

Short communication

Testing storage methods of faecal samples for subsequent measurement of helminth egg numbers in the domestic horse

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ABSTRACT

Parasite infection status, intensity and resistance have traditionally been quantified via flotation techniques, but the need for immediate analysis following defecation imposes limitations and has led to the use of several faecal storage techniques. However, their effect on nematode egg counts has not been systematically validated and is often generalised across taxa despite evidence of differences between species. Here, we take the domestic horse *Equus ferus caballus* as a model to examine the impact of commonly used storage techniques on egg recovery: 1) high and low concentrations of ethanol and formalin fixative solutions for up to four weeks and 2) refrigeration (3–5 °C) over a two-week period. We found a significant decline in faecal egg counts (FEC) following storage in high and low concentrations of both fixative solutions after two weeks, which stabilised after four weeks, and this pattern was uniform across replicates. FECs remained relatively stable over a week of refrigeration, but declined when refrigeration exceeded 8 days. Prior to FEC analysis, we recommend sample refrigeration for no more than one week. Storage in either fixative solution is sub-optimal for the preservation of nematode eggs, although the uniformity of the decline across samples could hold potential for projective calculation of parasite egg shedding when storage time is effectively controlled for.

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1. Introduction

Parasitic helminths are of increasing concern for captive livestock, both in terms of animal welfare and economic cost. Past blanket administration of anthelmintics has led to high levels of drug resistance in most livestock species (Waller, 1997; Kaplan and Vidyashankar, 2012). Although now advised against, blanket administration has continued, and likely will continue, in some holdings despite recommendations stressing the importance of individually-tailored anthelmintic regimes (Kaplan and Vidyashankar, 2012; Martínez-Valladares et al., 2015; Wilson et al., 2015). Under these regimes, heavily loaded individuals and those harbouring resistant parasites can be identified using faecal egg count (FEC) techniques and then selectively treated (Coles et al., 1992; Wilson et al., 2015).

The McMaster FEC technique is the most commonly practised method to determine helminth parasite egg shedding and resistance (Coles et al., 1992; Seivwright et al., 2004). However,

to minimise the chance of hatching eggs leading to under-representative counts (though egg destruction can also contribute to this), samples must be analysed soon after collection. This limitation has led to use of a variety of storage methods, yet the effect of these different methods on egg recovery is contested (McKenna, 1998; Jagla et al., 2013). Species-specific assessments of the effects of storage are recommended, to account for host-particular differences in faeces composition and parasite fauna (Stringer et al., 2014; Lynsdale et al., 2015). Although refrigeration of faecal matter is a common storage method for later analysis of helminth egg counts, time limit suggestions vary from three days to fifty days (Foreyt, 1986; Smith-Buijs and Borgsteede, 1986; Roepstorff and Nansen, 1998; Seivwright et al., 2004; Nielsen et al., 2010; Rinaldi et al., 2011). Fixative solutions such as ethanol or formalin are alternatives to refrigeration, and may be preferred for longer-term storage (Baines et al., 2015). The few studies testing fixative storage however suggest both ethanol and formalin may be poor at maintaining egg counts (Foreyt, 1986; Rinaldi et al., 2011; Jagla et al., 2013; Baines et al., 2015). Nonetheless, though uncommon in veterinary parasitology, studies on humans and some on wild animals continue to use fixatives to store samples without accounting for egg reduction (Vidya and Sukumar, 2002; Gillespie, 2006; Hing et al., 2013; East et al., 2015; Garg et al., 2005).

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In this study, we investigate the efficacy of faecal storage techniques for maintaining helminth egg counts, using a non-ruminant herbivorous host: the domestic horse *E. ferus caballus*. We test the effect of (a) different concentrations of the fixative solutions ethanol and formalin after two and four weeks of storage and (b) refrigeration over a period of two weeks.

2. Methods

We carried out the study in Sheffield, South Yorkshire, UK, selecting horses for participation in the study by FEC threshold; out of eighteen horses tested, we selected three adult horses ('A', 'B', 'C'), initially shedding over 200 eggs/g, for further collection and analysis. We collected samples over a ten day period in June–July 2014 (hosts A and B) and a three day period in June 2015 (C), with ambient average daily temperatures ranging between 15 and 26 °C.

