# Ambient temperature storage in DESS supports molecular studies of benzimidazole resistance from canine hookworm eggs

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**Abstract**

Emerging benzimidazole resistance in canine hookworms poses a growing concern for veterinary and public health. Molecular diagnostics targeting β-tubulin gene mutations are essential for resistance surveillance but traditionally rely on refrigerated faecal samples. This study evaluates dimethyl sulfoxide, EDTA, and saturated NaCl (DESS) as a room-temperature preservation medium for canine faeces. Using ITS-2 rDNA and *tubb-1* amplicon metabarcoding, we compared DNA integrity and diagnostic performance of DESS-preserved samples (4, 28, and 106 days) to refrigerated controls. No significant differences in PCR amplification or sequencing outcomes were observed. DESS enabled reliable detection of hookworm species and resistance-associated SNPs, including F167Y in *tubb-1*, with mutation frequencies consistent across treatments. Therefore, DESS can preserve samples from remote settings without cold chain logistics. Our findings validate DESS as a robust alternative for sample collection for molecular parasitology, facilitating expanded surveillance of anthelmintic resistance in field conditions.

Key words:

DESS, EDTA, DMSO, *Ancylostoma*, *Uncinaria*, MADR, Multi-anthelmintic drug resistance, nemabiome, SNP, tubulin

41 **Highlights**

42 1. DESS preserves canine faeces for DNA analysis without refrigeration.

43 2. Hookworm species and resistance SNPs detected reliably from DESS samples.

44 3. PCR and NGS results from DESS samples match refrigerated controls.

45 4. F167Y mutation found in *A. caninum* and *U. stenocephala* samples.

46 5. DESS enables remote hookworm surveillance without cold chain logistics.

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## 1. Introduction

Canine hookworms, *Ancylostoma caninum* and to a lesser extent *Uncinaria stenocephala*, are frequent and impactful gastrointestinal parasites of domestic dogs worldwide (Hawdon and Wise, 2021; Evason et al., 2023). In addition, they are both zoonotic (Bloomquist and Elston, 2024). Hookworm infections pose a serious health concern in both human and veterinary medicine, as they can cause potentially fatal disease in young dogs, and lead to conditions such as cutaneous larval migrans and eosinophilic enteritis in humans (Hawdon & Wise 2021). Treatment of canine hookworm relies on three key anthelmintic classes: benzimidazoles, avermectins and tetrahydropyrimidines (Geary et al., 2025; Sonmez et al., 2025). Benzimidazoles are the most widely accessible drugs for hookworm management in veterinary medicine, and any emerging resistance must be considered within the broader One Health context (Hawdon and Wise, 2021; Evason et al., 2023). Compelling evidence now demonstrates benzimidazole resistance in *A. caninum* infecting dogs in North America and eastern Australia (Venkatesan et al., 2023; Abdullah et al., 2025). Moreover, reports of multi-anthelmintic drug resistant *A. caninum* are increasing in the USA (Jimenez Castro et al., 2019; Kitchen et al., 2019; Geary et al., 2025).

For benzimidazoles, known mutations in the isotype-1 β-tubulin gene (*tubb-1*) lead to drug failure, and screening for their presence and frequency can identify populations and individuals where benzimidazoles are likely to be ineffective or their use is not warranted. For other drug classes, however, no specific molecular tests are currently available (Geary et al., 2025). Screening for hookworm *tubb-1* mutations is currently possible through in house amplicon metabarcoding using Illumina sequencing or even via commercially available diagnostic tests in the USA (Geary et al., 2025). Traditionally, this testing relies on fresh faecal samples, with concentrated eggs subjected to DNA isolation (Venkatesan et al., 2023; Abdullah et al., 2025). To keep hookworm eggs intact for concentration via flotation techniques, faecal samples must be

74 refrigerated to prevent embryonation. This requirement poses challenges in regions with limited
75 access to refrigeration and creates logistical constraints when cooled samples need to be
76 shipped. To address the need for refrigeration, we explored the use of faecal preservation with
77 dimethyl sulphoxide, EDTA, and saturated NaCl (DESS), which has previously been shown to
78 maintain DNA integrity in nematodes (Yoder et al., 2006; Beknazarova et al., 2017; Gonzálvez et
79 al., 2022). Originally developed to preserve avian tissues at room temperature, DESS is now
80 widely used across diverse specimens for genomic analyses and has been shown to outperform
81 storage in ethanol for DNA preservation (Seutin et al., 1991; Oosting et al., 2020).

82 This study aimed to evaluate the use of DESS solution for preserving canine faecal samples
83 at ambient temperature for molecular analysis of presumed strongyle/hookworm-like eggs. We
84 applied strongyle species identification using 'nemabiome' ITS-2 rDNA amplicon metabarcoding
85 and targeted hookworm beta-tubulin isotype 1 (*tubb-1*) assays to assess whether samples stored
86 in DESS for 4, 28 and 106 days yielded equivalent results to refrigerated canine faecal samples.

87

88 **2. Material and Methods**

89 **2.1. Samples and samples processing**

90 The use of residual clinical samples was in accordance with the University of Sydney
91 Animal Ethics Committee Protocol (2024/2508). Faecal samples (n=31) were provided by
92 registered veterinary practitioners in accordance with the Veterinary Practice and Animal
93 Welfare Acts for the purpose of diagnostics used in decision-making by the veterinary
94 practitioner. Samples were submitted for parasitological diagnostics; refrigerated residual
95 faecal samples that previously returned a positive result for hookworms at the clinical diagnostic
96 laboratory were received for molecular species characterisation. Material used for hookworm

97 species identification and benzimidazole susceptibility, and subsequent results were de-
98 identified.

