ORIGINAL ARTICLE

# Unambiguous identification of *Ancylostoma caninum* and *Uncinaria stenocephala* in Australian and New Zealand dogs from faecal samples

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Hookworms (Ancylostomatidae) are well-known parasites in dogs due to their health impacts and zoonotic potential. While faecal analysis is the traditional method for detection, improvements in husbandry and deworming have decreased their prevalence in urban owned dogs. Drug resistance in *Ancylostoma caninum* is becoming a discussion point in small animal practices across the region. This study aimed to identify hookworm species present in Australian and New Zealand dogs using molecular techniques. The ITS-2 and isotype-1 β-tubulin assays were used to identify and quantify hookworm species. Results showed absence of coinfection in Australian samples from Greater Sydney region belonging either to *A. caninum* or *Uncinaria stenocephala*, while New Zealand samples were a mixture of *A. caninum* and *U. stenocephala*. The amplified isotype-1 β-tubulin sequences exhibited susceptibility to benzimidazole drugs. Rare mutations were identified in *A. caninum* and *U. stenocephala* sequences, representing a small percentage of reads. This study highlights the importance of molecular techniques in accurately identifying and quantifying hookworm species in dog populations.

**Keywords** drug resistance; hookworms; ITS-2; nemabiome; tubulin

**Abbreviations** ASVs, amplicon sequence variants

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Hookworms (Ancylostomatidae) are frequently discussed parasites of dogs due to their haematophageous nature, subsequent health impacts, as well as zoonotic implications due to the cutaneous larva migrans and eosinophilic enteritis.[1–4] Traditional method for identification of infected dogs is the faecal analysis and observation of 'hookworm' eggs. Improvements of husbandry, diet and application of routine blanket deworming has seen decrease of hookworm detection over the past 40 years to the degree that they are rarely found in urban owned dogs unlike in free-roaming dogs.[5–7]

The development of drug resistance in *Ancylostoma caninum* (canine hookworm) or even multi-drug resistance is becoming a discussion point in small animal practice.[8] In Australia, *A. caninum* resistance to pyrantel embonate was experimentally confirmed already in 2008 and the authors urged "for greater vigilance and more judicious use of anthelmintics in small animal practice".[9] Recently two studies investigated the extent of benzimidazole resistance across the United States and one found over half of the *A. caninum* isolates had resistance allele frequency higher than 50% of that of the isotype-1 *β*-tubulin F167Y.[10,11] Besides *A. caninum*, Australian dogs are hosts for three other hookworms, including the tropical hookworms *Ancylostoma ceylanicum* and *Ancylostoma braziliense*, and the cooler climate hookworm *Uncinaria stenocephala*. Dogs in New Zealand are traditionally considered to be only infected by *U. stenocephala*, because *A. caninum* was assumed to be restricted to racing greyhounds. Smith and Hooke in 1976[12] warned that the importation of greyhounds into New Zealand would lead to an endemic population of *A. caninum*. Since then, there have been no published instances of *A. caninum* in New Zealand dogs other than a greyhound that was imported from Australia with hookworms resistant to pyrantel embonate[13] and recent case of severe infection with egg count of 154,800 in a greyhound.[14]

The aim of this study was to unambiguously identify species of hookworm eggs in canine faecal samples from Australia and New Zealand and to demonstrate the capacity of 'nemabiome' to identify their communities and genetic susceptibility to benzimidazoles.

## Material and methods

Six faecal samples (AU1–AU6) containing 'hookworm' eggs were sourced in 2019–2022 from Australian diagnostic laboratory (The University of Sydney, Australia). All samples were taken from Greater Sydney region, New South Wales, Australia. Eggs were collected via centrifugal flotation. AU1–AU4 samples were submitted for routine parasitological examination. AU5 was a sample with a suspect failure of NexGard Spectra (Boehringer Ingelheim) to clear hookworm infection. AU6 was a sample where repeated treatment with different 'wormers' did not lead to clearing of shedding 'hookworm' eggs; eggs measured 63.1 (min. 55, max. 75) × 39.3 (min. 35, max. 42, n = 30). For Australian samples, no attempt was

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made to discern species based on morphology and are only reported as 'hookworm' eggs.