We sampled ten distinct defecation events from each horse from indoor stables, with a combined total of 490 sub-samples. There was a maximum of 14 (A and B) and eight (C) hours potential lag between defecation and sample collection. For horses A and B, this potential lag was mostly overnight, when temperatures did not exceed 16 °C (checked via Wunderground.org, 2016). For these samples, eggs were less likely to be embryonated as the optimal temperature for development for equine strongyles is reported to be 25–33 °C (Nielsen et al., 2007). Though host C samples were collected during the day, recorded temperatures did not exceed 20 °C (Wunderground.org, 2016). We did not observe obvious embryonation during analysis. Samples were sealed into labelled bags immediately, refrigerated within two hours and analysed within four hours.

We carried out FECs using a special modification of the McMaster technique (MAFF, 1986) by mixing 4.5 g of faeces with 40.5 mL of saturated salt solution (NaCl) accepted to have a specific gravity of 1.18–1.20 (Cringoli et al., 2004; Rvc.ac.uk, 2016) before straining the solution through a sieve (aperture 1 mm) to remove large solid waste. We transferred 1 mL of solution to a double-chambered McMaster microscope slide. We then left the slide for five minutes to allow separation of faecal elements by mass before examining using a compound microscope, with all eggs observed in the entirety of both chambers of the slide counted to give a total FEC. The detection limit of the special modification of the McMaster technique is 10 eggs per gram (MAFF, 1986).

Following Taylor et al., 2007, eggs were visually identified through microscopic identification as strongyle and strongyloides-type nematode eggs. Identification to the generic and species levels was considered unreliable through microscopic identification, though future studies may benefit from including coprocultures to accurately identify the composition of nematode species and genera of tested populations.

2.1. Effect of fixative solutions

The efficacies of two different storage fixatives on preserving eggs were tested at low and high concentrations: 40% ethanol, 70% ethanol, 5% formalin and 10% formalin. Storage involved putting a 4.5 g sub-sample of faeces in 15 mL of fixative solution within 4 h of collection, ensuring full submersion of the sample for initial mixing. For each faecal pile, the FEC of one fresh sub-sample (n = 30) was used as the baseline point of comparison against two storage time points: two weeks and four weeks. Sample sizes for each solution, combining both time points, were as follows: formalin 5%, n = 40; formalin 10%, n = 40; ethanol 40%, n = 40; and ethanol 70%, n = 60.

After storage, sub-samples were mixed so that any eggs would be separated from solid faecal matter, which was then removed from solute using a sieve and discarded. The remaining solution

was centrifuged for five minutes at 1500 rpm, to separate faecal matter from fixative. The solute was removed and the solid pellet of faecal matter re-suspended in 40.5 mL of saturated salt solution, before being prepared and analysed as previously described.

2.2. Effect of refrigeration

We investigated the effect of refrigeration on daily FEC over a period of two weeks. Fresh faeces were separated into 4.5 g sub-samples and refrigerated in airtight zip-lock bags at 3–5 °C. Baseline 'fresh' subsamples and derived counts were the same samples as used for storage analysis. One subsample per defecation event was analysed as a baseline fresh control. FECs were measured on one subsample approximately every 24 h, up to 168 h (7 days) for 20 replicates (n = 140), and 336 h (14 days) for 10 replicates (n = 140).

2.3. Statistical analyses

All statistical analyses were conducted with R statistical software (R Core Team, 2015) version 3.1.3. We ran generalised linear mixed effects models (GLMMs) using the *glmer* function of the *lme4* package version 1.1–7 (Bates et al., 2015) and cumulative link mixed models (CLMMs) using the *clmm* function of the *ordinal* package version 2015.1–21 (Christensen, 2015).