99 Refrigerated samples received from diagnostic laboratories were partitioned into (i) 3 g for
100 faecal egg counting and egg concentration, and (ii) 3 g for preservation in DESS. Faecal egg
101 counts were performed with saturated salt (NaCl, specific gravity = 1.21) solution using Whitlock
102 Universal McMaster 4 chamber worm egg counting slide at the University of Sydney and eggs
103 concentrated and collected as previously described from the 3 g of faeces (Abdullah et al., 2025).
104 The limit of detection was 40 eggs per gram and the resultant egg pellet was stored at -20 °C until
105 further use or immediately processed for DNA isolation. DESS was prepared according Seutin et
106 al. (1991). We used the following chemicals: dimethyl sulfoxide (DMSO, cat. no. 2225, Ajax
107 Finechem), EDTA disodium salt (cat.no. 180, Ajax Finechem), sodium chloride (NaCl analytical
108 grade, cat. no. 465, Ajax Finechem) and sodium hydroxide (NaOH, analytical grade, cat. no. 482,
109 Ajax Finechem). DESS is 0.25M disodium EDTA pH 8.0 (pH adjusted using NaOH), 20% DMSO
110 and NaCl added to saturation. DESS was aliquoted into 15 mL tubes each containing 10 mL of
111 DESS. Faeces to be preserved in DESS were transferred (3 g) into the tube, vigorously shaken and
112 stored at room temperature (23-24 °C), aliquot was subsampled on day 4, day 28 and day 106.

113 **2.2. DNA isolation**

114 Genomic DNA was isolated using Isolate II Fecal DNA Kit (Meridian Bioscience, Australia).
115 DESS-preserved samples were homogenised by inverting the 15 mL tube with faeces in DESS
116 multiple times, then immediately a 1.5 mL aliquot was aspirated and transferred into 2 ml bead-
117 beating tube from Isolate II Fecal DNA Kit (Meridian Bioscience, Australia) and centrifuged at
118 3,000 ×g for 5 minutes. The supernatant was discarded and pellet flooded with 750 µL of lysis
119 buffer from the DNA kit. The pellet with lysis buffer was homogenised using a high-speed
120 benchtop homogeniser, FastPrep-24 (MP Biomedicals, Australia), for 40 s at 6.0 m/s. For
121 concentrated strongyle/hookworm-like eggs in 1.5 mL tube were first flooded with 750 µL of lysis

122 buffer and homogenised by repeated aspiration with a pipette, then all the contents were
123 transferred into bead-beating tube from Isolate II Fecal DNA Kit (Meridian Bioscience, Australia)
124 and the eggs disrupted and homogenised using FastPrep-24 using setting as described above.
125 Blank samples that only contained the lysis buffer we included throughout the process. Purified
126 genomic DNA samples and blanks were isolated into 100 µL of elution buffer (10 mM Tris-Cl, pH
127 = 8.5) and stored at −20 °C until further use.

128 **2.3. Amplification and comparison**

129 All isolated DNA was subjected to two independent real-time PCR amplifications. ITS-2
130 rDNA (ITS-2) was amplified with the NC-1 and NC-2 primers designed by Gasser et al. (1993), and
131 isotype-1 β-tubulin region BZ167 was amplified using primers developed by Jimenez Castro et al.
132 Jimenez Castro et al. (2019), which include key diagnostic SNPs conferring benzimidazole
133 resistance. All primers were adapted for Illumina metabarcoding using Next Generation
134 Sequencing (NGS) as previously described (Stocker et al., 2023; Abdullah et al., 2025). All PCR
135 reactions utilised SYBR-chemistry using SensiFAST™SYBR® No-ROX mix (Meridian Bioscience,
136 Australia). All PCR reactions were prepared using a Myra robotic liquid handling system (Bio
137 Molecular Systems, Australia). Each real-time PCR mix (25 µL) included 2 µL of template DNA
138 (neat) and primers at final concentrations of 400 nM. Reactions were run on a CFX Opus 96 Real-
139 Time PCR System with corresponding CFX Manager software (BioRad, Australia) to obtain cycle
140 threshold values (Ct). The protocol involved an initial denaturation at 95 °C for 3 min, followed by
141 up to 35 cycles of 95 °C for 5 s, 57 °C for 15 s, 72 °C for 15 s, and a final melt curve analysis. Each
142 run included a no-template control (dd$H_2O$) to monitor for potential contamination. Ct>35
143 samples were recoded as 35. Ct values for all samples were tabulated and analysed in GrapPad
144 Prism v10.4.1 (GraphPad Software). Data were analysed separately for ITS-2 and BZ167 assays.
145 Data passed tests for Gaussian normal distribution (D'Agostino-Pearson and Shapiro-Wilk tests).
146 Paired t-test was run for raw concentrated egg Ct compared to Ct from samples stored in DESS

147 for 4 days. Repeated measure (RM) one-way ANOVA with Geisser-Greenhouse correction and
148 Tukey's multiple comparison test was run for all treatments (raw concentrated, DESS 4 days,
149 DESS 28 days, DESS 106 days) per assay. RM one-way ANOVA was rerun within the data that has
150 potentially ambiguous data coded as Ct=35. The alfa (α) was set at 0.05.

151 **2.4. Next generation sequencing and data processing**

152 Amplicons (ITS-2 and BZ167) for raw concentrated, DESS 4 days and DESS 28 days as
153 described above were prepared in 96 well plate format and submitted for indexing, purification
154 and amplicon sequencing. In addition, for raw concentrated samples BZ200 amplicon was
155 amplified as described above using primers developed by Jimenez Castro et al. (2019) as
156 implemented previously (Stocker et al., 2023; Abdullah et al., 2025). The next generation
157 sequencing was performed at the Ramaciotti Centre for Genomics, University of New South
158 Wales, Sydney, Australia and sequenced on MiSeq i100 Plus 25M (300PE, Ilumina) and MiSeq
159 (250PE, Illumina). De-multiplexed FastQ files were generated via BaseSpace (Illumina).