Four faecal samples (NZ1–NZ4) containing 'hookworm' eggs were sourced in 2022 from New Zealand diagnostic laboratory (Massey University, New Zealand). Eggs were collected by centrifugal flotation into absolute ethanol prior to shipping to The University of Sydney. All the samples were submitted by the Society for the Prevention of Cruelty to Animals for routine parasitological examination. Hookworm eggs in samples NZ1–NZ3 were considered *U. stenocephala*. Sample NZ4 was from a greyhound with hookworm eggs measurement consistent with *A. caninum*.

Total DNA was isolated using the Isolate II Fecal DNA Kit (Bioline) as previously described.[15] The obtained DNA was subject to three polymerase chain reactions (PCRs) with primers adapted for Illumina metabarcoded next-generation sequencing. For nematode species assemblage, we used previously published primers targeting ITS-2 rDNA (ITS-2) region as per previously established 'nemabiome' protocols.[15,16] For isotype-1 $\beta$-tubulin amplification, two assays were used that cover nucleotide regions with diagnostic single nucleotide polymorphisms conferring benzimidazole resistance.[17] All PCRs were performed as previously described.[15] ITS-2 was amplified using 30 amplification cycles. For isotype-1 $\beta$-tubulin amplification, 40 cycles were used. Amplicons were submitted for amplicon sequencing using Illumina next generation sequencing at the Ramaciotti Centre for Genomics, University of New South Wales, Sydney, Australia, and sequenced on MiSeq using MiSeq Reagent Kits v2 (500 cycles, Illumina). Sequence data as FastQ files were analysed using R package 'dada2' (version 1.26).[18] Sequence reads were filtered, trimmed, merged reads and chimeras removed; ultimately 'amplicon sequence variants' (ASVs) were produced. ASVs were retained if they represented more than 0.05% of the total reads.

Within CLC Main Workbench 22.0 (CLC Bio, Qiagen, Clayton VIC, Aust), the ASVs were processed through NCBI's BLASTN GenBank database to identify and remove ASVs that were not consistent with a hookworm ITS-2 or isotype-1 $\beta$-tubulin sequences. Using a locally curated reference database with ITS-2 sequences for *A. caninum*, *A. ceylanicum*, *A. braziliense*, *Ancylostoma duodenale*, *Nectar americanus* and *U. stenocephala*, we assigned species to ASVs with the aid of phylogenetic analysis in CLC Main Workbench. For isotype-1 $\beta$-tubulin, ASVs were assembled against a reference *A. caninum* isotype-1 β-tubulin sequence (DQ459314) in CLC Main Workbench and frequency of mutations Q134H(CAA > CAT), F167Y(TTC > TAC), E198A(GAA > GCA), E198L(GAA > TTA), E198V (GAA > GTA) and F200Y(TTC > TAC) identified according to Venkatesan et al.[11] The data are available at LabArchives (https://doi.org/10.25833/91fj-cq08). Raw FastQ sequence data were deposited at SRA NCBI BioProject: PRJNA957571.

## Results

ITS-2 and isotype-1 $\beta$-tubulin ASVs were assigned to *A. caninum* and *U. stenocephala* (Figure 1). The ITS-2 assay produced on average 24,410 good-quality paired sequence reads (min. 15,050, max. 34,712) across all 10 samples that represented 13 ASVs. The two isotype-1 $\beta$-tubulin assays produced on average 12,523 (min. 9330, max. 18,556) and 12,139 (min. 7582, max. 20,051) good-quality paired sequence reads across four and nine samples (six and one samples failed to produce any hookworm isotype-1 $\beta$-tubulin

