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 isotype-1 β-tubulin gene (*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

42 **Highlights**

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

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

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

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

47 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

refrigerated to prevent embryonation. In ruminants, faeces with strongyle eggs can be also preserved by vacuum packing with storage at room temperature (Rinaldi et al., 2011). In dog hookworms the refrigeration requirement poses challenges in regions with limited access to refrigeration and creates logistical constraints when cooled samples need to be shipped. To address the need for refrigeration, we explored the use of faecal preservation with dimethyl sulphoxide, EDTA, and saturated NaCl (DESS), which has previously been shown to maintain DNA integrity in nematodes (Yoder et al., 2006; Beknazarova et al., 2017; Gonzálvez et al., 2022). Originally developed to preserve avian tissues at room temperature, DESS is now widely used across diverse specimens for genetic and genomic analyses, including preservation of high-molecular weight DNA, and has been shown to outperform storage in ethanol for DNA preservation (Seutin et al., 1991; Dawson et al., 1998; Yoder et al., 2006; Gaither et al., 2011; Beknazarova et al., 2017; Oosting et al., 2020; Sharpe et al., 2020; Gonzálvez et al., 2022).

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

2. Material and Methods

2.1. Samples and samples processing

The use of residual clinical samples was in accordance with the University of Sydney Animal Ethics Committee Protocol (2024/2508). Faecal samples (n=31, Table 1) were provided by registered veterinary practitioners in accordance with the Veterinary Practice and Animal Welfare Acts for the purpose of diagnostics used in decision-making by the veterinary

99 practitioner. Samples were submitted for parasitological diagnostics; refrigerated residual
100 faecal samples that previously returned a positive result for hookworms at the clinical diagnostic
101 laboratory were received for molecular species characterisation. Material used for hookworm
102 species identification and benzimidazole susceptibility, and subsequent results were de-
103 identified with data of samples collection, age, locality (postcode) and breed retained.

104 Refrigerated samples received from diagnostic laboratories were partitioned into (i) 3 g for
105 faecal egg counting and egg concentration, and (ii) 3 g for preservation in DESS. Faecal egg
106 counts and concentrations were performed with saturated salt (NaCl, specific gravity = 1.21)
107 solution using Whitlock Universal McMaster 4 chamber worm egg counting slide at the University
108 of Sydney and eggs concentrated and collected as previously described from the 3 g of faeces
109 via centrifugal flotation (800×g) in 50 mL Falcon-type plastic tube in saturated NaCl (Abdullah et
110 al., 2025). Faecal samples (3 g) were homogenised in 50 mL of NaCl solution and centrifuged at
111 800 × g for 5 minutes. The upper 10 mL of the supernatant was carefully transferred to a clean 50
112 mL Falcon-type plastic tube, topped up with distilled water, and centrifuged again under the
113 same conditions. Following centrifugation, the supernatant was aspirated and discarded. The
114 resulting pellet was transferred to a 1.5 mL microcentrifuge tube and washed with distilled water.
115 The limit of detection was 40 eggs per gram and the resultant concentrated strongyle/hookworm-
116 like egg pellets were stored in 1.5 mL tubes at -20 °C until further use or immediately processed
117 for DNA isolation. DESS was prepared according Seutin et al. (1991). We used the following
118 chemicals: dimethyl sulfoxide (DMSO, cat. no. 2225, Ajax Finechem), EDTA disodium salt
119 (cat.no. 180, Ajax Finechem), sodium chloride (NaCl analytical grade, cat. no. 465, Ajax
120 Finechem) and sodium hydroxide (NaOH, analytical grade, cat. no. 482, Ajax Finechem). DESS is
121 0.25M disodium EDTA pH 8.0 (pH adjusted using NaOH), 20% DMSO and NaCl added to
122 saturation. DESS was aliquoted into 15 mL Falcon-type plastic tubes each containing 10 mL of
123 DESS. Faeces to be preserved in DESS were transferred (3 g) into the 15 mL Falcon-type plastic

124 tube with 10 mL of DESS, vigorously shaken and stored at room temperature (23-24 °C), aliquot
125 (~1.5 mL of DESS with faeces) was subsampled on day 4, day 28 and day 106.

