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EVALUATION OF THE BAERMANN TECHNIC FOR RECOVERY OF LUNGWORM (NEMATODA, PROTOSTRONGYLIDAE) LARVAE FROM WILD RUMINANTS

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ABSTRACT

An evaluation of the Baermann technic, using first-stage larvae of Parelaphostrongylus odocoilei from mule deer and Protostrongylus spp. from bighorn sheep, was made to determine the effects of condition and amount of feces, time, funnel size, and water volume on the number of larvae recovered. Highest counts of P. odocoilei larvae occurred when 10 grams of fresh feces were put in 100 milliliters of water in a 10 or 15 centimeter glass funnel and allowed to stand 24 hours. Highest counts of Protostrongylus spp. larvae occurred when 10 grams of slightly-crushed, dry pellets were put in 280 or 490 milliliters water in a 15 centimeter glass funnel and allowed to stand 24 hours. A convenient, standardized technic for recovery of Protostrongylus spp. from bighorn sheep feces is presented based on results of this study, literature, and subjective assessments.

INTRODUCTION

Rocky Mountain bighorn sheep (Ovis canadensis) have had a history of periodic die-offs. High populations of lungworms (Protostrongylus stilesi and P. rushi), frequently complicated by bacterial and/or viral infections and known collectively as "the lungworm-pneumonia complex" (Buechner 1960, Stelfox 1971, Uhazy et al. 1973), have been associated with most of those die-offs. Infection of bighorns with Protostrongylus spp. is not a rare or isolated event; most sheep throughout their range are infected (see Forrester 1971 and many other papers).

The main method of diagnosing Protostrongylus infection in live bighorn sheep has been by recovery of first-stage larvae from feces utilizing the Baermann technic. The technic involves placing sheep in a water-filled funnel and, later, recovering larvae that have left the pellets and dropped to the stem of the funnel. The qualitative value of the technique (i.e., the ability to determine whether or not a sheep has Protostrongylus) has never been questioned. What has been questioned is whether or not the technic provides useful quantitative information given the

pellet-to-pellet, seasonal, and within and between pellet group variations in larval numbers.

Perhaps one reason little quantitative information has appeared in the literature is the lack of similar technic between laboratories in set-up of the Baermann apparatus and in the actual process. Many modifications of the basic technic are apparently employed, but these and other essential details are seldom provided in publications.

The objective of this paper is to recommend a standardized, convenient technic that provides repeatable results for recovery of Protostrongylus larvae from bighorn sheep feces. Data for a related "protostrongylid" nematode, Parelaphostrongylus odocoilei of mule deer, are provided for comparison.

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MATERIALS AND METHODS

COLLECTION-HANDLING OF FECES

Pelleted fresh feces from two, captive mule deer experimentally infected with P. odocoilei were collected from cement-floor, individual pens. Only feces less than one day old were used. They were mixed thoroughly within two hours of collection, separated into 10, 20 or 30 gram subsamples, bagged in plastic, and stored at 4C for up to two days or at -20C for up to eight days. Prior to running Baermann's, frozen feces were left one hour at 18C, then stored up to one day at 4C.

Frozen fecal pellet groups of bighorn sheep were collected from snow and frozen ground in Banff and Kootenay National Parks in late February, 1982. Additional samples were collected at the same time from Whiskey Mountain, approximately 10 kilometers south of Dubois in western Wyoming. Most pellets in the Park samples appeared freshly-deposited (i.e., a few days old), but in some cases this was difficult to determine. The samples from Wyoming appeared older. Feces from Banff and Kootenay were mixed thoroughly, separated into 10 gram portions, and stored at 4C for up to one week. Those from Wyoming were air-dried in paper bags for approximately one month. They were mixed thoroughly and separated into 10 gram portions prior to beginning the experiment.

THE BAERMANN TECHNIC

Pellet group subsamples (usually 10 grams, but see below) were placed on a double layer of cheesecloth (Grade 150) and put in water-filled glass funnels. Five water volumes (32, 100, 280, 490 and 780 millliliters)[1] were tested in three funnel sizes (10, 15, + 20 centimeters maximum diameter) at six time intervals (2, 4, 6, 12, 24, and 48 hours). Generally, three replicates of each condition were run.

