

Περιοδικό της Ελληνικής Κτηνιατρικής Εταιρείας

Τόμ. 67, Αρ. 3 (2016)



Ρυθμός μείωσης του αριθμού αυγών των νηματωδών παρασίτων και της εξέλιξης των προνυμφών τους στο τρίτο στάδιο σε κόπρανα προβάτων και αιγών που συντηρούνται στους 4οC

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doi: [10.12681/jhvms.15636](https://doi.org/10.12681/jhvms.15636)

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Βιβλιογραφική αναφορά:

DRIMTZIA, A., & PAPADOPOULOS, E. (2018). Ρυθμός μείωσης του αριθμού αυγών των νηματωδών παρασίτων και της εξέλιξης των προνυμφών τους στο τρίτο στάδιο σε κόπρανα προβάτων και αιγών που συντηρούνται στους 4οC. *Περιοδικό της Ελληνικής Κτηνιατρικής Εταιρείας*, 67(3), 177–182. <https://doi.org/10.12681/jhvms.15636>

■ Reduction rate of nematode egg counts and third-stage larvae development from sheep and goat faeces preserved at 4°C

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■ Ρυθμός μείωσης του αριθμού αυγών των νηματωδών παρασίτων και της εξέλιξης των προνυμφών τους στο τρίτο στάδιο σε κόπρανα προβάτων και αιγών που συντηρούνται στους 4°C

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ABSTRACT. Gastrointestinal nematodes cause major production losses to small ruminants. The most common way to diagnose or monitor the worm burdens in sheep and goats remains the quantitative parasitological examinations, i.e. the faecal egg counts. However, the reliability of the results of such methods depends greatly on the conditions and duration of the storage of the faecal samples prior to examination. The aim of this research was to evaluate the reduction rate and the maximum storage period, without significant losses, of nematode egg counts and third-stage larvae development from sheep and goat faeces preserved at 4°C. Towards this end, a pooled faecal sample was formed by collecting faeces from naturally infected sheep and goats (separately). Faecal egg counts and coprocultures were performed on fresh faeces and on preserved ones every week and up to 119 days post sampling. It was concluded that the preservation at 4°C, i.e. into a refrigerator, of fresh faeces from sheep and goats for parasitological examinations poses danger of misdiagnosis, if not performed in a period not exceeding 3 weeks of time. The rate of reduction of the faecal nematode egg counts starts to be significant lower than the ones performed with fresh samples, for both sheep and goats, after the third week of storage. The percentage of the gastrointestinal nematode larvae developing to the infective third-stage alters significantly for the *Haemonchus* genus, soon after the first week of storage ($p<0.05$).

Keywords: nematode egg counts, larvae development, storage, sheep, goats

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Date of initial submission: 08.04.2014
Date of revised submission: 29.05.2014
Date of acceptance: 31.05.2014

ΠΕΡΙΛΗΨΗ. Ο γαστρεντερικός παρασιτισμός προκαλεί σημαντικές απώλειες παραγωγής στα μικρά μηρυκαστικά. Ο συχνότερος τρόπος διάγνωσης ή παρακολούθησης του επιπέδου παρασιτισμού στα πρόβατα και τις αίγες είναι οι ποσοτικές παρασιτολογικές εξετάσεις κοπράνων. Ωστόσο, η αξιοπιστία των μεθόδων αυτών βασίζεται κατά πολύ στις συνθήκες και τη διάρκεια αποθήκευσης των κοπράνων πριν την εξέταση. Ο σκοπός της παρούσας έρευνας ήταν η αξιολόγηση του ρυθμού απώλειας των αυγών και ο καθορισμός του μεγίστου χρόνου συντήρησης των κοπράνων χωρίς σημαντική επίδραση στην εξέλιξη των προνυμφών στο 3° (μολύνον) στάδιο. Για το σκοπό αυτό συλλέχθηκε ένα ομαδικό δείγμα κοπράνων, ξεχωριστά για πρόβατα και αίγες, από φυσικώς μολυσμένα μικρά μηρυκαστικά. Τα κόπρανα εξετάσθηκαν φρέσκα κατά την ημέρα της συλλογής τους και σε εβδομαδιαία χρονικά διαστήματα, κατά τη συντήρησή τους στους 4°C, έως 119 ημέρες μετά τη συλλογή τους. Ταυτόχρονα μέρος των κοπράνων, τις ίδιες χρονικές στιγμές με την ποσοτική εξέταση για αυγά, καλλιεργούνταν για την εκκόλαψη και εξέλιξη των προνυμφών στο 3° (μολύνον) στάδιο. Διαπιστώθηκε ότι η συντήρηση των κοπράνων από πρόβατα και αίγες στους 4°C, για παράδειγμα στο ψυγείο, όταν προορίζονται για παρασιτολογικές εξετάσεις εγκυμονεί κινδύνους λανθασμένης διάγνωσης, εάν δεν πραγματοποιούνται σε χρονικό διάστημα το οποίο δεν υπερβαίνει τις 3 εβδομάδες. Ο ρυθμός της απώλειας των αυγών των παρασίτων στα κόπρανα αρχίζει να γίνεται σημαντικά χαμηλότερος συγκριτικά με τα φρέσκα δείγματα, μετά την 3η εβδομάδα συντήρησης στους 4°C. Το ποσοστό της εξέλιξης των προνυμφών στο 3° στάδιο μεταβάλλεται σημαντικά για το γένος *Haemonchus* αμέσως μετά την πρώτη εβδομάδα συντήρησης των κοπράνων ($p<0,05$).