160 All FastQ files were processed through R package 'dada2' v1.26.0 (Callahan et al., 2016) in
161 R v4.2.2 (R Core Team, 2022), to obtain amplicon sequence variants (ASV) and their abundance
162 per sequenced amplicon using pipeline developed previously (Abdullah et al., 2025). The pipeline
163 includes, primer removal with 'cutadapt' v 4.0 (Martin, 2011), followed by 'dada2' filtering, error
164 estimation and denoising, before paired-end reads were merged to reconstruct the full ASV and
165 removal of chimeras. The assignment of species based on ITS-2 ASVs was carried out using two
166 complimentary approaches. We used IdTaxa from 'DECIPHER' package v3.2.0 (Murali et al.,
167 2018) using ITS-2 databased from nemabiome.ca (Workentine et al., 2020) and assignTaxonomy
168 from 'dada2' using in house hookworm database. All ASVs were further imported into CLC Main
169 Workbench v25.0.1 (Qiagen Australia) and top blastn hits against 'nr' database at NCBI was
170 identified. ASVs multiple sequence alignment was subject to phylogenetic reconstruction with
171 reference ITS-2 hookworm sequences, where species identity was confirmed by manual

172 assignment of ASV to a species based on position on the phylogenetic tree relative to the
173 reference ITS-2 sequences. For the *tubb-1* data (BZ167, BZ200), the assignment to species was
174 carried as previously described (Abdullah et al., 2025). Briefly, all ASVs assignment was verified
175 in CLC Main Workbench v25.0.1 against curated set of *tubb-1* reference sequences from *A.*
176 *caninum*, *A. ceylanicum*, *A. duodenale* and *Necator americanus* from 'WormBase ParaSite'
177 (Howe et al., 2017; Bolt et al., 2018; Harris et al., 2020) and *U. stenocephala* (Stocker and
178 Šlapeta, 2025). The sequence comparison enabled us to identify the exon/intron boundary and
179 residues that code for susceptibility to benzimidazoles, amino acids amino acids Q134, F167,
180 E198 and F200 (Venkatesan et al., 2023). ASVs were then pooled according to species and raw
181 read counts were converted into relative abundance (percentages). Any species read counts with
182 less <25 reads were disregarded as spurious. Proportions of SNP for amino acids amino acids
183 Q134, F167, E198 and F200 were calculated per species for each of the amplicons of *tubb-1*
184 (BZ167, BZ200).

185 **2.5. Data availability**

186 The metadata and intermediate files for all samples are available at LabArchives
187 (https://dx.doi.org/10.25833/pxca-6w16). Raw FastQ sequence data was deposited at SRA
188 NCBI BioProject: PRJNA1300611.

189

190 **3. Results**

191 **3.1. Canine faecal samples stored in DESS solution at ambient temperature are non-inferior**
192 **to refrigerated samples using ITS2 rDNA assay targeting strongyles and *tubb-1* of**
193 **hookworms**

194 Residual diagnostic canine faecal samples (n=31), previously determined to be positive for
195 hookworm-like eggs (strongyle-type eggs) using faecal flotation technique, were used throughout

196 (Table 1). These samples were processed for molecular determination of the identity of the
197 hookworm-like eggs (ITS-2 assay) and potential carriage of the *tubb-1* alleles conferring
198 benzimidazole resistance in hookworms. Samples (n=15) were split into two treatment groups:
199 (i) for processing using centrifugal flotation to concentrate the hookworm-like eggs and
200 immediate DNA isolation, and (ii) for preserving the raw faecal samples in DESS solution and kept
201 at ambient temperature. From the DESS-preserved samples, an aliquot was collected and DNA
202 isolated on day 4, day 28 and day 106.

203 Amplification of ITS-2 from concentrated eggs' DNA (n=15) and DESS-preserved samples
204 DNA after 4 days preservation at ambient temperature (n=15) yielded a mean Ct of 23.41 (min.
205 15.67, max. 34.00, SD 5.95) and a mean Ct of 22.72 (min. 15.07, max. 34.81, SD 4.72),
206 respectively. There was no significant difference between the two sets of Ct in a paired t-test
207 (two-tailed P=0.64, t=0.48, df=14). Testing samples at 28 days and 106 days stored in DESS
208 resulted in no significant Ct difference between the concentrated eggs DNA and between any of
209 the DESS-preserved samples DNA, using RM one-way ANOVA with Geisser-Greenhouse
210 correction and Tukey's multiple comparison test. Removing ACH02, ACH04, ACH06 and ACH07
211 had no effect on the calculation, and the resulting comparisons between sample (n=11)
212 remained non-significant (Figure 1A).

213 Amplification of BZ167-product from *tubb-1* from concentrated eggs' DNA (n=15) and
214 DESS-preserved samples DNA after 4 days preservation at ambient temperature (n=15) yielded
215 a mean Ct of 28.83 (min. 23.23, max. 34.69, SD 3.84) and a mean Ct of 29.00 (min. 23.40, max.
216 34.06, SD 3.13), respectively. There was no significant difference between the two sets of Ct in a
217 paired t-test (two-tailed P=0.88, t=0.16, df=14). Similarly, Ct showed no significant
218 differences between concentrated egg DNA and DESS-preserved samples at 28 and 106 days,
219 based on RM one-way ANOVA with Geisser-Greenhouse correction and Tukey's test. Removing

220 ACH02, ACH04, ACH06, and ACH07 did not affect the outcome; comparisons among the
221 remaining samples (n = 11) remained non-significant (Figure 1B).

222 **3.2. Amplicon metabarcoding from refrigerated canine faecal samples is non-inferior in**
223 **detecting hookworm species and hookworm *tubb-1* SNP conferring benzimidazole**
224 **resistance**

225 The amplions from the ITS-2 assay were subject to deep-sequening ('nemabiome'
226 amplicon metabarcoing). For the 15 samples testes with DESS, we sequenced both the
227 ampliocns from the concentrated hookworm-like eggs DNA as well as amplicons from DNA
228 isolated from DESS stored samples for 4 and 28 days (Figure 2). In addition, we re-isolated eggs
229 from ACH06, isolated DNA and amplified ITS-2. An additional 16 suspect hookworm positive
230 samples were included (Figure 2). After quality control and taxonomic assignment, all but five
231 samples yealded >2,000 good quality sequence reads belonging to strongyles (Figure 2A). No
232 strongyle reads were produced for ACH04 samples and for one of the ACH06 samples. In
233 addition, ACH20 has only 115 strongyle reads. For the remainig samples sequening depth ranged
234 from 83,594 to 5,287 reads (Figure 2A).

