Widespread occurrence of benzimidazole resistance SNPs in the canine hookworm, *Ancylostoma caninum,* in Australia

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Abstract

Canine hookworm (*Ancylostoma caninum*), a gastrointestinal nematode of domestic dogs, principally infects the small intestine of dogs and has the potential to cause zoonotic disease. In greyhounds and pet dogs in the USA, *A. caninum* has been shown to be resistant to multiple anthelmintics. We conducted a molecular survey of benzimidazole resistance in *A. caninum* from dogs at veterinary diagnostic centers in Australia and New Zealand. First, we implemented an ITS-2 rDNA deep amplicon metabarcoding sequencing approach to ascertain the species of hookworms infecting dogs in the region. Then, we evaluated the frequency of the canonical F167Y and Q134H isoform-1 β-tubulin mutations, which confer benzimidazole resistance, using the same sequencing approach. The most detected hookworm species in diagnostic samples was *A. caninum* (90%; 83/92); the related Northern hookworm (*Uncinaria stenocephala*) was identified in 11% (10/92) of the diagnostic samples. There was a single sample with coinfection by *A. caninum* and *U. stenocephala*. Both isoform-1 β-tubulin mutations were present in *A. caninum*, 49% and 67% for Q134H and F167Y, respectively. Mutation F167Y in the isoform-1 β-tubulin mutation was recorded in *U. stenocephala* for the first time. Canonical benzimidazole resistance codons 198 and 200 mutations were absent. Egg hatch assays performed on a subset of the *A. caninum* samples showed significant correlation between IC_{50} to thiabendazole and F167Y, with an increased IC_{50} for samples with >75% F167Y mutation. We detected 14% of dogs with >75% F167Y mutation in *A. caninum*. Given that these samples were collected from dogs across various regions of Australia, the present study suggests that benzimidazole resistance in *A. caninum* is widespread. Therefore, to mitigate the risk of resistance selection and further spread,

adoption of a risk assessment-based approach to limit unnecessary anthelmintic use should be a key consideration for future parasite control.

Keywords:

Ancylostoma caninum, Multiple-drug resistance (MDR), Multiple anthelmintic drug resistant (MADR), Greyhounds, Anthelmintics, Canine health

Highlights:

- High prevalence of benzimidazole resistance mutations in Australian canine hookworms.
- *Ancylostoma caninum* detected in 90% of samples and *Uncinaria stenocephala* in 11% of diagnostic samples.
- F167Y and Q134H mutations found in *A. caninum* and F167Y in *U. stenocephala*.
- Egg hatch assay confirms correlation between F167Y mutation and benzimidazole resistance.

Introduction

For many years the prevailing dogma regarding the use of anthelmintics in small animals, such as dogs and cats, as well as in humans, had been that the development of anthelmintic resistance would be slow to occur, or may never occur or be limited in spread (Schulz et al., 2018; Marsh and Lakritz, 2023). The theory behind this dogma was that, at the time of deworming, the *refugia* - the population of susceptible nematodes infecting the host - remains substantial (von Samson-Himmelstjerna et al., 2021). By contrast, in ruminants, routine and indiscriminate mass treatment with anthelminthics has led to widespread drug resistance, because the *refugia* has not been retained (Vercruysse et al., 2012; von Samson-Himmelstjerna et al., 2021). However, despite these differences between ruminants and small animals, a number of reports over recent years have indicated that resistance in canine hookworms is indeed a widespread problem in North America (Jimenez Castro et al., 2019; Kitchen et al., 2019; Venkatesan et al., 2023).

Nowadays, the anthelminthics class of benzimidazoles is largely ineffective in ruminants, the result of well documented single nucleotide polymorphisms (SNPs) that lead to nematodes carrying alleles resistant to these anthelminthics (Kwa et al., 1993; von Samson-Himmelstjerna et al., 2007). In humans, hookworms are commonly treated using benzimidazoles, specifically albendazole and mebendazole (WHO, 2017). Between 1995 and 2015, the efficacy of both albendazole and mebendazole for the treatment of human hookworm infection has decreased by as much as 15%, but resistance as such has not been documented (Schulz et al., 2018).

83 In dogs, anthelminthics resistance was first documented 20 years ago in hookworms to
84 the pyrimidine-derivative anthelmintic pyrantel in Queensland, Australia (Kopp et al.,
85 2007), and currently this anthelmintic is considered ineffective for treating hookworms
86 in dogs in Australia (Dale et al., 2024). However, resistance to benzimidazoles and other
87 anthelmintic classes for dogs in Australia has not been reported. The first report of
88 resistance to benzimidazoles in canine hookworms was from the USA in 2019 (Kitchen
89 et al., 2019). Molecular, phenotypic, and experimental infections evidence
90 demonstrated benzimidazole resistance in *A. caninum* was published independently by
91 two groups in 2019 (Jimenez Castro et al., 2019; Kitchen et al., 2019). These reports first
92 emerged in North America and were followed by an extensive surveillance for the
93 canonical F167Y (TTC>TAC) isoform-1 β-tubulin mutation conferring benzimidazole
94 resistance (Leutenegger et al., 2023; Venkatesan et al., 2023; Leutenegger et al., 2024).
95 A new mutation in isoform-1 β-tubulin at Q134H (CAA>CAT) was experimentally
96 established as another mutation responsible for resistance to benzimidazoles in *A.*
97 *caninum* (Venkatesan et al., 2023).

98 It is speculated that benzimidazole resistance in *A. caninum* emerged in racing
99 greyhound dogs because of indiscriminate and frequent deworming in racing kennels
100 (Jimenez Castro et al., 2019; Kitchen et al., 2019; Leutenegger et al., 2024). Evidence of
101 multiple anthelmintic drug resistance (MADR) in *A. caninum* led the American
102 Association of Veterinary Parasitologists to establish a hookworm task force to address
103 this issue in 2021 (von Samson-Himmelstjerna et al., 2021; Marsh and Lakritz, 2023). The
104 screening of thousands of dogs positive to hookworms in the USA and Canada
105 demonstrated that the canonical F167Y (TTC>TAC) isoform-1 β-tubulin mutation was
106 present in 11.3% (715/6,329) of faecal *A. caninum* positive samples (Leutenegger et al.,

107 2024). In a smaller study of samples from the USA, 49.7% (156/314) of the individual
108 samples tested positive for the F167Y (TTC>TAC) mutation (Venkatesan et al., 2023).
109 Reanalysis of next-generation sequence data from Venkatesan et al. (2023) for the
110 presence of non-*A. caninum* sequences revealed the presence of other hookworms
111 (*Ancylostoma braziliense*, *Ancylostoma tubaeforme* and *Uncinaria stenocephala*), but
112 only the benzimidazole susceptible residues of isoform-1β-tubulin were found in these
113 species (Stocker et al., 2024).

