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Assessing DESS solution for the long-term preservation of nematodes from faecal samples

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ABSTRACT

Preservation of biological samples is a relevant issue for many scientific disciplines. Although traditional preservers, such as formaldehyde or ethanol, imply major disadvantages related to health risks, DNA degradation and distortion of structures, they are widely used. Hence, the search for viable alternatives preserving morphometry and genetics seems necessary. Here we assess the suitability of DESS solution to preserve adult nematodes and their eggs in faeces. Concretely, faecal samples of terrestrial tortoises with oxyurids were used to: (i) compare the 1-month storage efficacy of eggs from different conservation protocols (faeces without preserver at -20 °C, faeces with DESS solution at room temperature, faeces with DESS solution at -20 °C and faeces with ethanol 70% at room temperature); (ii) address morphological nematode identification after 2 years of storage with DESS. We also corroborated that nematode DNA remained viable after 2 years. Overall, our results showed that DESS solution at room temperature is an advisable alternative to conserve both parasite eggs and adult nematodes for morphological identification and genetic purposes. It also offers the advantages of being low-cost, safe and suitable for fieldwork conditions and shipments without refrigeration for nematode preservation.

1. Introduction

In sciences like veterinary, ecology or parasitology, the collection of biological samples is a routine, but critical procedure. Some animal samples are collected by invasive handling (e.g., vein puncture, biopsy, etc.), but others avoid stressful animal management (OIE, 2022). The samples obtained by non-invasive procedures are relevant for assessing the health status of wild populations because they are highly susceptible to the stress that derives from human management (Hing et al., 2016). Hence faecal samples are one of the most representative ones in this category because they are frequently used to diagnose many gastrointestinal or general disorders (e.g., metabolic diseases or tumour processes), and are especially useful for epidemiological studies on parasitic diseases (Broussard, 2003; Verocai et al., 2020).

As the preservation of faecal samples is difficult given their high contamination risk by organisms usually found in faeces (e.g., bacteria or fungi) (Goneau et al., 2019), the selection of the best preserver is an essential step (Crawley et al., 2016; Murphy et al., 2002). Traditionally, the preservation of these samples requires preservative solutions and/or low temperatures. As a counterpart to longer sample durability, some protocols have several handicaps. Two of the most widely used preservers are ethanol and formaldehyde. Ethanol maintains DNA, but alters morphometry by causing wrinkling and fragility, while formaldehyde fixation damages DNA and imposes human health risks (e.g., toxicity, irritability and carcinogenicity) (Naem et al., 2010; Swenberg et al., 2013). Other alternatives, such as plastination based on substituting body water for artificial polymers, is an expensive method that preserves microscopic structures, but DNA is totally degraded (Gonzálvez et al., 2018). Refrigerating or freezing samples that are combined or not with preserver solutions is also recommended for preserving samples, but is sometimes impractical under non-laboratory conditions (Kozlova et al., 2020). Other promising options are to use

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saturated solutions composed of dimethyl sulphoxide, disodium EDTA and saturated NaCl (hereinafter DESS), which have been successfully tested to preserve adult nematodes and their DNA for at least 1 year (Bik et al., 2009; Chałańska et al., 2016; Yoder et al., 2006). Therefore, the development and assessment of feasible preservation protocols should be promoted. This study aimed to assess the use of DESS solution as a preserver of faecal samples containing parasite eggs and adult nematodes after 1 month and 2 years, respectively. The presence of viable DNA in nematodes stored in DESS solution for 2 years was also corroborated.

