Effects of fecal collection and storage factors on strongylid egg counts in horses

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ARTICLE INFO

Article history:
Received 1 July 2009
Received in revised form 27 August 2009
Accepted 24 September 2009

Keywords:
Fecal egg count
Strongyle
Horse
Storage
Temperature
Time

ABSTRACT

Fecal analyses are becoming increasingly important for equine establishments as a means of parasite surveillance and detection of anthelmintic resistance. Although several studies have evaluated various egg counting techniques, little is known about the quantitative effects of pre-analytic factors such as collection and storage of fecal samples. This study evaluated the effects of storage temperature, storage time and airtight versus open-air storage on fecal egg counts. The experimental protocols were replicated in two study locations: Copenhagen, Denmark and Athens, Georgia, USA. In both locations, the experiment was repeated three times, and five repeated egg counts were performed at each time point of analysis. In experiment A, feces were collected rectally and stored airtight at freezer (−10 to −18 °C), refrigerator (4 °C), room (18–24 °C), or incubator (37–38 °C) temperatures. Egg counts were performed after 0, 6, 12, 24, 48, and 120 h of storage. In experiment B, feces were collected rectally and stored airtight or in the open air in the horse barn for up to 24 h. Egg counts were performed after 0, 3, 6, 12, and 24 h of storage.

In experiment A at both locations, samples kept in the refrigerator showed no decline in egg counts, whereas storage in the freezer and incubator led to significantly declining egg numbers over time. In contrast, storage at room temperature yielded marked differences between the two study locations: egg counts remained stable in the U.S. study, whereas the Danish study revealed a significant decline after 24 h. In experiment B, the Danish study showed no differences between airtight and open-air storage and no changes over time, while the U.S. study found a significant decline for open-air storage after 12 h. This difference was attributed to the different barn temperatures in the two studies. To our knowledge, this is the first study to evaluate the pre-analytic factors affecting egg counts in horses using an experimental protocol replicated in two contrasting geographic and climatic locations. Our results demonstrate that refrigeration is the best method for storage of fecal samples intended for egg count analysis, but that accurate results can be derived from fecal samples collected from the ground within 12 h of passage.

1. Introduction

The group of equine strongyle parasites is very diverse and consists of about 60 described species (Lichtenfels et al., 2008). Strongyles ubiquitously infect grazing horses under all types of climatic and geographical conditions world-over. Moreover, the species reported as most
common are generally the same worldwide, despite major differences in the climate of the study location (Bucknell et al., 1995; Ogbourne, 1976; Reinemeyer et al., 1984; Silva et al., 1999). Thus, one would expect that parameters affecting strongyle egg development in horse feces would also be similar; however, no studies have been done comparing the influence of storage conditions across continents and climates to see if common traits can be identified.

For the past several decades, control of equine parasites has been based on the administration of frequent anthelmintic treatments at fixed intervals year-round (Drudge and Lyons, 1966; Anonymous, 1999; Lloyd et al., 2000; Mellor et al., 2001; O'Meara and Mulcahy, 2002; Matthee et al., 2002; Comer et al., 2006; Lind et al., 2007). Coincident with this approach to parasite control in horses has been an increasing prevalence and distribution of anthelmintic resistance in several important equine parasites, most notably in the cyathostomin. Presently, cyathostomin populations are widely resistant to benzimidazoles and pyrantel salts (Kaplan, 2002, 2004). Reduced egg reappearance periods after treatment with ivermectin have been reported recently (von Samson-Himmelstjerna et al., 2007; Molento et al., 2008; Lyons et al., 2008, 2009) which is considered the first sign of developing resistance (Sangster, 1999).