114 The aim of this study was to investigate the extent of benzimidazole resistance SNPs in
115 canine hookworm (*A. caninum*) outside North America, specifically in Australia and New
116 Zealand. We utilised opportunistic sampling from four different sources in Australia and
117 New Zealand. All hookworm-positive samples were screened using ITS-2 rDNA PCR and
118 deep amplicon sequencing to determine hookworms' species present. To investigate
119 resistance to benzimidazoles, we used deep amplicon sequencing to evaluate the
120 nucleotide sequence of the isoform-1 β-tubulin mutations that are known to be
121 associated with resistance to this drug class, specifically the regions that encode amino
122 acids Q_{134}, F_{167}, E_{198} and F_{200}. We then undertook egg hatch assay (EHA) on a subset of the
123 samples to correlate the molecular signatures, specifically frequency of F_{167}, with
124 benzimidazole-resistance phenotype of these field isolates.

125

126 **Material and methods**

127 **Ethics statement**

128 The use of residual clinical samples was in accordance with the University of Sydney
129 Animal Ethics Committee Protocol (2024/2508). Dog faecal samples for larval cultures
130 were obtained in accordance with the University of Queensland Animal Ethics
131 Committee Protocol (2021/AE001169) and faecal egg count reduction test on one dog
132 was conducted in accordance with University of Queensland Animal Ethics Committee
133 Protocol (SVS/111/20).

134 **Faecal samples with hookworm infection**

135 Faecal material from New South Wales, Australia and New Zealand was provided for the
136 purpose of diagnostics used in decision-making by the veterinary practitioner. Results
137 and material used for hookworm species and benzimidazole susceptibility were de-
138 identified. Faecal samples were provided by registered veterinary practitioners in
139 accordance with the Veterinary Practice and Animal Welfare Acts. Samples were
140 submitted for parasitological diagnostics to Vetnostics (Laverty Pathology—North Ryde
141 Laboratory, Sydney, New South Wales, Australia) and Veterinary Pathology Diagnostics
142 Services (VPDS, The University of Sydney, New South Wales, Australia); refrigerated
143 residual faecal samples that previously returned a positive result for hookworms at the
144 clinical diagnostic laboratory were received for molecular species characterisation. In
145 addition, samples from Massey University, Palmerston North, New Zealand that
146 contained hookworm eggs were processed to isolate the eggs, which were stored in 80%
147 ethanol for transfer to VPDS.

148 Material from Queensland, Australia was obtained from dogs from adoption and shelter
149 facilities that receive rescued or surrendered animals from greyhound rescue
150 organizations and pounds across southeast Queensland. Dogs within these facilities are

151 kept in individual pens, which are cleaned and disinfected daily. Fresh faecal samples
152 were collected from each dog after natural defecation and screened for hookworm
153 infection using faecal flotation as described below.

154 **Faecal egg counts, hookworm egg recovery and DNA isolation**

155 Faecal egg counts were performed with saturated (NaCl) solution using Whitlock
156 Universal McMaster 4 chamber worm egg counting slide in two different laboratories. At
157 the University of Queensland, 3 g of faecal samples was homogenised with 42 ml of
158 saturated salt solution (specific gravity = 1.21) and filtered through a tea strainer and the
159 filtrate was immediately pipetted into the counting chamber (4 compartments each of
160 0.5 ml volume). Counting all compartments (2 ml) delivered minimum detection of 7 egg
161 per gram (absence of eggs indicates <7 eggs per gram in the sample). At the University of
162 Sydney, 3 g of faecal samples was homogenised with 60 ml of saturated salt solution
163 (specific gravity = 1.21) and filtered via coarse strainer. We counted 2 compartments
164 each of 0.5 ml volume resulting in minimum detection of 20 egg per gram (absence of
165 eggs indicates <20 eggs per gram in the sample). Only strongyle-like (=hookworm) eggs
166 were counted.

167 At the University of Sydney, the remainder of the filtered homogenate was poured into 50
168 ml Falcon tube and centrifuged at 800×g for 10 minutes for centrifugal floatation. The
169 upper most supernatant (~10 ml) was collected with a plastic Pasteur pipette into a new
170 clean 50 ml Falcon tube and topped with tap water and centrifuged at 800×g for 10
171 minutes. The supernatant was decanted and the pellet that contained the concentrated
172 eggs was retained. Then ~1 ml of tap water was added with a clean plastic Pasteur pipette
173 to disturb the pellet by repeated aspiration until a homogeneous solution formed. The

174 homogenate was transferred to a clean 1.5ml Eppendorf tube and spun at 8,000×g for 10
175 minutes. The supernatant was completely removed and the Eppendorf tube with the egg
176 pellet was stored at -20 °C until further use.

177 At the University of Queensland, eggs were purified for use in the egg hatch assay (see
178 later). The remaining eggs were suspended in distilled water in 1.5 ml microtubes,
179 incubated for 72 hours at 28 ˚C and allowed to hatch. The hatched larvae were stored at
180 -20 ˚C until further use.

181 The total genomic DNA was isolated from hookworm eggs using the Isolate II Fecal DNA
182 Kit (Meridian Bioscience, Australia) according to the manufacturer's instructions with
183 following homogenisation step. For purified eggs,750 µL of lysis buffer was added to the
184 Eppendorf tube with the pellet and homogenised by repeated aspiration with a pipette,
185 then all the contents were transferred into bead-beating tube and the eggs disrupted and
186 homogenised using a high-speed benchtop homogeniser, FastPrep-24 (MP Biomedicals,
187 Australia), for 40 s at 6.0 m/s. Every 11th sample was a blank sample that only contained
188 the lysis buffer. For hatched larvae, Monarch Genomic DNA Purification Kit (New England
189 Biolab, Australia) was used according to the manufacturer's instructions. Purified
190 genomic DNA samples and blanks were isolated into 100 µL of elution buffer (10 mM Tris-
191 Cl, pH = 8.5) and stored at −20 °C until further use.

192 **Amplicon metabarcoding and Illumina deep sequencing**

193 All isolated DNA samples were first screened for the presence of nematode DNA using
194 nematode ITS2 rDNA quantitative PCR and samples with C_t<35 were considered as a
195 suitable DNA material for follow up processes.