126 **2.2. DNA isolation**

127 Genomic DNA was isolated using Isolate II Fecal DNA Kit (Meridian Bioscience, Australia).
128 DESS-preserved samples were homogenised by inverting the 15 mL tube with faeces in DESS
129 multiple times, then immediately a ~1.5 mL aliquot was aspirated and transferred into 2 ml bead-
130 beating tube from Isolate II Fecal DNA Kit (Meridian Bioscience, Australia) and centrifuged at
131 3,000×g for 5 minutes. The supernatant was discarded and pellet flooded with 750 µL of lysis
132 buffer from the DNA kit. The pellet with lysis buffer was homogenised using a high-speed
133 benchtop homogeniser, FastPrep-24 (MP Biomedicals, Australia), for 40 s at 6.0 m/s. For
134 concentrated strongyle/hookworm-like eggs in 1.5 mL tube, they were first flooded with 750 µL
135 of lysis buffer and homogenised by repeated aspiration with a pipette, then all the contents were
136 transferred into bead-beating tube from Isolate II Fecal DNA Kit (Meridian Bioscience, Australia)
137 and the eggs disrupted and homogenised using FastPrep-24 using setting as described above.
138 Blank samples that only contained the lysis buffer we included throughout the process. Purified
139 genomic DNA from all samples and blanks were eluted into 100 µL of elution buffer (10 mM Tris-
140 Cl, pH = 8.5) and stored at −20 °C until further use.

141 **2.3. Amplification and comparison**

142 All DNA samples were subjected to two independent real-time PCR amplifications. ITS-2
143 rDNA (ITS-2) was amplified with the NC-1 and NC-2 primers designed by Gasser et al. (1993), and
144 isotype-1 β-tubulin region BZ167 was amplified using primers developed by Jimenez Castro et al.
145 Jimenez Castro et al. (2019), which include key diagnostic SNPs conferring benzimidazole
146 resistance. All primers were adapted for Illumina metabarcoding using Next Generation
147 Sequencing (NGS) as previously described (Stocker et al., 2023; Abdullah et al., 2025). All PCR
148 reactions utilised SYBR-chemistry using SensiFAST™SYBR® No-ROX mix (Meridian Bioscience,

149 Australia). All PCR reactions were prepared using a Myra robotic liquid handling system (Bio
150 Molecular Systems, Australia). Each real-time PCR mix (25 µL) included 2 µL of template DNA
151 (neat; from both the concentrated material and the DESS preserved material) and primers at final
152 concentrations of 400 nM. Reactions were run on a CFX Opus 96 Real-Time PCR System with
153 corresponding CFX Manager software (BioRad, Australia) to obtain cycle threshold values (Ct).
154 The protocol involved an initial denaturation at 95 °C for 3 min, followed by up to 35 cycles of 95
155 °C for 5 s, 57 °C for 15 s, 72 °C for 15 s, and a final melt curve analysis. Each run included a no-
156 template control (ddH_2O) to monitor for potential contamination. Ct>35 samples were recoded
157 as 35. Ct values for all samples were tabulated and analysed in GrapPad Prism v10.4.1
158 (GraphPad Software). Data were analysed separately for ITS-2 and BZ167 assays. Data passed
159 tests for Gaussian normal distribution (D'Agostino-Pearson and Shapiro-Wilk tests). Paired t-test
160 was run for raw concentrated egg Ct compared to Ct from samples stored in DESS for 4 days.
161 Repeated measure (RM) one-way ANOVA with Geisser-Greenhouse correction and Tukey's
162 multiple comparison test was run for all treatments (raw concentrated, DESS 4 days, DESS 28
163 days, DESS 106 days) per assay. RM one-way ANOVA was rerun within the data that has
164 potentially ambiguous data coded as Ct=35. The alfa (α) was set at 0.05.

165 **2.4. Next generation sequencing and data processing**

166 Amplicons (ITS-2 and BZ167) for raw concentrated, DESS 4 days and DESS 28 days as
167 described above were prepared in 96 well plate format and submitted for indexing, purification
168 and amplicon sequencing. In addition, for raw concentrated samples BZ200 amplicon was
169 amplified as described above using primers developed by Jimenez Castro et al. (2019) as
170 implemented previously (Stocker et al., 2023; Abdullah et al., 2025). The next generation
171 sequencing was performed at the Ramaciotti Centre for Genomics, University of New South
172 Wales, Sydney, Australia and sequenced on MiSeq i100 Plus 25M (300PE, Ilumina) and MiSeq
173 (250PE, Illumina). De-multiplexed FastQ files were generated via BaseSpace (Illumina).