In response to this growing crisis, new recommendations are being made to reduce the treatment intensity and thereby delay further development of resistance. A strategy that has gained increasing support among equine parasitologists is the principle of selective therapy, whereby fecal samples from all horses on the premises are analyzed, but only those individuals that exceed a pre-determined cut-off value for eggs per gram (EPG) are treated (Gomez and Georgi, 1991; Duncan and Love, 1991; Krecek et al., 1994; Kaplan, 2002). Likewise, it has become imperative for horse farms to routinely screen for anthelmintic resistance to ensure efficacy of the drugs chosen. In horses, the only practicable method for broad spectrum detection of resistance is the Fecal Egg Count Reduction Test (FECRT), in which fecal samples are collected pre- and post-treatment and the percent reduction of parasite eggs is calculated. Clearly, analyzing fecal samples is now the foundation for evidence-based equine parasite control. European Union countries such as Denmark, Sweden and The Netherlands have recently implemented prescription-only regulations for anthelmintic drug usage. As a result, veterinarians are once again becoming key players in designing parasite control strategies based on routine analysis of fecal samples (Nielsen et al., 2006).

Although recommendations are often given on how to handle and store equine fecal samples prior to analysis, there is little evidence in the scientific literature to support them. In sheep studies using the Egg Hatch Assay (EHA), it is important to keep strongyle eggs as fresh and undeveloped as possible. This has led to recommendations of keeping eggs at 4°C (Le Jambre, 1976; Smith-Buys and Borgsteede, 1986) or in airtight containers to produce an anaerobic environment (Hunt and Taylor, 1989). According to the cited studies, these storage conditions can prevent egg development for up to 7 days. It is therefore often assumed that equine fecal samples intended for fecal egg count analysis should be kept airtight and cool prior to analysis, but there is virtually no evidence behind this and it is unknown whether eggs can tolerate storage at other temperatures for shorter or longer periods.

With the increasing emphasis on fecal egg count (FEC) analysis, there is a need to revisit the methodology and identify procedures that can be implemented as good management practices. Although studies have been carried out evaluating and comparing various techniques for fecal egg counting (O’Grady and Slocombe, 1980; Egwang and Slocombe, 1982; Cringoli et al., 2004), little information has been gathered evaluating preanalytic factors such as collection and storage of fecal samples prior to analysis. Because it is impossible to measure egg counts accurately if the sample has been degraded prior to analysis, general knowledge of how best to collect and store a fecal sample is of great importance for both veterinary practitioners in the field and scientists investigating anthelmintic resistance. The studies described herein were performed to evaluate the consistency of egg counts obtained from equine fecal samples kept under various storage conditions for defined time intervals. Identical study protocols were followed under summer conditions in a warm temperate climate and autumn conditions in northern temperate climate.

2. Materials and methods

2.1. Fecal samples

At each study location, the FEC of several horses were evaluated and a candidate shedding 200–500 eggs per gram (EPG) was selected as a feces donor. All fecal samples were collected rectally in large portions. Larval cultures were performed to evaluate the presence of any large strongyle species.

2.2. Study locations

The study was carried out in two separate locations with different climates. The U.S. portion was carried out in Athens, Georgia situated at 33°96’ N, 83°40’ W, while the Danish portion was performed in Copenhagen at 55°96’ N, 12°35’ E. The U.S. portion of the study was carried out in the summer months of June and July, 2006 at the University of Georgia, Athens, GA. This location has a warm temperate climate with average monthly temperatures ranging between 5.6 and 26.7°C on a yearly basis. However, during the summer, the Georgia climate is subtropical with average daily high temperatures ranging from 30.6 to 32.4°C. The Danish portion of the study was performed in November 2008 at the University of Copenhagen, Denmark. Denmark has a northern temperate coastal climate with average monthly temperatures ranging from 0 to 15.7°C. In November, the average temperature range is 2.3–7.0°C.

