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EXTRACTING PROTOSTRONGYLID NEMATODE LARVAE FROM UNGULATE FECES

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ABSTRACT: A major weakness of the Baermann funnel technique for extracting nematode larvae from feces is the funnel. As many as 67% of *Parelaphostrongylus tenuis* first-stage larvae lodged on the sloping surface of glass Baermann funnels. The number of larvae collected after 24 hr was not significantly correlated with total numbers in the samples, whether feces were supported over tissue paper or over window screening. Instead, we collected about 8 times as many larvae and achieved a significant relationship between larvae collected and the total numbers present when pelleted fecal material was submerged over screening in vertical-sided beakers. The methodology of this more efficient and more accurate way of estimating numbers of protostrongylid larvae is described. Most larvae were located on and in the mucous layer covering fecal pellets and readily left fresh pellets emersed in water; 72% of these larvae left after 6 min and only 11% remained after 1 hr. Larvae in water at room temperature sank as fast as 6 cm/min, but those close to a vertical glass surface sank more slowly (97% sank 18.5 cm in 105 min).

Key words: Baermann funnel technique, fecal examination, nematode larval collection, *Parelaphostrongylus tenuis*.

INTRODUCTION

The Baermann funnel technique was originally employed to extract hookworm larvae from soil (Baermann, 1917), but it has since been variously modified and used particularly to detect the larvae of protostrongylid and trichostrongylid nematodes in the feces of ungulates (Pillmore, 1961; Forrester and Senger, 1964; Todd et al., 1970; Uhazy et al.; 1973; Samuel and Gray, 1982; Beane and Hobbs, 1983; Halvorsen and Wissler, 1983; Gajadhar et al., 1994). The technique involves placing feces over a supporting filter of tissue or screening in a water-filled funnel. Larvae must leave the fecal material, pass through the filter, and sink into the stem of the funnel from which they are drained and counted after 6 to 24 hr.

The technique is used commonly to generate quantitative data, especially the prevalence (proportion of animals passing larvae) and mean intensity of infection (average number of larvae passed per infected individual). The accuracy of prevalence data is limited by the sensitivity of the apparatus in detecting infections when only low numbers of larvae are being passed (Mason, 1989; Welch et al., 1991). Reliable mean intensity data require that the numbers of larvae that can be drained from funnels, consistently are proportional to the total numbers present in fecal samples. Apparently, the assumption that this is achieved when using the Baermann funnel technique to extract protostrongylid larvae has never been tested. In this paper, we evaluate the reliability of the Baermann funnel technique using feces from white-tailed deer (*Odocoileus virginianus*) containing first-stage larvae of the meningeal nematode, *Parelaphostrongylus tenuis*, and we describe a new method for examining ungulate feces that eliminates identified shortcomings of the convential Baermann technique.

MATERIALS AND METHODS

Fecal pellets containing first-stage larvae of *P. tenuis* were collected during the spring and summer of 1996 from experimentally infected captive white-tailed deer and from wild white-tailed deer wintering near Grand Marais, Minnesota (USA; 47°41′N, 90°35′W). Pellets were fresh or stored at 1 C ≤2 mo before use. All pellets used for a particular test were collected and stored in the same manner.

The location of first-stage larvae in deer pellets was investigated by submerging 10 replicate pellets, impaled individually on a pin, for 3, 3, 3, 10, 10 and 31 min (total 1 hr) in a series of six standard Petri dishes filled with water at room temperature. A grid (0.8 × 0.8 cm) was

etched on the bottom of the Petri dish using a dissecting pin. The number of larvae that came off the pellet in each dish was counted. Thereafter, the intact pellet was broken up finely and shaken in 100 ml of water, and a 25 ml subsample quickly pippetted into a gridded Petri dish and examined for any remaining larvae using a stereoscopic microscope at a magnification of 16 to 25×.

The rate at which larvae sink in water was first estimated by directly observing larvae falling a measured distance in a test tube held vertically in front of an horizontally positioned stereomicroscope head. Because larvae fell at varying rates, the following procedure was performed. *Parelaphostrongylus tenuis* larvae were gently inoculated into the top of each of eight glass graduated cylinders (18.5 cm high) filled with 100 ml of water. At 15 min intervals (for a total of 120 min) four 25 ml aliquots were pippetted sequentially, from top to bottom, from one of the cylinders and the number of larvae in each aliquot was counted.

An experiment was designed to compare the efficiency of two techniques for detecting protostrongylid larvae in feces. These were the Baermann funnel technique, using tissue paper or vinyl screening to support feces and filter larvae, and a new method using vertical-sided beakers with feces submerged over screening.