174 All FastQ files were processed through R package 'dada2' v1.26.0 (Callahan et al., 2016) in
175 R v4.2.2 (R Core Team, 2022), to obtain amplicon sequence variants (ASV) and their abundance
176 per sequenced amplicon using pipeline developed previously (Abdullah et al., 2025). The pipeline
177 includes, primer removal with 'cutadapt' v 4.0 (Martin, 2011), followed by 'dada2' filtering, error
178 estimation and denoising, before paired-end reads were merged to reconstruct the full ASV and
179 removal of chimeras. The assignment of species based on ITS-2 ASVs was carried out using two
180 complimentary approaches. We used IdTaxa from 'DECIPHER' package v3.2.0 (Murali et al.,
181 2018) using ITS-2 databased from nemabiome.ca (Workentine et al., 2020) and assignTaxonomy
182 from 'dada2' using in house hookworm database. All ASVs were further imported into CLC Main
183 Workbench v25.0.1 (Qiagen Australia) and top blastn hits against 'nr' database at NCBI was
184 identified. ASVs multiple sequence alignment was subject to phylogenetic reconstruction with
185 reference ITS-2 hookworm sequences, where species identity was confirmed by manual
186 assignment of ASV to a species based on position on the phylogenetic tree relative to the
187 reference ITS-2 sequences. For the *tubb-1* data (BZ167, BZ200), the assignment to species was
188 carried as previously described (Abdullah et al., 2025). Briefly, all ASVs assignment was verified
189 in CLC Main Workbench v25.0.1 against curated set of *tubb-1* reference sequences from *A.*
190 *caninum*, *A. ceylanicum*, *A. duodenale* and *Necator americanus* from 'WormBase ParaSite'
191 (Howe et al., 2017; Bolt et al., 2018; Harris et al., 2020) and *U. stenocephala* (Stocker and
192 Šlapeta, 2025). The sequence comparison enabled us to identify the exon/intron boundary and
193 residues that code for susceptibility to benzimidazoles, amino acids amino acids Q134, F167,
194 E198 and F200 (Venkatesan et al., 2023). ASVs were then pooled according to species and raw
195 read counts were converted into relative abundance (percentages). Any species read counts with
196 less <25 reads were disregarded as spurious. Proportions of SNP for amino acids amino acids
197 Q134, F167, E198 and F200 were calculated per species for each of the amplicons of *tubb-1*
198 (BZ167, BZ200).

199 **2.5. Data availability**

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

203

204 **3. Results**

205 **3.1. Canine faecal samples stored in DESS solution at ambient temperature are non-inferior**
206 **to refrigerated samples using ITS2 rDNA assay targeting strongyles and *tubb-1* of**
207 **hookworms**

208 Residual diagnostic canine faecal samples (n=31), previously determined to be positive for
209 hookworm-like eggs (strongyle-type eggs) using faecal flotation technique, were used throughout
210 (Table 1). These samples were processed for molecular determination of the identity of the
211 hookworm-like eggs (ITS-2 assay) and potential carriage of the *tubb-1* alleles conferring
212 benzimidazole resistance in hookworms. Samples (n=15) were split into two treatment groups:
213 (i) for processing using centrifugal flotation to concentrate the hookworm-like eggs and
214 immediate DNA isolation, and (ii) for preserving the raw faecal samples in DESS solution and kept
215 at ambient temperature. From the DESS-preserved samples, an aliquot was collected and DNA
216 isolated on day 4, day 28 and day 106.

217 Amplification of ITS-2 from concentrated eggs' DNA (n=15) and DESS-preserved samples
218 DNA after 4 days preservation at ambient temperature (n=15) yielded a mean Ct of 23.41 (min.
219 15.67, max. 34.00, SD 5.95) and a mean Ct of 22.72 (min. 15.07, max. 34.81, SD 4.72),
220 respectively. There was no significant difference between the two sets of Ct in a paired t-test
221 (two-tailed P=0.64, t=0.48, df=14). Testing samples at 28 days and 106 days stored in DESS
222 resulted in no significant Ct difference between the concentrated eggs DNA and between any of
223 the DESS-preserved samples DNA, using RM one-way ANOVA with Geisser-Greenhouse

224 correction and Tukey's multiple comparison test. Removing ACH02, ACH04, ACH06 and ACH07
225 (see Section 3.2; Table 1) had no effect on the calculation, and the resulting comparisons
226 between sample (n=11) remained non-significant (Figure 1A).

227 Amplification of BZ167-product from *tubb-1* from concentrated eggs' DNA (n=15) and
228 DESS-preserved samples DNA after 4 days preservation at ambient temperature (n=15) yielded
229 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.
230 34.06, SD 3.13), respectively. There was no significant difference between the two sets of Ct in a
231 paired t-test (two-tailed P=0.88, t=0.16, df=14). Similarly, Ct showed no significant
232 differences between concentrated egg DNA and DESS-preserved samples at 28 and 106 days,
233 based on RM one-way ANOVA with Geisser-Greenhouse correction and Tukey's test. Removing
234 ACH02, ACH04, ACH06, and ACH07 did not affect the outcome; comparisons among the
235 remaining samples (n = 11) remained non-significant (Figure 1B).