235 Considering only hookworm taxonomic asignment based on ITS-2, 11 of the 15 samples
236 had hookworm sequence reads across all the treatment (eggs purification and DESS
237 preseravation) (Figure 2B). Only *A. caninum* or *U. stenocephala* hookworm sequnces were
238 detected (Figure 2C). The ACH09 was mixed infection detected in all the treatmnets (eggs
239 purification and DESS preseravation for 4 days and 28 days). The remainig ten samples had only
240 *A. caninum* sequences consistently detected in all treatments. For the remainig non-hookworm
241 or variable samples, sequences belonged to kangaroo, rabbit or sheep strongyle nametodes.
242 None of the ACH02 and ACH07 samples yealded hookworom reads, instead they were assigned
243 to varing species and genera of strongyles from kangaroos (*Rugopharynx, Monilonema, Cloacina,*
244 *Cyclostrongylus, Parazoniolaimus, Arundelia, Hypodontus, Wallabinema, Labiomultiplex*).

245 Sample ACH06 yielded variable result, besides one sample not yielding any reads, it either had
246 reads belonging to (*Graphidium strigosum*, *Trichostrongylus retortaeformis*) or hookworm (*A.*
247 *caninum*); we re-purified eggs, re-isolated DNA and re-amplification yielded reads belonging to
248 rabbit nematodes similarly to those from DESS-preserved aliquot. For ACH20, there were only
249 46/115 reads belonging to hookworm (*A. caninum*), the remaining reads matched sheep
250 nematode ITS-2 sequences.

251 We then inquired, if the *tubb-1* BZ167 amplicons were suitable for identification of
252 hookworm species and SNPs coding for benzimidazole resistance (mutations Q134R and
253 F167Y), we processed the amplicons for amplicon metabarcoding and deep-sequencing. Using
254 hookworm *tubb-1* reads at BZ167 region, the results of samples (n=15) were compared between
255 concentrated hookworm-like eggs and those preserved in DESS corroborated the ITS2 rDNA
256 results. Samples ACH02, ACH04, ACH06 and ACH07 did not contain any hookworm *tubb-1*
257 sequence reads, although the samples produced sequencing reads not matching any known
258 targets (Figure 3A). The remaining 11 samples had >2,000 sequencing reads that matched *tubb-*
259 *1* of hookworms regardless of the treatment (Figure 3B). Similarly for samples ACH16-ACH31,
260 two samples (ACH20 and ACH27) did not yield any hookworm *tubb-1* sequences, and ACH20
261 was excluded because it yielded only 275 reads (<2,000 threshold). In total, there were 24
262 samples for which *tubb-1* (BZ167 amplicon) revealed hookworms; 21 with only *A. caninum tubb-*
263 *1*, one with only *U. stenocephala tubb-1* and two with *tubb-1* from both species (Figure 3C). For
264 the co-infection sample ACH09, there were 26.3% sequencing reads belonging to *U.*
265 *stenocephala tubb-1* from the concentrated eggs samples, while 53.1% and 31.1% reads
266 belonged to *U. stenocephala tubb-1* in the samples preserved in DESS for 4 days and 28 days,
267 respectively (Figure 3C). Sample ACH17 had 91.6% reads that belonged to *U. stenocephala tubb-*
268 *1*.

269 Mutation at *tubb-1* coding for amino acid change F167Y was present in 26% (6/23) and 33%
270 (2/3) of the samples with *A. caninum* and *U. stenocephala* using the samples from concentrated
271 eggs, respectively (Figure 3D). One of the six *A. caninum* positive samples, ACH10 with 9.5% of
272 F167Y, was part of the DESS treatment; ACH10 was consistently showing F167Y mutation in the
273 DESS-preserved samples for 4 and 28 days (17.4% and 14.7%). One of the two *U. stenocephala*
274 positive samples, ACH10 with 44.4% F167Y mutation, was part of the DESS treatment and
275 consistently showed F167Y mutation allele on day 4 and 28 (48.0% and 75.3%). ACH30 had 3.7%
276 of F167Y mutation allele (Figure 3E). Only one out of the six samples had >70% of the tubb-1 *A.*
277 *caninum* F167Y (95.5%; ACH19, Table 1) (Figure 3D). Mutation at *tubb-1* coding for amino acid
278 change Q134R was absent from *A. caninum* and *U. stenocephala*, except for sample ACH01
279 preserved for 4 days in DESS with *A. caninum tubb-1* Q134R allele at 2.6% (Figure 3F). However,
280 a functionally unknown Q134R mutation in *A. caninum tubb-1* was present in multiple samples
281 (Figure 3F).

282 Dogs that were found positive for *A. caninum* were predominantly greyhounds (39%, 9/23)
283 followed by kelpies (2/23), while the remaining samples came from individual dog breeds (Table
284 1). Four greyhounds (4/9), one Shar-Pei and one French Bulldog had the *A. caninum tubb-1*
285 mutation F167Y, with the Shar-Pei (ACH19) having the highest percentage of the mutation
286 (95.5%), followed by a greyhound (ACH25, 53.4%). A Kelpie, Labrador Retriever and Bull Arab
287 cross were the breeds that were infected with *U. stenocephala*, the Kelpie (ACH09) sample had
288 the highest percentage (44-75%) of the *U. stenocephala tubb-1* mutation F167Y (Table 1).

289 All samples screened at *tubb-1* BZ200 amplicons revealed E198 for both *A. caninum* and
290 *U. stenocephala tubb-1*. The F200 residue was conserved across all *U. stenocephala tubb-1*,
291 and majority of *A. caninum tubb-1*, with the exception of rare mutation in F200S (2.8%) in sample
292 ACH18 and F200L (0.1%) in ACH10.

