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The detection of three independent benzimidazole resistance mutations in a single *Ancylostoma caninum* population from a Golden Retriever breeding kennel in Sao Paulo, Brazil

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ABSTRACT

Multiple anthelmintic drug resistance (MADR) is widespread in the canine hookworm *Ancylostoma caninum* in kenneled greyhounds in the USA and Australia with benzimidazole resistance being associated with the isotype-1 β-tubulin SNPs Q134H(CAA > CAT) and F167Y(TTC > TAC) in both cases. These two SNPs have also been found to be widespread, often at high frequency, in *A. caninum* populations in pet dogs across the USA but the situation in domestic dogs in other countries is currently unknown. Here, we report the identification of a single *A. caninum* population, from a Golden Retriever breeding kennel in São Paulo Brazil, which contained three different benzimidazole resistance SNPs; Q134H(CAA > CAT), F167Y(TTC > TAC), and F200Y(TTC > TAC). These SNPs were confirmed by three different methodologies; allele-specific qPCR, Illumina and Oxford Nanopore Technologies (ONT) deep amplicon sequencing. This is the second report of the F200Y(TTC > TAC) SNP in *A. caninum* from Brazil, suggesting it may be established in this region and illustrating need to consider regional differences when applying molecular diagnostic tests of anthelmintic resistance in canine hookworms. Analysis of the ONT sequence data of the near full-length isotype-1 β-tubulin showed that these three separate canonical resistance SNPs were present on different haplotypes suggesting their genetic independence. The ONT sequencing also detected two additional, previously unreported, non-synonymous SNPs - S73N(AGT > AAT) and V368A(GTT > GCT) – whose potential role in benzimidazole resistance is currently unknown. There was a high degree of variance in the amplicon sequencing data between replicate PCRs illustrating the challenges for accurately determining allele frequencies from archived fecal samples. In conclusion, the identification of three independent benzimidazole resistance mutations in a single *A. caninum* population suggests a high risk of anthelmintic resistance emergence in high transmission and drug selection environments such as kennels. It also suggests regional differences in the suitability of specific molecular diagnostic tests to detect anthelmintic resistance in canine hookworms.

1. Introduction

Ancylostoma caninum is one of the most common gastrointestinal nematode infections in dogs in many regions of the world with heavy infections leading to anemia in young dogs and, in some cases, mortality (Epe, 2009). It is also zoonotic, resulting in cutaneous larval migrans and eosinophilic gastritis in humans (Bowman et al., 2010).

Transmission of *A. caninum* is particularly common in kenneled environments and hookworm infections have been a significant issue in the greyhound industry for many years due to sand exercise runs being a

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highly conducive environment for hookworm transmission (Dryden and Ridley, 1999). Kennel environments may be particularly conducive to the emergence of anthelmintic resistance because they combine several important risk factors, including relatively high host density, repeated exposure to infective larvae in contaminated environments, and frequent repeated anthelmintic treatment to control ongoing transmission. In greyhound facilities, for example, sand runs and other kennel-associated environments have been described as highly favourable for hookworm transmission, necessitating regular treatment and creating strong drug selection pressure (Evason et al., 2024; Kopp et al., 2008).In addition, movement of dogs out of such kennel environments may facilitate the wider dissemination of resistant parasites into the general pet dog population (Jimenez Castro et al., 2019, 2020). Consequently, hookworm control in racing greyhound kennels relies on regular, often monthly, anthelmintic treatments. This high drug selection pressure has resulted in the emergence of *A. caninum* which is resistant to all three major drug classes: benzimidazoles, macrocyclic lactones, and pyrantel. Multiple anthelmintic drug resistance (MADR) against these three drug classes is now widespread in *A. caninum* among kenneled and retired racing greyhounds in the USA and Australia (Abdullah et al., 2024; Jimenez Castro et al., 2019; Venkatesan et al., 2023).

An analysis of 39 million diagnostic reports revealed a 47% increase in hookworm prevalence among pet dogs across the USA from 2012 to 2018 (Drake and Carey, 2019). Although this increase likely reflects multiple contributing factors rather than anthelmintic resistance alone, it nevertheless, together with anecdotal reports of clinical cases unresponsive to treatment, raised the concern that resistance might not be confined to racing greyhounds but could also be emerging in pet dogs, despite the lower drug selection pressure in this population. This concern was subsequently supported by detailed phenotypic analyses confirming multiple-anthelmintic drug resistance in several *A. caninum* isolates from independent clinical cases in pet dogs across the USA (Jimenez Castro et al., 2019).

The development of molecular markers for benzimidazole resistance has significantly advanced the ability to conduct widescale surveillance for resistance to this drug class in *A. caninum*. Amino acid substitutions at codons 167, 198, and 200 of the isotype-1 β-tubulin have been shown to confer resistance in a variety of strongylid nematode species and more recently at codon 134 in *A. caninum* (Venkatesan et al., 2023). The underlying DNA sequence polymorphisms can now be detected using molecular techniques such as deep amplicon sequencing or qPCR (Jimenez Castro et al., 2021; Kitchen et al., 2019). These assays have enabled researchers to screen large numbers of hookworm-positive canine fecal samples and quantify the prevalence and relative frequency of benzimidazole resistance mutations. Deep amplicon sequencing of *A. caninum* eggs harvested from 685 hookworm-positive pet dog fecal samples submitted by veterinarians across the USA to diagnostic labs found the F167Y(TTC > TAC) or Q134H(CAA > CAT) isotype-1 β-tubulin SNPs to be present at prevalences of 49.7% (overall mean frequency 54.0%) and 31.1% (overall mean frequency 16.4%) respectively (Venkatesan et al., 2023). No canonical resistance polymorphisms were detected at codons 198 or 200 in any of these samples. A qPCR study detected the F167Y(TTC > TAC) mutation in 10.6% of 511 samples and at 12.5% in an large study of 262,872 canine stool samples submitted by veterinarians to diagnostic labs across the USA and Canada between March and December of 2022 using the KeyScreen™ commercial diagnostic assay (Leutenegger et al., 2023b, 2024). More recently, an even larger survey of 885,424 similar canine fecal diagnostic submissions from across the USA between March 2022, and December 2023 found a 14.4% prevalence of the F167Y(TTC > TAC) resistance marker. This study also provided a detailed risk assessment based on host demographic factors. The analysis revealed that intact (non-neutered) dogs, certain age groups (particularly juveniles under one year and seniors over seven years), and specific breeds had significantly higher odds of harboring benzimidazole-resistant *Ancylostoma* spp. infections. Regional variations were also observed, with higher prevalence rates detected in the Southeastern and Gulf Coast states. Additionally, the study reported sex-based differences, with male dogs exhibiting slightly higher infection risks (Jimenez Castro et al., 2025).

