# Freezing of sheep faeces invalidates *Haemonchus contortus* faecal egg counts by the McMaster technique

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**ABSTRACT**

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Faecal pellets from a sheep that was artificially infected with a monoculture of *Haemonchus contortus* were collected over a 2-h period in the morning. In the laboratory the faeces were thoroughly mixed by hand and 48 by 1 g aliquots of the pellets were sealed in plastic bags, from which the air had gently been expressed. The faecal worm egg count of the sheep was about 14 000 $g^{-1}$.

Varying numbers of the bags were either processed for faecal worm egg counting (FEC) by the McMaster technique on day 0, or were stored at one of the following temperatures: about 4 °C, –10 °C or –170 °C before processing. The faecal aliquots that were frozen were thawed at room temperature after having been frozen for either 2 h or 7 days, and processing of aliquots maintained at 4 °C proceeded shortly after the samples had been removed from the refrigerator.

A dramatic reduction in egg numbers was found in all the aliquots that were frozen at –170 °C before faecal worm egg counts were done, as well as in those frozen for 7 days at about –10 °C. Numerous empty, or partially empty, egg shells were observed when performing the counts in faeces that had been frozen. In contrast, there was no significant reduction in the numbers of eggs in aliquots maintained for 7 days in a refrigerator at ± 4 °C before examination, when compared with others examined shortly after collection of the faeces.

Since *H. contortus* eggs in faeces are damaged by freezing, some methods that can be used for short term preservation are outlined.

It is concluded that all nematode egg counts from cryopreserved faeces (whether in a freezer at –10 °C or in liquid nitrogen) should possibly be regarded as being inaccurate, unless the contrary can be demonstrated for different worm genera. However, exceptions are expected for the more rugged ova, such as those of the ascarids and *Trichuris* spp.

**Keywords**: Cryopreservation, frozen faeces, *Haemonchus contortus,* sheep, worm egg count

## INTRODUCTION

With minor modifications the McMaster method that was developed by Gordon & Whitlock in 1939 has been adopted practically universally for estimating the concentration of nematode eggs in the faeces of ruminants. This technique for counting nematode eggs in faeces comprises flotation of the nematode eggs in fluids of high relative density, such as saturated NaCl or sucrose solution for counting in specially-designed (so-called McMaster) counting chambers.

At ambient temperatures during the peak worm season many nematode eggs hatch within about 12 h, and the faecal egg count (FEC) is reduced accord-

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ingly, since the shells of the eggs that have hatched are seldom detected during the count. However, egg hatching is progressively delayed in relation to the extent to which the temperature drops in autumn and winter and it eventually ceases at low temperatures, in accordance with the worm species concerned (Levine 1980).

When frozen, faeces can be preserved practically indefinitely, but the question has arisen as to whether the freezing of faeces has any effect on nematode eggs contained in them and hence the FEC when they are subsequently thawed.

## MATERIALS AND METHODS

Faeces were obtained from a donor sheep wether that was artificially infected with *Haemonchus contortus* monoculture in the laboratory. It was maintained in a separate concrete-floored pen that was swept daily to prevent unintended nematode infection. Faecal pellets were obtained by hanging faecal collecting bags on the sheep for a period of approximately 2 h in the morning.

In the laboratory the well-formed pellets which were of "normal" consistency were thoroughly mixed by hand without crushing them. Thereafter 48 by 1 g aliquots of the pellets were placed in individual plastic bags, approximately 10 x 10 cm in size. Before being hermetically sealed, the bags were gently flattened by hand to remove as much air as possible.

The faecal aliquots were randomly apportioned to the treatments listed in Table 1. Immediately after the faecal bags had been sealed, eight of them were placed in a refrigerator at ± 4 °C, 14 in a freezer at ± –10 °C, 16 in the gas phase of liquid nitrogen at about –170 °C, and ten were left at room temperature (± 25 °C). Thereafter the various aliquots were examined at the intervals indicated in Table 1.

The aliquots of faeces that were frozen were allowed to thaw at room temperature (± 25 °C) for about 2 h before the processing for egg counting commenced. However, only one of the three aliquots that had been frozen for 2 h in the freezer and similarly only one of the five placed for the same period in liquid nitrogen were examined on day 0 (Tables 1 and 2). The remaining "day 0" aliquots (two from the freezer and four from the liquid nitrogen) were placed in a refrigerator at ± 4 °C after they had thawed at room temperature, and were processed only on day 1 (Tables 1 and 2).