196 Total DNA was subjected to three independent real-time PCR amplifications at the
197 University of Sydney as previously described (Stocker et al., 2023). ITS-2 rDNA (ITS-2) was
198 amplified by the NC-1 and NC-2 primers designed by Gasser et al. (1993), and two
199 isoform-1 β-tubulin regions were amplified using assays BZ167 and BZ200 developed by
200 Jimenez Castro et al. (2019), which include key diagnostic SNPs conferring
201 benzimidazole resistance. All primers were adapted for Illumina metabarcoding using
202 Next Generation Sequencing (NGS). The ITS-2 target was amplified using 32-36
203 amplification cycles, while 40 amplification cycles were used for isoform-1 β-tubulin
204 (BZ167, BZ200). All reactions utilized SYBR-chemistry using SensiFAST™SYBR® No-ROX
205 mix (Meridian Bioscience, Australia). Each real-time PCR mix (25 µL) included 2 µL of
206 template DNA (neat) and primers at final concentrations of 400 nM. All PCR reactions
207 were prepared using a Myra robotic liquid handling system (Bio Molecular Systems,
208 Australia). Reactions were run on a CFX96 Real-Time PCR Detection System with
209 corresponding CFX Manager software (BioRad, Australia). The protocol involved an initial
210 denaturation at 95 °C for 3 min, followed by 32 or 40 cycles of 95 °C for 5 s, 60 °C for 15
211 s, 72 °C for 15 s, and a final melt curve analysis. Each run included a no-template control
212 (ddH_2O) to monitor for potential contamination. Amplicons were submitted for amplicon
213 sequencing using Illumina NGS at the Ramaciotti Centre for Genomics, University of New
214 South Wales, Sydney, Australia and sequenced on MiSeq using MiSeq Reagent Kits v2
215 (250PE, Illumina). De-multiplexed FastQ files were generated via BaseSpace (Illumina).

216 **Analysis of the Illumina sequenced amplicons**

217 All FastQ files were processed through R package 'dada2' v1.26.0 (Callahan et al., 2016)
218 in R v4.2.2 (R Core Team, 2022), to obtain amplicon sequence variants (ASV) and their

219 abundance per sequenced amplicon. The dada2 pipeline at the University of Sydney
220 includes filtering, error estimation and denoising, before paired-end reads were merged
221 to reconstruct the full ASV before removal of chimeras. Prior to processing through the
222 dada2 pipeline, primers were removed from all forward and reverse FastQ reads using
223 'cutadapt' version 4.0 (Martin, 2011).

224 The assignment of species based on ITS-2 ASVs was carried out in CLC Main Workbench
225 v22.0 (Qiagen Australia) as previously described (Stocker et al., 2024). All ASVs were
226 imported into CLC Main Workbench and top blastn hits against 'nr' database at NCBI
227 was identified. ITS-2 sequences were discarded for any ASVs where the top blastn hit
228 was not hookworm (Ancylostomatidae). For species assignment we constructed
229 alignment of AVSs with an in-house curated reference ITS-2 hookworm sequence library
230 consisting of publicly available sequences (KP844736; MT345056; JQ812692; LC036567;
231 JX220891; EU344797; AB793527). The resulting multiple sequence alignment was
232 subject to phylogenetic reconstruction, where species identity was based on manual
233 assignment of ASV to a species based on position on the phylogenetic tree relative to the
234 reference ITS-2 sequences.

235 For the β-tubulin sequence data (BZ167, BZ200 assay), the assignment of species was
236 carried with the aid of CLC Main Workbench v22.0 (Qiagen Australia) as previously
237 described in Stocker et al. (2024). Briefly, all ASVs were imported into CLC Main
238 Workbench and top blastn hits against 'nr' database at NCBI was identified. All ASVs
239 where the top blastn hit was a hookworm (Ancylostomatidae) β-tubulin were retained for
240 further analysis. The reference hookworm alignments of BZ167 and BZ200 amplicons
241 according to Stocker et al. (2024) was used to unambiguously identify ASVs identity and

242 only those ASVs that were confirmed based on phylogenetic analysis were included in
243 further analyses. The sequence alignment included β-tubulin reference sequences from
244 *A. caninum* (DQ459314) (Schwenkenbecher et al., 2007), *Ancylostoma duodenale*
245 (EF392850), *U. stenocephala* (Stocker et al., 2023), and *N. americanus* (EU182348),
246 which enabled us to identify the exon/intron boundary and residues that code for
247 susceptibility to benzimidazoles, amino acids amino acids Q_{134}, F_{167}, E_{198} and F_{200}
248 (Venkatesan et al., 2023). Our reference alignment included sequence data for isoform-
249 1 β-tubulin from *A. caninum*, *A. ceylanicum*, *A. duodenale* and *Necator americanus* at
250 'WormBase ParaSite' (Howe et al., 2017; Bolt et al., 2018; Harris et al., 2020).

251 Following the dada2 pipeline, and species assignment raw read counts were converted
252 into relative abundance (percentages) through. Any species read counts with less <25
253 reads were disregarded as spurious. Proportions of SNP for amino acids amino acids
254 Q_{134}, F_{167}, E_{198} and F_{200} were calculated per species for each of the amplicons of isoform-
255 1 β-tubulin (BZ167, BZ200).

256 The number of samples, and number of *A. caninum* samples with Q134H (CA<u>A</u>>CA<u>T</u>) and
257 F167Y (T<u>T</u>C>T<u>A</u>C) mutations separately, were summed per postcode in New South
258 Wales. Proportional symbol maps were created by joining this data to postcodes of
259 Australia shapefile (Geocentric Datum of Australia, GDA 1994) in ArcGIS® Pro. Spearman
260 rank correlations (r_{SP}) were calculated between EPG per sample and both percentages of
261 Q134H (CA<u>A</u>>CA<u>T</u>) and F167Y (T<u>T</u>C>T<u>A</u>C) mutation. In addition, the EPG between
262 resistant versus not resistant status for both mutations was assessed using a Kruskal-
263 Wallis non-parametric analysis of variance. Spearman rank correlations were also
264 calculated between EPG, percentage of Q134H (CA<u>A</u>>CA<u>T</u>) mutation, percentage of

265 F167Y (TTC>TAC) mutation and age. Finally, the association between breed (greyhound
266 versus other) and EPG, percentage of Q134H (CAA>CAT) mutation and percentage of
267 F167Y (TTC>TAC) mutation was assessed using a Kruskal-Wallis non-parametric
268 analysis of variance. Type I error for all statistical tests was set at 0.05 (IBM SPSS
269 Statistics v 28).

270 ***In vitro* egg hatch assay (EHA)**

271 Eggs were extracted from fresh faecal samples from hookworm-positive dogs for use in
272 *in vitro* egg hatch assays (EHAs). Saturated sugar solution (specific gravity = 1.30), three
273 times the weight of individual faecal samples, was added to the faecal samples in a
274 shaker bottle. The bottle was manually agitated to form a slurry, which was then filtered
275 through a 200 µm sieve into a beaker to remove large debris. All of the filtered solution
276 was then transferred into 50 ml Falcon tubes, filling each tube up to the 40 ml, and then
277 10 ml of distilled water was slowly and carefully poured along the edge of the tube to
278 avoid mixing with the faecal solution. Each tube was then screw capped and centrifuged
279 at 2000 rpm (400×g) for 10 minutes. Following centrifugation, the 'fuzzy' layer (water-
280 sucrose interface) of each tube was removed with a pipette and pooled into a 200 mL
281 beaker. The solution was passed through a 75 µm sieve, retaining the filtrate, which was
282 further passed through a 20 µm sieve, which retained the hookworm eggs. This sieve was
283 then retrograde washed with 20 mL of distilled water into a glass Petri dish. The recovered
284 eggs were carefully poured into a two 15 mL centrifuge tubes, leaving the debris in the
285 Petri dish. The eggs were concentrated into 2 mL of water by centrifugation for 5 minutes
286 at 2000 rpm (400×g) and discarding the supernatant. The egg concentration was
287 estimated by thoroughly mixing the 2 mL egg solution and counting the eggs in three 20µl

288 aliquots. The egg numbers were adjusted to approximately 2 eggs per microlitre either
289 by dilution with distilled water or by concentration using centrifugation as described
290 above.