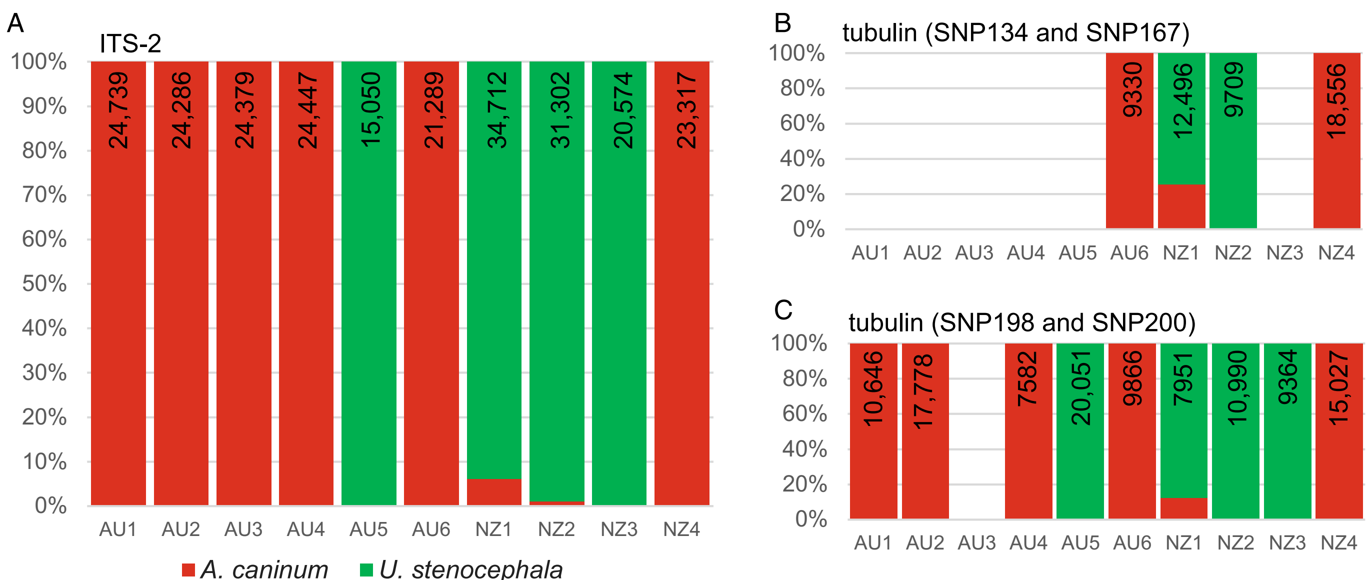


**Figure 1.** **Deep sequencing and identification of hookworms in Australian (AU1–AU6) and New Zealand (NZ1–NZ4) dog faeces. Amplification and deep sequencing based on ITS-1 assay (A) and two isotype-1 $\beta$-tubulin assays (B, C). Coinfection of *Ancylostoma caninum* and *Uncinaria stenocephala* was detected in three (NZ1–NZ3) samples from New Zealand. Presence of *A. caninum* sequences was demonstrated for all three amplicons for sample NZ1 (A, 6.1%; B, 25.4%; C, 12.4%), and only ITS-2 detected *A. caninum* sequences in 1.0% and 0.1% for NZ2 and NZ3, respectively. Sequencing depth is indicated by the number within each bar.**


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sequences, respectively; see Figure 1) that represented 25 and 63 ASVs, respectively.

The Australian material based on ITS-2 in samples AU1–AU4 and AU6 belonged to *A. caninum*, while the AU5 sequences of ITS-2 belonged to *U. stenocephala* (Figure 1). No mixed infection was detected in the Australian material. The New Zealand material NZ1–NZ3 based on ITS-2 was a mixture of *U. stenocephala* (99.9%–93.9%) and *A. caninum* (0.1%–6.1%) (Figure 1). NZ4 sample included only *A. caninum* ITS-2 sequences.

Most of the amplified isotype-1 $\beta$-tubulin sequences exhibited benzimidazole susceptible sequence at position 134 ($Q_{[CAA]}$), 167 ($F_{[TTC]}$), and 200 ($F_{[TTC]}$) across both *A. caninum* and *U. stenocephala* sequences and 198 ($E_{[GAG]}$) in *A. caninum* or 198 ($E_{[GAA]}$) in *U. stenocephala*. Within *A. caninum* (AU6), a rare E198K (GAG > AAG) was detected on single ASV, representing 0.36% of all *A. caninum* reads. Within *U. stenocephala* (NZ2), a rare E198V (GAA > GTA) was detected on single ASV, representing 2.28% of all *U. stenocephala* reads.