293

294 **4. Discussion**

295 Ensuring optimal data quality in parasitological studies requires careful evaluation of
296 preservation methods. Proper collection and storage are critical for maintaining sample integrity
297 prior to laboratory analysis, yet they often demand considerable effort from field personnel and
298 involve complex logistics to transport samples under suitable conditions. This study
299 demonstrates that preserving canine faecal samples in DESS performs comparably to
300 refrigeration, eliminating the need for a cold chain or reliance on ethanol or proprietary
301 preservatives. Samples stored in DESS remained fully compatible with standard DNA-based
302 molecular workflows, including PCR amplification and NGS-based amplicon sequencing,
303 supporting both hookworm species identification and detection of benzimidazole-resistance
304 associated SNPs.

305 Using repeated measures one-way ANOVA, we found no significant difference between
306 concentrated egg samples and faecal samples stored in DESS for up to 106 days. DESS preserves
307 DNA by deactivating metal-dependent enzymes (e.g., DNases) via EDTA, with this effect
308 enhanced by the alkaline pH (8) created by NaOH, which dissolves EDTA and provides a buffering
309 capacity. The solution is saturated with sodium chloride (NaCl) to stabilise DNA, and contains
310 DMSO which facilitates the transport of compounds (e.g., EDTA and NaCl) across cell
311 membranes while also acting as a cryoprotectant (Seutin et al., 1991). DESS has been shown to
312 perform well across a broad temperature range, including room temperature. Theoretically, we
313 expected higher Ct values for DESS-preserved samples compared to concentrated samples, as
314 the latter contained the equivalent of 3 g of eggs, whereas only one-tenth of that material was
315 used for the DESS-preserved samples. However, the observed non-inferiority may be explained
316 by material loss during the egg concentration process using saturated salt (Zajac and Conboy,
317 2011). This represents an additional benefit of DESS, as it suggests egg concentration may not
318 be necessary, potentially streamlining diagnostic workflows using sample volumes similar to

319 those in this study. Although the tested samples had relatively high egg counts (mean: 4,896 eggs
320 per gram; range: <40 to 23,280), samples with lower counts (<500 eggs per gram; ACH01, ACH05)
321 performed comparably to those with higher counts. Nonetheless, caution is warranted when
322 interpreting results from low egg count samples, as insufficient sampling could lead to
323 overinterpretation. Sample-to-sample variability or uneven egg distribution in faecal aliquots
324 was evident in mixed infection samples (ACH09, ACH10). While no studies have investigated the
325 spatial distribution of hookworm eggs in canine faeces, human studies have shown no clear
326 spatial pattern for hookworm and *Schistosoma mansoni* eggs in faecal samples (Krauth et al.,
327 2012).

328 The presence of *tubb-1* mutations in hookworm eggs from canine faecal samples does not
329 necessarily equate to clinical drug failure. However, a prevalence>75% of the F167Y mutation
330 in *tubb-1* has been correlated with resistance in the Egg Hatch Assay and in vitro benzimidazole
331 resistance, and is currently considered the threshold for predicting resistance based on this
332 mutation alone (McKean et al., 2024; Abdullah et al., 2025). One of our samples, from a Shar-Pei
333 (ACH19), carried 95% of the F167Y mutation, suggesting likely clinical resistance to
334 benzimidazole. Samples with <75% prevalence of the mutation are less likely to lead to
335 observable treatment failure, though continued benzimidazole use may select for this resistant
336 genotype within the hookworm population. In this context, attention should also be given to
337 additional mutations such as Q134H in *tubb-1*, which has been shown to confer benzimidazole
338 resistance even in the absence - or at low frequency - of the F167Y mutation in *A.*
339 *caninum* (Venkatesan et al., 2023).

340 Consistent with findings from Abdullah et al. (2025), greyhounds were the most frequently
341 hookworm-positive breed in our study. In North America, analysis of faecal samples from
342 17,671,724 individual dogs revealed that greyhounds had a significantly higher risk of hookworm
343 infection detected via faecal flotation (odds ratio = 15.3) (Burton et al., 2024). Abdullah et al.

344 (2025) also reported a crossbreed dog infected with *Ancylostoma caninum* carrying >75% of the
345 F167Y mutation, adding to evidence that benzimidazole-resistant *A. caninum* is not restricted to
346 greyhounds. Although resistance was initially regarded as a breed-specific issue, it now appears
347 to have spread beyond greyhounds. A recent North American survey of diagnostic samples found
348 that greyhounds were only the fourth most frequently infected breed with the *tubb-1* F167Y
349 mutation, with poodles, Bernese mountain dogs, and cocker spaniels showing even higher
350 prevalence (Leutenegger et al., 2024).

351 The *tubb-1* F167Y allele has recently been reported in *U. stenocephala* in Australia,
352 providing an early warning of potential benzimidazole resistance (Abdullah et al., 2025). The
353 allele was detected in a 10-week-old Labrador Retriever with an egg count of 200 (TS024 in
354 Abdullah et al., 2025), where 50.7% of the eggs carried the F167Y mutation. In this study, we
355 report two additional *U. stenocephala* samples carrying the F167Y allele: one from a 10-week-
356 old Kelpie with a mutation frequency between 44–75%, and another with a low frequency (<5%).
357 To date, six *U. stenocephala*-positive samples from southeastern Australia (New South Wales
358 and the Australian Capital Territory) and three from New Zealand have been screened for the
359 F167Y allele (this study; Stocker et al., 2023; Abdullah et al., 2025). In contrast, reanalysis of a
360 nationwide study in the USA by Venkatesan et al. (2023) identified *U. stenocephala* in two
361 individual dog faecal samples and four pooled samples, but all carried the benzimidazole-
362 susceptible F167 allele (Stocker et al., 2024). No studies have yet investigated *U.*
363 *stenocephala* in dogs from regions where it is common, such as Canada and continental Europe.
364 The presence of the *tubb-1* F167Y allele in *U. stenocephala* is a strong indicator of potential
365 clinical drug failure. Establishing in vivo evidence that benzimidazoles fail to clear *U.*
366 *stenocephala* infections carrying the F167Y allele is now urgently needed.

367 **Conclusions**

This study confirms that DESS is a robust and practical preservation medium for canine faecal samples used in molecular parasitology. It maintains DNA integrity over extended periods without refrigeration, supports PCR and NGS workflows, and enables reliable detection of hookworm species (*A. caninum* and *U. stenocephala*) as well as resistance-associated SNPs. DESS is particularly valuable for remote sampling, where immediate refrigeration is not feasible and shipment on ice is prohibitive. Its compatibility with molecular diagnostics and ability to eliminate cold chain requirements make it an important tool for expanding parasitological research and surveillance in field settings.

