Veterinary Parasitology 221 (2016) 130–133

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

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

Jennie A.H. Crawley[a,*,1], Simon N. Chapman[a,1], Virpi Lummaa[a,b], Carly L. Lynsdale[a]

[a] Department of Animal and Plant Sciences, Alfred Denny Building, University of Sheffield, Western Bank, Sheffield S10 2TN, UK
[b] Department of Biology, University of Turku, FIN-20014 Turku, Finland

ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form 11 March 2016
Accepted 15 March 2016

Keywords:
Refrigeration
Faecal egg count
Formalin
Ethanol
Nematode
Parasite

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.

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 underrepresentative 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).

* Corresponding author.
E-mail address: jahcrawley1@gmail.com (J.A.H. Crawley).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.vetpar.2016.03.012

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 subsamples 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.