## Discussion

The presence of 'hookworm' eggs in dog faeces can be erroneously considered as drug failure, as noted in the case of the dog AU5 from Australia that was apparently on monthly NexGard Spectra (afoxolaner and milbemycin oxime). On the label, it states that only *A. caninum* and *A. ceylanicum* are the target species. Milbemycin oxime is the nematocidal active known to have poor efficacy against *U. stenocephala*[19] yet was the only hookworm species identified in the AU5 sample. Unambiguous hookworm species identification enabled resolution of this case and recommendation to use an alternative product (including pyrantel, fenbendazole or moxidectin) that treats *U. stenocephala*.

Our small-scale study demonstrated absence of detectable alleles in *A. caninum* and *U. stenocephala* associated with benzimidazole resistance. Recently Venkatesan et al[11] demonstrated widespread β-tubulin F167Y(TTC > TAC) mutation present in 50% (156/314) dogs infected with *A. caninum* using the 'nemabiome' approach[11] and Leutenegger et al[10] in 11% (54/511) using *A. caninum* allele-specific qPCR tests. In AU6 *A. caninum*, the persistent shedding of *A. caninum*, despite repeated treatment reported by the owner, is therefore attributed to re-infection or non-compliance. The rare presence of E198K allele in AU6 *A. caninum* sample is not unexpected given that E198K has been identified as a benzimidazole-resistant allele in *Caenorhabditis elegans*,[20] fungi,[21] *Giardia*[22] and *Haemonchus contortus*.[23] Significance of E198K or E198V allele, however, in *A. caninum* and *U. stenocephala* remains to be experimentally confirmed. The isotype-1 β-tubulin E198V mutation, however, was speculated to provide low level of benzimidazole resistance in ruminant trichostrongyle species.[24]

Traditionally, trained parasitology technicians are considered able to differentiate two commonly occurring hookworms (*A. caninum* and *U. stenocephala*) in dogs based on the size of their eggs. Eggs of *U. stenocephala* (70–90 × 40–50 μm) are markedly larger compared to eggs of *A. caninum* (55–74 × 37–43 μm).[25] Training of the technician requires constant exposure to material with 'hookworm' eggs that become rare in urban dogs limiting such training and practice. During an Australian national study in 2004/2005, only 3.9% (95% CI 2.6 to 5.2%) dogs visiting veterinary practices were found positive for 'hookworm' eggs.[6] The presence of additional species, *A. ceylanicum* and *A. braziliense*, in Australia further complicates species identification based on egg size differences.[26,27] Development of multiplex real-time PCR assay for the identification of hookworm species[28] or application of 'nemabiome'[16] as implemented in this study enables detection of both coinfections as well as species proportions.

In this study, we confirmed the presence of *A. caninum* in New Zealand dogs, demonstrating what is likely now an endemic agent of disease in New Zealand.[12,13] To our knowledge, *A. caninum* was believed to be limited to greyhounds in New Zealand and not present in the general dog population (I Scott personal communication). The only recent published study within the past 20 years demonstrated 12% (95% CI 7 to 17) prevalence of hookworms in working dogs from New Zealand but did not differentiate between *A. caninum* and *U. stenocephala*.[29] There also exists a record of hookworms in 10% of 185 farm dogs from the Hawke's Bay region on the east coast of New Zealand's North Island (Matthews M. 2016; Survey of nematode parasites of farm working dogs in Hawke's Bay; Proceedings of the Society of Sheep and Beef Cattle Veterinarians of the NZVA, 141–145) and 4% of owned pet/shelter dogs in New Zealand (Woollett B, Forsyth M and Beugnet F. 2016; Newsletter of the Companion Animal Society of the NZVA, 27 (1), 32–38). In other recent as yet unpublished observations comparing the size of hookworm eggs appearing in the faeces of greyhounds and other dogs in New Zealand, less than 1% of the eggs present in the general dog population were small enough to be consistent with *A. caninum*. Considering *A. caninum*'s pathogenicity, its presence in New Zealand is a concern to the health of dogs and potentially humans.[30,31]

The presented approach that includes hookworm species identification coupled with benzimidazole resistance alleles will inevitably enable the screening of whole Australia and New Zealand for these mutations and so understanding of the extent of benzimidazole resistance in *A. caninum* and other species.

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