**CRediT authorship contribution statement**

**Yi-Jou Chen:** Investigation, Methodology, Validation, Data curation. **Vanessa Li:** Writing – review & editing, Investigation, Data curation. **Michelle Suwandy:** Investigation, Methodology, Data curation. **Ioana Bianca Mitrea:** Investigation, Methodology, Data curation. **Douglas Hayward:** Resources, Investigation, Data curation. **Susan Jaensch:** Resources, Investigation, Data curation. **Emily Kate Francis:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Jan Šlapeta:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

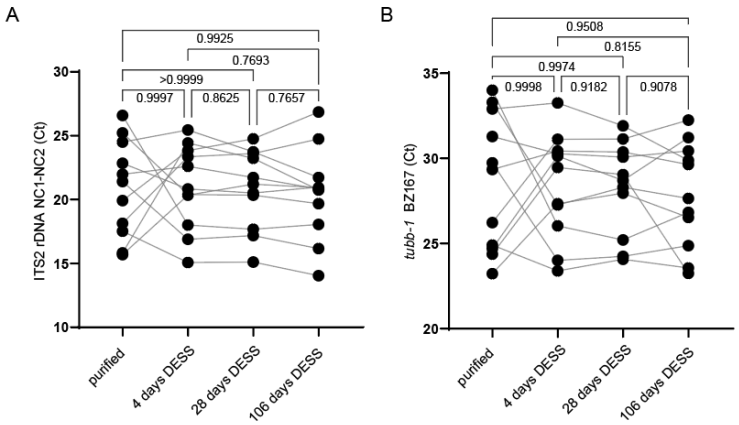
**Acknowledgements**

Funding was provided by the Betty and Keith Cook Canine Research Bequest (Sydney School of Veterinary Science, The University of Sydney, Australia), and by the UEFISCDI - Executive Agency for Higher Education, Research, Development and Innovation Funding (Romania): PN-IV-P2-2.2-MC-2024 and 131 PED. This research was in part funded by the Sydney School of Veterinary

392 Science Research & Enquiry Unit of Study 2025 fund. This work was completed in partial
393 fulfillment for the requirements of the Doctor of Veterinary Medicine degree, The University of
394 Sydney (VL).

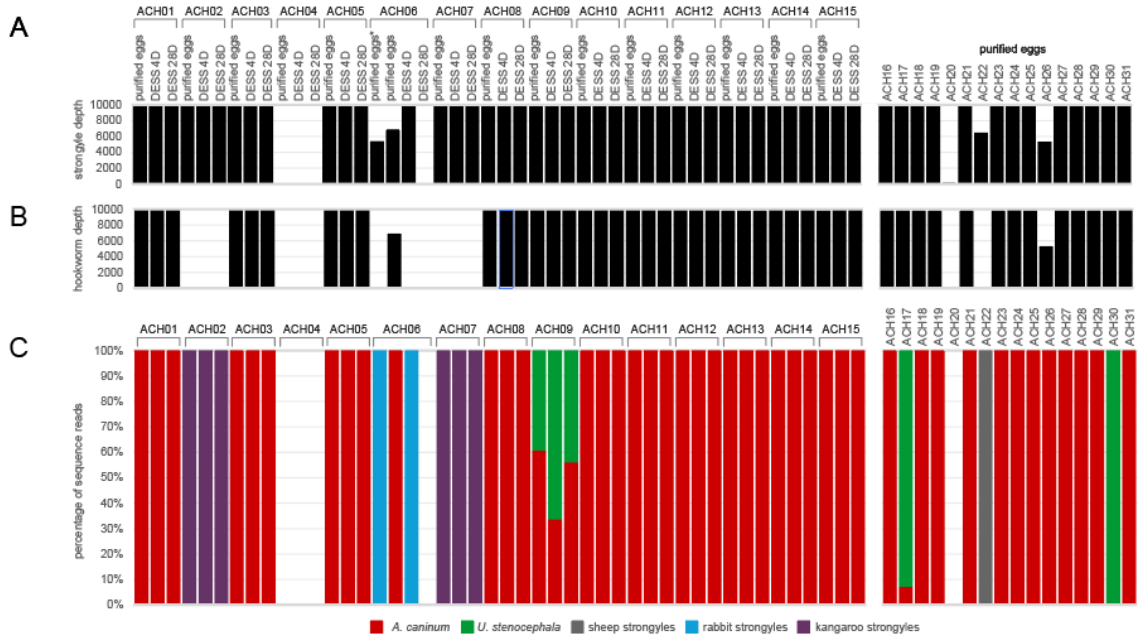
395

396 Figure legends




397

398 **Figure 1.** Real-time PCR amplifications of (A) internal transcribed spacer (ITS)-2 rDNA and (B)
399 isotype-1 β-tubulin gene (*tubb-1*) from dog faecal samples. Sampled with hookworm-like eggs
400 were amplified and cycle threshold (Ct) was recoded. The *tubb-1* amplicon BZ167 spans the
401 mutations responsible for Q134R and F167Y amino acid changes. Only samples that were
402 confirmed to contain hookworm species are shown. Each sample was processed as either
403 concentrated eggs (purified eggs) or preserved in DESS for different length of time - 4 days, 28
404 days and 106 days. Repeated measure (RM) one-way ANOVA with Geisser-Greenhouse
405 correction and Tukey's multiple comparison test demonstrated no significant difference
406 between the treatments (P>0.05).




