METHODS FOR ESTIMATING THE DENSITY OF *Elaphostrongylus rangiferi* Mitskevich (Nematoda, Metastrongyloidea) LARVAE IN FAECES FROM REINDEER, *Rangifer tarandus* L.

Metoder for å estimere tettheten av hjernemarklarver i avføring fra reinsdyr.

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Summary: A method for estimating the density of *Elaphostrongylus rangiferi* larvae in reindeer faeces that have been deep frozen is described. The method involves the use of an inverted microscope with plankton counting chambers. Statistical data on the efficiency and sensitivity of the method are given. With fresh faeces, the results obtained with the method were not significantly different from those obtained with the Baermann technique. With faeces that had been stored in deep freeze, the method detected on average 30 per cent more larvae than the Baermann technique.

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Sammendrag: En metode for å estimere tettheten av hjernemarklarver i avføring som har vært dypfryst blir beskrevet. Anvendelse av et invertert mikroskop med plankton tellekammer inngår i metoden. Det blir gitt statistiske data for metodens effektivitet og følsomhet. Ved undersøkelse av fersk avføring skilte ikke de resultatene metoden ga seg fra de som ble oppnådd med Baermanns metode. Ved undersøkelse av avføring som hadde vært lagret dypfrosset ga metoden i gjennomsnitt 30 prosent flere larver enn Baermanns metode.

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INTRODUCTION

Established methods for faecal examination are most suitable when samples are easily obtained and may be examined without prolonged storage. Often in field investigations of reindeer herds the maximum number of faecal samples must be taken when the opportunity arises and storage of samples before processing may be necessary. It is often difficult to prevent faecal samples from freezing in the field, in which case subsequent storing in deep freeze is very convenient. This is generally a clean and durable way of storing faecal samples collected in tightly sealed polythene bags.

Storing at deep freeze temperatures will, however, cause death in the larval population. It was therefore assumed that such storage would make the Baermann technique less suitable in quantitative studies. For this reason we have developed a method which is less influenced by the vitality of the larvae at the time of examination. Properties of this method have been tested on fresh and deep frozen faecal samples.

MATERIAL AND METHODS

We have used inverted microscopes equipped for plankton investigations in the examination of faeces for nematode larvae.

The equipment for plankton investigations consists of cylinders (counting chambers) of volumes from about 5 cm^3 to 100 cm^3. The bottom area of the cylinder is large enough to allow the dispersal of a high number of sedimented small particles and is fitted with a bottom plate which functions as a cover slip for the microscope. Objectives up to 100X (oil) may be used. Some counting chambers are constructed with a removable cylinder so that most of the liquid may be discarded after sedimentation leaving a column only a few millimeters high on the bottom plate. This facilitates the penetration of light through unclear liquid. We used permanently mounted cylinders with volumes of 5 and 25 cm^3.

An inverted microscope with photographic attachment and counting chambers is shown on Figure 1.

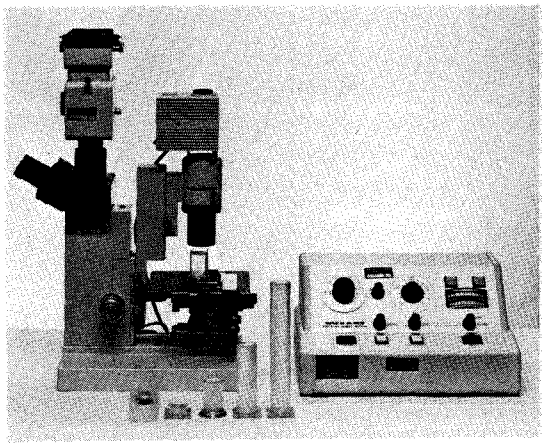

Figure 1. Inverted microscope with photographic attachment and counting chambers. Counting chambers of various sizes are displayed.

Invertert mikroskop med fotoutstyr og plankton tellekammere.