The tissue paper used was Kimwipes (Kimberly-Clarke, Mississauga, Ontario, Canada) with irregular pore sizes up to 0.4 × 0.6 mm. The vinyl window screening had a mesh opening of 1.0 × 1.4 mm. Glass funnels used for the Baermann test had a top diameter of 15 cm, straight sides angled at 30° to the vertical, and were filled with 400 ml of tap water at room temperature. The funnel stem (10 cm long, inside diameter 11 mm) was fitted with a 4 cm length of neoprene tubing stoppered with a Mohr pinchcock clamp (Fig. 1). The new beaker technique involved placing fecal pellets into an "envelope" formed by folding a piece of vinyl window screening (12 × 12 cm) and stapling the open edges to contain the pellets (Fig. 2a). The envelope was then submerged in a 250 ml beaker filled with tap water for 24 hr (Fig. 2b).

A fecal sample of 21 to 30 pellets was divided equally into thirds with 7 to 10 (1.9 to 2.75 grams dry) used in each method. After 24 hr, the inner glass surface of the Baermann funnels and beakers was examined directly for larvae adhering to the glass surface using a horizontally positioned stereomicroscope head. Then, 30 ml of solution were drained from each Baermann funnel and larvae counted. The pellets and filters were removed from each funnel and the remaining solution swirled to free any larvae stuck to the glass. The entire contents were then drained into a 400 ml beaker, allowed to settle for 1 hr, decanted to a volume of 50 ml using gentle vacuum suction, and examined for larvae (Fig. 2c). Similarly, for the beaker method, the screen envelope and pellets were removed and the solution allowed to sit for 1 hr before decanting to a volume of 50 ml and examining for larvae. Upon removal of the fecal solution, the filters used in both the Baermann and beaker methods were inverted and placed in an 18 cm diameter petri dish filled with water for 24 hr to recover any larvae stuck to the filter material. To estimate the number of larvae remaining on or in feces, pellets were macerated in a 500 ml beaker filled with water and three 25 ml aliquots of solution were removed and examined. Each technique was replicated 15 times. All glassware was cleaned between trials following the method of Whitlaw and Lankester (1995).

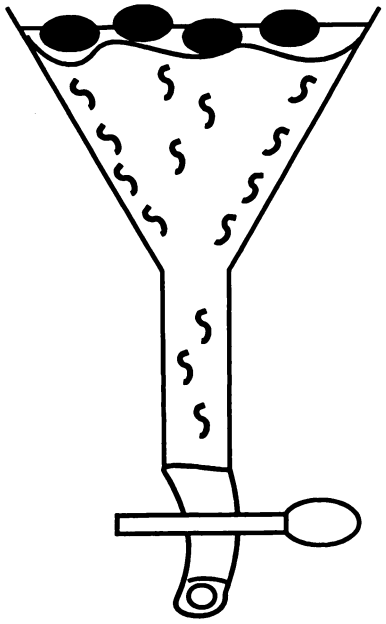

FIGURE 1. The Baermann funnel technique for collecting nematode larvae from feces.

Data were analyzed using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, Illinois, USA). Larval counts were either log or square root transformed to achieve normal distribution. Counts from each technique were compared using a one-way analysis of variance. The relationship between larvae collected in 24 hr and the total larvae in the fecal sample, for each technique, was tested using regression analysis (Zar, 1984). P-values <0.01 were considered significant.

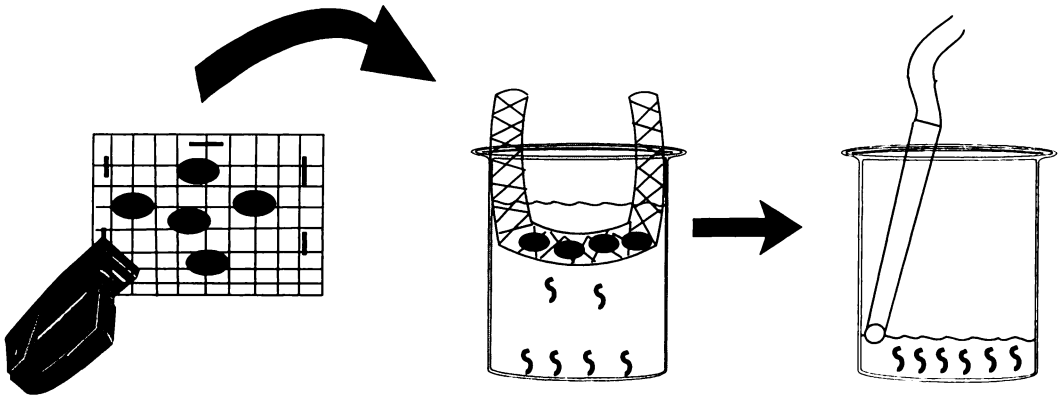

FIGURE 2. The beaker method for collecting nematode larvae from feces. A. Feces contained in a screen envelope. B. Envelope placed into a beaker filled with water. C. Solution decanted by vacuum suction to a volume of 50 ml.