Αξεις ευρετηριασμού: Αυγά νηματωδών, εξέλιξη προνυμφών, συντήρηση κοπράνων, πρόβατα, αίγες

INTRODUCTION

Gastrointestinal nematode parasitism is one of the most common infections in livestock and it remains worldwide one of the greatest limiting factors in any successful sustainable ruminant livestock production system. It can cause major production losses to small ruminant industry, along with a significant decrease of animal welfare, if this condition is not diagnosed and managed properly (Mavrot et al., 2015).

The most common way to diagnose or monitor the worm burdens in sheep and goats remains the quantitative parasitological examination for the faecal nematode egg counts, expressed as 'eggs per gram' (EPG) of faeces (Taylor et al., 2007). This number is an indication of the adult worm burdens in the gut, though it should be stressed that no precise and direct correlation exists between EPG counts and the adult worm burdens within the host. Additionally, the estimation of the faecal EPG counts is a useful tool to check if a drug still remains effective, to indicate the relative susceptibility of individual animals to parasite infection and, most important, to be used in conjunction with other information to design and evaluate sustainable parasite control programs for small ruminants. This is of significant need in order to improve parasite management in sheep and goats, particularly in face of challenges, such as the development of anthelmintic resistance (Jackson and Coop, 2000; Papadopoulos, 2008; Jackson, 2009; Morgan and Coles, 2010; Learmount et al., 2015). Modern parasite control approaches of ruminants,

i.e. targeted selective treatments, are based largely on the faecal nematode egg counts estimated individually in order to select the animals in need for treatment. In such cases the reliable nematode faecal egg counts estimation is of vital importance to conclude and design correct action steps towards the effective parasite control (Gallidis et al., 2009; Kenyon et al., 2009; Papadopoulos et al., 2012).

The reliability of the results of the parasitological methods depends greatly on the conditions and duration of the storage of the faecal samples prior to examination, when for various reasons they cannot be performed immediately after the sampling. It is known that eggs from various nematode species hatch in different temperatures and the larvae development is also greatly influenced by the temperature (McKenna, 1998; Nielsen et al., 2010).

The aim of this research was to evaluate the reduction rate and the maximum storage period, without significant losses, of nematode egg counts and third-stage larvae development from sheep and goat faeces preserved at 4°C.

MATERIALS AND METHODS

Faecal samples and parasitological methods

Three semi-extensive mixed flocks, i.e. keeping sheep and goats together, were selected from the same area near Thessaloniki, Northern Greece ($40^{\circ}38'27''N$, $23^{\circ}01'29''E$) under the criterion to be naturally heavily infected with a mixture of gastrointestinal nematodes including different species, as known from previous

examinations. All animals of the above flocks were of dairy breeds/cross-breeds grazing daily and they have not been treated recently with anthelmintics for a period of more than 6 months.

A pooled faecal sample was formed by collecting faeces directly from the rectum of randomly selected animals within the 3 flocks. Faecal samples from sheep were pooled separately from the ones from goats. As soon as they were collected, within 2 hours, they were transferred to the Laboratory of Parasitology and Parasitic Diseases of the School of Veterinary Medicine, for further proceeding.

The nematode egg counts per g of faeces (EPG) were carried out using the modified McMaster method (MAFF, 1986). On each examination day, a faecal sample was also put for coproculture for 10 days at 25°C (MAFF, 1986) in order to allow eggs to develop to the 3rd-stage larvae, which were collected using the Baermann method and identified according to morphological keys (Van Wyk et al., 2004).