A short piece of rubber tubing was attached to the stem of the funnel and clamped. Tap water used in the funnels was 24-28C.

A maximum of 100 milliliters of fluid was withdrawn from the funnel and put into two 50 millliliter centrifuge tubes. Tubes were centrifuged at 1100 r.p.m. for 10 minutes and all but five milliliters of supernatant suctioned from each tube. The two 5 millilter portions of fluid were pooled in a plastic petri dish 50 millimeters in diameter. This dish was placed in a glass petri dish 200 millimeters in diameter which had a grid etched on the bottom to facilitate counting. The fluid was examined using a dissecting scope at 12X (see below) and larvae separated into one of four morphologic types (see below).

If larvae were very numerous and overlapping in the petri dish so that it was obvious that counting would be difficult, the 10 milliliter suspension was added to 90 milliliters of water, mixed thoroughly, and a 10 milliliter subsample removed, and larvae counted. Generally, counts were converted to larvae per gram (LPG) wet weight feces, however, the fecal samples from Whiskey Mountain were air-dried and, therefore, converted to LPG dry weight feces.

The frequency distribution of the numbers of larvae per gram of wet or dry feces in bighorn sheep samples is not random (Uhazy et al. 1973, and unpub.). For all bighorn samples we've studied, except for the Whiskey Mountain samples of this study, a natural logarithmic transformation of the counts results in a normal distribution. Thus, for statistical analysis, this transformation was made. Normal distributions of Whiskey Mountain larvel counts were not achieved by natural logarithmic or square root transformations. Thus, nonparametric statistics were used in data analysis of this sample.

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1 Added details of choices:

funnel size (centimeters)	water volume (milliliters)	diameter of funnel at water line (centimeters)
10	32	5
10	100	7
15	100	7
15	280	10
15	490	13
20	490	13
20	780	15

LARVAL TYPES

First-stage larvae of the two Protostrongylus lungworms of bighorn sheep are indistinguishable. The lungworms are P. Stilesi Dikmans, 1931 and P. rushi Dikmans, 1937. The mule deer nematode is Parelaphostrongylus odocoilei (Hobmaier and Hobmaier, 1934) a parasite of the dorsal musculature. First-stage larvae of both genera are similar in size and morphology, but Protostrongylus has a straight, symmetrically-tapered, pointed tail and P. odocoilei has a curved tail with a dorsal spine in the anterior part of the curve.

In this study, "straight-tailed" larvae belonging to Protostrongylus spp. predominated in sheep feces. Larvae with dorsal spines that closely resembled those of Parelaphostrongylus were present. They are geographically widespread in feces of bighorns and possibly comprise a number of protostrongylid lungworms one of which is Muellerius capillaris (Muller, 1889) (Pybus, unpub.). There is recent evidence that mountain sheep and goat have several protostronglylid nematodes with spined larvae not reported from these hosts previously (Pybus et al., unpub.) We separated dorsal-spined larvae from Protostrongylus larvae at 120X or 250X depending on experience of the observer. (Those new to the method should examine tails of larvae at 400X until a "feel" for identification is acquired). Of the three areas sampled in this study, only sheep from Banff yielded dorsal-spined larvae.

Large and small free-living mematode larvae were also recovered; the latter were similar in size to dorsal-spined and Protostrongylus larvae. Free-living larvae were granule-filled (Protostrongylus were not), light green (Protostrongylus were pale), and did not have a dorsal spine.

EXPERIMENTAL PROTOCOL

Experiment 1.

This expermiment, which was designed to determine the optimum amount of feces to use in the Baermann technic, was divided into two trails. In trail one, each of 10 groups of mule deer fecal pellets between 3 and 100 grams (wet weight) were placed in a 15 centimeter funnel with 350 milliliters water and run 15-19 hours. In trial two, two each of 10, 20, and 30 grams of deer pellets were placed in 150 milliliters water in 15 centimeter funnels and baermannized for 4 and 24 hours, respectively.