291 The egg hatch assay was performed using 96-well plates containing thiabendazole
292 embedded in agar, as described previously for assays with human hookworms (Kotze et
293 al., 2009). A stock solution of thiabendazole (5 mg/mL, Sigma-Aldrich Australia) was
294 prepared in dimethyl sulfoxide (DMSO) and serially diluted 2-fold in the same solvent.
295 Aliquots (2 µL) from a series of dilutions were added to 96-well microtiter plates, such
296 that each row of the plate comprised a double dilution, ranging from 0.003 µg/mL up to
297 1.359 µg/mL or 0.011 µg/mL to 5.437 µg/mL. The first two wells of each row were used as
298 control wells (received 2 µL of DMSO only). Molten agar (200 µL at 2% w/v) (Davis
299 Gelatine, Australia; powdered agar Grade J) was dispensed into each well of the plate
300 and allowed to set. Plates were placed into plastic press-seal bags stored at 4°C.

301 Prior to establishing the assays, the plates were allowed to warm in an incubator set to
302 28˚C. 30µL of concentrated hookworm egg solution (~2 eggs/µL) was pipetted into each
303 well. Plates were incubated for 48 hours at 28˚C, whereupon the assay was terminated
304 by addition of Lugol's iodine solution. The numbers of unhatched eggs and larvae in each
305 well were counted with the aid of a stereo microscope (Stemi 508, Carl Zeiss, Australia).
306 All assays were performed in triplicate for the sample isolated from each dog sample.

307 The number of larvae in each well was expressed as a percentage of the total numbers of
308 larvae and unhatched eggs in order to give the hatch rate in each well, and then this was
309 expressed relative to the mean hatch rate in the control wells. The data were then
310 analysed by non-linear regression using GraphPad Prism software (GraphPad Software

311 Inc. USA). This analysis yielded IC_{50} values representing the concentration of
312 thiabendazole that resulted in an egg hatch of 50% compared to control assays. The IC_{50}
313 values for the dogs examined in this study were compared to those reported previously
314 for drug-susceptible *A. caninum* isolates: 0.046 and 0.048 µg/mL (Jimenez Castro et al.,
315 2019) and 0.045 µg/mL (Diawara et al., 2013). An Australian fenbendazole susceptible
316 isolate of *A. caninum* ('Dougie', from February 2021) served as local positive control;
317 demonstrated faecal egg reduction of 100% 10 days post administration from initial 525
318 EPG.

319 The left-over eggs were suspended in distilled water in 1.5 ml microtubes, incubated for
320 72 hours at 28˚C and allowed to hatch. The hatched larvae were stored at -20˚C and
321 subjected to DNA isolation and follow up processes described earlier.

322 **In vivo efficacy measurements**

323 One dog (sample D6) was treated by the shelter facilities and faecal egg count monitored
324 throughout the treatment. The dog was treated according to its weight and product label
325 on day 0 with Drontal Allwormer (Vetoquinol, Australia; actives: febantel, praziquantel
326 and pyrantel embonate) and on day 21 with Credelio Plus (Elanco, Australia: milbemycin
327 oxime and lotilaner). In animals, febantel is a prodrug that is actively hydrolyzed to the
328 active metabolite fenbendazole. Egg counts were conducted within the period 10-21
329 days after Drontal Allwormer treatment to calculate faecal egg reduction percentage
330 (>95% reduction is considered effective product). Only a single sample was obtained at
331 day 8 after Credelio Plus because the dog was then rehomed.

332 **Data availability**

333 The EHA raw data, FEC reduction data and metadata for all samples are available at
334 LabArchives (https://dx.doi.org/10.25833/hmvy-jn79). Raw FastQ sequence data was
335 deposited at SRA NCBI BioProject: PRJNA1149426.

336

337 **Results**

338 **Dominance of *A. caninum* across south-eastern Australia using metabarcoded ITS-**
339 **2 rDNA deep sequencing in dogs**

340 We used 106 faecal samples that were considered hookworm positive. There were 20
341 samples from New Zealand from dogs. For 61 of the 106 samples the worm faecal egg
342 count (FEC) ranged from 20 to 4,920 (avg. 526) eggs per gram (EPG). The majority of the
343 Australian samples came from a commercial diagnostic laboratory (n=50) that were
344 collected over period of 13 months between March, 2023 and March, 2024.

345 There were 86 samples from south-eastern Australia, 85 samples from dogs and one
346 from a cat (TS42). Good quality DNA was obtained from 98% (84/86) yielding C_t<35 (11.8
347 to 31.6) at ITS-2 rDNA assay targeting nematode DNA. All 84 samples yielded
348 metabarcoded and deep sequenced ITS2 rDNA sequence data for nematode species
349 identification with the sequencing depth (n=84; avg. 21,323, 794 to 32,899) (Fig. 1A). Two
350 samples yielded low ITS-2 rDNA sequence depth, 794 (TS100) and 1,537 (TS95)
351 sequencing reads; removing those the remaining yielded >2,000 reads per sample (n=82;
352 avg. 21,815, range 2,736 to 32,899). The cat hookworm sample contained sequences
353 that matched *Ancylostoma tubaeforme* (TS42). The remaining dog samples (n=81)
354 contained sequences of *A. caninum* (n=77, 95%) and *U. stenocephala* (n=4, 5%). One

355 sample (TS35) had both *A. caninum* (96%) and *U. stenocephala* (4%) ITS-2 rDNA
356 sequences. The additional two samples with <2,000 ITS-2 rDNA sequences (TS95, TS100)
357 included only *A. caninum* sequences.

358 **Widespread and high frequency of *A. caninum* canonical F167Y (TTC>TAC) isoform-**
359 **1 β-tubulin benzimidazole resistance mutation in Australia**

360 For 81 Australian samples, the isoform-1 β-tubulin BZ167 amplicons were obtained with
361 an average sequencing depth for confirmed hookworm sequences between 81 to 31,265
362 (Fig. 1B). Excluding six samples (TS6, TS7, TS39, TS55, TS63, TS90; sequence depth 81 to
363 1,699 depth) with <2,000 sequences, the average sequencing depth was 11,092
364 sequences (n=75; 2,213 to 31,265). There were 72 samples with >2,000 isoform-1 β-
365 tubulin BZ167 amplicon sequences belonging to *A. caninum* (avg. depth 11,271) (Fig. 1B).