Faecal samples stored frozen in polythene bags were brought to room temperature about 12 h before the sample was processed. The amount of faeces to be examined was weighed to the nearest 0.1 g. Often only small quantities of faeces are obtained from single animals, especially calves in the field, and therefore we have been using about 1.0 g of faeces (1-4 intact pellets) as our subsample unit. Larvae were extracted from the faeces as follows:

The pellets were soaked in about 3 ml of water in a small glass tube (6 drams vial) for 30 minutes. The tube was then gently shaken and the fluid decanted into a plankton cylinder leaving the pellets in the tube. An additional 3 ml of water was added to the faeces which were further soaked for 10 minutes. The tube was again shaken and the water decanted into the counting chamber. This procedure was repeated an additional 3 times, thus allowing faeces to be «washed» 5 times.

Depending on the density of larvae and the softness of the faeces, the water from the 5 subsequent washings could be collected in one or several counting chambers. After identification and counting the samples were easily transferred to vials for fixation and preservation.

Larval counts from repeated faecal examinations of two reindeer, No 119 and No 138 were used to test the method. The properties of the method were investigated using fresh faecal samples followed by a comparison with frozen samples and with the Baermann technique.

For the Baermann technique we used glass funnels fitted with a rubber tube and a clamp. A circular sieve of synthetic fibers of mesh size 90 um was placed in the funnel. The faeces were placed on the sieve and enough tap water added to submerge them. The extraction was carried out at room temperature over night, and the entire volume of water was transferred to the counting chamber.

RESULTS

Table 1 shows the percentage fraction contributed by each of the five washings to the total number of larvae extracted from each sample. The results are given as the mean percentages of 100 separate samples from each of the two reindeer.

Despite the relatively large difference in the mean absolute numbers of larvae in the samples from the two animals, 91.2 and 36.4 respectively, there is a high degree of consistency in the relative efficiency of larval extraction. The cumulative sum of larvae is increasing asymtotically, with the fifth washing contributing on average about 3 per cent to the total. The mean absolute number of larvae in the fifth washing was 2.2, in the samples from reindeer 138, and 1.3 from reindeer 119. In the samples from 138, 39 per cent of the fifth washing contained no larvae compared to 54 per cent for 119.

The sensitivity of the method is revealed in the results from long time monitoring of larval output from the two reindeer. Samples were taken from reindeer 138 on 43 different days. From 17 of these daily samples, 5 subsamples were taken while 10 subsamples were taken from the remaining 26. Larvae were found in all 345 subsamples.

Reindeer 119 was sampled on 42 different days. Five subsamples were drawn from 18 of these daily samples and 10 subsamples from the remaining 24. In 5 of these 330 subsamples (1.5%) no larvae were found. The 5 negative subsamples stem from 4 different daily samples when the other parallels yielded from 1 to 8 larvae. Faeces were softer than usual on all of these 4 days.

Table 2 lists the mean number of larvae per gram faeces in the first five ($\bar{x}_1$), the next five ($\bar{x}_2$), and in all ten ($\bar{x}_{1,2}$) parallel subsamples from 25 daily faecal samples from reindeer 138. The statistical relationship among means for different daily

samples is analysed further in Table 3, and the statistical relationship between means based on the two sets of subsamples from the same daily sample is analysed further in Table 4.

Table 3 shows that the means based on the first five subsamples are statistically different for samples 1-5 and 21-25 but not for samples 10-14. By increasing the number of subsamples on which the means are based to ten, the differences among samples 10-14 were statistically verified. In Table 4 it is shown, however, that the means of the first five and the next five subsamples from the same daily sample also were significantly different in 4 out of 15 cases. The tendency for this to occur seems to be inversely related to the number of larvae per gram faeces.

Statistically the method therefore distinguished between daily samples over the entire range of larval densities examined here when the means are based on five subsamples. The efficiency of this increases when the number of subsamples are increased to ten.

Means that are based on two sets of five subsamples from the same sample may also be statistically different, particularly when the larval density is low. This is an effect of the overdispersion of larvae in the faeces and demonstrates that caution must be applied in drawing conclusions about difference in real density when density is low, even if statistically significant differences may be demonstrated among samples of different origins (i.e. difference in host individual or season).

Table 2 also shows that the range between the lowest and highest density of larvae is considerable in parallel subsamples. This is reflected in the confidence interval which has a mean size of ± 19.1 per cent of the mean it represents when 10 subsamples are used.