2. Material & methods

2.1. Study design and parasitological analyses

In June 2019, faecal samples from nine spur-thighed tortoises (*Testudo graeca*) were collected at the regional wildlife rescue centre "El Valle" (Murcia, SE Spain). Samples were stored refrigerated (4 °C) until they were handled in the Department of Animal Health (University of Murcia, Spain). All the samples were mixed to constitute a pool within 24 h after collection. A coprological analysis and egg counting were performed in a McMaster chamber to know the abundance of oxyurid eggs (6307 eggs per gram). The pool was distributed into four study groups with six replicates each: (1) faeces without preserver, but maintained at −20 °C; (2) faeces with DESS solution at room temperature; (3) faeces with DESS solution at -20 °C; (4) faeces with 70% ethanol at room temperature. The quantification and morphometrical evaluation of eggs from each study group were performed 30 days after preservation, as described for the initial faeces pool. To evaluate differences in egg counts among the study groups, Wilcoxon tests with Bonferroni correction were applied. Significance was assessed with alpha = 5% ($p < 0.05$). The R software v3.6.0 was employed for all the descriptive and statistical analyses (R Core Team, 2022).

Of the adult specimens, the 37 faeces sampled in wild *T. graeca* populations in spring 2019 in SE Spain were stored for 2 years in DESS solution at -20 °C. After this time, all the adult specimens were collected and cleaned with distilled water. Three selected representative structures (spicule, tail, oesophagus) of all the preserved nematodes were measured by a photomicroscopy system (Leica EZ Camera 2.4.1) associated with specific morphometry software (LAS EZ 2.1.0). This information was compared to the morphological and morphometrical data reported in the scientific literature, which was carried out by two experts in Parasitology to maximise the objectivity of identification based on morphological keys (Bouamer et al., 2003, 2001; Bouamer and Morand, 2005, 2003, 2002; Petter, 1966).

2.2. DNA extraction, PCR and sequencing

In order to corroborate that DNA remained viable in the samples stored for 2 years in DESS (as reported by Bik et al. (2009) for unmounted nematodes stored for 6 months), one specimen was randomly selected to be analysed. Genomic DNA was firstly extracted from faecal samples by the NZY Tissue gDNA Isolation kit (NZYTech). Subsequently, an 18S fragment of approximately 900 bp in length was amplified using primers Nem_18S_F (5'CGCGAATRGCTCATTACAACAGC3') and Nem_18SR_R (5'GGGCGGTATCTGATCGCC3') from Floyd et al. (2005). PCR reactions were performed in a total volume of 20 μL that comprised 1 unit of Taq polymerase (Biotaq, Ecogen, Spain) with the buffer recommended by the supplier at a final concentration of 1.6 mM $MgCl_2$, 0.2 mM of each dNTP (dNTPs Mix, Ecogen, Spain), 0.4 μM of each primer and 1 μL of the extracted DNA. PCR conditions consisted in: 1 step of 3 min at 95 °C; 35 iterations of 40s at 95 °C; 40s at 54 °C; 60s at 72 °C; a final extension step at 72 °C for 10 min. The PCR product was purified and sequenced in an ABI 3130xl sequencer (Applied Biosystems, Foster City, CA, USA). The obtained sequence was visually cleaned and matched the sequences in the GenBank database by BLAST.