The current hypothesis for the emergence of anthelmintic resistance in *Ancylostoma caninum* across the USA suggests that it originated in greyhound kennels, due to the high drug selection pressures applied by frequent anthelmintic treatments and near ideal conditions for hookworm transmission. Greyhounds, particularly those involved in racing, are housed in environments that require regular, often monthly, treatment with benzimidazoles, macrocyclic lactones, and pyrantel to control hookworm infections (Evason et al., 2024; Kopp et al., 2008). It is hypothesized that as resistant *A. caninum* populations emerged in racing greyhound kennels, resistant parasites then spread into the pet dog population across the USA through the adoption of retired greyhounds as pets. The resulting environmental contamination across the USA with drug resistant hookworm eggs, such as in dog parks, would have facilitated this spread. Canada has also experienced increasing reports of *A. caninum* resistance, particularly among imported dogs. A study conducted in Quebec reported 100% prevalence of *A. caninum* in imported Greyhounds from the USA and 30.77% in shelter dogs (Nezami et al., 2024). Molecular analysis further revealed the presence of the F167Y (TTC > TAC) single nucleotide polymorphism (SNP) in the isotype-1 β-tubulin gene in one of the imported Greyhounds, indicating potential benzimidazole resistance (Nezami et al., 2024). Subsequent testing of a larger number of dogs (n = 32,205) in May and April of 2023 detected a F167Y(TTC > TAC) prevalence of 6.0% (11 of 184 hookworm positive dogs). Four of the 11 dogs did not have any travel history outside of Canada, indicating that benzimidazole-resistant *A. caninum* is already endemic in Canada (Evason et al., 2023). The role of imported dogs from the USA, including adopted racing greyhounds, may have been an important source of resistant *A. caninum* in Canada. Interestingly, studies have recently detected high frequencies of the F167Y(TTC > TAC) and Q134H(CAA > CAT) isotype-1 β-tubulin SNPs in greyhounds, and also in a number of pet dogs, in SE Australia suggesting potentially a similar situation may pertain there although larger scale surveys are needed (Abdullah et al., 2024).

Little is known about anthelmintic resistance in *A. caninum* in countries outside the USA, Canada, and Australia. One study in Brazil reported the presence of the F200Y(TTC > TAC) isotype-1 β-tubulin SNP in *A. caninum* from Minas Gerais in Brazil (Furtado et al., 2014). Among 234 worms collected from naturally infected dogs, only one worm harboured this mutation, representing a low frequency of 0.8% (Furtado et al., 2014).

In this manuscript, we report the identification of three canonical isotype-1 β-tubulin benzimidazole resistance SNPs, F167Y(TTC > TAC), Q134H(CAA > CAT), and F200Y(TTC > TAC), in a single *A. caninum* population from a Golden Retriever breeding kennel in São Paulo, Brazil. To the authors' knowledge, this finding is the first report of an *A. caninum* population simultaneously harbouring all three mutations on three different haplotypes and suggests there is both a high degree of drug selection and hookworm transmission in this breeding kennel. These results further support the concern of anthelmintic-resistant *A. caninum* emerging in kenneled dogs where hookworm transmission is high and anthelmintic treatments are frequent and underscores the importance of monitoring high-risk environments for the presence of drug-resistant parasites.

2. Materials and methods

2.1. Sample collection and nucleic acid extraction

A fecal sample was received by the TECSA Laboratories, Belo Horizonte, Brazil, VCA Brazil, a division of VCA and MVH (Mars Veterinary Health), as a routine diagnostic submission from a Golden Retriever breeding kennel in São Paulo, Brazil in July 2023 for fecal flotation and tested positive for hookworm eggs. The sample was collected from a

kennel housing a dam with a 42-day-old litter and likely included fecal material from both the dam and litter. Total nucleic acids (TNA) were extracted from a 0.15g aliquot of the fresh fecal sample using the previously described protocol, which includes mechanical bead-beating disruption of parasite eggs to maximize TNA yield (Leutenegger et al., 2023b). This initial TNA preparation was used for the KeyScreen™ GI Parasite PCR Test (Antech Diagnostics). The remainder of the fecal sample was stored at 4 °C without fixation and additional TNA samples were prepared in October 2023 (TNA re-extraction 1) and January 2024 (TNA re-extraction 2); 99 days and 176 days after the original TNA extraction from fresh fecal material used for the KeyScreen™ GI Parasite PCR Test, respectively (Fig. 1).

A positive control TNA sample was made for the Oxford Nanopore Technologies (ONT) amplicon sequencing by pooling equal amounts of TNA preparations made from 12 different hookworm positive canine fecal samples from Orlando, Florida to Antech. The individual control TNA samples were prepared from feces whilst it was still fresh (stored at 4 °C for ~1 week prior to TNA preparation). These individual TNA preparations used to make the positive control pool TNA had been previously determined as positive for the Q134H(CAA > CAT) and F167Y(TTC > TAC) SNPs by QPCR.

2.2. Fecal zinc sulfate flotation

Fecal flotation was performed using a centrifugal flotation technique with zinc sulfate as the flotation medium on the submitted fecal samples, and the results were reported qualitatively (Reichard and Anne, 2021).

2.3. qPCR assays

The KeyScreen™ GI Parasite PCR Test (Antech Diagnostics) was applied to the first TNA extraction from the newly submitted sample on 5/7/2023 (Evason et al., 2024; Leutenegger et al., 2023a).

Individual qPCR assays targeting a species-specific ITS-1 marker and the separate Q134H(CAA > CAT), F167Y (TTC > TAC), and F200Y(TTC > TAC) SNPs were later performed using TNA re-extraction 1 as template (Leutenegger et al., 2024). The qPCR conditions were 13.15 µL of molecular grade water, 5 µL of KAPA buffer, 0.75 µL of dNTPs, 0.75 µL of forward primer, 0.75 µL of reverse primer, 0.5 µL of KAPA HiFi HotStart DNA polymerase, 0.1 µL of BSA (Bovine Serum Albumin), 3 µL of DNA template. The thermocycling conditions were 95 °C for 3 min, followed by 40 cycles of 98 °C for 20 s, 70 °C for 30 s, 72 °C for 2 min and 30 s, and the final extension for 72 °C for 2 min and 30 s.