2.3. Egg count technique

Fecal egg counts (FECs) were performed using a modified Stoll technique (Stoll, 1923) with a 5 eggs per
gram detection limit. Ten (10.0) grams of feces were measured into a tared plastic beaker, and then tap water was added to a total weight of 100.0 g. The feces were mixed thoroughly with a tongue depressor, strained through a single layer of cheesecloth, and the fecal debris was pressed with a tongue depressor to remove excess water. Immediately after straining, the mixture was stirred and then exactly 2 ml were pipetted into a conical tube. Flotation solution was added until a convex meniscus was formed. Cover slips were placed on the tubes and the tubes were centrifuged in closed swing buckets at 300 × g for 10 min. After centrifuging, the cover slips were removed from the tubes and each was placed on a glass microscope slide. Each tube was topped with a second cover slip for 5 min, with no additional centrifugation. Finally, the second cover slip was placed on the slide with the first, and the eggs under both cover slips were counted with a compound microscope at 100 × total magnification. The eggs from both cover slips were totaled and multiplied by five to obtain EPG. Each egg count performed during the study was repeated five times. There was one minor difference between the Danish and U.S. egg counting procedures. The Danish laboratory used a saturated sugar–salt solution with a specific gravity of 1.27, while the U.S. laboratory used a Sheather’s saturated sugar solution with a specific gravity of 1.26. However, the centrifuge tubes used in the Danish laboratory were 10 ml, while the U.S. laboratory used 15 ml tubes. The respective dilution factors yielded effective specific gravities of about 1.22 in both laboratories.

2.4. Experiment A: temperature and storage time

A fecal sample exceeding 400 g was collected rectally. Feces were placed in a 4 l resealable bag and homogenized thoroughly using manual manipulation before dividing into four 100 g portions. Each portion was placed in a 4 l resealable freezer bag and all air was squeezed out by using a rolling technique to produce an airtight storage medium. The four 100 g portions were placed in a freezer, a refrigerator, an incubator, or held at room temperature (see Table 1 for specific temperature ranges). Fecal egg counts were carried out after 0, 6, 12, 24, 48 and 120 h of storage. Egg counts were repeated five times at each time point. Temperatures were measured at the time of each fecal analysis. The experiment was repeated on three occasions in each country. Temperatures in the two barns differed markedly. In the U.S. experiment, temperatures ranged from 25.6 to 29.1 °C, whereas the range was 11.8 to 16.9 °C in the Danish counterpart.

2.5. Experiment B: time after defecation

Feces were collected rectally and subdivided into two 100 g samples. One was put in a closed airtight 4 l resealable bag, while the other was left uncovered for open-air exposure. Both were left on the stall floor in a horse barn. Egg counts were performed after 0, 3, 6, 12 and 24 h of storage. Egg counts were repeated five times at each time point. Temperatures were measured at the time of each fecal analysis. The experiment was repeated on three occasions in each country. Temperatures in the two barns differed markedly. In the U.S. experiment, temperatures ranged from 25.6 to 29.1 °C, whereas the range was 11.8 to 16.9 °C in the Danish counterpart.

2.6. Statistical analyses

All statistical analyses were carried out using SAS software (SAS, 2009) version 9.2. Data were analyzed with mixed linear models evaluating the influence of storage time, treatment (temperature or airtight versus open-air storage), egg count repeat, trial and country (USA or Denmark). In addition, interactions between variables were evaluated in the model. Egg count data at time 0 were treated as baseline. Residuals were tested for normality with the Kolmogrov–Smirnov (KS) test. Effects observed over time were evaluated with Tukey’s sequential trend test. All statistical comparisons were evaluated at the 5% significance level (p < 0.05) after accounting for the baseline. Since time 0 data represented egg counts using fresh feces, all time 0 data within a trial were averaged and used as baseline. Least square mean (lsmean) values at each time point were used for inferential purposes. These are calculated using the mean FEC at each time point and subtracting an adjustment term for baseline values. Sequential trend tests, which use weighted averages of lsmeans, were used to evaluate treatment effects across time.

Statistical models used for the analyses are presented below. These are written in a non-technical form as typically used in standard software like SAS to make the exposition simple. In all models below, asterisks (*) represent interaction effects.