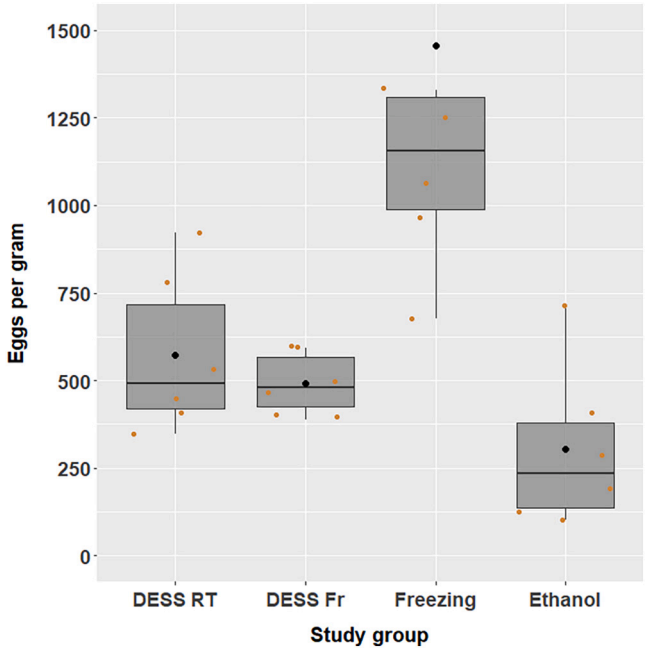

Fig. 1. Boxplots representing the quantification results (eggs of oxyurids per gram of faeces) 30 days after preservation for each study group [DESS RT (DESS at room temperature), DESS Fr (DESS freezing), Freezing and 70% ethanol)]. The horizontal line and the black dot represent the median and mean value, respectively. Gold dots depict all the numerical measures obtained per group (an outlier value of the "Freezing" group was omitted in the graphic to facilitate interpretation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

On the 1-month egg preservation, our analyses showed that freezing was the best method to maintain the number (median; rank) of oxyurid eggs originally present in faeces (1156.0 eggs/g; 675.2–3457.9), followed by DESS solution at room temperature (491.1 eggs/g; 347.8–920.7) and DESS solution upon freezing (480.8 eggs/g; 388.8–593.4); 70% ethanol (235.3 eggs/g; 102.3–716.1) obtained the worst results. The statistical analyses showed marginal (p-value <0.06)* and significant differences in the egg concentration between the freezing group and the other study groups [DESS at room temperature (p-value: 0.052)*, DESS upon freezing (p-value: 0.030) and 70% ethanol (p-value: 0.026)] (Fig. 1). Freezing and DESS solution (regardless of temperature) preserved the morphology and structures of nematode eggs similarly to those in the fresh samples. However, ethanol brought about several morphological alterations, namely cuticle deformation and lack of content in all the eggs, which hindered morphological identification despite the characteristic oxyurid egg shape.

Regarding adult specimens, the morphometrical evaluation revealed the presence of 11 different parasitic species in the sampled tortoises (Table S1) belonging to genus *Tachygonetria* ($n = 9$ species), *Mehdiella* ($n = 1$ species) and *Alaeuris* (n = 1 species). The morphometrical features of the nematodes collected in the present study (preserved in DESS) and the data obtained from the scientific literature revealed an evident homology among all the compared parasitic species (Fig. S1) (Table S1).

The genetic analyses successfully amplified the extracted sample. The obtained sequence (697 bp) produced a BLAST match with 98% identity to three nematode species available in GenBank: *Ozolaimus linstowi* (KJ632671.1), *Aspiculuris tetraptera* (MH215350.1, KY462828.1, KY462827.1, MT755640.1) and *Thelandros tinerfensis* (KJ778073.1). These oxyuirid species belong to the family Pharyngodonidae and infest reptiles (*O. linstowi* and *T. tinerfensis*) and rodents (*A. tetraptera*).

Table 1
General advantages and disadvantages of five alternative preservers according to different criteria.

	Economic profitability	Safety for health[1]	DNA preservation[2]	Respects morphometry parasite eggs[3]	Suitable field conditions	Suitable border transport[4]
DESS	Yes	Yes	Yes	Yes	Yes	Yes
Ethanol (70%)	Yes	Yes	Yes	No	Yes	No*
Freezing (-20 °C)	Yes	Yes	Yes	Yes	No	No
Formaldehyde (10%)	Yes	No	No	Yes	Yes	No*
Plastination	No	Yes	No	Unknown	No	Yes

Based on: the authors' previous experience and the scientific literature: [1] (Swenberg et al., 2013), [2] (Beknazarova et al., 2017; Carew et al., 2018; Guyard et al., 2017; Romanazzi et al., 2015), [3](Jagla et al., 2013; Koudela and Bodeček, 2006), [4](IATA, 2022).