236 **3.2. Amplicon metabarcoding from refrigerated canine faecal samples is non-inferior in**
237 **detecting hookworm species and hookworm *tubb-1* SNP conferring benzimidazole**
238 **resistance**

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

249 Considering only hookworm taxonomic asignment based on ITS-2, 11 of the 15 samples
250 had hookworm sequence reads across all the treatments (eggs purification and DESS
251 preseravation) (Figure 2B). Only *A. caninum* or *U. stenocephala* hookworm sequnces were
252 detected (Figure 2C). The ACH09 was mixed infection detected in all the treatmnets (eggs
253 purification and DESS preseravation for 4 days and 28 days). The remainig ten samples had only
254 *A. caninum* sequences consistently detected in all treatments. For the remainig non-hookworm
255 or variable samples, sequences belonged to kangaroo, rabbit or sheep strongyle nametodes.
256 None of the ACH02 and ACH07 samples yealded hookworom reads, instead they were assigned
257 to varing species and genera of strongyles from kangaroos (*Rugopharynx, Monilonema, Cloacina,*
258 *Cyclostrongylus, Parazoniolaimus, Arundelia, Hypodontus, Wallabinema, Labiomultiplex*).
259 Sample ACH06 yielded variable result, besides one sample not yielding any reads, it either had
260 reads belonging to (*Graphidium strigosum*, *Trichostrongylus retortaeformis*) or hookworm (*A.*
261 *caninum*); we re-purified eggs, re-isolated DNA and re-amplification yielded reads belonging to
262 rabbit nematodes similarly to those from DESS-preserved aliquot. For ACH20, there were only
263 46/115 reads belonging to hookworm (*A. caninum*), the remaining reads matched sheep
264 nematode ITS-2 sequences.

265 We then inquired, if the *tubb-1* BZ167 amplicons were suitable for identification of
266 hookworm species and SNPs coding for benzimidazole resistance (mutations Q134H and
267 F167Y), we processed the amplicons for amplicon metabarcoding and deep-sequencing. Using
268 hookworm *tubb-1* reads at BZ167 region, the results of samples (n=15) were compared between
269 concentrated hookworm-like eggs and those preserved in DESS corroborated the ITS2 rDNA
270 results. Samples ACH02, ACH04, ACH06 and ACH07 did not contain any hookworm *tubb-1*
271 sequence reads, although the samples produced sequencing reads not matching any known
272 targets (Figure 3A). The remaining 11 samples had >2,000 sequencing reads that matched *tubb-*
273 *1* of hookworms regardless of the treatment (Figure 3B). Similarly for samples ACH16-ACH31,
274 two samples (ACH20 and ACH27) did not yield any hookworm *tubb-1* sequences, and ACH20

275 was excluded because it yielded only 275 reads (<2,000 threshold). In total, there were 24
276 samples for which *tubb-1* (BZ167 amplicon) revealed hookworms; 21 with only *A. caninum tubb-*
277 *1*, one with only *U. stenocephala tubb-1* and two with *tubb-1* from both species (Figure 3C). For
278 the co-infection sample ACH09, there were 26.3% sequencing reads belonging to *U.*
279 *stenocephala tubb-1* from the concentrated eggs samples, while 53.1% and 31.1% reads
280 belonged to *U. stenocephala tubb-1* in the samples preserved in DESS for 4 days and 28 days,
281 respectively (Figure 3C). Sample ACH17 had 91.6% reads that belonged to *U. stenocephala tubb-*
282 *1*.

283 Mutation at *tubb-1* coding for amino acid change F167Y was present in 26% (6/23) and 33%
284 (2/3) of the samples with *A. caninum* and *U. stenocephala* using the samples from concentrated
285 eggs, respectively (Figure 3D). One of the six *A. caninum* positive samples, ACH10 with 9.5% of
286 F167Y, was part of the DESS treatment; ACH10 was consistently showing F167Y mutation in the
287 DESS-preserved samples for 4 and 28 days (17.4% and 14.7%). One of the two *U. stenocephala*
288 positive samples, ACH10 with 44.4% F167Y mutation, was part of the DESS treatment and
289 consistently showed F167Y mutation allele on day 4 and 28 (48.0% and 75.3%). ACH30 had 3.7%
290 of F167Y mutation allele (Figure 3E). Only one out of the six samples had >70% of the tubb-1 *A.*
291 *caninum* F167Y (95.5%; ACH19, Table 1) (Figure 3D). A mutation in the *tubb-1* gene resulting in
292 the amino acid substitution Q134H was detected in multiple *A. caninum* DNA samples but was
293 absent in *Uncinaria stenocephala* DNA (Figure 3F). Additionally, a Q134R substitution of
294 unknown functional significance was identified in one of the *A. caninum* samples (ACH01), with
295 a variant allele frequency of 2.6% (Figure 3F).

296 Dogs that were found positive for *A. caninum* were predominantly greyhounds (39%, 9/23)
297 followed by kelpies (2/23), while the remaining samples came from individual dog breeds (Table
298 1). Four greyhounds (4/9), one Shar-Pei and one French Bulldog had the *A. caninum tubb-1*
299 mutation F167Y, with the Shar-Pei (ACH19) having the highest percentage of the mutation

300 (95.5%), followed by a greyhound (ACH25, 53.4%). A Kelpie, Labrador Retriever and Bull Arab
301 cross were the breeds that were infected with *U. stenocephala*, the Kelpie (ACH09) sample had
302 the highest percentage (44-75%) of the *U. stenocephala tubb-1* mutation F167Y (Table 1).