2.4. Effect of fixative solutions

To test the effect of storage in fixative on FEC, we fitted a GLMM accounting for a poisson distribution of the response variable (FEC). The GLMM assessed an interactive effect between the treatment fixed term (four level factor: ethanol 40%; ethanol 70%; formalin 5%; formalin 10%), and the time fixed term (three level factor: fresh; two weeks; four weeks). We included random effects of horse identity (three level factor) and replicate (10 level factor) to control for between- and within-individual variation respectively. We also tested whether any change in FEC over time was uniform across replicates, using a likelihood ratio test to compare models with and without a random slope effect of replicate nested within horse ID.

2.5. Effect of refrigeration

Threshold models were fitted using the CLMM function to determine whether there was a significant decrease in daily FEC after one week and two weeks of refrigeration. We fitted threshold models as we expected there to be a certain time point at which eggs start to decline. The response variable of FEC was split into an ordinal factor with categories of five. The two models included day as a continuous fixed effect ranging from 0 to 7 and 0 to 14 respectively. Random effects again accounted for horse ID and replicate ID, in the one-week model. Only a random term of replicate ID was included in the two-week model, as data originated from one individual horse. To test which day fridge storage became unviable, we fitted a GLMM with 'day' as a fixed factor (15 levels: fresh, days 1–14), and both 'replicate' and a 'replicate*day' interaction as random effects. We used this model with a Tukey test for pairwise comparison of fresh egg counts against egg counts from refrigerated subsamples.

3. Results

Faecal egg counts of collected samples differed between horses, and varied over the collection period, ranging from 7 to 23 for host A, 60–208 for B, and 62–93 for C. Subsequent results account for these between-individual differences by including a random term for individual ID in all analyses.

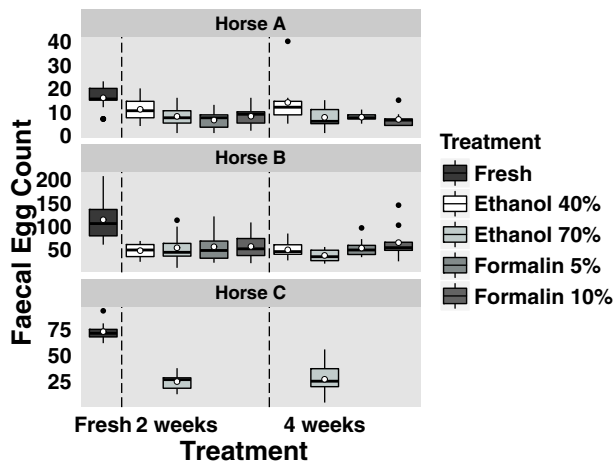


Fig. 1. Comparison of faecal egg counts from fresh faecal samples ($n = 30$), and from those stored in fixative solutions for two and four week periods, across three horses (ethanol 40%, $n = 40$; ethanol 70%, $n = 60$; formalin 5%, $n = 40$; formalin 10%, $n = 40$). Box limits show upper and lower quartiles, vertical lines show range, midline the median and white points show the mean.

3.1. Effect of fixative solutions

Storage in fixative solutions significantly decreased nematode egg recovery over both two-week ($n = 270$, $\beta = -8.00e^{-1} \pm 4.99e^{-2}$, $z = -16.04$, $p < 0.001$) and four-week periods ($n = 270$, $\beta = -7.25e^{-1} \pm 4.86e^{-2}$, $z = -14.92$, $p < 0.001$) (Fig. 1). This impact on FEC was the same across all fixatives (using ethanol 40% as reference: 70% ethanol: $\beta = 1.92e^{-2} \pm 3.76e^{-2}$, $z = 0.51$, $p = 0.610$; 5% formalin: $\beta = -4.27e^{-7} \pm 3.93e^{-2}$, $z = 0.00$, $p = 1.00$; 10% formalin: $\beta = -4.16e^{-7} \pm 3.93e^{-2}$, $z = 0.00$, $p = 1.00$). There was no further significant decrease from week two to week four for any of the fixative solutions ($n = 270$, $\beta = 0.07 \pm 0.06$, $z = 1.29$, $p = 0.196$). The decline in FEC after two weeks was uniform across replicates for all treatments, as the slope did not vary significantly: samples stored in 5% formalin showing an average 55.4% decrease ($n = 40$, $X^2_4 = 4.253$, $p = 0.373$); 10% formalin a 49.8% decrease ($n = 40$, $X^2_4 = 2.739$, $p = 0.602$); 40% ethanol a 44.6% decrease ($n = 40$, $X^2_4 = 7.751$, $p = 0.101$), and 70% ethanol a 56% decrease ($n = 60$, $X^2_4 = 7.685$, $p = 0.104$) compared to freshly analysed samples.