2.6.1. Experiment A

FEC changes across temperature, time, and the country (Denmark, U.S.) were statistically evaluated using the model:

\[
\text{FEC} = \text{Baseline} \times \text{Treatment} \times \text{Country} + \text{Time}
\]

\[
+ \text{Treatment} \times \text{Country} + \text{Treatment} \times \text{Time}
\]

\[
+ \text{Country} \times \text{Trial} \times \text{Treatment} \times \text{Country} \times \text{Error},
\]

where Treatment refers to the storage type. Baseline represents a slope term for the baseline response and the slopes are assumed to be different for each treatment and country combination. The remaining variables were treated as class variables where a parameter is associated for each level of the variable. The resulting model is an analysis of covariance model. Error was modeled to be independent and normally distributed with different variances for each combination of time, country, and treatment. Additionally, to account for variability among

| Table 1: Storage temperature conditions during experiment A. |
|-----------------|---------------------------------|-----------------|
| **Storage type** | **Denmark** | **USA** |
| Freezer         | −18.3 to −16.8 °C | −10 °C |
| Refrigerator    | 4 °C | 4 °C |
| Room temperature| 18.0 to 21.3 °C | 24 °C |
| Incubator       | 37 °C | 38 °C |

* Room was air-conditioned.
trials, countries, and treatments, a three-way random effect was introduced where the random effect variances were different for each choice of country and treatment combination. Time trends were evaluated for each of the four storage types: freezer, refrigerator, room temperature and incubator in each study location separately. For this analysis the following model was used:

$$\text{FEC} = \text{Baseline} + \text{Time} + \text{Trial} + \text{Error},$$

where Trial refers to the three experiment repeats performed in each study location. Error was assumed to be normally distributed with mean 0 and variance that was different at each time point. Additionally, to account for variability among replicates, Trial was modeled to be a random effect which was assumed to be normally distributed.

### 2.6.2. Experiment B

Statistical analysis was performed using an analysis of covariance model given below:

$$\text{FEC} = \text{Baseline} + \text{Time} + \text{Treatment} + \text{Treatment} \times \text{Time} + \text{Error},$$

where Treatment refers to the method of storage (aerobic or anaerobic). Baseline refers to the slope parameter using the baseline data. As described previously, Treatment and Time are class variables and hence Treatment by Time interaction is a class variable. Time trends within the two storage types were subsequently evaluated using the model:

$$\text{FEC} = \text{Baseline} + \text{Time} + \text{Error},$$

where we assumed the errors to be normally distributed with mean 0 and different variances for different time points.

### 3. Results

No large strongyle species were identified in the larval cultures.

For all analyses in experiments A and B, diagnostics with predicted values and residuals indicated that the normality assumption on the error and the random effects were reasonable. In all cases, the Kolmogrov–Smirnov (KS) test for normality yielded a p-value above 0.05. Point slope estimates for each storage type are shown in **Table 2** with corresponding standard errors and p-values evaluating whether the slopes were statistically different from 0.

#### 3.1. Experiment A: temperature and storage time

Fixed effect two-way interaction between time and country, and the three-way interaction between time, treatment and country were significant ($p < 0.0001$). Hence, separate analyses were performed for each country and treatment. Results from experiment A are presented in **Fig. 1**.

### 3.1.1. Freezer

The time effect was significant for both Denmark and U.S. data ($p < 0.0001$). Tukey’s sequential trend test revealed that both Denmark and U.S. data showed significant decreasing trends from the beginning of 6 h and 12 h to 120 h ($p < 0.0001$). However, these trends did not persist from the beginning of 24 h ($p = 0.3873$ for Denmark and 0.6523 for U.S.). Thus, the decreasing trend is caused due to reduction in mean FEC during the first 24 h.

![Fig. 1. Fecal egg counts during storage in the freezer (•), refrigerator ( ), room temperature (•) and incubator (−−) in USA (left) and Denmark (right). Results are presented as least square means calculated for each time point (6, 12, 24, 48, and 120 h). Error bars designate standard error. FECs at time 0 represent mean values prior to storing the feces. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)](image-url)
3.1.2. Refrigerator
The mean FECs across time were significantly different for Denmark ($p = 0.0095$) while it was not the case for U.S. data ($p = 0.1243$). Using Tukey’s sequential trend test, both Denmark and U.S. data showed no significant decreasing trends ($p > 0.0837$) except that Denmark data showed a significant drop from 48 to 120 h ($p = 0.0217$). Thus, the time effect seen in Denmark data is due to this drop in the average egg counts during the last time period. However, there were no decreasing trends for both U.S. and Denmark data.