Experiment 2.

Funnel size, water volume and duration of baermannization were evaluated for larvae of P. odocoilei and Protostrongylus spp. from pelleted feces (Table 1).

RESULTS

EXPERIMENT 1. OPTIMUM AMOUNT OF FECES.

There was little to choose from when varying amounts of mule deer feces were baermannized for 15-19 hours. Results for the varying weights of feces (with LPG's in parentheses) were similar: 3 grams (3038), 5 grams (3340), 8 grams (3467), 10 grams (4225), 15 grams (3804), 20 grams (3460), 30 grams (3910), 50 grams (3607), 70 grams (2576), and 100 grams (3355). Recoveries were highest for the 10, 15 and 30 gram samples.

Results for the second trial, likewise, gave little to choose from between 10, 20 and 30 gram samples although 10 and 20 gram samples were slightly superior to 30 gram samples. LPG from 10, 20, and 30 grams of feces baermannized 4 and 24 hours were 1569, 1304, 1133 and 2952, 2956 and 2533, respectively.

EXPERIMENT 2. EFFECT OF TIME, FUNNEL SIZE, WATER VOLUME AND CONDITION OF FECES ON LARVAL RECOVERY.

Levels of infection for experiments outlined in Table 1 as reflected by LPG's were subjectively assessed as being moderate.

P. odocoilei

With one exception (32 milliliters of water in the 10 centimeter funnel), more larvae were recovered at 24 hours than at any other time interval regardless of funnel size or amount of water in the funnel (Table 2). However, statistically, time of baermannization was generally not an important factor (pairwise comparisons were all $P > 0.05$ except for 2 vs. 24 hours, $P < 0.001$). There was no significant interaction between duration of baermannization and funnel size ($P > 0.05$) or water volume ($P > 0.05$).

Larval yields were significantly higher ($P < 0.001$) from the 10 and 15 centimeter funnels than from the 20 centimeter funnel, however, as shown clearly on Table 2, water volume, not funnel size was the key treatment parameter of the two. More LPG feces were recovered using 100 milliliters water than with 32, 280, 490 or 780 milliliters water ($P < 0.001$). Also, there was no difference ($P > 0.05$) in LPG feces between the 10 and 15 centimeter funnels with 100 milliliters water or the 15 and 20 centimeter funnels with 490 milliliters water.

In summary, we recommend filling a 10 or 15 centimeter funnel with 100 milliliters of water and baermannizing for 24 hours (less time in the funnel is acceptable).

Protostrongylus spp. (Canada-source feces)

Time had the greatest influence on the number of larvae recovered from

fresh feces (Table 2). More LPG feces ($P < 0.001$) were recovered at 24 hours than at any other time except 48 hours (24 vs. 48 hours, $P > 0.05$).

As with P. odocoilei, larval yields were higher from the 10 and 15 centimeter funnels than from the 20 (actually, the following comparison were significantly different, 10 vs. 20, 15 vs. 20). Also, as with P. odocoilei, there was no difference $P > 0.05$) in LPG feces from the 10 and 15 centimeter funnels with 100 milliliters water or the 15 and 20 centimeters with 490 milliliters water. Unlike results for P. odocoilei, water volume did not generally have a significant influence on the number of larvae recovered. In fact, at 24 hours there were no significant differences ($P > 0.05$) between the various water volumes used. The highest counts at 24 hours were achieved when 100 milliliters of water were used. Thus, for counting larvae of Protostrongylus spp. from fresh, pelleted feces, we recommend filling a 15 centimeter funnel with 100 milliliters of water and baermannizing for 24 hours.

Protostrongylus spp. (Wyoming-source feces)

Cutting the dried fecal pellet into quarters (halved along its length and width) greatly ($P < 0.001$) improved numbers of larvae recovered when compared to results from intact pellets (Table 2). Although highest LPG "cut" feces were acheived at 24 hours of baermannization at all three water volumes (100, 280, 490 milliliters) in the 15 centimeter funnel, there were few statistically significant differences (2 hours vs. 12 hours and 12 hours vs. 24 hours). Likewise, although lower LPG "cut" feces were recorded when 100 milliliters of water were used than when 280 or 490 milliliters were used, there were no significant differences.