3.2. Effect of refrigeration

We found that seven days of refrigeration did not significantly impact FEC ($n = 210$, $\beta = -0.02 \pm 0.05$, $p = 0.676$), but FEC significantly declined after two weeks by an average of 44% ($n = 70$, $\beta = -0.18 \pm 0.04$, $z = -5.08$, $p < 0.001$; Fig. 2). A pairwise comparison of each day against the fresh control suggests that this decline becomes marginally non-significant at day eight and significant at day 10 ($n = 10$, see Table 1).

4. Discussion

Our results using the domestic horse as a model show refrigeration is viable for short-term storage, but long-term storage in fixatives does not sustain egg counts (Supplementary Table S1 in the online version at DOI: [10.1016/j.vetpar.2016.03.012](https://doi.org/10.1016/j.vetpar.2016.03.012)).

Refrigeration of faecal samples is often used for maintaining FEC for later analysis, though it has not previously been agreed at what point egg detectability declines. Egg counts have been found to remain consistent for up to five days of refrigeration (Nielsen et al., 2010), but in longer-term methodological studies, samples were tested weekly, rather than daily (Seivwright et al., 2004). We

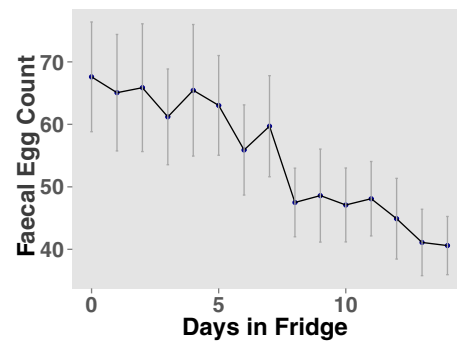


Fig. 2. Faecal egg counts of samples taken over a two-week period. Samples were taken from three individuals over the first week ($n = 210$) and one over the second week ($n = 70$). Points show average FEC. Error bars show standard error.

Table 1

Pairwise comparison of FEC of samples kept in fridge for x number of days against freshly analysed samples.

day	estimate \pm S.E	z value	p value
1	-0.145 ± 0.14	1.035	0.9995
2	-0.159 ± 0.14	1.14	0.9985
3	-0.242 ± 0.14	1.726	0.9249
4	-0.298 ± 0.14	2.118	0.7223
5	-0.209 ± 0.14	1.491	0.9777
6	-0.335 ± 0.14	2.38	0.5303
7	-0.345 ± 0.14	2.445	0.4799
8	-0.473 ± 0.14	3.328	0.0605
9	-0.478 ± 0.14	3.361	0.0555
10	-0.491 ± 0.14	3.45	0.0414*
11	-0.468 ± 0.14	3.296	0.0670*
12	-0.549 ± 0.14	3.849	0.0100*
13	-0.626 ± 0.14	4.365	<0.01**
14	-0.624 ± 0.14	4.357	<0.01**

* $p < 0.05$.

** $p < 0.01$.

tested samples daily for fourteen days, finding no decline in the first week. The decline in egg count was large from day eight of storage, and significantly lower than fresh counts from day 10. Our results support the recommendation of Nielsen et al. (2010) that eggs can be stored in a fridge for at least a week without significant declines in detectability of eggs. Samples were stored between 3 and 5 °C, below the minimum hatching temperature (6 °C), and above freezing which is known to decrease detectability of eggs (Nielsen et al., 2010; Jagla et al., 2013). Declines are therefore most likely due to biological degradation (Nielsen et al., 2010).