407

408 **Figure 2.** Internal transcribed spacer (ITS)-2 rDNA molecular profiling of dog faecal samples.
409 Sampled with hookworm-like eggs were profiled using metabarcoded deep Illumina amplicon
410 sequencing at ITS-2 rDNA. ACH1-15 have been profiled as concentrated eggs (purified eggs),
411 faeces preserved in DESS for 4 days (DESS 4D) and 28 days (DESS 28D). ACH06 was reisolated
412 as purified eggs and reamplified/re-sequenced (*). Samples ACH16-31 were only processed as
413 concentrated purified eggs. (A) The bar chart representing the number of sequencing reads
414 belonging to strongyle ITS-2 sequences for each sample, the maximum upper scale is set to show
415 10,000 reads. (B) The bar chart showing sequencing reads belonging to hookworms
416 (Ancylostomatidae) ITS-2 sequences for each sample, the maximum upper scale is set to show
417 10,000 reads. (C) Percentage of strongyle species within each sample (depicted as proportion).
418 Amplicon sequence variants (ASVs) were pooled according to species of nematodes. Proportion
419 of each hookworm species (*Ancylostoma caninum*, *Uncinaria stenocephala*) and spurious
420 species is colour coded in the stacked bar chart. Samples are vertically aligned across the bar
421 charts.

422

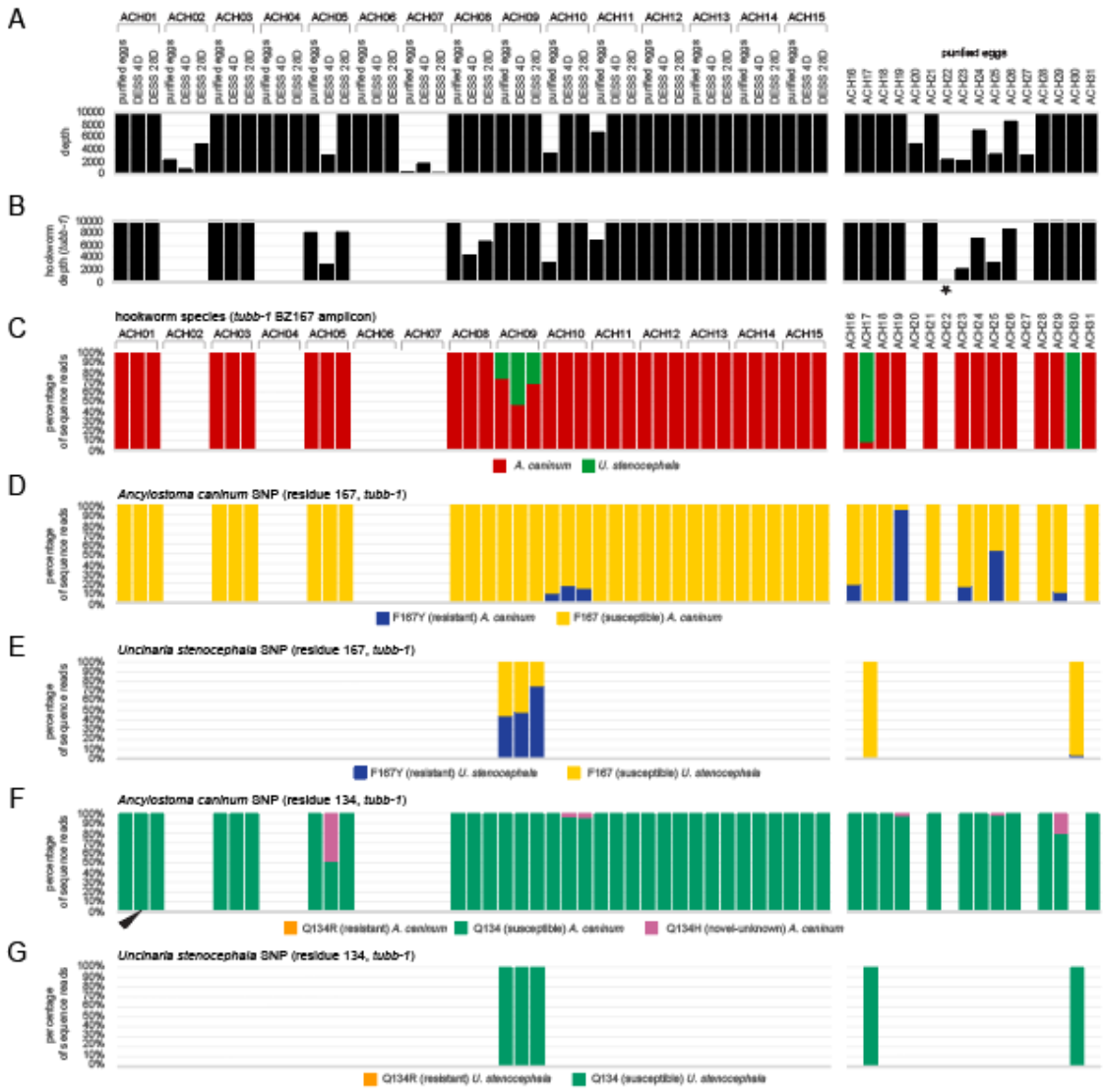



**Figure 3.** Isotype-1 β-tubulin gene (*tubb-1*) molecular profile of dog faecal samples. Sampled with hookworm-like eggs were profiled using metabarcoded deep Illumina amplicon sequencing at *tubb-1* amplicon BZ167 that spans the mutations responsible for Q134R and F167Y amino acid changes. ACH1-15 have been profiled as concentrated eggs (purified eggs), faeces preserved in DESS for 4 days (DESS 4D) and 28 days (DESS 28D). Samples ACH16-31 were only processed as concentrated purified eggs. (A) The bar chart representing the total number of sequencing reads for each sample, the maximum upper scale is set to show 10,000 reads. (B) The bar chart showing sequencing reads belonging to hookworms (Ancylostomatidae) *tubb-1* sequences for each

sample, the maximum upper scale is set to show 10,000 reads. Sample depicted with * (star) had <2,000 reads and was excluded. (C) Percentage of strongyle species within each sample (depicted as proportion) based on the *tubb-1* sequence data. Amplicon sequence variants (ASVs) were pooled according to species of nematodes. Proportion of each hookworm species (*Ancylostoma caninum*, *Uncinaria stenocephala*) is colour coded in the stacked bar chart. (D) Percentage of SNP (single nucleotide polymorphism) that codes for the mutation F167Y in *tubb-1* of *A. caninum*. (E) Percentage of SNP that codes for the mutation F167Y in *tubb-1* of *U. stenocephala*. (F) Percentage of SNP (single nucleotide polymorphism) that codes for the mutation Q134R in *tubb-1* of *A. caninum*. The arrowhead points to a small percentage of the nutation in ACH01 (DESS 4D) sample. An alternative, novel, mutation leading to Q134H is also shown. (G) Percentage of SNP that codes for the mutation Q134R in *tubb-1* of *U. stenocephala*. Samples are vertically aligned across the bar charts.