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

307

308 **4. Discussion**

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

319 Using repeated measures one-way ANOVA, we found no significant difference between
320 concentrated egg samples and faecal samples stored in DESS for up to 106 days. DESS preserves
321 DNA by deactivating metal-dependent enzymes (e.g., DNases) via EDTA, with this effect
322 enhanced by the alkaline pH (8) created by NaOH, which dissolves EDTA and provides a buffering
323 capacity. The solution is saturated with sodium chloride (NaCl) to stabilise DNA, and contains

324 DMSO which facilitates the transport of compounds (e.g., EDTA and NaCl) across cell
325 membranes while also acting as a cryoprotectant (Seutin et al., 1991). DESS has been shown to
326 perform well across a broad temperature range, including room temperature. Theoretically, we
327 expected higher Ct values for DESS-preserved samples compared to concentrated samples, as
328 the latter contained the equivalent of 3 g of eggs, whereas only one-tenth of that material was
329 used for the DESS-preserved samples. However, the observed non-inferiority may be explained
330 by material loss during the egg concentration process using saturated salt (Zajac and Conboy,
331 2011). This represents an additional benefit of DESS, as it suggests egg concentration may not
332 be necessary, potentially streamlining diagnostic workflows using sample volumes similar to
333 those in this study. Although the tested samples had relatively high egg counts (mean: 4,896 eggs
334 per gram; range: <40 to 23,280), samples with lower counts (<500 eggs per gram; ACH01, ACH05)
335 performed comparably to those with higher counts. Nonetheless, caution is warranted when
336 interpreting results from low egg count samples, as insufficient sampling could lead to
337 overinterpretation. Sample-to-sample variability or uneven egg distribution in faecal aliquots
338 was evident in mixed infection samples (ACH09, ACH10). While no studies have investigated the
339 spatial distribution of hookworm eggs in canine faeces, human studies have shown no clear
340 spatial pattern for hookworm and *Schistosoma mansoni* eggs in faecal samples (Krauth et al.,
341 2012). An alternative explanation is that eggs preserved in DESS may continue to embryonate,
342 leading to an increase in cell number and, consequently, a higher number of genomic copies.
343 Similar observations were made with ruminant strongyle eggs preserved in lysis buffer, where
344 embryonation was visually evident and more pronounced compared to eggs stored in ethanol
345 (Francis and Šlapeta, 2022).

346 The presence of *tubb-1* mutations in hookworm eggs from canine faecal samples does not
347 necessarily equate to clinical drug failure. However, >75% of the F167Y mutation in *tubb-1* has
348 been correlated with in vitro benzimidazole resistance in the Egg Hatch Assay, and can be
349 considered the threshold for predicting clinical resistance based on this mutation alone (McKean

350 et al., 2024; Abdullah et al., 2025). One of our samples, from a Shar-Pei (ACH19), carried 95% of
351 the F167Y mutation, suggesting likely clinical resistance to benzimidazole. Samples with <75%
352 of the F167Y mutation are less likely to lead to observable treatment failure, though continued
353 benzimidazole use may select for this resistant genotype within the hookworm population. In this
354 context, attention should also be given to additional mutations such as Q134H in *tubb-1*, which
355 has been shown to confer benzimidazole resistance even in the absence - or at low frequency -
356 of the F167Y mutation in *A. caninum* (Venkatesan et al., 2023).

357 Consistent with Australian findings from Abdullah et al. (2025), greyhounds were the most
358 frequently hookworm-positive breed in our study. In North America, analysis of faecal samples
359 from 17,671,724 individual dogs revealed that greyhounds had a significantly higher risk of
360 hookworm infection detected via faecal flotation (odds ratio = 15.3) (Burton et al., 2024).
361 Abdullah et al. (2025) also reported a crossbreed dog infected with *Ancylostoma caninum*
362 carrying >75% of the F167Y mutation, adding to evidence that benzimidazole-resistant *A.*
363 *caninum* is not restricted to greyhounds in Australia. Although resistance was initially regarded
364 as a breed-specific issue, it now appears to have spread beyond greyhounds (Burton et al., 2024;
365 Geary et al., 2025; Jimenez Castro et al., 2025). A recent North American survey of diagnostic
366 samples found that greyhounds were only the fourth most frequently infected breed with the
367 *tubb-1* F167Y mutation, with poodles, Bernese mountain dogs, and cocker spaniels showing
368 even higher prevalence (Leutenegger et al., 2024; Jimenez Castro et al., 2025).