The table demonstrates, however, that the method is capable of distinguishing among samples with different levels of larval densities, and that very little may be gained by increasing the number of subsamples from five to ten in this respect. Even single subsamples may suffice to document differences when these are large enough, as for example among faeces with 1-15, 100, 200, 500 and 1000 larvae per gram. Only differences of this magnitude are likely to be of epidemiological significance.

Ten subsamples of about 1.0 g were drawn from each of 12 fresh faecal samples collected from reindeer. Five subsamples were examined with the washing method while five were examined with the Baermann technique. The results are shown in Table 5. The two methods gave comparable results, and the one was not consistently more efficient than the other.

Faecal samples that had been stored deep frozen since early 1977 were subsampled in the same way, and the results of washing and baermannisation in 1980 were compared with the results of washing in 1977 (Table 6). Washing gave a much higher number of larvae (mean 70% of 1977 result) than the Baermann technique (mean 40% of 1977 result).

DISCUSSION

Deep freezing offers a clean and efficient way of storing faecal samples, but for quantitative examination, methods must be developed that are not significantly affected by the reduced vitality of the parasites in the stored faeces. With the «washing method» described here the vitality of the nematode larvae involved influenced the results far less than when the Baermann technique was used. The difference between the two methods is likely to be even greater when species that are less tolerant of freezing than *E. rangiferi* are involved.

When dealing with fresh faeces the two methods gave comparable results. The washing method may produce results faster, especially when only one subsample is taken from the faeces. When more subsamples are taken, a stage is reached where the washing method will require more labour input than the Baermann technique.

Neither method offers a way of estimating the true number of reproducing nematodes in the host animal, and both methods have built in errors which stem from the highly overdispersed distribution of larvae in the faeces. The first problem can only be analysed separately for each host/parasite system. The second must be taken into consideration whenever results of faecal examinations are compared.

A standard light microscope can deal with only small volumes at the required magnification, and a McMaster counting chamber with a volume of 0.15 ml is about the upper practical limit. Even this chamber does not allow the use of more powerful objectives than about 10X on most microscopes.

When using a standard microscope small subsamples often must be drawn from larger ones in which

Table 1. The relative number of larvae of *Elaphostrongylus rangiferi* in each of five subsequent washings. The results are the means from 100 samples of approx. 1 g from each reindeer.

Tabell 1. Den prosentvise andel av hjernemarklarver som ble påvist i 5 påfølgende utvaskinger av faecesprøver på omkring 1 gram. Resultatene er gjennomsnittet av 100 prøver fra hvert av reinsdyrene nr. 138 og nr. 119.

	Mean percentage of larvae in each washing		Standard deviation	
	No 138	No 119	No 138	No 119
1. washing	62.1	59.2	24.41	27.62
2. washing	21.0	23.9	15.47	20.86
3. washing	8.7	8.3	8.21	10.50
4. washing	5.4	5.8	8.09	8.71
5. washing	2.8	3.0	3.76	4.62
Mean number of larvae per sample	91.2	36.4	99.00	41.34

Table 3. One way variance analysis on log transformed data for statistical differences among means (i.p.g.) of different daily samples. Sample No as in Table 2.

Tabell 3. En-veis varians analyse av log transformerte data for statistisk forskjell mellom gjennomsnittlig antall hjernemarklarver pr. gram avføring i forskjellige dagsprøver. Prøvenr. som i Tabell 2.

Sample No	Sub-samples	Mean l.p.g.	F P	Sign.
1	First	15.7	4.0564	+
2	five	28.5		
3		18.8	0.05<P<0.01	
4		23.1		
5		54.1		
10	First	97.6	1.0431	Not
11	five	105.6		sign.
12		121.0	P>0.05	
13		129.2		
14		107.2		
10	All	82.9	3.6897	+
11	ten	109.2		
12		118.8	0.05>P>0.01	
13		122.3		
14		123.8		
21	First	187.6	19.7815	+++
22	five	201.9		
23		241.6	P<0.001	
24		367.6		
25		372.9		

Table 2. The mean number of larvae of *Elaphostrongylus rangiferi* per gram faeces in the first five (x_1), the next five (x_2) and in all ten ($x_{1,2}$) parallel subsamples from 25 different daily faecal samples from reindeer No 188. 95% confidence intervals and ranges are given. The samples are numbered aooording to size of the mean of all fen subsampies ($x_{1,2}$).