Experimental design

The individual faecal samples, separately per animal species, were pooled together and homogenised thoroughly by means of an electric mixer. They were put into a plastic bag each, which was clearly marked as sheep or goats, closed firmly to maintain moisture and left for storage. Both bags were stored into the same refrigerator at 4°C, which was monitored to record any accidental big deviation of the temperature.

Before each later sub-sampling from the storage bag, a careful mixing of the faeces was performed and 4 samples of 3g each, from different sites of the bag content, were taken for EPG counts (a total of 12g). These 4 samples were tested separately and each of them was used to fill 2 McMaster plates. In this way, a mean EPG number per day of examination was calculated from 8 different recorded counts. All the EPG counts were made by the same person (DA).

In parallel, a portion of 5g was similarly taken (a total of 20g) from each bag and put for coproculture, according to the methods described above (MAFF, 1986).

The faecal EPG counts and the relevant coprocultures started on the faecal collection day (fresh faeces –D0), they were repeated every week for the next 2

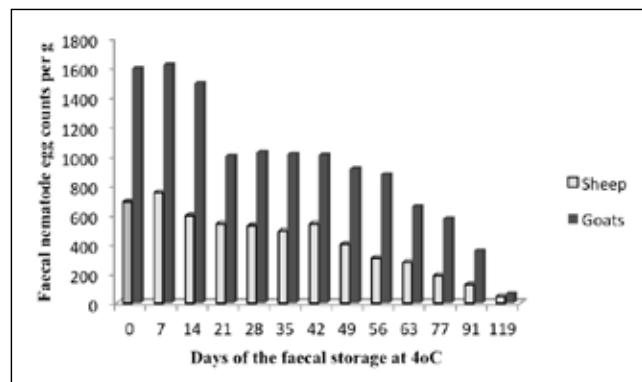


Figure 1: Nematode egg counts per g of faeces during the study period (D0-D119)

months (D7 up to D63) and thereafter every two weeks (D77 up to D91), while the last ones were performed on D119 (end of the experiment).

Statistical analysis

The faecal egg counts of the fresh faeces were compared with the ones of the preserved faeces at each examination point using the t test (SPSS v16.0) with confidence intervals of 95%.

RESULTS

Both, sheep and goats, pooled faecal samples were found to contain high nematode egg counts, as confirmed by the parasitological examination. More precisely, the fresh faeces, when examined on the day of collection (D0), contained a mean number of 687.5 and 1593.75 nematode eggs per g of sheep and goats faeces, respectively (Figure 1). Nematode eggs could be recovered from the stored faeces at 4°C during the whole study period (up to D119). However, after 3 weeks (D26) of storage the EPG counts started to decrease significantly for both animal species ($p<0.05$). The reduction of the recovery rate of nematode eggs from the sheep and goats faeces preserved at 4°C is presented in Figure 1. Furthermore, goats faeces were found to contain significantly higher EPG counts than sheep, despite the fact that both animal species were kept together ($p<0.05$).

The coprocultures from the fresh faeces contained third-stage larvae from the most common gastrointestinal nematode genera, i.e. *Teladorsagia* 32.7% and 35.6%, *Haemonchus* 22.5% and 19.0%, *Trichostrongylus* 18.9%

Table 1: Percentage (%) of third-stage larvae identified in the coprocultures of sheep faeces stored at 4 °C for different time periods

Nematode genera (L3)	Days of storage at 4 °C												
	0	7	14	21	28	35	42	49	56	63	77	91	119
<i>Haemonchus</i>	22.5	19.1	11.1	10.0	8.1	8.3	6.6	5.2	4.1	4.6	2.3	2.0	2.2
<i>Teladorsagia</i>	32.7	34.2	34.9	34.8	36.5	44.2	39.2	38.4	35.2	33.7	38.0	37.0	39.1
<i>Trichostrongylus</i>	18.9	18.2	21.2	25.8	27.3	26.1	25.3	26.6	22.9	20.8	21.1	18.2	20.0
<i>Cooperia</i>	7.5	7.6	11.7	8.4	8.3	6.1	7.8	7.0	5.0	7.5	6.5	8.0	8.5
Others*	18.4	20.9	21.1	21.0	19.8	15.3	21.1	22.8	32.8	33.4	32.1	34.8	30.2