2.4. Illumina ITS2 metabarcoding and isotype-1 β-tubulin deep amplicon sequencing

To confirm the *Ancylostoma* species identification, ITS-2 Illumina short-read metabarcoding was performed using NC1 and NC2 primers as previously described (Avramenko et al., 2015; Venkatesan et al., 2023). To identify any isotype-1 β-tubulin SNPs at codons 134, 167, 198, and 200, Illumina short-read deep amplicon sequencing was performed (Avramenko et al., 2019; Jimenez Castro et al., 2019, 2021). Briefly, a 293 bp fragment, encompassing codons 134 and 167, and a 340 bp fragment encompassing codons 198 and 200 were amplified as previously described (Jimenez Castro et al., 2021; Venkatesan et al., 2023). The only methodological difference from those previous studies was that, instead of the DNA template being prepared from hookworm eggs harvested by flotation, TNA preparations from fecal material were used. Multiple 1 µl aliquots of the neat TNA re-extraction 1 and TNA re-extraction 2 preparations were used as PCR templates. For Illumina deep sequencing, all the steps, including sample purification, indexing, and library preparation, were employed as previously described (Avramenko et al., 2019). The Illumina MiSeq platform was used to sequence the libraries with the 2 x 250 bp V2 sequencing chemistry (Illumina Inc., USA). DADA2 was used to analyze the raw Illumina FASTQ sequencing files following the tutorials on DADA2 and Nemabiome.ca website (Callahan et al., 2016; DADA2 ITS Pipeline Workflow, n.d.; Nemabiome.ca). Primer sequences were trimmed from forward and reverse reads with Cutadapt (4.9) using the parameters "–times 2", "–discard-untrimmed", and "–error-rate 0.2" (Martin, 2011). Quality filtering was applied to the trimmed reads with R package dada2 (1.36.0) "filterAndTrim" function using the parameters "truncLen = c (200,150)", "maxN = 0", "maxEE = c(2, 5)", "truncQ = 2", "rm.phix = TRUE", and "compress = TRUE". Error rates of the filtered sequencing reads were learned with dada2 "learnErrors" function and denoised with dada2 "dada" function. Then the denoised forward and reverse reads were merged with dada2 "mergePairs" function to obtain Amplicon Sequence Variants (ASVs). Frequency table of ASVs per sample was obtained by dada2 "makeSequenceTable" function and chimeric sequences were removed from the frequency table using dada2 "removeBimeraDenovo" function with "method = "consensus"" setting. Each sequencing run was processed separately using the described DADA2 method. ASV sequences were then exported to FASTA format from the frequency table using Biostrings R package (2.76.0) "writeXStringSet" function for species identification via blastn (2.16.0) against NCBI nucleotide database with the following settings: "-db nt", "-remote", "-max_target_seqs 5", "-qcov_hsp_perc 25", "-perc_identity 0", "-task megablast" (Camacho et al., 2009). Only ASVs with *A. caninum* isotype-1 β-tubulin references sequences as top hit and ≥ 1% in at least one

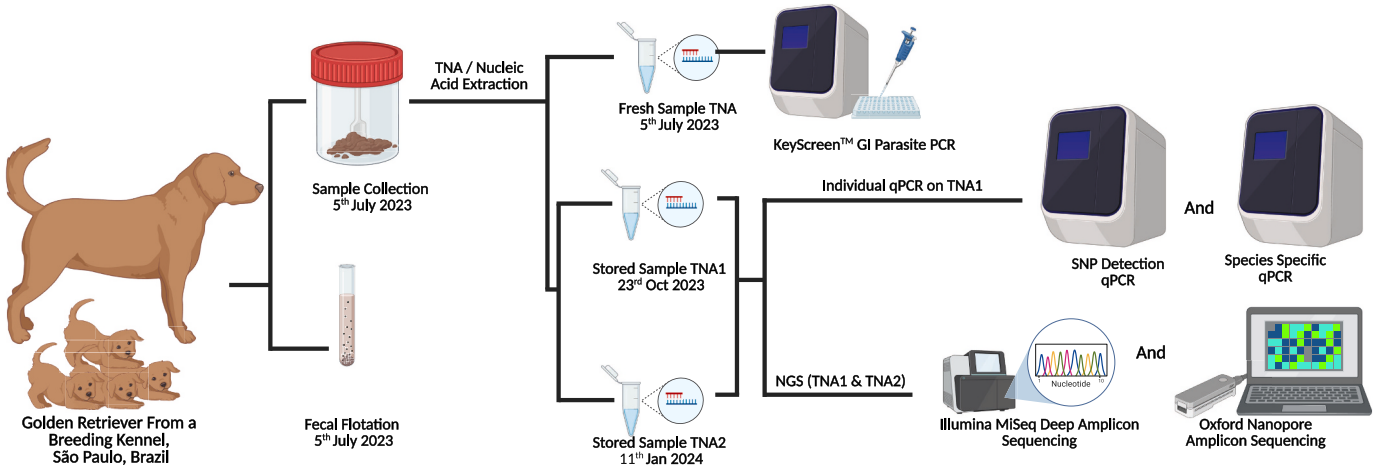


Fig. 1. Schematic flow chart of the application of qPCR, Illumina and ONT deep amplicon sequencing to an archived fecal stool material.

replicate were kept for downstream frequency analysis.

2.5. Oxford Nanopore amplicon (ONT) sequencing

Primer pairs were designed to amplify a 3547 bp near full-length fragment of the *A. caninum* isotype-1 β-tubulin gene. The forward and reverse primer sequences are as follows: ACWG_4_Fwd: 5′-TGCAATCATGCGTGAGATCG-3′ and ACWG_4_Rev: 5′-GGTGGTCTACTCCTCGGGG-3'. The near full-length isotype-1 β-tubulin gene was PCR amplified from multiple 3 μl aliquots of the neat TNA re-extraction 1 and TNA re-extraction 2 preparations. In addition, a pool of 12 different canine fecal TNA samples from Orlando, Florida, with a Ct value of 21.9 for the *A. caninum* ITS1 qPCR, and positive for the Q134H (CAA > CAT), and F167Y (TTC > TAC) SNPs was used as a positive control for the ONT amplicon sequencing.

The near full-length *A. caninum* isotype-1 β-tubulin gene was PCR amplified and sequenced using Nanopore MinION R10.4.1 flow cell. POD5 files containing raw sequencing signal were basecalled using Dorado (v0.9.0) and a super-accuracy base calling model (dna_r10.4.1_e8.2_400bps_sup@v5.0.0) (Oxford Nanopore Technologies, 2025). The basecalled reads were output into BAM format and converted to FASTQ format using SAMTools (1.18) (Danecek et al., 2021). Cutadapt (4.9) was used to remove primer sequences from each read and low-quality reads (Martin, 2011). Trimmed and filtered reads were then aligned to full-length *A. caninum* isotype-1 reference sequence (DQ459314.1) using Minimap2 (2.28-r1209) (Li, 2018, 2021), and SNP frequency at each position was calculated with Bam-readcount (0.8) (Khanna et al., 2022) SNPs resulting in nonsynonymous mutations with average base quality >40 and read depth >2500 were extracted with an R script using tidyverse (1.1.5) and Biostrings (2.72.0) packages (DebRoy and Hervé, 2024; Pagès et al., 2024; Wickham et al., 2019).

2.6. Haplotype analysis

The error rate of ONT sequencing is too great to use DADA2 to generate Amplified Sequence Variants (ASVs) (Callahan et al., 2017, 2016, Nanopore consensus sequencing and DADA2). Therefore, in order to determine whether the detected Q134H (CAA > CAT), F167Y (TTC > TAC), and F200Y (TTC > TAC) SNPs were in cis or trans, we took a different approach similar to the PHARE pipeline recently developed for ONT sequence haplotype analysis in *Plasmodium* (Hosch et al., 2024). Aligned reads were converted to multiple sequence aligned FASTA format with gofasta (1.2.1) (Jackson, 2022). Each read was then annotated with its genotype at codons 134, 167, 198, and 200 with an R script using tidyverse (1.1.5) and Biostrings (2.72.0) packages and reads with the same resistance genotype at the multiple codon positions were collapsed into a single haplotype.