Each aliquot was processed for egg counting by a modification of the method of Reinecke (1973): The faecal pellets were gently crumbled by means of a spatula in 59 mℓ 40 % sugar solution at ambient temperature, before being homogenised for a short burst per sample with an Ultraturrax homogeniser. A few drops of amyl alcohol were added to each of the bottles containing the faecal suspensions to break up bubbles that resulted from the homogenisation. After thorough mixing of each suspension by swirling and then repeatedly drawing it into and expelling it from a bulb pipette, two chambers of each McMaster slide were filled from the pipette. The mixing was repeated before each chamber was filled.

The slides were allowed to stand for at least 5 min after filling before counting commenced. The aliquots were processed in batches so that each counting procedure would be completed within 2 h. All the worm eggs within the grid of each counting chamber were counted using 10x objective and ocular lenses of a compound microscope. The numbers were summed per slide, which gave a sensitivity of 200 eggs $g^{-1}$ of faeces.

Statistical analyses comprised comparisons of the counts of the different sets of faecal samples with one another, using the Mann-Whitney U test.

## RESULTS

The FEC of the sheep from which faeces were obtained for the trial was 14 200 $g^{-1}$ (mean count of ten aliquots of 1 g each).

Both intact and damaged ova were encountered. The latter were of two main types, namely empty eggshells and those of which the contents had partially been lost.

When the initial preparations were examined on day 0, only intact eggs were counted (Table 2) but when the first aliquot that was thawed after freezing in liquid nitrogen was examined, no intact and only some damaged ova were seen. It was decided to count both intact and damaged ova in all the aliquots examined thereafter.

No intact ova were recovered from any of the faecal aliquots frozen at ± –170 °C. In contrast, a mean of 22 intact ova was counted per aliquot of the faeces thawed after having been in the freezer at –10 °C for 2 h, but in those frozen for 7 d there was a dramatic drop to a mean of only 0.2 intact ova in the 11 aliquots examined.

TABLE 1 Trial design

| Aliquots (No.) | Storage method | Temperature (°C) | Period | Examination |
|---|---|---|---|---|
| 10 | Unfrozen[a] | ± 20 | 2 h | Day 0 |
| 1 | Freezer | ± –10 | 2 h | Day 0 |
| 2 | Freezer[b] | ± –10 | 2 h | Day 1 |
| 1 | Liquid nitrogen | ± –170 | 2 h | Day 0 |
| 4 | Liquid nitrogen[b] | ± –170 | 2 h | Day 1 |
| 1 | Unfrozen | ± 4 | 1 day | Day 1 |
| 7 | Unfrozen | ± 4 | 7 days | Day 7 |
| 11 | Freezer | ± –10 | 7 days | Day 7 |
| 11 | Liquid nitrogen | ± –170 | 7 days | Day 7 |

[a] Unfrozen: Maintained either at room temperature (day 0) or at ± 4 °C in a refrigerator (days 1 and 7)
[b] Thawed on day 0 at room temperature, but then stored at ± 4 °C and processed on day +1

TABLE 2 Faecal worm egg counts (count x 200 = eggs $g^{-1}$ of faeces)

| Day when thawed and/or processed | No. of aliquots | Worm egg counts[b] | | | |
|---|---|---|---|---|---|
| | | Intact | Damaged | Total | |
| | | (mean) | (mean) | Mean | Range |
| Day 0 | | | | | |
| Unfrozen[a] | 10 | n/r | n/r | 71.0 | 46–108 |
| Freezer[c] | 1 | n/r | n/r | 27.0 | n/a |
| Freezer[d] | 2 | 22.0 | 28.0 | 50.0 | 39–61 |
| Liquid N[c] | 1 | 0.0 | 3.0 | 3.0 | n/a |
| Liquid N[d] | 4 | 0.0 | 20.5 | 20.5 | 0–38 |
| Day 1 | | | | | |
| Unfrozen | 1 | 64.0 | 3.0 | 67.0 | n/a |
| Day 7 | | | | | |
| Unfrozen | 7 | 58.4 | 4.6 | 63.0 | 37–108 |
| Freezer | 11 | 0.2 | 0.8 | 1.0 | 0–3 |
| Liquid N | 11 | 0.0 | 0.7 | 0.7 | 0–4 |
| Overall means[e] | | | | | |
| Unfrozen | n/a | 59.1 | 4.4 | 67.7 | 37–108 |
| Freezer | n/a | 3.6 | 5.0 | 9.8 | 0–61 |
| Liquid N | n/a | 0.0 | 5.8 | 5.8 | 0–38 |

