 and DQ438079. Experimental haplotypes E2 – E5 contain novel substitutions, deletions and/or polymorphisms.

In our analyses, *A. caninum* does not form a monophyletic clade, as *A. caninum* Genbank haplotype G1 falls outside of Clade C2, appearing as parallel to the Genbank *A. tubaeforme* haplotype G8, from which it is separated by a genetic distance of 0.6% (Table 4). The identity of sample 19 (experimental haplotype E5), derived from a 545 bp band primed with RTABCR1, is uncertain as it also falls outside clade C2 and is not included in a clade with any other *Ancylostoma* species. This means that the genetic distance from the other species included in the analysis is: *A. caninum* – 0.45%, *A. duodenale* – 0.60%, *A. tubaeforme* – 0.65%, *A. ceylanicum* – 0.81% and *A. braziliense* – 1.72%. As this sample is genetically most similar to the *A. caninum* samples, it may be a variant of *A. caninum*. Our phylogenetic analyses of sequences of a ~673 bp band, primed with RTGHF1 and RTAYR1 strongly support identification of *A. braziliense*, consistent with Traub et al. (2008), although we did not have a positive control to further support this conclusion. This is based on the strongly-supported monophyly of all RTAYR1-primed experimental samples and Genbank-derived *A. braziliense* samples, as well as the low genetic distance (0.1%) between these groups. In contrast, the mean genetic distances between Genbank *A. braziliense* samples and *A. caninum* and *A. duodenale* were 2.4 and 2.1% respectively. Further, experimental *A. brasiliense* haplotype E6, found in two samples, was identical to Genbank *A. brasiliense* haplotype G6, represented by sample DQ438054.

In general, our study supports the monophyly of *A. ceylanicum*, *A. braziliense*, *A. caninum*, *A. duodenale* and our study samples. We found no evidence for the occurrence of the Indian species, *A. ceylanicum,* in our sample set, despite the presence of a large human population of Indian origin in Durban, or of *A. tubaeforme*. The phylogeny we retrieved (Figure 2) as well as analyses of genetic distances between groups (Table 4) show *A. braziliense* to be genetically the most distinct *Ancylostoma* species (mean distance of 2.0% to the other *Ancylostoma* spp), whereas the other *Ancylostoma* spp. are genetically less distinct from each other. The distances between *A. caninum*, *A. duodenale*, *A. ceylanicum* and *A. tubaeforme*, which do not form strongly-supported phylogenetic groups, range between 0.6 and 1%. *A. ceylanicum* and *A. caninum* are separated by 0.7%, which possibly accounts for some of the difficulties in distinguishing them genetically (Traub et al., 2007). It is interesting to note that *A. caninum* and *A. ceylanicum* were considered synonyms before 1951 and that this issue is still being debated (Palmer et al., 2007; Yoshida, 1971). Future works might include analyses of a faster-evolving DNA region in order to elucidate relationships among hookworm species infecting dogs in southern Africa.

Conclusion

Our data indicated that dogs in the Durban metropole harbour *A. caninum* (95% of our infected sample), *A.*

braziliense (26%) or both species (21%) and have revealed the presence of unique South African ITS haplotypes of *A. caninum* and *A. braziliense*. Consistent with data from other studies (Palmer et al., 2007; Traub et al., 2004), *A. caninum* appears to be the dominant species. The presence of *Ancylostoma* species in dogs poses a risk to humans of acquiring zoonoses, although human cases have not been investigated in Durban (Mukaratirwa and Singh, 2010). An increase in the sample size will yield more reliable prevalence rates, and may reveal the presence of further *Ancylostoma* species in the region. *A. ceylanicum* was not identified in any of the samples used in the current study, but might be expected to have been introduced in Durban by the immigration of people from India, where this species is endemic (Yoshida, 1971). Extension of this work may lead to the development of a PCR-RFLP test which can be used with confidence in further screening analyses aimed at identifying the presence of *A. braziliense*, *A. caninum* and perhaps other *Ancylostoma* species in dogs in South Africa.

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