369 The *tubb-1* F167Y allele has recently been reported in *U. stenocephala* in Australia,
370 providing an early warning of potential benzimidazole resistance (Abdullah et al., 2025). The
371 allele was detected in a 10-week-old Labrador Retriever with an egg count of 200 (TS024 in
372 Abdullah et al., 2025), where 50.7% of the eggs carried the F167Y mutation. In this study, we
373 report two additional *U. stenocephala* samples carrying the F167Y allele: one from a 10-week-
374 old Kelpie with a mutation frequency between 44–75%, and another with a low frequency (<5%).

375 To date, six *U. stenocephala*-positive samples from southeastern Australia (New South Wales
376 and the Australian Capital Territory) and three from New Zealand have been screened for the
377 F167Y allele (this study; Stocker et al., 2023; Abdullah et al., 2025). In contrast, reanalysis of a
378 nationwide study in the USA by Venkatesan et al. (2023) identified *U. stenocephala* in two
379 individual dog faecal samples and four pooled samples, but all carried the benzimidazole-
380 susceptible F167 allele (Stocker et al., 2024). No studies have yet investigated *U. stenocephala*
381 in dogs from regions where it is common, such as Canada and continental Europe. The presence
382 of the *tubb-1* F167Y allele in *U. stenocephala* is a strong indicator of potential clinical drug failure.
383 Establishing in vivo and in vitro evidence that benzimidazoles fail to clear *U. stenocephala*
384 infections carrying the F167Y allele is now urgently needed.

385 **Conclusions**

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

394

395 **CRediT authorship contribution statement**

396 **Yi-Jou Chen:** Investigation, Methodology, Validation, Data curation. **Vanessa Li:** Writing – review
397 & editing, Investigation, Data curation. **Michelle Suwandy:** Investigation, Methodology, Data
398 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.

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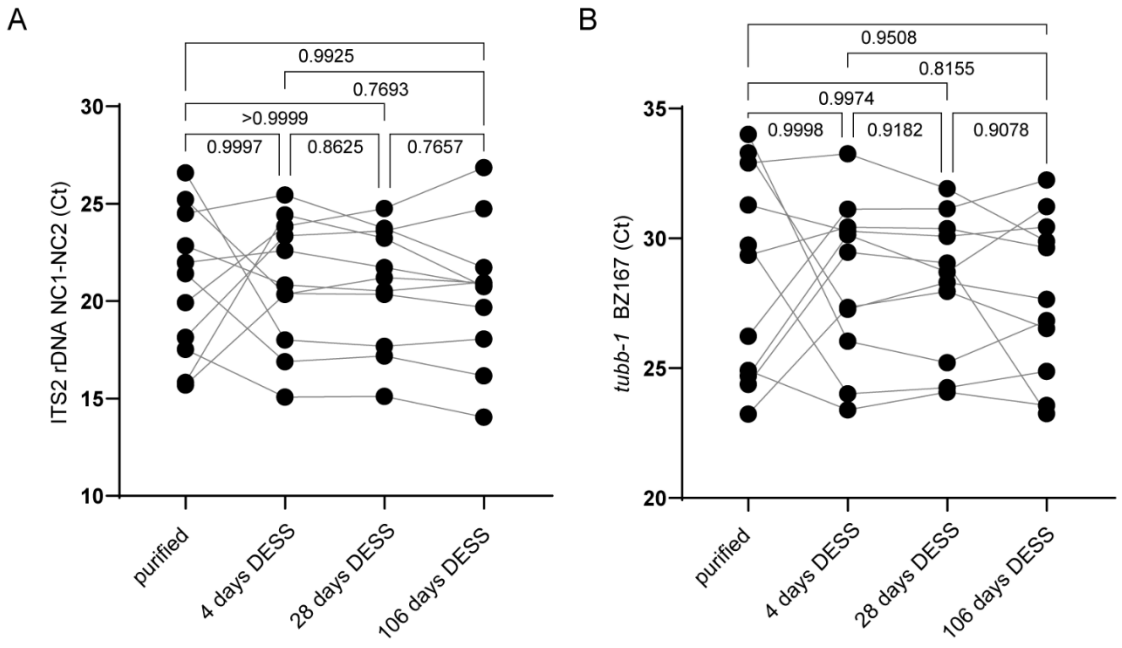
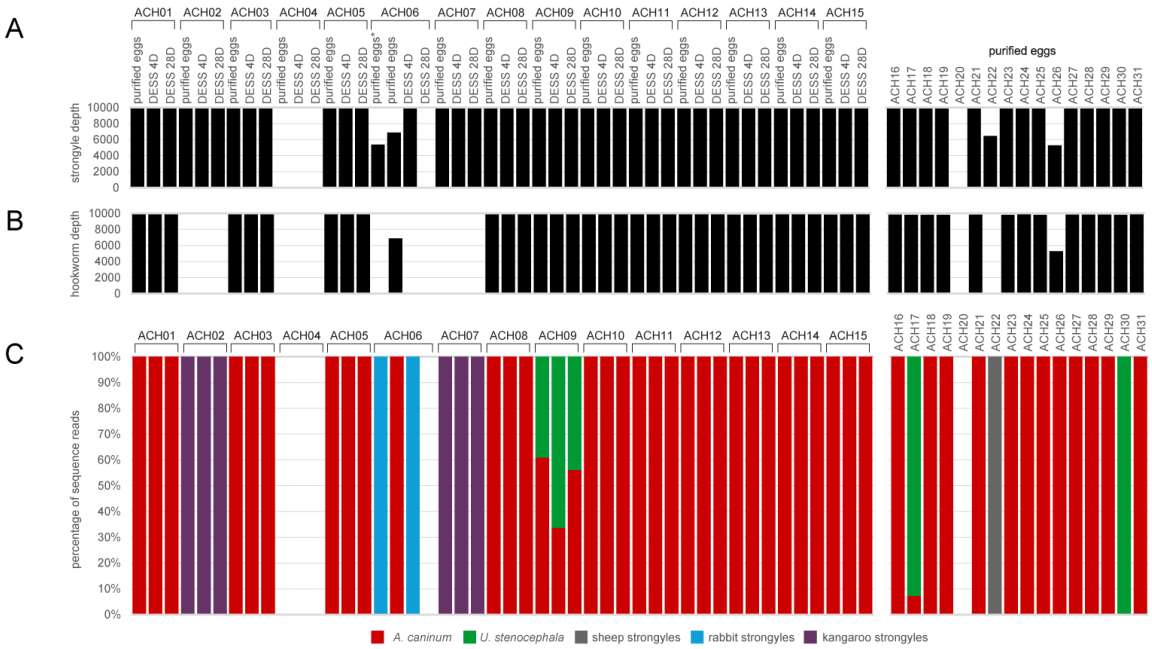
414 Figure legends