366 There were 35 (49%, 35/72) samples that harboured on average 10% (1% to 45%; 95%
367 confidence interval 7-13%) of the Q134H (CAA>CAT) resistant allele and 48 (67%, 48/72)
368 samples that harboured on average 41% (1% to 100%; 95% confidence interval 33-51%)
369 of the F167Y (TTC>TAC) benzimidazole resistant allele (Fig. 1C). There were 18 (25%,
370 18/72) and 14% (10/72) samples with >50% and >75% of the F167Y (TTC>TAC) (Fig. 1C).
371 There were 26 (36%, 26/72) samples that included both Q134H (CAA>CAT) and F167Y
372 (TTC>TAC) benzimidazole resistant alleles. The six samples with <2,000 sequences for
373 *A. caninum* had only the susceptible (coding for Q_{134}, F_{167}) alleles, except TS90 with 100%
374 of F167Y (TTC>TAC) mutation across its BZ167 sequences. Sequence analysis of
375 isoform-1 β-tubulin sequence (amino acids E_{198} and F_{200}) revealed no evidence of
376 benzimidazole resistance signatures.

377 There were four *U. stenocephala* and one (1/4) of the samples TS24 included F167Y
378 (TTC>TAC) in the isoform-1 β-tubulin mutation in 51% of the sequence reads. The
379 remaining isoform-1 β-tubulin of *U. stenocephala* matched benzimidazole susceptible
380 sequence residues. The cat TS42 sample with *A. tubaeformae* and its isoform-1 β-tubulin
381 included only the benzimidazole susceptible sequences (amino acids Q_{134}, F_{167}, E_{198} and
382 F_{200}).

383 For the New Zealand samples only 50% (10/20) of DNA yielded C_t<35 (20.2 to 33.6); nine
384 samples yielded ITS-2 rDNA sequence data to consider species identification (TS76-
385 TS84) with the sequencing depth (avg. 18,488; 1,118 to 27,624) (Fig. 1A). Four of the nine
386 samples contained sequence data that belonged to non-hookworm sequence including
387 ITS-2 rDNA sequencing matched to parasites of sheep and rabbits; three DNA samples
388 contained only ASVs that matched pseudoparasite DNA sequences. Subtracting the
389 pseudoparasite sequences to retain hookworm sequence data revealed presence of
390 uniquely *U. stenocephala* sequences, five samples with sequence depth for hookworms
391 from 8,027 to 27,624 and one sample (TS76) with only 118 sequences. Sequence
392 analysis of isoform-1 β-tubulin (amino acids Q_{134}, F_{167}, E_{198} and F_{200}) revealed no evidence
393 of benzimidazole resistance signatures in New Zealand samples.

394 **High frequency of Q134H (CAA>CAT) or F167Y (TTC>TAC) mutation in *A. caninum* in**
395 **greyhounds**

396 We then focused on 55 dog samples from two diagnostic labs (Vetnostics, VPDS) for
397 which we had *A. caninum* Q134H (CAA>CAT) and F167Y (TTC>TAC) mutation frequency.
398 There were 53 dogs with defined location to a postcode from New South Wales, Australia

399 (one dog from New South Wales did not have a postcode and the other dog was from
400 Queensland). Dogs came from 12 distinct New South Wales postcodes (Fig. 2).

401 There was no significant correlation between Q134H (CAA>CAT) or F167Y (TTC>TAC) and
402 EPG (correlation coefficient=-0.169, P=0.217; correlation coefficient=0.03, P=0.82).
403 Considering the association of presence or absence of either Q134H (CAA>CAT) or
404 F167Y (TTC>TAC) and EPG, there was no significant association for either SNP (Kruskal-
405 Walis Test: H=0.126, degrees of freedom=1, P=0.72; H=0.56, degrees of freedom=1,
406 P=0.45). We then considered the threshold of >40% for F167Y (TTC>TAC) mutation and
407 recalculated the association, but the rest remained not significant (Kruskal-Walis Test:
408 H=0.02, degrees of freedom=1, P=0.89). There was only a single sample with >40% of the
409 Q134H (CAA>CAT) mutation, therefore preventing calculation of these test statistics.
410 Considering the age of the dogs for which the age was available (n=41) there was no
411 significant correlation between age and EPG, Q134H (CAA>CAT) or F167Y (TTC>TAC)
412 (Spearman correlation, P>0.05).

413 The median resistance in Greyhounds for both the Q134H (CAA>CAT) and F167Y
414 (TTC>TAC) *A. caninum* mutation (0.035 and 0.090), respectively were significantly higher
415 than that for other breeds (median 0.000; Kruskal Wallis Test, P≤0.01) (Fig 2). There was
416 no significant difference between EPGs for the two breed categories (Kruskal-Wallis Test,
417 P=0.22).

418 **Phenotypic evidence of benzimidazole resistance in *A. caninum* field sample with**
419 **>75% F167Y (TTC>TAC) mutation**

420 We tested 13 *A. caninum* samples (D1, D2, D3, D5, D6, D8, D9, D10, D12, D15, D16, D17,
421 D18/20) with EHA that had their frequency of Q134H (CAA>CAT) and F167Y (TTC>TAC) *A.*

422 *caninum* SNP established (Fig. 1, Table 1). Dose response curves and IC_{50} formed two
423 groups, those that had high IC_{50}>0.15 µg/mL and those that had low IC_{50}<0.15 µg/mL of
424 thiabendazole (Fig. 3A). Three greyhounds (D1/D2: TS87/88, D3: TS89, D17: TS103) and
425 the crossbreed dog (D6: TS92, D18/20: TS104/106) were in the high IC_{50} group and they
426 all had >75% of F167Y (TTC>TAC) *A. caninum* mutation in the isoform-1 β-tubulin (Fig. 1,
427 Table 1). The IC_{50} of the remaining seven greyhound samples ranged from 0.02 to 0.06
428 µg/mL of thiabendazole and frequency of *A. caninum* F167Y (TTC>TAC) was <40% (Fig.1,
429 Table 1). For one dog (represented by two samples D6 and D18/20 in Fig. 1), we
430 established an *in vitro* EHA on eggs purified from dog's faeces on day 1 and day 9,
431 showing IC_{50} of 0.24 (0.19 to 0.30) µg/mL and 0.17 (95%CI 0.12 to 0.24) µg/mL of
432 thiabendazole, respectively. There was no significant difference between the response
433 curves for these two samples, demonstrated by single response curve fitting both
434 sample responses to thiabendazole (P=0.19; F=1.735, DFn=2, DFd=45). The
435 benzimidazole susceptible control IC_{50} was 0.11 (95%CI 0.10 to 0.12) µg/mL (Fig. 3), and
436 the *A. caninum* DNA was devoid of canonical F167Y (TTC>TAC) and Q134H (CAA>CAT)
437 isoform-1 β-tubulin mutations which confer benzimidazole resistance; the BZ167 and
438 BZ200 amplicons coded for amino acids Q_{134}, F_{167}, E_{198} and F_{200}. There was significant
439 correlation of IC_{50} for thiabendazole in EHA and F167Y (TTC>TAC) mutation in the
440 isoform-1 β-tubulin (Spearman's r=0.83 (95%CI=0.52 to 0.95, P-value < 0.001) (Fig. 3B).