4. Discussion

Although previous scientific studies have evaluated the potential role of DESS as a preserver of nematodes, this is the first report to jointly consider the preservation of egg and adult specimens, and it also describes the longest time (2 years) for nematode preservation using DESS solution (Beknazarova et al., 2017; Chałańska et al., 2016; Yoder et al., 2006).

According to our results, DESS solution allows all egg structures with a similar morphology to those of fresh samples to be preserved, as described using traditional methodologies like formaldehyde and low temperatures (Aldeen et al., 1993; Crawley et al., 2016). However, it has clear benefits because it avoids having to employ irritant and carcinogenic chemicals (e.g., formaldehyde), as well as technologies and infrastructures that can be difficult to apply outside labs (e.g., freezer, plastination) (Crawley et al., 2016; Gonzálvez et al., 2018; Swenberg et al., 2013) (Table 1). As ethanol is commonly associated with gross alterations in the morphology and count of parasite eggs, its use in parasitological surveys based on morphological identification should be avoided (Crawley et al., 2016; Jagla et al., 2013).

Concerning the long-term preservation of adults, the nematode species identified in the present study matched a parasitological survey that previously addressed the same study area (Chávarri et al., 2012). We demonstrated that DESS solution preserves microscopic key-structures of nematodes (e.g., spicules, tail, oesophagus), which is relevant for morphometrical identifications for at least 2 years. Our results showed that using DESS to preserve adult nematodes is highly recommended for long-term surveys, where samples are stored for months, or even years, under optimal morphometrical conditions, which is essential to obtain reliable results (Table 1). We were able to extract viable DNA from one sample stored for 2 years in DESS. DNA preservation can facilitate individual or massive species identification (metabarcoding); although this was not our objective because neither *Tachygonetria*, *Mehdiella* nor *Alaeuris* have available 18S sequences in GenBank (only *Tachygonetria* spp. has one available 28S sequence). Our purpose was to corroborate that nematode DNA would remain viable. Further studies should quantify PCR and sequencing success in more samples. The combination of morphological and genetic approaches is especially useful for wildlife epidemiological surveys. However, the sequences of many parasitic species are still not registered in genetic databases like GenBank and more efforts need to be made in this line.

To summarise, we found that faecal samples can be preserved for at least 2 years in DESS, which is a much longer period than those previously described (Bik et al., 2009; Chałańska et al., 2016; Yoder et al., 2006). This finding makes it a convenient tool for epidemiological surveys because it avoids problems associated with other traditional preservers like formaldehyde, which have well-known negative effects on handlers' health and on the quality of DNA parasites (Guyard et al., 2017). Moreover, storage protocols using DESS enable the economic profitable preservation of parasites, preserve morphometry and DNA from parasite specimens, and allow work to be performed under field conditions and samples to be quickly shipped worldwide without restrictions.

Ethical statement

No approval of research ethics committees was required to accomplish the goals of this study because the collection of samples did not require animal manipulation.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.10.010.

References

Aldeen, W.E., Shisenant, J., Hale, D., Matsen, J., Carroll, K., 1993. Comparison of pooled formalin-preserved fecal specimens with three individual samples for detection of intestinal parasites. J. Clin. Microbiol. 31, 144–145. https://doi.org/10.1128/jcm.31.1.144-145.1993.

Beknazarova, M., Millsteed, S., Robertson, G., Whiley, H., Ross, K., 2017. Validation of DESS as a DNA preservation method for the detection of *Strongyloides* spp. in canine feces. Int. J. Environ. Res. Public Health 14, 624. https://doi.org/10.3390/ijerph14060624.

Bik, H.M., Hawkins, L.E., Hughes, J.A., Lambshead, J.D., 2009. Rapid decline of PCR amplification from genomic extracts of DESS-preserved, slide-mounted nematodes. Nematology 11, 827–834. https://doi.org/10.1163/156854109X422922.