3. Results

3.1. The detection of three different isotype-1 β-tubulin benzimidazole resistance SNPs in a single-species infection of Ancylostoma caninum by qPCR

A fresh environmental fecal sample from a kennel housing a single Golden Retriever bitch with its litter was submitted for routine diagnostic testing from a canine breeding establishment in São Paulo, Brazil. The commercial KeyScreen™ GI Parasite PCR test run on this sample was positive for both *Ancylostoma* spp and benzimidazole resistance. Given that benzimidazole resistance in canine hookworms from Brazil has been rarely reported, this sample was subject to further investigation. The presence of *A. caninum* was confirmed by an ITS-1 species-specific qPCR assay applied to the same fecal TNA preparation that had been used for the original KeyScreen™ assay. Three different allele-specific isotype-1 β-tubulin qPCR assays targeting different benzimidazole resistance SNPs -Q134H(CAA > CAT), F167Y(TTC > TAC), and F200Y(TTC > TAC)- were also positive when applied to this same TNA preparation.

3.2. Confirmation of the three isotype-1 β-tubulin benzimidazole resistance SNPs by deep amplicon illumina sequencing

Confirmation of the qPCR results and further investigation of case 8027 was performed by deep amplicon Illumina sequencing. This methodology was applied to two independent TNA preparations that were re-extracted from the original fecal sample after 99 days and 176 days storage at 4 °C respectively (Fig. 1). ITS2 amplicons were successfully generated and deep sequenced from 6 to 6 replicate PCRs from TNA re-extraction 1 and TNA re-extraction 2 respectively. A total of 1,118,226 Illumina reads were generated across the replicates of which all of them mapped to nematode ITS reference sequences. 100% of these nematode ITS2 reads mapped to *A. caninum* reference sequences (100% identity) confirming this case was a single-species hookworm infection.

Two isotype-1 β-tubulin fragments were amplified from each of the two fecal re-extraction TNA preparations to test for the presence of the known canonical benzimidazole resistance SNPs, A 293bp "exon 4" fragment encompassing codons 134/167 and a 340bp "exon 5" fragment encompassing codons 198/200 (Figs. 1 and 2A). The generation of PCR amplicons for these isotype-1 β-tubulin fragments from the two TNA re-extractions was challenging with variable success of amplicon and sequence generation between 10 or 12 replicate PCRs performed on each of the two TNA re-extraction preparations (Fig. 2B–C).

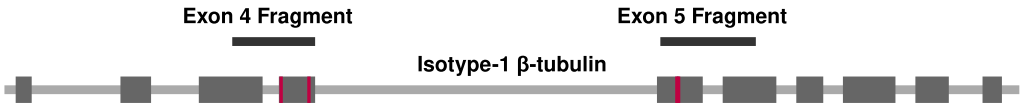
In the case of the "exon 4" fragment, amplicons were successfully generated from 5/6 and 2/4 of the replicate PCRs performed on TNA 1 and TNA2 fecal re-extraction preparation respectively (Fig. 2B). Illumina deep sequencing of these replicate amplicons (676-28,959 reads/sample) revealed a high degree of variance of the ASVs, and their relative frequencies, between the sequenced replicate amplicons (Fig. 2B). ASVs carrying the Q134H(CAA > CAT), and F167Y(TTC > TAC) SNPs were detected in 3/6 replicate PCRs (frequency range 18.3-21.4%) and 4/6 (frequency range 12.7-100%) of the amplicons generated from TNA re-extraction 1 (Fig. 2B).

In the case of the "exon 5" fragment, amplicons were successfully generated from 5/5 and 7/7 of the replicate PCRs performed on TNA 1 and TNA 2 fecal re-extraction preparations respectively (Fig. 2C). Illumina deep sequencing of these replicate amplicons (44,870–154,402 reads/sample) again revealed a high degree of variance in ASVs with the F200Y(TTC > TAC) SNP being detected in 4/5 (frequency range of 10.3-34.3%) and 3/7 (frequency range of 8.7-46.0%) of the amplicons generated from TNA re-extraction 1 and re-extraction 2 respectively (Fig. 2C). No canonical benzimidazole resistance SNPs were detected at codon 198 from any of the ASVs in any replicate PCR amplicons.

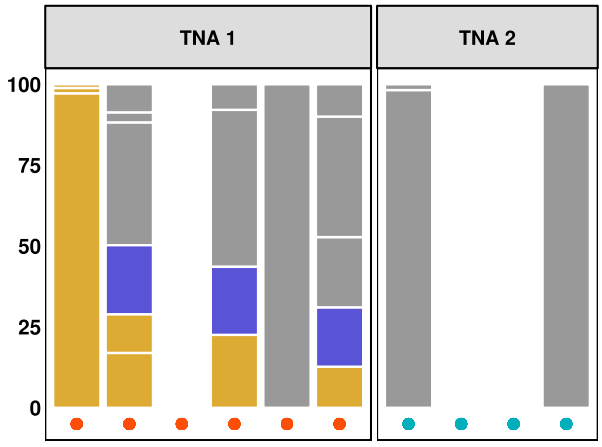
3.3. Oxford Nanopore amplicon sequencing of the near-full length A. caninum isotype-1 β-tubulin gene confirms the three canonical benzimidazole resistance SNPs and detects two additional non-synonymous SNPs

We also undertook ONT long-read amplicon sequencing on case 8027 to further confirm the presence of the Q134H(CAA > CAT), F167Y (TTC > TAC) and F200Y(TTC > TAC) SNPs and to identify any additional non-synonymous SNPs in the *A. caninum* isotype-1 β-tubulin gene. Amplicons of the near-full length *A. caninum* isotype-1 β-tubulin gene (3,547bp) were successfully generated from 7/10 and 6/10 of the replicate PCRs performed on TNA re-extraction 1 and TNA re-extraction 2 preparations respectively (Fig. 3). ONT deep sequencing of these replicate amplicons was performed and the reads aligned to the *A. caninum* isotype-1 β-tubulin reference sequence (DQ459314.1) (range of 20,970–117,433 reads per amplicon). Since the error rate of ONT sequencing is too high to fulfill the minimum requirements for the DADA2 model, it was not possible to generate ASVs for the 3547 bp amplicon. Consequently, SNPs were detected at each nucleotide