*Others include the genera of *Chabertia*, *Bunostomum* and *Oesophagostomum*

Table 2: Percentage (%) of third-stage larvae identified in the coprocultures of goat faeces stored at 4 °C for different time periods

Nematode genera (L3)	Days of storage at 4 °C												
	0	7	14	21	28	35	42	49	56	63	77	91	119
<i>Haemonchus</i>	19.0	20.2	14.0	7.1	4.4	5.1	3.2	2.9	3.5	2.7	1.2	1.9	2.9
<i>Teladorsagia</i>	35.6	33.8	38.2	35.2	37.7	36.9	44.8	39.8	38.8	41.3	42.0	42.8	42.2
<i>Trichostrongylus</i>	22.1	20.0	24.0	27.4	28.1	27.5	17.9	21.1	20.9	22.0	25.6	20.0	23.1
<i>Cooperia</i>	14.0	11.2	11.7	9.4	11.9	8.8	9.8	11.9	6.5	8.5	8.0	6.0	4.5
Others*	9.3	14.8	12.1	20.9	17.9	21.7	24.3	24.3	30.3	25.5	23.2	29.3	27.3

*Others include the genera of *Chabertia*, *Bunostomum* and *Oesophagostomum*

and 22.1%, *Cooperia* 7.5% and 14.0%, *Chabertia/Bunostomum/Oesophagostomum* 18.4% and 9.3% for sheep and goats, respectively. All genera could be developed and detected in the coprocultures up to the end of the study period (D119). The only dramatic reduction in the overall percentage composition was noticed for *Haemonchus* larvae, which were significantly decreased after the first week (D7) of storage ($p<0.05$). On the contrary, *Teladorsagia* L3 seem to remain unaffected by the different storage periods. Not a valid conclusion could be drawn for the rest of genera, since such a study requires monospecific coprocultures and it was outside of the scope of this study. The detailed results for the L3 identification throughout the study period are presented in Tables 1 and 2 for sheep and goat faecal samples, respectively.

DISCUSSION

The aim of this research study was to estimate the maximum period of storage of sheep and goat faeces

prior to examination, at 4°C, without significant reduction on the gastrointestinal nematode egg counts. It was also investigated the possible influence of such preservation on the egg hatching and the development of larvae, belonging to different genera, up to the infective 3rd-stage.

It is a common practise worldwide to store faecal samples after collection into the refrigerator in order to preserve them (reduce hatching) and avoid missing parasitic elements leading to the underestimation of the level of parasitism. In some cases, such samples may be preserved prior to examination for long periods (McKenna, 1998; Nielsen et al., 2010). The pooled faecal samples used in this study were found to have significant different nematode egg counts between sheep and goats. This is a common observation, even in cases where both animal species are kept under the same management system (Papadopoulos et al., 2003).

The parasitological examination of sheep and goat faeces still remains the most commonly used diagnostic tool of gastrointestinal parasitism, compared to other

more sophisticated and expensive alternatives, which use modern molecular methodologies (Taylor et al., 2007; Roeber et al, 2012). Furthermore, the number of nematode eggs per g of faeces represents important information to base most anthelmintic control strategies in order to avoid the development of anthelmintic resistance (Papadopoulos, 2008; Sargison, 2011). Such strategies require the knowing of the level of parasitism in order to differentiate the heavily infected animals, which need anthelmintic treatment, from the non or very little infected ones, which do not need treatment and maintain a part of the worm population *in refugia*, i.e. not exposed to anthelmintics (Kenyon et al., 2009).

On the other hand, the differentiation of the strongyle eggs according to the parasite genera is an important complement to the parasitological examination of faeces (Wan Wyk et al., 2004). This can be done by culturing the faeces to allow the egg hatching and the development of the larvae to the third-stage, which can be identified to species (Banks et al., 1990; Wan Wyk et al., 2004). The nematode larvae identified in this study represent the most common genera parasitizing small ruminants in Greece. The most dominant small ruminant gastrointestinal nematode parasites in our country are *Teladorsagia circumcincta* and *Haemonchus contortus*, followed by other genera in smaller percentages, such as *Trichostrongylus*, *Chabertia*, *Cooperia* and others (Papadopoulos et al., 2003; Gallidis et al., 2009). Among them, *Teladorsagia* and *Haemonchus* genera have developed anthelmintic resistant strains in Greece, a fact that makes their monitoring most important in regional epidemiological surveys or parasite control schemes (Gallidis et al., 2009, 2012). According to the findings of this study, *Teladorsagia* spp. development was not significantly affected by the storage period ($p>0.05$). On the contrary, *Haemonchus* spp. larvae failed significantly to develop after one week of storage at 4 °C, a fact that partly may be due to the tropical or subtropical character of this parasite (Taylor et al., 2007).