RESULTS

Most *P. tenuis* larvae quickly left fresh fecal pellets submerged on a pin in water. Seventy-two percent left within 6 min and only 11% remained on or in the pellet after 1 hr. Also, the total numbers of larvae varied among pellets from the same grouping (mean ± SE = 728 ± 58; range = 498–1,010 larvae/pellet).

We determined that some larvae in water sank as rapidly as 6 cm/min, but sinking was slowed and variable when larvae were close to the sides of the test tube. Ninety-seven percent of larvae inoculated into the top of an 18.5 cm cylinder took 105 min to settle into the bottom 25 ml of water.

Using an inverted steromicroscope head to examine the sides of the Baermann funnel, we observed numerous larvae suspended along the sloped surface. No larvae were found sticking to the sides of the beakers.

Using Baermann funnels, only 13 and 14% of the total number of larvae were collected in 30 ml of solution drained from the funnel stems when pellets were supported over tissue paper and screen filters, respectively (Table 1). About two-thirds of the larvae in all samples became lodged on the sloping glass of the funnels, rather than sinking into the funnel stem where they could have been drained off. Greater numbers of larvae were retained in the tissue paper than screening. The number of larvae in 30 ml of solution drained from funnels did not correlate with total numbers of larvae in fecal samples, whether feces were supported over tissue ($r^2 = 0.29$; $P = 0.0389$) (Fig 3a) or screening ($r^2 = 0.12$; $P = 0.208$) (Fig 3b).

Using a beaker with pellets submerged in a screen envelope, 87% of the larvae present in samples settled to the bottom of the beaker and could be counted. Two percent were retained by the screen filter and 11% did not leave the pellet. The number of larvae collected after 24 hr using the beaker method correlated significantly with the total number of larvae in fecal samples ($r^2 = 0.98$; $P < 0.01$) (Fig. 3c).

DISCUSSION

A major weakness of the Baermann funnel technique is the funnel. The number of first-stage *P. tenuis* larvae present on fe-

TABLE 1. Comparison of numbers of *Parelaphostrongylus tenuis* larvae collected from white-tailed deer feces using the Baermann funnel and beaker techniques.

	Different methods for collecting *P. tenuis* first-stage larvae from feces														
	Test using funnel with tissue paper					Test using funnel with screening					Test using beaker with screening				
Sample	I[a]	II[b]	III[c]	IV[d]	Total	I[a]	II[b]	III[c]	IV[d]	Total	I[a]	II[b]	III[c]	IV[d]	Total
1	42	99	28	47	216	19	176	5	40	240	146	0	3	13	162
2	25	166	52	53	296	60	125	4	53	242	148	0	3	40	191
3	52	692	188	113	1,045	29	644	16	107	796	1,251	0	23	100	1,374
4	40	245	38	67	390	25	370	9	33	437	323	0	6	100	429
5	63	621	86	33	803	30	697	17	27	771	926	0	18	80	1,024
6	6	115	32	93	246	5	141	4	120	270	157	0	3	73	233
7	77	433	102	0	612	71	287	9	47	414	543	0	3	7	553
8	11	72	33	33	149	13	33	3	60	109	124	0	4	0	128
9	99	273	67	60	499	44	322	1	80	447	460	0	4	73	537
10	88	208	61	40	397	120	352	4	27	509	520	0	20	27	567
11	49	308	101	33	491	155	273	2	27	457	431	0	3	27	461
12	80	304	45	27	456	47	297	11	7	362	308	0	3	0	311
13	75	127	44	20	266	31	74	3	7	115	164	0	6	20	190
14	35	130	33	26	225	27	88	6	33	154	65	0	2	13	80
15	47	94	17	20	178	65	147	0	27	239	310	0	1	27	338
Mean*	53[f]	259[h]	62[l]	44[k]	418[l]	49[f]	268[h]	6[j]	46[k]	371[l]	392[g]	0	7[j]	40[k]	439[l]
SE[e]	7	49	11	8	64	11	50	1	9	54	84		2	9	91
Mean %	13	62	15	11		14	67	2	17		87	0	2	11	
SE	2	3	1	3		3	4	0.3	4		2		0.3	2	