Tabell 2. Det gjennomsnittlige antall hjernemarklarver påvist pr. gram avføring i de første 5 (x_1), de neste 5 (x_2), og i alle 10 ($x_{1,2}$) delprøver fra 25 forskjellige dagsprøver fra reinsdyr nr. 138. Prøvene er nummerert etter størrelsen på $x_{1,2}$. 95% konfidensintervall og største og minste resultat for hver prøve er også oppgitt.

Sample No	First five subsamples x_1	Conf.int.	Range	Next five subsamples x_2	Conf.int.	Range	All ten susamples $x_{1,2}$	Conf.int.
1	15.7	9.1- 22.3	9- 28	12.1	3.5- 20.7	3- 31	13.9	8.8- 19.0
2	28.5	16.2- 41.4	9- 35	12.3	9.1- 15.5	7- 15	20.5	12.5- 28.5
3	18.8	10.9- 26.7	7- 30	22.6	17.6- 27.6	16- 30	20.7	16.3- 25.1
4	23.1	21.0- 25.2	21- 27	33.2	30.0- 36.4	28- 39	28.2	24.5- 31.9
5	54.1	27.2- 81.0	18-108	36.7	18.5- 54.9	13- 63	45.4	29.5- 61.3
6	58.8	37.2- 80.4	22- 90	46.1	34.2- 58.0	35- 79	52.4	40.4- 64.4
7	48.8	15.5- 82.1	8- 91	64.7	43.2- 86.2	27- 92	56.7	37.8- 75.6
8	58.0	52.4- 63.6	49- 73	76.2	49.3-103.1	46-114	67.1	53.2- 81.0
9	82.5	72.6- 92.6	84-115	80.2	66.9- 93.5	72-106	81.4	73.7- 89.1
10	97.6	29.3-165.9	36-243	68.1	29.8-106.4	33-142	82.9	45.8-120.0
11	105.6	58.8-152.4	58-212	112.8	89.9-135.7	87-133	109.2	85.2-133.2
12	121.0	97.9-144.1	93-176	116.7	91.1-142.3	63-150	118.8	102.9-134.7
13	129.2	103.8-154.6	102-177	115.5	75.9-155.1	80-157	122.3	100.3-144.3
14	107.2	95.8-118.6	73-139	140.5	100.8-180.2	74-170	123.8	102.1-145.5
15	136.8	106.7-166.9	101-189	121.2	98.6-143.8	114-161	129.0	111.0-147.0
16	119.1	109.9-133.3	177-127	154.3	141.2-167.4	140-166	136.7	122.3-151.1
17	164.2	136.2-192.2	115-183	155.3	128.3-182.3	118-176	159.7	141.7-177.7
18	147.5	106.7-188.3	109-213	174.5	149.8-199.2	146-195	161.0	137.5-184.5
19	140.7	128.8-152.6	133-183	182.7	165.3-200.1	159-198	161.7	145.0-178.4
20	174.8	135.0-214.6	99-236	194.7	168.5-220.9	144-226	184.8	162.0-207.6
21	187.6	149.7-225.5	133-265	201.7	168.1-235.3	165-229	194.6	170.9-218.3
22	201.9	173.9-229.9	159-240	246.7	187.5-305.9	117-279	224.3	191.0-257.6
23	241.6	201.6-281.6	197-281	228.8	191.2-266.4	172-274	235.2	209.3-260.7
24	367.6	327.2-408.0	344-436	369.7	312.8-426.6	295-481	368.6	336.6-400.6
25	372.9	331.2-414.6	310-478	450.7	396.1-505.3	360-622	411.8	371.5-452.1

Table 4. t-tests on log transformed data for statistical differences between means (l.p.g.) of the first five (x_1) and next five (x_2) subsamples from the same daily sample. Sample No as in Table 2.

Tabell 4. t-test av log transformerte data for statistisk forskjell mellom gjennomsnittlig antall hjernemarklarver pr. gram avføring i de første 5 og de neste 5 delprøver fra de samme dagsprøver. Prøvenr. som i tabell 2.