441 *In vivo* evidence based on repeated FEC for one dog (D6 and D18/20 in Fig. 1) suggested
442 decreased (<95%) ability of febantel/pyrantel to reduce egg shedding post treatment.
443 This crossbreed dog (D6, D18/20) initially shed 540 EPG of *A. caninum* hookworms on
444 day 1 (day of treatment). The hookworm isolate carried 83% of F167Y (TTC>TAC)
445 mutation in the isoform-1 β-tubulin (D6: TS092; Fig. 1), and was treated with 'Drontal

446 Allwormer', but the faecal egg reduction only reached 88.8% (60 EPG) and 72.2% (195
447 EPG) on day 10 and day 14 post treatment, respectively. The same dog was re-treated
448 with macrocyclic lactone ('Credelio Plus') on day 20 (90 EPG) post original treatment that
449 resulted in 100% *A. caninum* egg reduction in faeces on day 8 post second treatment (0
450 EPG).

451 **Discussion**

452 This survey of dogs from south-east Australia infected with hookworms demonstrated a
453 high frequency of *A. caninum* with canonical mutations F167Y and Q134H in the isoform-
454 1 β-tubulin that are known to be associated with benzimidazole resistance. Both F167Y
455 and Q134H mutations have been conclusively confirmed to lead to benzimidazole
456 resistance using *Caenorhabditis elegans* as a surrogate (Kitchen et al., 2019; Venkatesan
457 et al., 2023). The extent of both mutations, 67% for F167Y and 49% for Q134H in *A.*
458 *caninum* tested in this study, exceeds even the recent USA report by Venkatesan et al.
459 (2023) that utilised the same methodology based on NGS amplicon metabarcoding. Our
460 samples from New South Wales, Australia correspond to hookworm-positive samples
461 tested by a commercial diagnostic laboratory (n=49) over a 13-month period. Our
462 sampling effort for New South Wales represents approximately 1,700 dog samples,
463 assuming a conservative *A. caninum* prevalence of 2.9% in temperate and subtropical
464 regions of Australia (Massetti et al., 2022).

465 Using our samples from New South Wales, Australia, we show that the isoform-1 β-
466 tubulin F167Y mutation is present in canine hookworms across the state and
467 predominantly in greyhounds that formed most of the samples. In North America, the
468 isoform-1 β-tubulin F167Y mutation was present in all geographic areas with detected

469 hookworms, including Canada (Leutenegger et al., 2024). Initially the F167Y
470 polymorphism was recognised in greyhounds; currently the highest isoform-1 β-tubulin
471 F167Y mutation prevalence was shown to be in poodles (29%), followed by Bernese
472 mountain dogs (25%), cocker spaniels (23%) and greyhounds (22%) that are infected with
473 *A. caninum* in North America (Jimenez Castro et al., 2019; Kitchen et al., 2019;
474 Leutenegger et al., 2024). Using faecal samples from 17,671,724 individual dogs in North
475 America, greyhounds were found to have a significantly higher risk of testing positive for
476 hookworms on faecal flotation (odds ratio = 15.3) (Burton et al., 2024). Additionally, the
477 interval to test negative was 71–72 days for presumably anthelminthic-treated
478 greyhounds, compared to just 1–2 days for other breeds (Burton et al., 2024). In this study
479 from Australia, in addition to greyhounds, one crossbreed dog from Queensland was
480 infected with *A. caninum* and had 83% of the isoform-1 β-tubulin F167Y mutation, along
481 with a reduced in vitro IC_{50} for thiabendazole.

482 The EHA tests for *in vitro* susceptibility to benzimidazoles thus phenotype including *A.*
483 *caninum* resistance to benzimidazole (Kotze et al., 2005; Kotze et al., 2009; Diawara et
484 al., 2013). Importantly, we demonstrated correlation of EHA and the isoform-1 β-tubulin
485 F167Y mutation, and those samples with elevated EHA (IC_{50}) all showed frequency of
486 F167Y mutation >75%. Known *in vitro* resistant *A. caninum* with IC_{50} of 8.76 μM (=1.76
487 μg/mL, KGR) and 40.08 μM (=8.07 μg/mL, BCR) for thiabendazole had 100% and 76% of
488 the F167Y mutation (McKean et al., 2024). Our resistant *A. caninum* had IC_{50} from 0.17 to
489 0.43 μg/mL of thiabendazole and are closer to the range (0.55 to 0.67 μg/mL) detected
490 for resistant *A. caninum* by Jimenez Castro et al. (2019). The *in vitro* resistance was
491 further confirmed by FEC reduction in one of the Australian dogs *in vivo*. There was no

492 significant correlation between F167Y (TTC>TAC) frequency and IC_{50} for thiabendazole in
493 a similar small study based on 15 *A. caninum* samples (Jimenez Castro et al., 2021).

494 The detection of the isoform-1 β-tubulin F167Y mutation in *U. stenocephala*, the
495 Northern hookworm that commonly infects dogs in cooler climates, is unexpected, as
496 drug resistance has not previously been reported for *U. stenocephala* (Leutenegger et al.,
497 2023; Venkatesan et al., 2023; Stocker et al., 2024). In our samples from Australia, we
498 detected *U. stenocephala* F167Y mutation in one out of four dog samples. More
499 frequently in Australia, *U. stenocephala* is a parasite of red foxes which likely serves as
500 the reservoir (Ryan, 1976). In a study of 930 foxes from New South Wales, Australia, the
501 most prevalent nematodes were *Toxocara canis* (35.2%) and *U. stenocephala* (30.6%),
502 while *A. caninum* was detected in only 70 (7.5%) foxes (Ryan, 1976). Although *U.*
503 *stenocephala* is often considered relatively non-pathogenic, it has been acknowledged
504 as a cause of subacute enteritis and hypoproteinaemia in dogs (Gibbs, 1958; Miller,
505 1968). The significance of the F167Y mutation in *U. stenocephala* in conferring
506 benzimidazole resistance in the presence of a large *refugium* in red foxes will require
507 further confirmation from *in vitro* and *in vivo* drug response studies.