Worms of the various genera produce different number of eggs per day. For example, *Haemonchus contortus* is one of the most fecund nematode (individual females are capable of producing thousands of eggs per day), while *Teladorsagia circumcincta* females are less fecund than *H. contortus*, with an average egg production of 100–200 eggs per female per day (Roeber et al., 2013). In such cases, the storage of the faecal samples

seems to be of high importance. Furthermore, the effects of cold storage of infective third-stage larvae (L3) of different isolates of the parasitic nematode *Haemonchus contortus* were studied with respect to infectivity, pre-patent period and propensity for larval arrestment. Two complementary experiments were conducted with 2 groups of lambs, where each animal was inoculated with 2000 *H. contortus* L3, of either Swedish or Kenyan origin. In a first experiment, L3s were cold treated at 5 °C for 9 months prior to infection, whereas in a second experiment larvae were newly hatched. Individual faecal egg counts and worm burdens were determined for each experiment. The results showed that the greatest differences were associated with the pre-treatment of larvae. The pre-patent period and the FECs differed significantly between the experiments but not between the isolates used in each experiment. However, the extent of hypobiosis was significantly different between the two isolates when fresh larvae were used (36% Kenyan isolate and 70% Swedish). The storage of *H. contortus* at 5 °C had no apparent effect on the infectivity of L3s, as high establishment ranging from 43 to 74% were observed, irrespective of isolates used. This study showed that *H. contortus* exhibited similar phenotypic traits regardless of geographical origin. Thus, there was limited evidence for adaptations to temperate climatic conditions (Troell et al., 2006). Nevertheless, the results of the above experiments provide useful information on the treatment (cold storage) of the faecal material which is going to be used for further experimental trials including artificial inoculation of animals.

Additionally to ruminants and horses (McKenna, 1998; Nielsen et al., 2010), fresh faecal specimens from deer were also examined for nematode eggs (primarily of the genera *Haemonchus* and *Ostertagia*), using a flotation technique, and then were re-examined for up to 200 days after storage in 2.5, 5 or 10% formalin, absolute methyl alcohol, or 70% ethyl alcohol at room temperature (20 °C) or after storage without preservative at 4, 0, or -20 °C (Foreyt, 1986). For long-term storage, 10% formalin was the best fixative for recovery of eggs (compared with the rate of recovery of eggs from fresh faeces). Approximately 50% of the strongyle eggs were detected in faeces stored in formalin for 200 days. However, between days 3 and 10 of storage, the recovery rate was low (less than 50%), presumably due to ion binding. Alcohols were unsuitable for preservation, and storage at 0 or -20 °C resulted in an

egg recovery rate of less than 50%. Storage at 4 °C for 50 days resulted in approximately 90% recovery of nematode eggs. Number of *Parelaphostrongylus tenuis* larvae recovered from faecal specimens stored in 10% formalin for 24 days was greater than that recovered from fresh faecal specimens (Foreyt, 1986). In our case, the duration of the recovery of nematode eggs from small ruminant faeces, in similar numbers to the fresh ones, were 3 weeks as after 4 weeks of storage the recovery rate started to reduce significantly ($p<0.05$). However, it is useful to state that the nematode eggs (though in smaller numbers) could be detected in the faeces of sheep and goats up to the end of the study period. In other words, faecal samples stored in the refrigerator for long periods, can be used to diagnose the presence of parasite eggs, but not the numbers of them. On the contrary, in case we need to identify the larvae genera present in the faecal samples, particularly

Haemonchus spp., it is strongly recommended, according to our findings, to perform the coproculture using fresh faecal material.

CONCLUSIONS

It was concluded that the preservation at 4°C, i.e. into a refrigerator, of fresh faeces from sheep and goats for parasitological examinations poses danger of misdiagnosis, if performed in a period exceeding 3-4 weeks of time post sampling. The rate of reduction of the faecal nematode egg counts starts to be significant lower than the ones performed with fresh samples, for both sheep and goats, after the third week of storage. The percentage of the gastrointestinal nematode larvae developing to the infective third-stage alters significantly for the *Haemonchus* genus, soon after the first week of storage. ■

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