3.1.3. Room temperature
The mean FECs across time were different for Denmark ($p < 0.0001$) but not for U.S. data ($p = 0.0568$). Using Tukey’s sequential trend test, FEC from Denmark showed significant decreasing trend during the entire study duration ($p < 0.0001$) while the FEC in U.S. remained stable across time ($p \geq 0.0515$).

3.1.4. Incubator
The time effect was significant for both Denmark and U.S. data ($p < 0.0001$). Using Tukey’s sequential trend test, both Denmark and U.S. data showed significant decreasing trends during the entire study duration ($p \leq 0.0003$).

3.1.5. Summary for experiment A
Thus, in summary there were significant differences in the mean FEC across time and different storage conditions. Our results show decreasing trend during the entire study duration in mean FEC for feces stored in incubator uniformly across the two countries. A similar decreasing trend was also observed for feces stored in the freezer during the first 24 h. There were no decreasing trends in mean FEC when feces were stored in Refrigerator for both countries.

The only significant difference between the two countries arose when eggs were stored in the room temperature. While in U.S. the mean FEC remained the same across time, the mean FEC from Denmark exhibited a significant decreasing trend.

3.2. Experiment B: time after defecation
Fixed effect three-way interaction between Time, Country and Baseline was significant ($p < 0.0001$). However, there were no significant Baseline*Treatment inter-actions within each country ($p > 0.621$). Results from experiment B are presented in Fig. 2.

3.2.1. Danish experiment details
There were no significant differences in FEC between aerobic and anaerobic method of storage ($p = 0.8453$). There were no significant differences in FEC across time ($p = 0.1047$) and the treatment by time interaction was also non-significant ($0.9241$).

3.2.2. U.S. experiment details
There were significant differences in FEC between aerobic and anaerobic method of storage ($p = 0.0002$). There were significant differences in both FEC across time ($p < 0.0001$) and the treatment by time interaction ($p < 0.0001$). Further analysis of the data from anaerobic storage was performed to evaluate if there are significant time effects. The point estimate of the slope was determined to be $0.2534$ with a standard error of $0.0647$. The slope was significantly different from zero ($p = 0.0001$) and the least squares mean FEC at various times were not statistically from each other ($p = 0.0563$).

3.2.3. Summary for experiment B
The Danish part of the study revealed no statistical changes over time, and no differences between the airtight and open-air storage for the entire 24 h. In contrast, the U.S. counterpart revealed a significant decline in egg counts for open-air storage after 12 h. However, egg counts from feces in airtight storage remained unchanged for the entire 24 h.

4. Discussion
This study has demonstrated the impact of conditions during collection and storage of fecal samples on fecal egg count analysis. To our knowledge, this is the first study to compare data from two different locations. This approach has generated strong evidence, on which recommendations can now be given.

When fecal samples were kept at artificial temperatures in the freezer, refrigerator or incubator, the outcomes were very similar in both study locations. However, large differences resulted from room temperature storage. Although room temperature conditions differed between the two countries, this does not explain these findings.
because the U.S. study had superior preservation of eggs despite higher temperatures. However, U.S. temperatures remained relatively constant because of air-conditioning. Since air-conditioning is uncommon in Denmark, temperatures fluctuated a few degrees. Whether such minor temperature excursions can stimulate eggs to hatch is unknown, but there are no biological explanations for this phenomenon.