[a] Unfrozen: Stored either at room temperature, or in refrigerator (± 4 °C)
[b] n/r: intact and damaged ova not recorded separately; n/a: not applicable
[c] Thawed and examined on day 0
[d] Thawed on day 0, but then stored at ± 4 °C and examined on day 1
[e] Calculated from the individual egg counts (not from the means above)

In the faecal aliquots that were not frozen (of which ten were processed on day 0, commencing 2 h after collection and seven after storage for 7 d in a refrigerator at ± 4 °C), there were no statistically significant differences between the FECs on day 0 and day 7 ($P > 0.05$). In contrast, despite only small numbers of aliquots having been processed after freezing for 2 h at either –10 °C or –170 °C, the differences between the total counts (intact and damaged ova) of these aliquots and those thawed and

processed on day 7 were significant ($P < 0.01$). The differences on day 7 between the aliquots frozen at the two different temperatures were not significant ($P > 0.05$). On that day, however, the counts of the 22 aliquots that had been frozen were highly significantly lower than the seven unfrozen ones ($P < 0.001$).

## DISCUSSION

To facilitate the observation of the worm eggs after flotation in each faecal suspension, 1 g instead of the customary 2 g of faeces was used in the trial per 60 ml suspension. For this reason faeces from a relatively heavily infected sheep were used, to obtain sufficiently high counts for statistical analysis without having to examine an inordinately large number of counting chambers. Furthermore, 100x magnification was used for the counting, instead of the 40x usually employed for routine nematode egg counting in our laboratory. It was considered that the use of both the lower density of the faecal suspensions and the higher magnification would increase the chances that eggs would not be missed when viewed under the microscope.

In common with usual results in our laboratory, considerable variation occurred between the FECs of the different aliquots of faeces taken at random from a single collection and examined on the same day (viz. 46–108 in the unfrozen faeces of day 0) (Table 2).

It is not known whether the initial high FEC could have played a role in the effect of freezing of the faeces on the counts obtained, but it seems unlikely, particularly since the faeces consisted of well-formed pellets, despite the high egg count. On the other hand, it is possible that different results would have been obtained had the faeces been diarrhoeic.

It is uncertain why, in the faeces that had been frozen either at ± –10 °C or –170 °C, relatively large numbers of damaged ova were seen only on day 1, while much smaller numbers were encountered on day 0 and day 7. In the first aliquot that was frozen in liquid nitrogen and examined on day 0, the low count can possibly be ascribed to the fact that the damaged ova were difficult to detect, and were missed. This surmise is supported by comparing the egg counts of the three aliquots that were frozen in the freezer at –10 °C and thawed after 2 h on day 0. While one of these aliquots was processed for counting on day 0, the remaining two were kept at ± 4 °C after having been thawed and were processed for counting only on day 1 (Table 2). The total egg count of the aliquot of faeces examined on day 0 (before damaged ova were noticed for the first time) was similar to the mean FEC of intact ova observed in the other two examined on day 1, excluding the considerable numbers of damaged ova that were detected when specifically and diligently searched for. On the other hand, despite careful examination, very low numbers of ova, either intact or damaged, were encountered on day 7 in any of the aliquots that had been frozen.

From the results of this trial there is little if any doubt that faeces of sheep containing the eggs of *H. contortus* cannot be frozen without invalidating subsequent FECs determined by the McMaster technique. The question arises as to whether this is valid only for the eggs of the single worm species tested, or whether it also holds true for those of other nematode species. In addition, it is possible that different methods of thawing frozen faeces may perhaps give more satisfactory results. For example, in contrast to the slow thawing method that was used in the present trial, vials of cryopreserved L3 of the common gastrointestinal nematodes of small ruminants and cattle are thawed very quickly in water at ± 55 °C in our laboratory until merely the smallest trace of ice remains, at which stage the vials are transferred to cold tap water. With this method excellent viability of the larvae is obtained (Van Wyk, Gerber & Van Aardt 1977; Van Wyk, Gerber & De Villiers 2000). Another factor may be the use of 40 % sucrose solution for flotation of the ova in the faecal suspension, while concentrated solutions of $NaNO_3$, NaCl, $MgSO_4$ and $ZnSO_4$ solution are commonly used elsewhere (Reinecke 1983) and their effects should be similarly investigated.