Bouamer, S., Morand, S., 2002. Description of *Tachygonetria combesi* n. sp. and redescriptions of four species of *Tachygonetria* Wedl, 1862 (Nematoda: Pharyngodonidae), with a new diagnosis of the genus. Syst. Parasitol. 53, 121–139. https://doi.org/10.1023/a:1020443905905.

Bouamer, S., Morand, S., 2003. Descriptions of two new species of the genus *Tachygonetria* Wedl, 1862 (Nematoda-Pharyngodonidae) and discussion of the relationships among the species of the genus. Parasitol. Res. 91, 68–73. https://doi.org/10.1007/s00436-003-0932-4.

Bouamer, S., Morand, S., 2005. Descriptions of two new species of the genus *Tachygonetria* Wedl, 1862 (Nematoda, Pharyngodonidae) and redescriptions of five species parasites of Palaearctic Testudinidae. Zoosystema 27, 193–209.

Bouamer, S., Morand, S., Bourgat, R., 2001. Oxyuroids of Palearctic Testudinidae–new definition for *Alaeuris* Seurat, 1918 (Nematoda: Pharyngodonidae) and redescription of *Alaeuris numidica* (Seurat, 1918). J. Parasitol. 87, 128–133. https://doi.org/10.1645/0022-3395(2001)087[0128:OOPTND]2.0.CO;2.

Bouamer, S., Morand, S., Kara, M., 2003. Redescription of four species of *Mehdiella* from Testudinidae, with a key to the species and discussion on the relationships among the species of this genus. Parasite 10, 333–342. https://doi.org/10.1051/parasite/2003104333.

Broussard, J.D., 2003. Optimal fecal assessment. Clin. Tech. Small Anim. Pract. 18, 218–230. https://doi.org/10.1016/S1096-2867(03)00076-8.

Carew, M.E., Coleman, R.A., Hoffmann, A.A., 2018. Can non-destructive DNA extraction of bulk invertebrate samples be used for metabarcoding? PeerJ 6, e4980. https://doi.org/10.7717/peerj.4980.

Chałańska, A., Bogumił, A., Malewski, T., Kowalewska, K., 2016. The effect of two fixation methods (TAF and DESS) on morphometric parameters of *Aphelenchoides ritzemabosi*. Zootaxa 4083, 297–300. https://doi.org/10.11646/zootaxa.4083.2.9.

Chávarri, M., Berriatua, E., Giménez, A., Gracia, E., Martínez-Carrasco, C., Ortiz, J.M., de Ybáñez, R.R., 2012. Differences in helminth infections between captive and wild spur-thighed tortoises *Testudo graeca* in southern Spain: a potential risk of reintroductions of this species. Vet. Parasitol. 187, 491–497. https://doi.org/10.1016/j.vetpar.2012.02.007.

Crawley, J.A.H., Chapman, S.N., Lummaa, V., Lynsdale, C.L., 2016. Testing storage methods of faecal samples for subsequent measurement of helminth egg numbers in the domestic horse. Vet. Parasitol. 221, 130–133. https://doi.org/10.1016/j.vetpar.2016.03.012.

Floyd, R.M., Rogers, A.D., Lambshead, P.J.D., Smith, C.R., 2005. Nematode-specific PCR primers for the 18S small subunit rRNA gene. Mol. Ecol. Notes 5, 611–612. https://doi.org/10.1111/j.1471-8286.2005.01009.x.

Goneau, L.W., Mazzulli, A., Trimi, X., Cabrera, A., Lo, P., Mazzulli, T., 2019. Evaluating the preservation and isolation of stool pathogens using the COPAN FecalSwabTM transport system and walk-away specimen processor. Diagn. Microbiol. Infect. Dis. 94, 15–21. https://doi.org/10.1016/j.diagmicrobio.2018.11.020.

Gonzálvez, M., Ortiz, J., Navarro, M., Latorre, R., 2018. Preservation of macroparasite species via classic plastination: an evaluation. Folia Parasitol. 65 https://doi.org/10.14411/fp.2018.019.