A. Amplicon Fragment

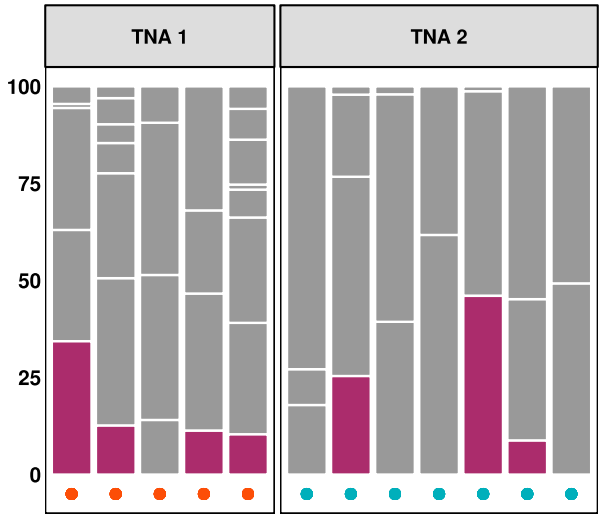


B. Exon 4 Fragment (Codons 134 / 167)



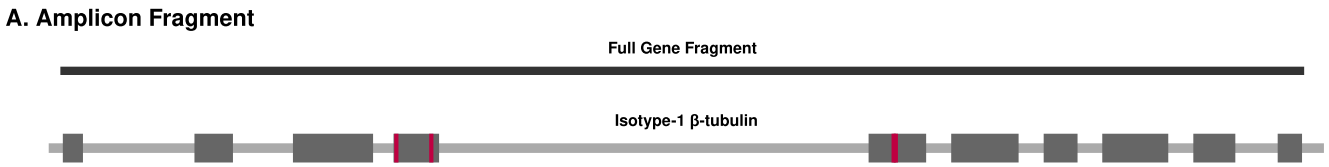
Sample			167Y TAC	134H CAT
ID	Aliquot	Read #	SNP %	SNP %
1	TNA 1	25,419	100.0	0.0
2	TNA 1	28,959	28.9	21.4
3	TNA 1	0	NA	NA
4	TNA 1	17,256	22.5	21.0
5	TNA 1	676	0.0	0.0
6	TNA 1	23,136	12.7	18.3
7	TNA 2	25,482	0.0	0.0
8	TNA 2	0	NA	NA
9	TNA 2	0	NA	NA
10	TNA 2	24,066	0.0	0.0

C. Exon 5 Fragment (Codons 198 / 200)

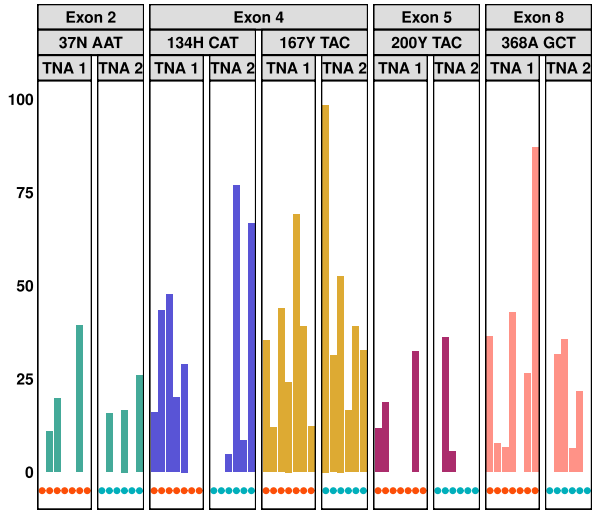


Sample			200Y TAC
ID	Aliquot	Read #	SNP %
1	TNA 1	70,030	34.3
2	TNA 1	85,692	12.6
3	TNA 1	44,870	0.0
4	TNA 1	154,402	11.3
5	TNA 1	107,674	10.3
6	TNA 2	66,974	0.0
7	TNA 2	51,163	25.4
8	TNA 2	45,239	0.0
9	TNA 2	128,350	0.0
10	TNA 2	124,214	46.0
11	TNA 2	78,240	8.7
12	TNA 2	130,804	0.0

Fig. 2. Illumina sequencing of amplicons from the isotype-1 β-tubulin gene encompassing codons 134, 167, 198 and 200.
Panel A: Schematic representation of the *A. caninum* isotype-1 β-tubulin gene model and positions of the two amplified fragments; "exon 4, codons 134/167 fragment" and "exon 5, codons 198/200 fragment". The vertical red bars on exons 4 and 5 indicate the positions of codons 134, 167, 198 and 200 respectively.
Panel B: "Exon 4, codons 134/167 fragment" amplicon deep Illumina sequencing data. An amplicon was successfully generated from the TNA re-extraction 1 and TNA re-extraction 2 preparations in 6/10 replicate PCR reactions (indicated by the orange dots at the base of the chart) and 4/10 replicate PCR reactions (indicated by the blue dots at the base of the chart) respectively. Each of these amplicons was deep sequenced and the chart shows the relative proportions of the ASVs generated for each amplicon expressed as percentages (Y-axis). ASVs carrying the Q134H(CAA > CAT) SNP are indicated in blue, ASVs carrying the F167Y(TTC > TAC) SNP are indicated in yellow and the remaining ASVs with susceptible genotypes at these two codons are indicated in grey. The table on the right shows the total number of reads mapping to the reference *A. caninum* isotype-1 β-tubulin sequence (DQ459214.1) for each amplicon ordered as in the chart and percentage of the reads corresponding to the ASV carrying either the Q134H(CAA > CAT) or F167Y(TTC > TAC) SNPs.
Panel C: "Exon 5, codons198/200 fragment" amplicon deep Illumina sequencing data. An amplicon was successfully generated from the TNA re-extraction 1 and TNA re-extraction 2 preparations in 5/10 replicate PCR reactions (indicated by the orange dots at the base of the chart) and 7/10 replicate PCR reactions (indicated by the blue dots at the base of the chart) respectively. Each of these amplicons was deep sequenced and the chart shows the relative proportions of the ASVs generated for each amplicon expressed as percentages (Y-axis). ASVs carrying the F200Y(TTC > TAC) SNP are indicated in red and the remaining ASVs with susceptible genotypes at codons 198 and 200 are indicated in grey. The table on the right shows the total number of reads mapping to the reference *A. caninum* isotype-1 β-tubulin sequence (DQ459314.1) for each amplicon ordered as in the chart and percentage of the reads corresponding to the ASV carrying the F200Y(TTC > TAC) SNP.