415

416 **Figure 1.** Real-time PCR amplifications of (A) internal transcribed spacer (ITS)-2 rDNA and (B)
417 isotype-1 β-tubulin gene (*tubb-1*) from dog faecal samples. Samples with hookworm-like eggs
418 were amplified and cycle threshold (Ct) was recoded. The *tubb-1* amplicon BZ167 spans the
419 mutations responsible for Q134H and F167Y amino acid changes. Only samples that were
420 confirmed to contain hookworm species are shown. Each point represents an individual Ct value.
421 Each sample was processed as either concentrated eggs (purified eggs) or preserved in DESS for
422 different length of time - 4 days, 28 days and 106 days. Each faecal sample is connected by thin
423 line. Repeated measure (RM) one-way ANOVA with Geisser-Greenhouse correction and Tukey's
424 multiple comparison test demonstrated no significant difference between the treatments
425 (P>0.05).



426

427 **Figure 2.** Internal transcribed spacer (ITS)-2 rDNA molecular profiling of dog faecal samples.
428 Sampled with hookworm-like eggs were profiled using metabarcoded deep Illumina amplicon
429 sequencing at ITS-2 rDNA. ACH1-15 have been profiled as concentrated eggs (purified eggs),
430 faeces preserved in DESS for 4 days (DESS 4D) and 28 days (DESS 28D). ACH06 was reisolated
431 as purified eggs and reamplified/re-sequenced (*). Samples ACH16-31 were only processed as
432 concentrated purified eggs. (A) The bar chart representing the number of sequencing reads
433 belonging to strongyle ITS-2 sequences for each sample, the maximum upper scale is set to show
434 10,000 reads. (B) The bar chart showing sequencing reads belonging to hookworms
435 (Ancylostomatidae) ITS-2 sequences for each sample, the maximum upper scale is set to show
436 10,000 reads. (C) Percentage of strongyle species within each sample (depicted as proportion).
437 Amplicon sequence variants (ASVs) were pooled according to species of nematodes. Proportion
438 of each hookworm species (*Ancylostoma caninum*, *Uncinaria stenocephala*) and spurious
439 species is colour coded in the stacked bar chart. Samples are vertically aligned across the bar
440 charts.