508 The absence of resistant SNPs that would code for E_{198} and F_{200} in the isoform-1 β-tubulin
509 of *A. caninum* in Australia agrees with reports from the USA (Venkatesan et al., 2023). The
510 F200Y SNP has been documented once in *A. caninum* dog samples from Brazil, but the
511 *in vitro* or *in vivo* phenotype was not measured (Furtado et al., 2014). The F200Y was
512 detected in human *N. americanus* from Haiti with frequency of 20-90%, and in an *in vitro*
513 EHA assay these hookworm isolates were less sensitive to thiabendazole (IC_{50} = 0.12 to
514 0.26 μg/μL) (Diawara et al., 2013). Molecular dynamics simulations using isotype-1 β-

515 tubulin showed that *A. caninum* and *Ancylostoma ceylanicum* both shared the same
516 drug binding profile, while *A. duodenale* and *N. americanus* both had different binding
517 profiles with no overlap except for the E_{198} interaction that is shared with all nematodes
518 (Jones et al., 2022).

519 With growing evidence of anthelmintic resistance in *A. caninum*, the question arises
520 whether the fitness and ability of resistant strains to spread across the dog population
521 differ from non-resistant strains. Recently, a strain of triple-resistant *A. caninum* (BCR)
522 was shown to exhibit reduced 'larval activation', an initial step in the transformation of
523 infective non-feeding larvae into migrating and feeding larvae in a permissive host
524 (McKean et al., 2024). Larval activation *in vitro* is initiated when third-stage infective
525 larvae (iL3) of *A. caninum* are incubated with a host-mimicking signal, such as dog
526 serum, to resume feeding over a period of 24 hours (Hawdon and Schad, 1990; Hotez et
527 al., 1993). The consequences of the 40-50% reduced initial activation of the *A. caninum*
528 triple-resistant strain (BCR) reported by McKean et al. (2024) are not yet known but
529 suggest a possible reduced ability to establish infection. However, we did not find any
530 association between the SNPs and FEC in the dogs we studied. Our study does not
531 permit the examination of relationships between FECs and other variables (e.g., worm
532 fitness) because the infection histories (such as exposure to larvae and drug treatment
533 history) of the dogs are completely unknown. Our samples were neither tested for larval
534 activation nor for the presence of pyrantel and macrocyclic lactone resistance, so
535 reduced 'larval activation' may either be restricted to the triple-resistant strains, or it may
536 not lead to lower infection rate and changes in egg output.

537

538 **Conclusion**

539 The molecular tools employed in the present study enable agile moderate to high
540 throughput tracking of anthelmintic resistance mutations. This offers advantages in
541 terms of speed and cost compared to phenotypic *in vivo* and *in vitro* measurements of
542 sensitivity to anthelmintic drugs. We identified the hookworm species and
543 simultaneously discovered widely distributed isoform-1 β-tubulin SNPs across Eastern
544 Australia. Hookworms, whipworm and roundworms in dogs and cats are traditionally
545 managed by sustained routine administration of anthelminthics. The recent discovery of
546 MADR in canine hookworm (*A. caninum*) has raised the concerns of the veterinary
547 profession (von Samson-Himmelstjerna et al., 2021; Marsh and Lakritz, 2023). Adopting
548 a risk assessment-based approach to limit unnecessary use of parasiticides has the
549 potential to mitigate the risk of resistance selection and spread, and should be an
550 important consideration in responsible parasite control (Selzer and Epe, 2021; Bagster
551 and Elsheikha, 2022).

552

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557

558 **Table 1:** Summary of *Ancylostoma caninum* egg hatch assay with frequency of Q134H and F167Y polymorphisms in isoform-1 β-tubulin.

559

Sample ID	Breed	EPG	IC50 µg/mL *	IC_{50} CI95% µg/mL *	Q134H[&]	F167Y[&]
High IC_{50}>0.15 µg/mL of thiabendazole						
D3:TS89	Greyhound	1,275	0.429	0.344 to 0.534	0%	89.91%
D1:TS87[^]	Greyhound	1,050	0.298	0.216 to 0.412	0%	85.47%
D2:TS88[^]	Greyhound	645	0.262	0.237 to 0.289	0%	88.80%
D6:TS92[^^]	Crossbreed	540	0.239	0.201 to 0.285	0%	83.11%
D18/D20:TS104/106[^^]	Crossbreed	225	0.170	0.121 to 0.239	0%/0%	100%/77.54%
D17:TS103	Greyhound	5,535	0.194	0.150 to 0.250	2.55%	81.23%
Low IC_{50}<0.15 µg/mL of thiabendazole						
D5:TS91	Greyhound	14,910	0.056	0.048 to 0.066[#]	0%	10.69%

D9:TS95	Greyhound	930	0.050	0.043 to 0.059[#]	18.27%	33.51%
D10:TS96	Greyhound	4,080	0.043	0.040 to 0.046[#]	0%	22.67%
D8:TS94	Greyhound	390	0.039	0.031 to 0.048[#]	0%	29.26%
D12:TS98	Greyhound	3,930	0.037	0.035 to 0.039[#]	0%	0%
D15:TS101	Greyhound	720	0.036	0.031 to 0.041[#]	0%	6.56%
D16:TS102	Greyhound	885	0.021	0.013 to 0.033[#]	7.43%	22.53%

560 [*] thiabendazole concentration in µg/mL

561 [#] drug-susceptible *A. caninum* - 0.046 and 0.048 µg/mL (Jimenez Castro et al., 2019) and 0.045 µg/mL (Diawara et al., 2013).

562 [&] percentage of canonical benzimidazole resistance codons on the isoform-1 β-tubulin

563 [^,^^] same dog

564 Notes: EPG – *A. caninum* eggs per gram; IC_{50} - 50% inhibition concentration; CI95% - 95% confidence interval.

565

566 **Figure 1 | Molecular profiling of hookworm species using ITS-2 rDNA and**
567 **benzimidazole susceptibility using isoform-1 β-tubulin.**

568 (A) Sampled with hookworm-like stages that were profiled using metabarcoded deep
569 Illumina amplicon sequencing at ITS-2 rDNA. All but one sample came from dogs. Th
570 single, cat single sample (TS042) is on the very right. The broad scale of the samples is
571 indicated above the bar chart. Amplicon sequence variants (ASVs) were pooled
572 according to species of nematode they belonged using reference sequence alignment.
573 Proportion of each species (*Ancylostoma caninum*, *A. tubaeforme, Uncinaria*
574 *stenocephala*) is colour coded in the stacked bar chart; ASVs that belonged to unrelated
575 nematodes are depicted as 'pseudoparasites' and represented trichostrongylid species
576 from sheep and rabbits. The bar chart underneath represents sequence depth for each
577 sample, and samples with <2,000 sequence reads are depicted with a star. Samples are
578 vertically aligned across the bar charts. (B) Dog samples profiled using targeting BZ167
579 amplicon of the isoform-1 β-tubulin, specifically region covering amino acids Q_{134} and
580 F_{167} conferring benzimidazole resistance. Only ASVs of hookworm are used and the
581 sequencing depth is depicted underneath. Samples that did not amplify hookworms are
582 indicated by square and samples with <2,000 sequence reads are depicted with a star.
583 (C) Bar chart for Q_{134} and F_{167} isoform-1 β-tubulin of *A. caninum* where proportion of an
584 alternative residue coding for amino acid that confers benzimidazole resistance are
585 show. Samples with no bar chart are those that did not amplify *A. caninum* and so there
586 are no *A. caninum* ASVs.