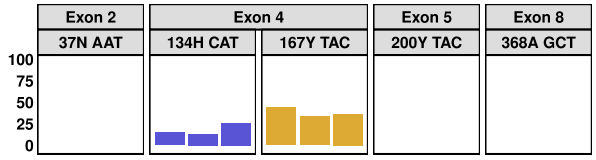


B. Sample 8027



Sample		37N AAT		134H CAT		167Y TAC		200Y TAC		368A GCT	
ID	Aliquot	SNP %	Read #	SNP %	Read #	SNP %	Read #	SNP %	Read #	SNP %	Read #
1	TNA 1	0.0	0	16.0	11,849	35.4	26,230	11.6	8,588	36.2	26,874
2	TNA 1	10.8	5,061	43.4	20,336	11.9	5,574	18.6	8,725	7.7	3,586
3	TNA 1	19.7	9,061	47.6	21,875	43.7	20,074	0.0	0	6.6	3,050
4	TNA 1	0.0	0	20.1	4,209	24.2	5,062	0.0	0	42.7	8,961
5	TNA 1	0.0	0	29.0	15,390	69.0	36,613	0.0	0	0.0	0
6	TNA 1	39.3	32,316	0.0	0	39.1	32,091	32.2	26,462	26.5	21,780
7	TNA 1	0.0	0	0.0	0	12.3	3,015	0.0	0	87.1	21,323
8	TNA 2	0.0	0	0.0	0	98.4	43,190	0.0	0	0.0	0
9	TNA 2	15.7	18,388	0.0	0	31.3	36,706	36.1	42,330	31.5	36,967
10	TNA 2	0.0	0	4.6	2,863	52.6	32,466	5.5	3,374	35.6	22,009
11	TNA 2	16.7	7,892	76.8	36,388	16.6	7,856	0.0	0	6.2	2,941
12	TNA 2	0.0	0	8.3	4,359	39.0	20,359	0.0	0	21.5	11,233
13	TNA 2	26.0	7,247	66.6	18,614	32.7	9,133	0.0	0	0.0	0

C. Positive Control



Sample		37N AAT		134H CAT		167Y TAC		200Y TAC		368A GCT	
ID	Aliquot	SNP %	Read #	SNP %	Read #	SNP %	Read #	SNP %	Read #	SNP %	Read #
1	NA	0.0	0	15.0	6,948	44.1	20,449	0.0	0	0.0	0
2	NA	0.0	0	13.3	6,659	33.6	16,774	0.0	0	0.0	0
3	NA	0.0	0	25.8	11,498	36.4	16,239	0.0	0	0.0	0

Fig. 3. Oxford Nanopore amplicon sequencing of the near-full length *A. caninum* isotype-1 β-tubulin gene
Panel A: Schematic representation of the *A. caninum* isotype-1 β-tubulin gene model. The vertical red bars on exons 4 and 5 indicate the positions of codons 134, 167, 198 and 200 respectively
Panel B: An amplicon corresponding to the near-full length *A. caninum* isotype-1 β-tubulin gene was successfully generated from the TNA re-extraction 1 and TNA re-extraction 2 preparations in 7/10 replicate PCR reactions (indicated by the orange dots at the base of the chart) and 6/10 replicate PCR reactions (indicated by the blue dots at the base of the chart) respectively. Each of these amplicons was deep sequenced using Oxford Nanopore Technologies (ONT) sequencing. Since the error rate of ONT sequencing is too high to fulfill the minimum requirements for the DADA2 model, it was not possible to generate ASVs for the 3547 bp amplicon. Consequently, SNPs were detected at each nucleotide position, relative to the *A. caninum* isotype-1 β-tubulin reference sequence (DQ459314.1) using Minimap2 (2.28-r1209) (Li, 2018, 2021) and their frequencies calculated with Bam-readcount (0.8) (Khanna et al., 2022). The histogram chart shows the frequencies all the non-synonymous SNPs detected in the *A. caninum* isotype-1 β-tubulin gene in each of the replicate PCRs. The table on the right shows the total number of reads mapping to the reference *A. caninum* isotype-1 β-tubulin sequence and the frequencies of each of the non-synonymous SNPs ordered as in the chart.
Panel C: An amplicon corresponding to the near-full length *A. caninum* isotype-1 β-tubulin gene was generated from a positive control sample. This single positive control pooled sample was made from individual TNA samples prepared from 12 different hookworm positive canine fecal samples from Orlando, Florida prepared within 1 week of fecal collection. The amplicon was successfully generated from 3/3 replicate PCR and the Q134H(CAA > CAT) or F167Y(TTC > TAC) SNPs detected at similar frequencies in each case.

position, relative to the *A. caninum* isotype-1 β-tubulin reference sequence (DQ459314.1) using Minimap2 (2.28-r1209) (Li, 2018, 2021) and their frequencies calculated with Bam-readcount (0.8) (Khanna et al., 2022). For the TNA1 and TNA2 fecal re-extraction preparations respectively, the Q134H(CAA > CAT) SNP was detected in 5/7 and 4/6 PCR replicates (frequency range 4.6-76.8%), the F167Y (TTC > TAC) SNP was detected in 7/7 and 6/6 PCR replicates (frequency range 11.9-98.4%) and the F200Y(TTC > TAC) SNP in 3/7 and 2/6 PCR replicates (frequency range 5.5-36.1%) (see Fig. 4).

Additional non-synonymous SNPs, outside of the known canonical benzimidazole resistance codons, were also detected. For the TNA1 and TNA2 fecal re-extraction preparations respectively, a S37N(AGT > AAT) SNP (exon 2) was detected in 3/7 and 3/6 PCR replicates (frequency range 10.8- 39.3%) and a V368A (GTT > GCT) SNP (exon 8) in 6/7 and 6/8 (frequency range 7.7-87.1%). No canonical benzimidazole resistance SNPs were detected at codon 198 in any of the replicate PCR amplicons.

As described above, there was significant variability between replicate PCRs applied to the two TNA fecal re-extraction preparations both in terms of success of amplicon generation and in allelic dropout for both the Illumina and ONT amplicon deep sequence data. Consequently, we applied ONT amplicon sequencing of the near full-length *A. caninum* isotype-1 β-tubulin gene to a TNA preparation made from fresh fecal material as a comparative control. Triplicate PCRs and ONT sequencing were applied to a TNA sample comprising of a pool of 12 individual TNA preparations from canine fecal samples from Orlando, Florida stored for less than 7 days at 4 °C prior to TNA preparation. These individual samples had previously been shown by QPCR to be positive for the 134H (CAT) and 167Y(TAC) SNPs and -ve for any other canonical benzimidazole resistance SNPs at codons 198 or 200. Amplicons were generated