441

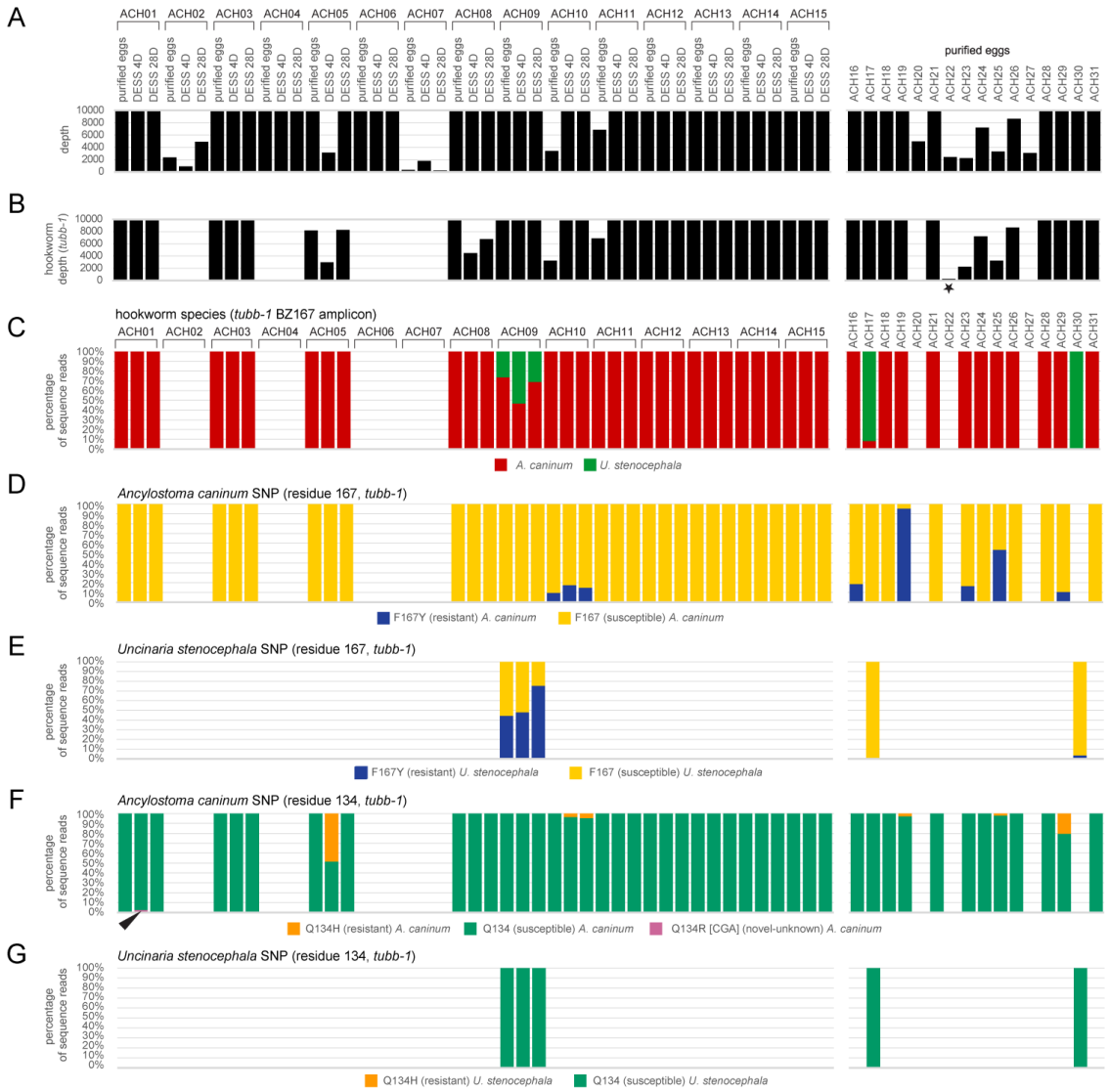


Figure 3. Isotype-1 β-tubulin gene (*tubb-1*) molecular profile of dog faecal samples. Sampled with hookworm-like eggs were profiled using metabarcoded deep Illumina amplicon sequencing at *tubb-1* amplicon BZ167 that spans the mutations responsible for Q134H and F167Y amino acid changes. ACH1-15 have 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

452 <2,000 reads and was excluded. (C) Percentage of strongyle species within each sample
453 (depicted as proportion) based on the *tubb-1* sequence data. Amplicon sequence variants (ASVs)
454 were pooled according to species of nematodes. Proportion of each hookworm species
455 (*Ancylostoma caninum*, *Uncinaria stenocephala*) is colour coded in the stacked bar chart. (D)
456 Percentage of SNP (single nucleotide polymorphism) that codes for the mutation F167Y in *tubb-*
457 *1* of *A. caninum*. (E) Percentage of SNP that codes for the mutation F167Y in *tubb-1* of *U.*
458 *stenocephala*. (F) Percentage of SNP (single nucleotide polymorphism) that codes for the
459 mutation Q134H in *tubb-1* of *A. caninum*. An alternative, novel, mutation leading to Q134R is also
460 shown (arrowhead). (G) No SNP that codes for the mutation Q134H in *tubb-1* of *U. stenocephala*.
461 Samples are vertically aligned across the bar charts.

462

463

464

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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* (%)	
						Q134H	F167Y	Q134H	F167Y
ACH01*	Staghound cross	3 yrs	February 2025	<40	QLD 4869	0, 0, 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, 48.6, 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, 3.5, 4.6	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	2.7	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	2.1	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	20.5	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.