Guyard, A., Boyez, A., Pujals, A., Robe, C., Tran Van Nhieu, J., Allory, Y., Moroch, J., Georges, O., Fournet, J.C., Zafrani, E.S., Leroy, K., 2017. DNA degrades during storage in formalin-fixed and paraffin-embedded tissue blocks. Virchows Arch. 471, 491–500. https://doi.org/10.1007/s00428-017-2213-0.

Hing, S., Narayan, E.J., Thompson, R.C.A., Godfrey, S.S., 2016. The relationship between physiological stress and wildlife disease: consequences for health and conservation. Wildl. 43, 51–60. https://doi.org/10.1071/WR15183.

IATA, 2022. Dangerous Goods Regulations [Accessed: 15 Feb 2022]. Available at: https://www.iata.org/en/publications/dgr/.

Jagla, E., Spiewak, J., Zalesny, G., Pipiolek, M., 2013. Effect of storage and preservation of horse faecal samples on the detectability and viability of strongylid nematode eggs and larvae. Vet. Inst. Pulawy 57, 161–165. https://doi.org/10.2478/bvip-2013-0030.

Koudela, B., Bodeček, Š., 2006. Effects of low and high temperatures on viability of *Parascaris equorum* eggs suspended in water. Vet. Parasitol. 142, 123–128. https://doi.org/10.1016/j.vetpar.2006.05.031.

Kozlova, V.A., Pokrovskaya, M.S., Meshkov, A.N., Drapkina, O.M., 2020. Actual approaches to the transportation of biological samples at low temperatures. Klin. Lab. Diagn. 65, 619–625. https://doi.org/10.18821/0869-2084-2020-65-10-619-625.

Murphy, M.A., Waits, L.P., Kendall, K.C., Wasser, S.K., Higbee, J.A., Bogden, R., 2002. An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. Conserv. Genet. 3, 435–440. https://doi.org/10.1023/A:1020503330767.

Naem, S., Pagan, C., Nadler, S.A., 2010. Structural restoration of nematodes and acanthocephalans fixed in high percentage alcohol using DESS solution and rehydration. J. Parasitol. 96, 809–811. https://doi.org/10.1645/GE-2402.1.

OIE (World Organisation for Animal Health), 2022. OIE Terrestrial Manual [Accessed: 15 Feb 2022]. Available at: https://www.oie.int/es/que-hacemos/normas/codigos-y-manuales/acceso-en-linea-al-manual-terrestre/.

Petter, A.J., 1966. Équilibre des espèces dans les populations de nématodes parasites du colon des tortues terrestres. Scientific publications of the Muséum national d'Histoire naturelles, París.

R Core Team, 2022. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Romanazzi, V., Traversi, D., Lorenzi, E., Gilli, G., 2015. Effects of freezing storage on the DNA extraction and microbial evaluation from anaerobic digested sludges. BMC Res. Notes 8, 420. https://doi.org/10.1186/s13104-015-1407-2.

Swenberg, J.A., Moeller, B.C., Lu, K., Rager, J.E., Fry, R., Starr, T.B., 2013. Formaldehyde carcinogenicity research: 30 years and counting for mode of action, epidemiology, and cancer risk assessment. Toxicol. Pathol. 41, 181–189. https://doi.org/10.1177/0192623312466459.

Verocai, G.G., Chaudhry, U.N., Lejeune, M., 2020. Diagnostic methods for detecting internal parasites of livestock. Vet. Clin. N. Am. - Food Anim. Pract. 36, 125–143. https://doi.org/10.1016/j.cvfa.2019.12.003.

Yoder, M., Ley, I.T.D., King, I.W., Mundo-Ocampo, M., Mann, J., Blaxter, M., Poiras, L., Ley, P.D., 2006. DESS: a versatile solution for preserving morphology and extractable DNA of nematodes. Nematology 8, 367–376. https://doi.org/10.1163/156854106778493448.