We do not recommend using 100 milliliters of water when baermannizing air-dried "cut" feces. Variable amounts of water are absorbed by the feces and 100 milliliters of water is not available for removal after 24 hours.

In summary, when counting larvae of Protostrongylus spp. from air-dried feces stored in paper bags (the most often-used method), we recommend first cutting the pellets, adding them to a 15 centimeter funnel filled with 280 or 490 milliliters of water, and letting them stand for 24 hours.

DISCUSSION

It has long been recognized that "diagnosis of lungworm infection in bighorn sheep is best accomplished by finding first-stage protostrongylid larvae in fresh fecal samples" (Forrester, 1971). A variety of modifications of Baermann's original method (Baermann, 1917) for doing so has been described (Pillmore, 1958, 1959, 1961; Forrester and Senger, 1964; Uhazy et al., 1973). Pillmore's version (1961) received some recognition (Forrester, 1971) and may be very reliable as he contends, but it has not been adopted widely.

Adoption of a standardized version of the Baermann technic that would permit comparisons of larval counts between areas, investigators, laboratories, etc. has not occurred. It appears that, over the years, each laboratory using the Baermann technic has discovered and employed modifications of the basic technic the details of which are never published.

Our work may not improve the situation, but it has substantiated that a number of specific factors influences the efficiency of recovering larvae from the Baermann apparatus. Based on results of the present study, published literature (for example, Forrester and Senger, 1963 and Uhazy et al., 1973), and general impressions of what is practical to accomplish in the wildlife laboratory, we recommend the following as a standard technic for diagnosis and quantification of Protostrongylus infections in bighorn sheep:

1. Collect 10 to 20 grams of freshly-deposited sheep feces. Highest counts of larvae have been reported (Uhazy et al., 1973; Gates, 1975) from January to April and very low counts from May to October, so collect accordingly.

2. Store feces in paper bags at room temperature (20-21C) for 3-4 weeks.

3. Crush from 4 to 10 grams dried pellets by either quartering each pellet as described in this paper or by squeezing each pellet gently lengthwise with large, blunt forceps.

4. Add 280 millliliters of warm (24-28C) water to a glass funnel 15 centimeters in diameter.

5. Place the feces on a double layer of cheesecloth, then place both in the funnel for 24 hours. We usually setup Baermanns in mid-day and make no changes to usual office lighting; thus, samples overnight in dark.

6. Remove 100 millliliters of fluid from the funnel stem into two 50 millliliter centrifuge tubes.

7. Centrifuge 10 minutes at approximately 1100 r.p.m.'s (or allow tube to stand for 1 to 2 hours) and remove all but 5 millliliters of the supernatant from each tube.

8. Place the 10 millliliters of fluid suspension in a disposable 50 millimeter plastic petri dish and put that into a 100 millimeter glass petri dish with a grid etched on the bottom.

9. Examine at 12-25X (depending on experience) using sub-stage illumination.

10. Distinguish between and count larvae as described in the Materials and Methods section. Convert counts to larvae per gram (LPG) dry-weight feces.

Clean glassware (funnels, centrifuge tubes, pipettes, and slides) is essential. All glassware is washed thoroughly with hot water. Plastic petri dishes are used only once.

Contamination of glassware by larvae between samples occurs readily. Some sheep in a herd may be passing 600-1000 LPG or more while others may be passing none. If such samples are run consecutively in improperly washed glassware or in funnels side by side, contamination is possible. Consequently, care must be taken in handling glassware, etc. when running many samples at once.

Adoption of the technic as described here is appropriate for laboratories where other modifications of the Baermann technic have been used in the past. This entails dividing a pellet group in half and following procedures that have been used and the one described here. We have found (Wishart and Jorgenson, pers. comm. and W.M.S., unpub.) relatively consistent, similar differences between technics enabling use of a multiplication factor to update and unify data.

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Table 1. Design for Experiment 2.