For longer-term preservation, fixative solutions are often used to preserve eggs. However, egg counts have been reported to decrease after storage in low concentrations of fixative (40% ethanol; 4% formalin; Jagla et al., 2013), and the efficacies of higher concentrations are less known. We found that neither concentration of either ethanol or formalin is able to preserve FEC for two weeks. After this initial decline there was no further significant decrease between two and four weeks. This suggests storage in fixative, rather than time in storage, may drive declines, but it is unclear as to what could cause such effects. Storage in the fixative may result in eggs sinking in the flotation solution (Baines et al., 2015), which would reduce egg counts, but to what extent sinking affected our samples is unknown. It would be of interest for future studies to compare treated samples at time point 0 to assess the effect of immediate storage in fixative. The decrease in detectability - regardless of cause - suggests storage in formalin or ethanol is not favourable for accurate assessment of parasite burdens using flotation-based detection methods. Fixatives may still be useful for other egg counting techniques, as it is unknown in our study if such declines are potentially caused by fixative-induced changes in egg flotation

ability, rather than egg loss. Moreover, in all four storage treatments the decline in helminth eggs was uniform across the different samples, suggesting that it may be possible to account for decreases in egg count to calculate the level of egg shedding, though further research is required. These calculations cannot be used to estimate prevalence due to downward bias of estimates; low original counts could drop to zero following storage. Calculations can however be used to estimate an infection intensity to an extent, as relative infection between individuals would stay constant; highly infected individuals should have higher counts post storage than less infected.

5. Conclusion

In conclusion, we have shown that storage of faecal samples may preserve egg counts for a short period of time, though fresh analysis should always be conducted if possible. Refrigeration for maximum of a week will not significantly affect counts, whilst storage in fixatives is not advisable until the uniformity in declines is quantified in more individuals and populations.