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**Table 1: Summary of dog samples with hookworm-like eggs used in this study**

| Identifier | Breed | Age | Date | EPG | Locality | *A. caninum tubb-1* (%) | | *U. stenocephala tubb-1* (%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Q134R | F167Y | Q134R | F167Y |
| ACH01* | Staghound cross | 3 yrs | February 2025 | <40 | QLD 4869 | 0, 2.6, 0 | 0, 0, 0 | n/d | n/d |
| ACH02* | Chihuahua | n/a | November 2024 | 200 | TAS 7000 | n/d | n/d | n/d | n/d |
| ACH03* | Cavoodle | n/a | November 2024 | 3,500 | NSW 2030 | 0, 0, 0 | 0, 0, 0 | n/d | n/d |
| ACH04* | Italian Greyhound | 3 yrs | November 2024 | 400 | NSW 2290 | n/d | n/d | n/d | n/d |
| ACH05* | Greyhound | 5.9 yrs | November 2024 | 240 | NSW 2620 | 0, 0, 0 | 0, 0, 0 | n/d | n/d |
| ACH06* | Beaglier | 1.2 yrs | November 2024 | 80 | NSW 2102 | n/d | n/d | n/d | n/d |
| ACH07* | Miniature Fox Terrier | 0.5 yrs | April 2020 | 80 | NSW 2570 | n/d | n/d | n/d | n/d |
| ACH08* | Kelpie | 0.2 yrs | December 2024 | 2,200 | NSW 2312 | 0, 0, 0 | 0, 0, 0 | n/d | n/d |
| ACH09* | Kelpie | 0.2 yrs | December 2024 | 1,600 | NSW 2312 | 0, 0, 0 | 0, 0, 0 | 0, 0, 0 | 44.4, 48.0, 75.3 |
| ACH10* | Greyhound | 2 yrs | February 2025 | 7,160 | ACT 2904 | 0, 0, 0 | 9.5, 17.4, 14.7 | n/d | n/d |
| ACH11* | Pug | 1 yr | January 2024 | 2,360 | NSW 2594 | 0, 0, 0 | 0, 0, 0 | n/d | n/d |
| ACH12* | Border Collie | 0.1 yr | January 2025 | 7,680 | QLD 4067 | 0, 0, 0 | 0, 0, 0 | n/d | n/d |
| ACH13* | Miniature Schnauzer | 11 yrs | January 2025 | 1,560 | NSW 2444 | 0, 0, 0 | 0, 0, 0 | n/d | n/d |
| ACH14* | Jack Russell Terrier | 5 yrs | December 2024 | 4,240 | NSW 2321 | 0, 0, 0 | 0, 0, 0 | n/d | n/d |
| ACH15* | Fox Hound | 4 yrs | December 2024 | 23,280 | QLD 4067 | 0, 0, 0 | 0, 0, 0 | n/d | n/d |
| ACH16 | Greyhound | 4 yrs | February 2025 | 160 | NSW 2795 | 0 | 18.5 | n/d | n/d |
| ACH17 | Labrador Retriever | 2 yrs | February 2025 | 125 | NSW 2711 | 0 | 0 | 0 | 0 |
| ACH18 | Doberman | 0.6 yr | March 2025 | 750 | QLD 4390 | 0 | 0 | n/d | n/d |
| ACH19 | Shar-Pei | 6.6 yrs | April 2025 | 400 | NSW 2460 | 0 | 95.5 | n/d | n/d |
| ACH20 | Greyhound | 2 yrs | April 2025 | 560 | NSW 2430 | n/d | n/d | n/d | n/d |
| ACH21 | Griffan Bruxelloxs | 2 yrs | March 2025 | 12,040 | QLD 4124 | 0 | 0 | n/d | n/d |
| ACH22 | Staffordshire Bull Terrier | 0.6 yrs | August 2024 | 440 | NSW 2621 | n/d | n/d | n/d | n/d |
| ACH23 | French Bulldog | 1 yr | September 2024 | 1,680 | NSW 2711 | 0 | 16.4 | n/d | n/d |
| ACH24 | Greyhound | 2 yrs | July 2024 | 20,000 | NSW 2460 | 0 | 0 | n/d | n/d |
| ACH25 | Greyhound | 0.6 yr | May 2024 | 240 | QLD 4570 | 0 | 53.4 | n/d | n/d |
| ACH26 | Greyhound | 1.6 yr | July 2024 | <40 | NSW 2590 | 0 | 0 | n/d | n/d |
| ACH27 | Labrador Retriever | 3.5 yrs | July 2024 | <40 | NSW 2794 | n/d | n/d | n/d | n/d |
| ACH28 | Greyhound | 1.9 yrs | May 2024 | 40 | NSW 2594 | 0 | 0 | n/d | n/d |
| ACH29 | Greyhound | 3.9 yrs | May 2024 | <40 | NSW 2460 | 0 | 10.6 | n/d | n/d |
| ACH30 | Bull Arab cross | 0.3 yr | October 2024 | 600 | NSW 2037 | n/d | n/d | 0 | 3.7 |
| ACH31 | Greyhound | adult | September 2024 | n/a | NSW 2460 | 0 | 0 | n/d | n/d |

*, samples processed as raw concentrated, DESS-preserved for 4 days and DESS-preserved for 28 days; n/a, not known; EPG, strongyle hookworm-like eggs per gram; QLD, Queensland, Australia; NSW, New South Wales, Australia; TAS, Tasmania, Australia; ACT, Australian Capital Territory, Australia; n/d, not detected.