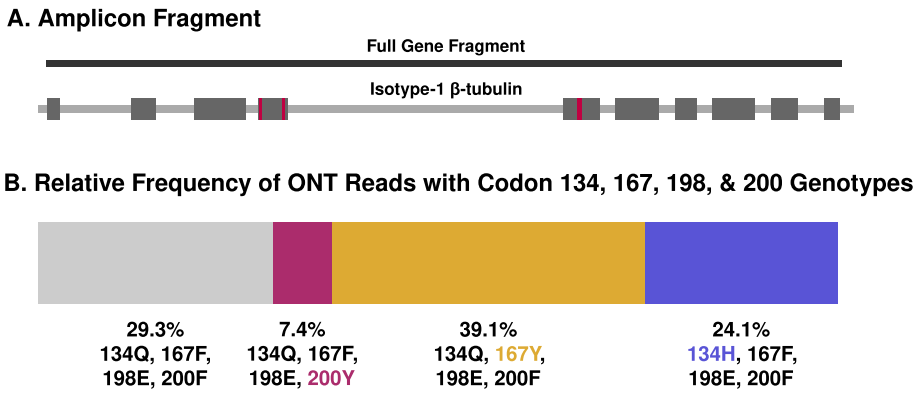


Fig. 4. Haplotypic relationship of the 134H(CAT), 167Y(TAC) and 200Y(TAC) benzimidazole resistance SNPs as determined by ONT sequencing of the near full-length isotype-1 β-tubulin gene.
The ONT sequence reads from the TNA re-extraction 1 and 2 preparations that aligned to the *A. caninum* isotype-1 β-tubulin reference sequence (DQ459314.1) were converted to multiple sequence aligned FASTA format with gofasta (1.2.1) (Jackson, 2022). Each read was then annotated with its genotype at codons 134, 167, 198, and 200 with an R script using tidyverse (1.1.5) and Biostrings (2.72.0) packages and reads with the same resistance genotype at the multiple codon positions were collapsed into a single haplotype. The bar shows the relative proportions of ONT sequence reads (as percentages) with the different codon 134, 167,198 and 200 genotypes. The resistance genotypes are shown in red, yellow and blue text for codons 200, 167 and 134 respectively and susceptible genotypes shown in black text.

and the 134H(CAT) and 167Y(TAC) SNPs were detected in 3/3 replicate amplicons from the pooled TNA sample at consistent frequences between the replicates (frequency ranges 15 - 25.8% and 33.6 - 44.1% respectively). The S37N(AGT > AAT) and V368A(GTT > GCT) SNPs were not detected in this pooled TNA control from the USA.

3.4. The Q134H(CAA > CAT), F167Y(TTC > TAC), and F200Y(TTC > TAC) isotype-1 β-tubulin SNPs are on separate haplotypes in the A. caninum population

The generation of ONT sequence reads spanning all three codons provided us with an opportunity to investigate whether the Q134H(CAA > CAT), F167Y(TTC > TAC), and F200Y(TTC > TAC) SNPs were located on the same haplotype (in cis) or on different haplotypes (in trans). The error rates of ONT sequencing precluded us from generating ASVs with DADA2 for this purpose. Consequently, we used a similar approach recently described for ONT sequence haplotype analysis in *Plasmodium* (Hosch, 2024). Each aligned read was annotated with its genotype at codons 134, 167, 198, and 200, and reads with the same genotype at the 4 codon positions were collapsed into a single haplotype. The Q134H (CAA > CAT), F167Y(TTC > TAC), and F200Y(TTC > TAC) resistance SNPs were found to be on separate haplotypes with mean overall frequencies of 24.1%, 39.1%, and 7.4% respectively (Fig. 3). No haplotypes carrying more than one of the three resistance SNPs were detected showing each of the three benzimidazole resistance mutations were in different haplotypes.

4. Discussion

Molecular diagnostics of companion animal gastrointestinal parasites is a rapidly advancing field and is set to revolutionize both clinical diagnostics and research. The canine KeyScreen™ GI Parasite PCR Test (Antech Diagnostics, Inc.) detects 20 different canine gastrointestinal parasite species and includes a combined detection of the Q134H(CAA > CAT) and F167Y(TTC > TAC) benzimidazole resistance SNPs (Jimenez Castro et al., 2025; Leutenegger et al., 2023b). In this paper, we present a detailed characterization of the benzimidazole resistance alleles present in a single parasite population originally detected by the KeyScreen™ PCR panel in a diagnostic submission from a Golden Retriever breeding kennel in Sao Paulo, Brazil.

There are two important discussion points resulting from this work. Firstly, the detection of three benzimidazole resistance mutations, present on different sequence haplotypes, in a single *A. caninum* population. This is a unique finding to date, likely the consequence of a high selection pressure environment, and the practical implications of this are discussed below. Secondly, the work illustrates the value of integrating multiple methodologies to confirm anthelmintic resistance SNPs, and characterise resistance alleles, when only archived fecal stool material of sub-optimal quality is available for investigation. There are some important lessons to consider when interpreting deep amplicon sequencing data from samples of this type which will be discussed.

4.1. The detection of three genetically independent benzimidazole resistance mutations in a single A. caninum population and the implications for anthelmintic drug use in kenneled dog communities

A. caninum infection and the presence of three canonical benzimidazole resistance SNPs - Q134H(CAA > CAT), F167Y(TTC > TAC), and F200Y(TTC > TAC)- were initially detected by targeted qPCR assays. Given the unusual finding of three different benzimidazole resistance SNPs in a single *A. caninum* population, we sought to confirm the original qPCR results using deep amplicon sequencing. There was the additional challenge of having to work with fecal material archived for several months and consequent low quality DNA preparations.

We first used ITS-2 Illumina metabarcoding to confirm that the *A. caninum* infection was monospecific with no other hookworm species present in the sample. The presence of different three canonical benzimidazole resistance SNPs at codons Q134H(CAA > CAT), F167Y(TTC > TAC), and F200Y(TTC > TAC) in this monospecific infection was then confirmed by both short-read Illumina and long-read ONT amplicon sequencing. The deep amplicon sequencing also provided additional genetic information. Firstly, it showed that the three resistance SNPs each occurred on separate haplotypes (i.e. in trans). The Illumina sequence revealed the exon 4 Q134H(CAA > CAT) and F167Y(TTC > TAC) benzimidazole resistance SNPs were located on separate ASVs but, due to the need to generate a separate amplicon spanning exon 5 for short read sequencing, it could not determine the genetic relationship with F200Y(TTC > TAC) SNP. Consequently, ONT deep amplicon sequencing of the near full-length isotype-1 β-tubulin gene was performed and revealed the F200Y(TTC > TAC) SNP was present on a separate haplotype to the other two SNPs. The "trans" relationship of the three benzimidazole resistance SNPs in all the ONT amplicon sequence reads generated suggests separate origins rather than haplotypic co-occurrence.

The discovery of three different, genetically independent benzimidazole resistance SNPs in *A. caninum* from a breeding kennel in Sao Paulo,

Brazil, is concerning since puppies are being distributed into the wider community from this establishment. Although information on the treatment regime for this particular breeding kennel was not available, the high transmission found in such environments typically leads to puppies being dewormed weekly up to three months of age, then once every three weeks until six months, and monthly thereafter even as adults (Jimenez Castro et al., 2019, 2022). This situation is similar to that suggested as the reason behind the widespread occurrence of MADR *A. caninum* in pet dogs across the USA. In that case, intensive anthelmintic drug use in greyhound kennels, followed by the rehoming of racing greyhounds, is proposed to have led to distribution of MADR *A. caninum* into the wider environment and the pet dog community across the country (Jimenez Castro et al., 2020; Venkatesan et al., 2023). Consequently, our findings suggest the potential for an analogous scenario in Sao Paulo, Brazil particularly since the transmission of *A. caninum* is known to be high in that region particularly in urban areas where stray dogs, public spaces, and soil contamination contribute to the parasite's transmission cycle. For example, studies have reported infection rates of up to 39% in domestic dog populations from environmental surveys in cities such as São Paulo and Rio de Janeiro (Ugalde et al., 2023).