The most likely factors leading to egg loss during storage are hatching and biological degradation. Hatching is known to be an aerobic process, which does not occur at temperatures below 6 °C, takes 10–14 days at 7–10 °C, 2–5 days at 10–20 °C, and about 1 day at 20–30 °C (Ogbourne, 1972; Mfitilodze and Hutchinson, 1987; Rupashinge and Ogbourne, 1978). Although we attempted to remove as much air as possible from the fecal samples, they likely were not completely anaerobic. Fecal moisture content was not measured in this study, but differences in moisture level are expected to affect the availability of oxygen. Wet feces would be more likely to produce an anaerobic state which prevented eggs from hatching. November is outside the typical grazing season in Denmark, while in Georgia, USA horses are kept on pasture in June and July. The fecal donor horse in Denmark was fed a diet of mostly hay, whereas the fecal donor in the U.S. consumed a diet of mostly fresh grass. We observed that feces from the grass-fed U.S. horses appeared wetter than the Danish hay-fed counterpart. Thus, it is likely that some or most of the differences observed during room temperature storage can be attributed to egg loss (Danish data only) due to hatching. Furthermore, high degrees of embryonation were seen after 6 and 12 h of storage in the incubator and at room temperature, respectively (Danish data only, not shown). This suggests that eggs were not subject to biological degradation, but were able to develop and probably also hatch in an environment that was not sufficiently anaerobic. One way to evaluate the occurrence of hatching in future studies would be to combine egg counts with Baermann analysis to retrieve any hatched first stage larvae in the feces. After storage in the freezer, it was noted that many eggs were distorted with ruptured egg shells, which suggests that crystallization mechanically disrupted the eggs thereby decreasing their abilities to float. Although it has been reported that strongyle eggs tolerate freezing for short periods (von Ober-Blöbaum, 1932; Lucker, 1941), this study has shown that freezing largely reduces egg numbers during the first 12 h. Refrigerating fecal samples for up to 120 h had no effect on quantitative egg counts, and thus can be recommended for storage.

It is often assumed that manual recovery of feces per rectum is a superior method for collecting fecal samples. However, this can be hazardous in some situations, and even impossible in smaller breeds and in foals. Thus, a common approach is to clean out the stall and collect feces from the floors the following morning. This can be carried out by the horse owners or stable managers, and is much quicker and easier than rectal collection. This study is the first to provide evidence supporting the validity of this approach. Experiment B revealed that if feces are less than 12 h old, samples can be collected from the floor and analyzed, even under very warm conditions such as in Georgia, USA. Cooler temperatures during the Danish autumn appeared to preserve eggs very well for at least 24 h, regardless of airtight or open-air storage (Fig. 2).

Larval cultures did not reveal the presence of eggs from any large strongyle species. However, the composition of cyathostomin species in the mixed strongyle burdens was unknown. Larval culture does not allow differentiation of cyathostomins to the species level, and molecular tools capable of identifying these species were not used in this study. In future studies, it could be relevant to apply recently developed assays such as the PCR-ELISA (Hodgkinson et al., 2001, 2003) or the reverse line blot hybridization (Traversa et al., 2007) for species characterization of the strongyle eggs. The evidence generated from a large number of studies, however, suggests that the same species seem to prevail under various climatic and geographic conditions (Ogbourne, 1976; Anderson and Hasslinger, 1982; Reinemeyer et al., 1984; Torbert et al., 1986; Krecek et al., 1989; Mfitilodze and Hutchinson, 1990; Bucknell et al., 1995; Silva et al., 1999; Gawor, 1995; Lichtenfels et al., 2001; Lind et al., 2003; Boxell et al., 2004; Kuzmina et al., 2005; Slivinska et al., 2006; Gawor et al., 2006). Although cyathostomin eggs appear virtually identical, it cannot be ruled out that eggs of different species respond differently to various storage conditions. Larger scale studies involving several individual horses with different egg shedding levels and a number of different geographical locations would provide more information on the apparent ubiquitous nature of cyathostomin species and the uniform characteristics of their eggs shed in the feces.

In summary, this study generated useful information that can be disseminated to parasitologists, equine veterinarians, stable managers and horse owners. Fecal samples collected from the ground can be used for fecal egg count analysis if they are less than 12 h old. Samples can be stored for at least 120 h in the refrigerator, which is considered the best storage method evaluated. If fecal samples are intended for fecal egg counts, exposure to hot temperatures approaching 40 °C, as well as freezing temperatures both should be avoided. Airtight storage is recommended as a usual practice, but this will not ensure the prevention of hatching in all situations. Thus, samples should be cooled with ice packs or refrigerated as soon as possible after collection. Lastly, no matter how samples are collected, handled, and stored when performing a FECRT, it is important to handle and process pre- and post-treatment samples similarly to reduce unnecessary variability in the counts.

Acknowledgement

The authors are highly grateful to Dr. Craig R. Reinemeyer, East Tennessee Clinical Research for critically reviewing the manuscript.

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