587 **Figure 2 | Distribution of *Ancylostoma caninum* and benzimidazole resistant**
588 **isoform-1 β-tubulin single nucleotide polymorphism.**

589 Proportional symbol maps of (A) the number of *A. caninum* positive samples, and (B)
590 number of Q134H (CAA>CAT) and (C) number of F167Y (TTC>TAC) isoform-1 β-tubulin of
591 *A. caninum* positive samples. Samples were summed per postcode in New SouthWales,
592 Australia. Samples are joined to postcodes of Australia shapefile (Geocentric Datum of
593 Australia, GDA 1994) in ArcGIS® Pro.

594 **Figure 3 | *Ancylostoma caninum* IC_{50} for thiabendazole in egg hatch assays.**

595 (A) Correlation IC_{50} values for *A. caninum* samples for thiabendazole agar-based egg
596 hatch assay (EHA) from Queensland, Australia with percentage of F167Y (TTC>TAC)
597 isoform-1 β-tubulin of *A. caninum* present in the sample. Simple linear regression is
598 depicted as solid line with 95% confidence interval depicted as dotted line. (B) Histogram
599 of IC_{50} values for *A. caninum* samples from Queensland, Australia. A susceptible *A.*
600 *caninum* samples, that was confirmed to have 100% faecal egg count (FEC) reduction is
601 colour coded (black). The samples with >0.15 µg/mL thiabendazole IC_{50} values have their
602 identifiers above the bars.

603

604

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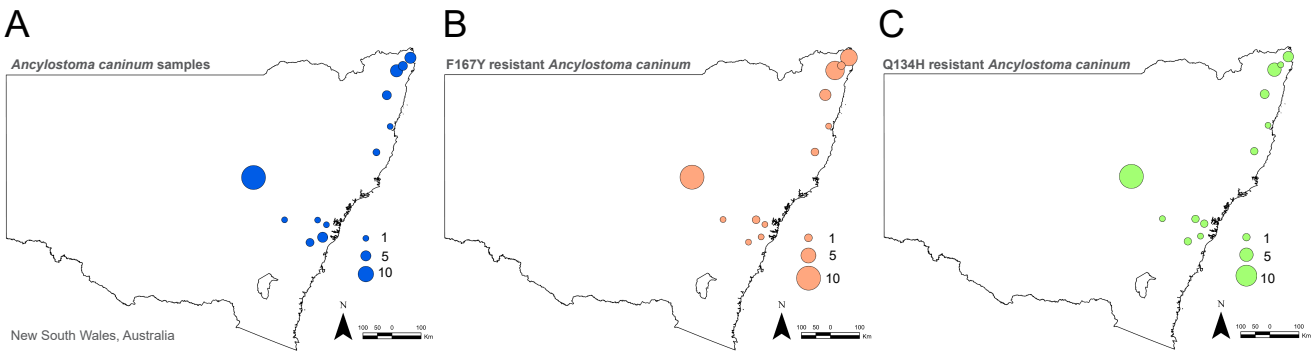
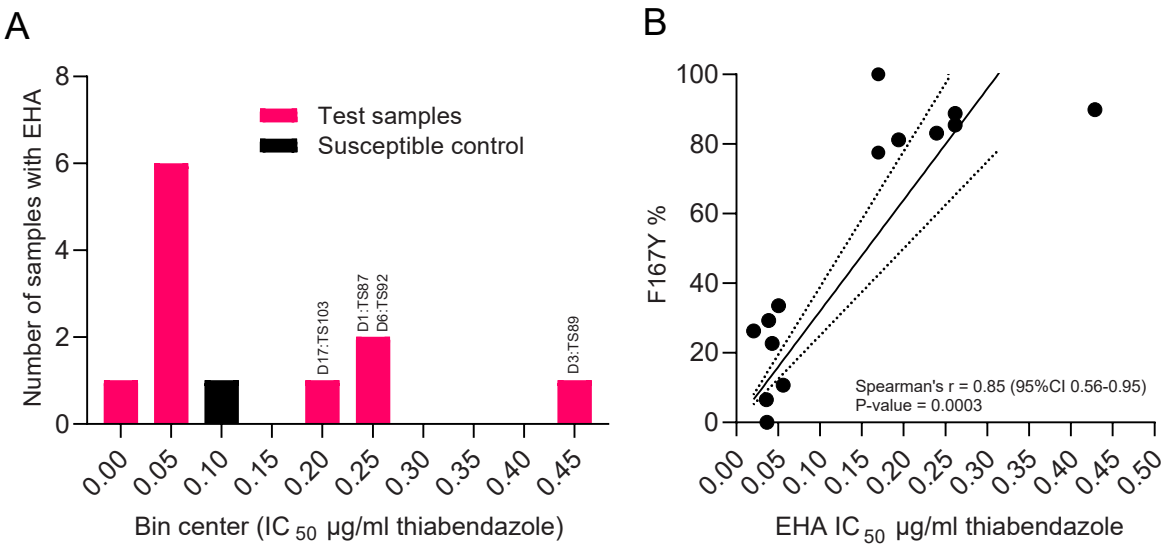
A
south-eastern Australia
(faecal samples)
New Zealand
(faecal samples)
Queensland, Australia
(cultured L3)
A. caninum
A. tubaeforme
U. stenocephala
pseudoparasites
<2000 sequence reads
B
BZ167 not amplified
C
Q134H
Q134 susceptible (Ancylostoma caninum)
F167Y
F167 susceptible (Ancylostoma caninum)
EHA susceptible
EHA resistant


A
Ancylostoma caninum samples
1
5
10
N
New South Wales, Australia
B
F167Y resistant Ancylostoma caninum
1
5
10
N
C
Q134H resistant Ancylostoma caninum
1
5
10
N


A
Number of samples with EHA
Test samples
Susceptible control
D17:TS103
D1:TS87
D6:TS92
D3:TS89
Bin center (IC 50 µg/ml thiabendazole)
B
F167Y %
Spearman's r = 0.85 (95%CI 0.56-0.95)
P-value = 0.0003
EHA IC 50 µg/ml thiabendazole