Using the McMaster method, Cabaret (1981) also found a decrease in FECs when comparing the numbers of eggs of *Ostertagia* (*Teladorsagia*) *circumcincta* in aliquots of sheep faeces that had been preserved in either a refrigerator or in a freezer at ± –10 °C, compared to other aliquots that were processed on day 0 without having been frozen. In contrast to the results obtained in the present trial, however, the reduction in numbers was similar in the faeces subjected to both methods of preservation.

With a "brine flotation" technique, Goldman & Johnson (1950) reported that there was a marked reduction of (predominantly hookworm) egg counts in human faeces thawed after having been frozen for

10 days to 3.5 months in a "deep-freeze locker", compared to the FECs obtained for control stools that were examined unfrozen within 24 h. Some of the ova that did float were distorted. They concluded that the freezing of stools is unsatisfactory for preserving human stools for worm egg counts "by direct examination or by $ZnSO_4$ and brine flotation techniques". On the other hand, Carney & Cross (1981) obtained similar egg counts, using the Kato thick smear method on the faeces of *Macaca cyclopis* monkeys infected with the trematode, *Schistosoma japonicum*, irrespective of the faeces being fresh, or frozen and thawed before examination. It is probable that the results obtained in our trial and those of Goldman & Johnson (1950) and Cabaret (1981) differed from those of Carney & Cross (1981) because of differences in the fragility of the ova of nematode and trematode parasites.

Pandey (1972) tested the survival of *Ostertagia ostertagi* ova in cattle faeces after various periods of freezing at –10 °C. He did not, however, compare FECs of fresh and frozen faeces, but only tested the survival of the ova by culturing the aliquots of faeces after they had been thawed, and then recovering the infective larvae (L3) that developed in the cultures. These recoveries were compared to larval numbers from cultures of fresh faeces that had not been frozen and that originated from the same batch. Interestingly, during the first 2 days of his trial similar numbers of L3 were recovered from both fresh faeces and aliquots that had been frozen and thawed. Only thereafter did fewer L3 develop in the faeces that had been frozen than in the aliquots that were kept in a refrigerator. In contrast to what is to be expected from the present trial, in Pandey's (1972) experiment the counts of L3 after 2 weeks at –10 °C were still 36% of the control count of L3 from unfrozen faeces. It is possible that the ova of *O. ostertagi* in bovine faeces are better able to survive freezing at this temperature than are those of *H. contortus* in sheep pellets.

It is not clear why there was a significant reduction in the FECs of the aliquots stored at –10 °C, between the "day 0" faeces (Table 2) examined on day 1 and those aliquots thawed and examined on day 7. While not investigated, it was taken for granted that the faeces would have been frozen completely after 2 h in a freezer, but this may not have been the case.

FECs of *H. contortus* in faeces stored for at least 21 days in a refrigerator at about 4 °C were found to be similar to others done on fresh faeces of the same faecal batches (F.S. Malan & J.A. van Wyk, unpublished observations 2000). However, when it is not possible to cool the faeces, the best approach for preserving them for FECs or egg hatch assays (for estimating anthelmintic susceptibility of worm populations), is to store them anaerobically, e.g. by placing them in water in a container sealed with an airtight cap or lid (Hunt & Taylor 1989). With this method 10 g of faeces and ten glass beads are added to about 90 mℓ of water in a 100-mℓ bottle with a screw cap. The container is shaken vigorously to break up the faeces and set the entrapped worm eggs free. The contents of the bottle become anaerobic within 3 h, and the faeces can be stored at ambient room temperature for up to 7 days (Hunt & Taylor 1989). Furthermore, R. Somers (personal communication 2000) found that the faecal pellets of sheep with a patent infection of *H. contortus* could be preserved by mixing the pellets with a little water and compacting them in a plastic hypodermic syringe that is sealed thereafter with adhesive tape before being stored in an ice-cooled, insulated container. Just sufficient water is added during the process of compaction to fill spaces left between imperfectly squashed pellets. He found such samples to be suitable for hatching eggs for a larval development test after a period of up to 7 days.

In conclusion, we suggest that all nematode egg counts from cryopreserved faeces (whether frozen in a freezer, dry ice or liquid nitrogen) should be regarded as possibly being inaccurate, unless the contrary can be demonstrated for each worm species, or at least for each worm genus. Probable exceptions are counts of the more rugged ova, such as those of the ascarids and whipworms, but it may be advisable to similarly investigate even these before accepting that their egg counts are not deleteriously affected by freezing.

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