The identification of the F200Y(TTC > TAC) SNP in a canine hookworm sample from Sao Paulo, Brazil, is also noteworthy in itself. Although this SNP is widespread in many ruminant strongylid nematode species, such as *Haemonchus contortus,* it was undetected in a deep amplicon sequencing study of 625 hookworm-positive samples from pet dogs across the USA (Venkatesan et al., 2023). In fact, this SNP has only ever been previously reported in *A. caninum* once before in a study conducted over 10 years ago from Minas Gerais, a region ~600 km North of São Paulo. In that study, 234 adult worms were collected from naturally infected dogs, including 110 worms from Piauí and 124 from Minas Gerais (Brazilian states approximately 2,300 km apart).The F200Y(TTC > TAC) SNP was detected in just one worm from Minas Gerais, using an amplification refractory mutation system (ARMS-PCR) assay, representing a very low prevalence (0.8%). Interestingly, no benzimidazole resistance SNPs were identified at codons 134 or 167 in that study (Furtado et al., 2014). The finding of this F200Y(TTC > TAC) resistance SNP in a second Brazilian population suggests it could be more widespread in Brazil than previously assumed which is worthy of further investigation through larger scale surveys. Furthermore, our finding suggests that although this F200Y(TTC > TAC) SNP has not yet been found in the USA, its incorporation into diagnostic assays and surveillance studies maybe be important in the future and already in other countries.

The use of ONT deep amplicon sequencing allowed us to scan the whole *A. caninum* isotype-1 β-tubulin gene for non-synonymous SNPs additional to the known canonical benzimidazole resistance SNPs at codons Q134H(CAA > CAT), F167Y(TTC > TAC), F200Y(TTC > TAC). We detected two such SNPs; S37N(AGT > AAT) and V368A(GTT > GCT). At present, the significance of these SNPs with respect to benzimidazole resistance is unknown. Interestingly, these were absent from the pooled control samples from Orlando, Florida, and it will be interesting to screen larger numbers of samples from Brazil, USA, and other regions to assess how widely these additional non-synonymous SNPs are distributed. To our knowledge, there have been no previous reports of the S37N(AGT > AAT) SNPs in any nematode β-tubulin or any suggestion that it is associated with benzimidazole resistance in any other organism. Interestingly, non-synonymous mutations have been previously described at codon 368 in *H. contortus*; specifically, Ile368Val or Ile368Leu in ivermectin resistant isolates (de Lourdes Mottier and Prichard, 2008). This suggests this codon is tolerant of amino acid substitutions in the otherwise highly conserved isotype-1 β-tubulin polypeptide. It would be interesting to determine the impact of these substitutions on the benzimidazole resistance phenotype of *Caenorhabditis elegans* using CRISPR-Cas-9 gene editing, in future work.

4.2. The interpretation of deep amplicon sequence data when used to detect anthelmintic resistance SNPs from archived fecal stool DNA

The aim of this work was to use deep amplicon sequencing to further investigate an unusual qPCR finding that suggested the presence of three different benzimidazole resistance SNPs in a single *A. caninum* population. However, the only material available for this work was fecal stool stored at 4 °C for a prolonged period of time. qPCR is highly sensitive and can successfully detect target even from complex mixtures of low amounts of degraded template DNA. However, targeted deep sequencing first requires the generation of amplicons by conventional end-point PCR prior to library preparation. Consequently, it is less tolerant of low amounts and quality of template DNA and of inhibitor molecules often present in fecal material.

Total Nucleic Acid (TNA) preparations were prepared from the archived fecal stool material after 99 days and 176 days storage (re-extractions TNA 1 and 2 respectively) (Fig. 1). Multiple replicate PCRs were performed to generate amplicons for Illumina and ONT deep sequencing. Amplicons were only successfully generated by some of the PCR replicates reflecting the poor quality of the template DNA prepared from the archived fecal material. All three resistance SNPs were detected in multiple replicate PCR amplicons by both sequencing methods confirming their presence in this single *A. caninum* population (Figs. 2 and 3). However, for the Illumina sequencing there was a high degree of variability in the ASV profiles generated between replicate amplicons demonstrating significant allelic dropout, including of those ASVs carrying the resistance SNPs (Fig. 2). Similarly, for the ONT sequencing the frequencies of the resistance SNPs varied considerably between replicate amplicons again indicating significant allelic dropout from the low-quality template DNA (Fig. 3B). This latter result contrasted with the much more consistent detection of the codon 134 and 167 resistance SNPs, at frequencies similar between replicates, from control template DNA which had been freshly prepared from canine fecal material within 8 days of collection (Fig. 3C).

These results illustrate the challenges of applying deep amplicon sequencing to template DNA prepared from archived fecal samples where significant DNA degradation may have occurred. It also illustrates the care that needs to be taken when interpreting deep amplicon sequencing data from such samples. For example, both negative results and specific allelic frequencies should be treated with caution, particularly if sufficient replication has not been performed. Accordingly, when analysing archived fecal material, multiple independent replicate PCRs should be performed wherever possible, and negative results should be interpreted cautiously. Nevertheless, the results show the potential to successfully use deep amplicon sequencing on even poor-quality DNA to confirm the presence of anthelmintic resistance SNPs and investigate their haplotypic relationships.

5. Conclusion

In summary, we have identified three independent benzimidazole resistance mutations in a single *A. caninum* population from a Golden Retriever breeding establishment in Sao Paulo Brazil. This provides a concerning example of the risk of the emergence of anthelmintic-resistant canine hookworms in environments where transmission and drug selection are high. The work also highlights the value of using next-generation amplicon sequencing to further investigate diagnostic results, such as commercial PCR tests, in companion animal parasitology. However, it also raises some challenges of working with archived fecal material and the need for careful quality control, replication and data interpretation.

CRediT authorship contribution statement

Mahya Dini: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation,

Formal analysis, Data curation, Conceptualization. **Rebecca Chen:** Writing – original draft, Software, Data curation. **Elizabeth Redman:** Methodology. **Christian M. Leutenegger:** Writing – review & editing, Methodology, Funding acquisition. **Jeffrey Tereski:** Methodology. **Samantha Loo:** Methodology. **Christian Savard:** Methodology. **Pablo D. Jimenez Castro:** Writing – review & editing, Methodology. **Samantha Ive Miyashiro:** Methodology. **John Gilleard:** Writing – review & editing, Validation, Supervision, Resources, Investigation.

Declaration of competing interests

Christian M. Leutenegger, Jeffrey Tereski, Samantha Loo, and Pablo D. Jimenez Castro are affiliated with Antech Diagnostics (Mars Petcare Science & Diagnostics), and Christian Savard is affiliated with Biovet Inc. (an Antech Diagnostics, Mars Petcare Science & Diagnostics company). This study received financial support from Antech Diagnostics. The remaining authors declare no other competing interests.

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Data availability

Raw sequencing reads have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA1422157 (BioSample SAMN55298341).

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