Sample No	x_1	x_2	t	P	Sign.
1	15.7	12.1	1.05	0.4>P>0.3	Not
2	28.8	12.3	2.67	0.03>P>0.02	+
3	18.8	22.6	5.00	P≈0.001	++
4	23.1	33.2	5.19	P<0.001	+++
5	54.1	36.7	0.97	0.4>P>0.3	Not
10	97.6	68.1	0.63	0.6>P>0.5	Not
11	105.6	112.8	0.61	0.6>P>0.5	Not
12	121.0	116.7	4.00	0.01>P>0.002	++
13	129.2	115.5	0.78	0.5>P>0.4	Not
14	107.2	140.5	1.40	0.4>P>0.3	Not
21	187.6	201.7	0.59	0.6>P>0.5	Not
22	201.9	246.7	1.16	0.2>P>0.1	Not
23	241.6	228.8	0.48	0.7>P>0.6	Not
24	367.6	369.7	0.006	P>0.9	Not
25	372.9	450.7	2.30	P≈0.5	Not

Table 5. Mean number of larvae of *Elaphostrongylus rangiferi* per gram faeces found in 5 subsamples by the Baermann technique and in 5 parallel subsamples by washing. Each subsample weighed approx. 1 g. Fresh faeces.

Tabell 5. Gjennomsnittlig antall hjernemarklarver funnet pr. gram fersk avføring ved Baermanns metode og ved vasking. Hver delprøve veide omkring 1 g. Det var 5 delprøver i hver prøve.

Sample No	Reindeer No	Baermann		Washing		
		x_1	Range	x_2	Range	x_1/x_2
1	42/72	633.2	932-396	487.2	776-258	1.3
2	8/80	582.5	847-409	796.0	931-695	0.7
3	42/72	318.9	426-223	224.1	283- 94	1.4
4	8/80	311.5	370-268	404.4	533-241	0.8
5	42/72	239.6	313-142	289.8	336-248	0.8
6	42/72	152.8	217-121	131.8	188- 84	1.2
7	42/72	130.9	181- 25	92.5	190- 63	1.4
8	7/79	35.7	41- 29	22.9	36- 7	1.6
9	7/79	12.2	17- 9	15.3	24- 9	0.8
10	33/78	12.0	18- 7	11.5	17- 9	1.0
11	7/79	10.7	16- 4	12.9	22- 4	0.8
12	2/79	3.8	6- 1	12.5	22- 6	0.3

Table 6. Mean number of larvae of *Elaphostrongylus rangiferi* per gram faeces in subsamples from faeces collected and deep frozen in 1977. First group of subsamples examined by washing in 1977. Second and third groups of subsamples are parallels examined by washing and the Baermann technique in 1980. Results rounded to whole numbers.

Tabell 6. Gjennomsnittlig antall hjernemarklarver pr. gram avføring. Avføringsprøvene ble lagret i dypfryser i 1977 og undersøkt igjen i 1980 med Baermanns metode og vasking.

Sample No	Washing 1977 x_1	Washing 1980 x_2	Baermann 1980 x_3	x_2/x_1	x_3/x_1
1	727	374	249	0.5	0.3
2	605	540	159	0.9	0.3
3	383	277	140	0.7	0.4
4	361	272	74	0.8	0.2
5	316	297	70	0.9	0.2
6	313	235	149	0.8	0.5
7	274	64	70	0.2	0.3
8	179	79	25	0.4	0.1
9	73	60	35	0.8	0.5
10	61	67	44	1.0	0.7
Mean				0.7	0.4

the parasites are contained after the extraction process. Furthermore, faeces for analysis are normally taken from a larger quantity of host faeces. These methods, therefore, involve «double sampling» and carry with them the increased statistical variance built into such an approach. Whilst steps are often taken to randomize the distribution of the parasites in a sample before subsamples are drawn it is probably rarely successful. Because of the fewer subsampling steps involved, application of the inverted microscope and plankton counting chambers may reduce this error. The analysis of the «washing method» presented here may be used as a basis for interpreting results obtained by this method in experimental and epidemiological studies.

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