Parasite (Source) Condition of feces	Funnel Size*	Water Volume	Duration of baermannization in hours	Number of replicates at each time
Protostrongylus spp.				
(Alberta-British Columbia)				
Pelleted:	10	32 ml.	2,4,6,12,24	3
	10	100 ml.	2,4,6,12,24	3
	15	100 ml.	2,4,6,12,24,(48)	3(2)
	15	280 ml.	2,4,6,12,24	3
	15	490 ml.	2,4,6,12,24	3
	20	490 ml.	2,4,6,12,24,(48)	3(1)
	20	780 ml.	2,4,6,12,24	3
Parelaphostrongylus odocoilei				
(Alberta)				
Pelleted:		as above**		
Protostrongylus spp.				
(Wyoming)				
Pelleted:	15	100 ml.	2,6,12,24,48	3
	15	280 ml.	2,6,12,24,48	3
	15	490 ml.	2,6,12,24,48	3
Pellets cut:***	15	100 ml.	2,6,12,24,48	3
	15	280 ml.	2,6,12,24,48	3
	15	490 ml.	2,6,12,24,48	3

* Maximum diameter in centimeters.
** Delete 48 hr.
*** Each pellet was cut once along its length and width with scissors.

Table 2. Effect of funnel size, water volume and time on recovery of first-stage P. odocoilei and Protostrongylus spp. larvae.

Funnel Size (max. dia.)	Water Volume	Time in Funnel in hr.	Larvae Per Gram Feces*			
			P. odocoilei	Protostrongylus spp.		
				Canadian Sheep	Wyoming Sheep	
					Pellets Intact	Pellets Cut
10 cm.	32 ml.	2	380 ± 26	33 ± 4	-	-
		4	384 ± 9	44 ± 7	-	-
		6	353 ± 11	50 ± 23	-	-
		12	430 ± 70	51 ± 14	-	-
		24	425 ± 95	94 ± 37	-	-
	100 ml.	2	480 ± 85	45 ± 7	-	-
		4	453 ± 54	43 ± 23	-	-
		6	499 ± 115	51 ± 29	-	-
		12	538 ± 115	59 ± 13	-	-
		24	566 ± 37	108 ± 61	-	-
13 cm.	100 ml.	2	479 ± 32	41 ± 14	7 ± 1	111 ± 46
		4	491 ± 60	60 ± 8	-	-
		6	472 ± 30	45 ± 23	64 ± 41	287 ± 96
		12	481 ± 59	63 ± 35	60 ± 8	264 ± 27
		24	527 ± 41	206 ± 77	229 ± 92	330 ± 110
		48	-	257, 138	252 ± 114	354 ± 121
	280 ml.	2	353 ± 40	26 ± 6	9 ± 6	90 ± 2
		4	361 ± 44	47 ± 9	-	-
		6	394 ± 36	58 ± 10	84 ± 23	285 ± 204
		12	390 ± 44	62 ± 30	134 ± 23	441 ± 52
		24	472 ± 20	138 ± 53	239 ± 32	641 ± 137
		48	-	-	247 ± 88	564 ± 244
13 cm.	490 ml.	2	240 ± 27	17 ± 6	6 ± 4	82 ± 14
		4	269 ± 23	26 ± 6	-	-
		6	257 ± 17	27 ± 2	59 ± 28	323 ± 181
		12	325 ± 25	36 ± 8	70 ± 42	374 ± 110
		24	359 ± 50	113 ± 31	134 ± 30	572 ± 110
		48	-	97	153 ± 9	553 ± 125
20 cm.	490 ml.	2	155 ± 84	27 ± 7	-	-
		4	290 ± 98	29 ± 3	-	-
		6	318 ± 64	29 ± 13	-	-
		12	278 ± 39	48 ± 24	-	-
		24	327 ± 58	153 ± 109	-	-
	780 ml.	2	78 ± 21	10 ± 2	-	-
		4	52 ± 17	15 ± 2	-	-
		6	149 ± 15	9 ± 5	-	-
		12	194 ± 64	29 ± 15	-	-
		24	258 ± 86	104 ± 84	-	-

* Mean ± SD