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References

- Baines, L., Morgan, E.R., Ofthile, M., Evans, K., 2015. Occurrence and seasonality of internal parasite infection in elephants, *Loxodonta africana*, in the Okavango Delta Botswana. *Int. J. Parasitol. Parasites Wildl.* 4, 43–48.
- Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* 67, 1–48.
- Christensen, R.H.B., Regression Models for Ordinal Data, 2015. 1–22.
- Coles, G.C., Bauer, C., Borgsteede, F.H., Geerts, S., Klei, T.R., Taylor, M., a Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Vet. Parasitol.* 44, 35–44.
- Cringoli, G., Rinaldi, L., Veneziano, V., Capelli, G., Scala, A., 2004. The influence of floatation solution: sample dilution and the choice of McMaster slide area (volume) on the reliability of the McMaster technique in estimating the faecal egg counts of gastrointestinal strongyles and *Dicrocoelium dendriticum* in sheep. *Vet. Parasitol.* 123, 121–131.
- East, M.L., Otto, E., Helms, J., Thierer, D., Cable, J., Hofer, H., 2015. Does lactation lead to resource allocation trade-offs in the spotted hyaena? *Behav. Ecol. Sociobiol.*, 805–814.
- Foreyt, W.J., 1986. Recovery of nematode eggs and larvae in deer: evaluation of fecal preservation methods. *J. Am. Vet. Med. Assoc.* 189, 1065–1067.
- Garg, P.K., Perry, S., Dorn, M., Hardcastle, L., Parsonnet, J., 2005. Risk of intestinal helminth and protozoan infection in a refugee population. *Am. J. Trop. Med. Hyg.* 73, 386–391.
- Gillespie, T.R., 2006. Noninvasive assessment of gastrointestinal parasite infections in free-ranging primates. *Int. J. Primatol.* 27, 1129–1143.
- Hing, S., Othman, N., Nathan, S.K.S.S., Fox, M., Fisher, M., Goossens, B., 2013. First parasitological survey of endangered Bornean elephants *Elephas maximus borneensis*. *Endanger. Species Res.* 21, 223–230.
- Jagla, E., Spiewak, J., Zalesny, G., Popiolek, M., 2013. Effect of storage and preservation of horse faecal samples on the detectability and viability of strongylid nematode eggs and larvae. *Bull. Vet. Inst. Pulawy* 57, 161–165.
- Kaplan, R.M., Vidyashankar, A.N., 2012. An inconvenient truth: global worming and anthelmintic resistance. *Vet. Parasitol.* 186, 70–78.
- Lynsdale, C.L., Santos, D.J.F., Dos Hayward, A.D., Mar, K.U., Htut, W., Aung, H.H., Soe, A.T., Lummaa, V., 2015. A standardised faecal collection protocol for intestinal helminth egg counts in Asian elephants, *Elephas maximus*. *Int. J. Parasitol. Parasites Wildl.* 4, 307–315.
- Martinez- Valladares, M., Geurden, T., Bartram, D.J., Martinez-Perez, J.M., Robles-Perez, D., Bohorquez, A., Florez, E., Meana, A., Rojo-Vazquez, F.A., 2015. Resistance of gastrointestinal nematodes to the most commonly used anthelmintics in sheep, cattle and horses in Spain. *Vet. Parasitol.* 211, 228–233.
- McKenna, P.B., 1998. The effect of previous cold storage on the subsequent recovery of infective third stage nematode larvae from sheep faeces. *Vet. Parasitol.* 80, 167–172.
- Ministry of Agriculture, Fisheries and Food, 1986. *Manual of Veterinary Parasitological Laboratory Techniques*. HMSO, London, pp. 7–15.
- Nielsen, M.K., Kaplan, R.M., Thamsborg, S.M., Monrad, J., Olsen, S.N., 2007. Climatic influences on development and survival of free-living stages of equine strongyles: implications for worm control strategies and managing anthelmintic resistance. *Vet. J.* 174, 23–32.
- Nielsen, M.K., Vidyashankar, A., Andersen, U.V., DeLisi, K., Pilegaard, K., Kaplan, R.M., 2010. Effects of fecal collection and storage factors on strongylid egg counts in horses. *Vet. Parasitol.* 167, 55–61.
- Rinaldi, L., Coles, G.C., Maurelli, M.P., Musella, V., Cringoli, G., 2011. Calibration and diagnostic accuracy of simple flotation: McMaster and FLOTAC for parasite egg counts in sheep. *Vet. Parasitol.* 177, 345–352.
- Roepstorff, A., Nansen, P., 1998. *Epidemiology, Diagnosis and Control of Helminth Parasites of Swine*, 3. FAO Animal Health Manual.
- Rvc.ac.uk, 2016. Royal Veterinary College, RVC, [online] Available at: <http://www.rvc.ac.uk> (accessed 10.03.2016).
- Seivwright, L.J., Redpath, S.M., Mougeot, F., Watt, L., Hudson, P.J., 2004. Faecal egg counts provide a reliable measure of *Trichostrongylus tenuis* intensities in free-living red grouse *Lagopus lagopus scoticus*. *J. Helminthol.* 78, 69–76.
- Smith-Buijs, C.M., Borgsteede, F.H., 1986. Effect of cool storage of faecal samples containing *Haemonchus contortus* eggs on the results of an in vitro egg development assay to test anthelmintic resistance. *Res. Vet. Sci.* 40, 4–7.
- Stringer, A.P., Smith, D., Kerley, G.I.H., Linklater, W.L., 2014. Reducing sampling error in faecal egg counts from black rhinoceros (*Diceros bicornis*). *Int. J. Parasitol. Parasites Wildl.* 3, 1–5.
- Taylor, M.A., Coop, R.L., Wall, R.L., 2007. *Veterinary Parasitology*. Blackwell Publishing, Oxford, pp. 798–810.
- Vidya, T.N.C., Sukumar, R., 2002. The effect of some ecological factors on the intestinal parasite loads of the Asian elephant (*Elephas maximus*) in southern India. *J. Biosci.* 27, 521–528.
- Waller, P.J., 1997. Anthelmintic resistance. *Vet. Parasitol.* 72, 391–412.
- Wilson, L., Rhodes, A.P., Dodunski, G., 2015. Parasite management extension—challenging traditional practice through adoption of a systems approach. *NZ. Vet. J.* 63, 292–300.
- Wunderground.org., 2016 Weather Forecast & Reports—long Range & Local | Wunderground | Weather Underground [online] Available at: <http://www.wunderground.org> (accessed 10.03.2016).