* Mean values followed by the same letter are not significantly different ($P > 0.01$).
[a] Number of larvae drained off in 24 hr.
[b] Number of larvae adhering to glass surface.
[c] Number of larvae trapped in filter.
[d] Number of larvae remaining in feces.
[e] SE = Standard error.

cal pellets that became lodged on the sloping sides of glass funnels was ≤67%; these could not be collected by the standard procedure. In comparison, the new method of submerging pellets over screening in straight-sided beakers yielded almost 8 times as many larvae and consistently extracted 87% of those present in samples.

Of previous studies utilizing the Baermann method to collect nematode larvae, only Todd et al. (1970) determined its efficiency (number of larvae collected compared to that actually present in a sample). Efficiency of recovering third-stage *Haemonchus contortus* larvae from herbage and soil was as high as 87% but varied with the type of filter, temperature, weight of herbage samples and soil type. Efficiency was inversely related to funnel diameter; it was 83% using 7 cm funnels, but only 27% using 30 cm funnels. Although no explanation for this difference was offered by Todd et al. (1970), our results suggest that one reason for the reduced recovery rates probably was a greater proportion of larvae being retained on the increased sloping surface area of the larger funnels. Samuel and Gray (1982) also reported that funnel size inversely affected the number of *Parelaphostrongylus odocoilei* larvae collected using the Baermann technique, as did Beane and Hobbs (1983) for *Protostrongylus* spp. The latter studies did not determine efficiency, but it was probably less than that obtained by Todd et al. (1970) for infective larvae of *H. contortus* which are 2 to 3 times larger and more mobile than first–stage protostrongylid larvae.

As well as being inefficient in extracting *P. tenuis* larvae, the Baermann funnel tech-

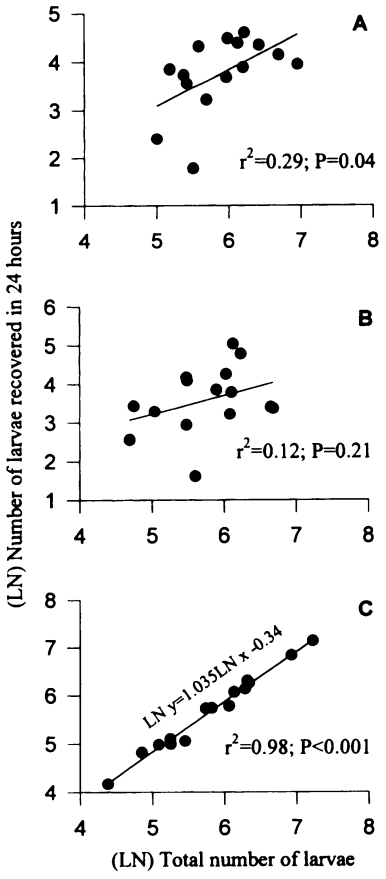

FIGURE 3. Linear regression of the number of nematode larvae collected after 24 hr versus the total number of larvae in the fecal sample. A. Using a Baermann funnel with tissue paper. B. Using a Baermann funnel with vinyl screening. C. Using a beaker with vinyl screening.

nique collected a variable proportion of the total larvae present and produced an unreliable estimate of actual numbers of larvae. A major source of this variation in our test probably resulted from the position of pellets floating in the funnel. More larvae freeing themselves from pellets near the center of the funnel will fall straight down and enter the funnel stem compared to pellets situated more towards the periphery of the funnel. Smaller funnels and those with less water will position more pellets closer to the center and over the opening of the funnel stem. This may explain why Samuel and Gray (1982) maximized the number of *P. odocoilei* larvae from 15 cm diameter funnels when they were filled with only 100 ml of water. Additional variation will result from between pellet differences in total larvae and the random position of pellets relative to the center of the funnel. Finally, depending on the moisture content, some pellets will float for varying periods before becoming totally submerged and evaporation of water in funnels over the 24 hr test period may expose parts of the once submerged pellets. Most *P. tenuis* first-stage larvae are on the surface of deer pellets (Lankester and Anderson, 1968; present study) and probably are able to leave only from the portion of the surface that is submerged. Keeping pellets submerged in a vertical-sided beaker avoids all of these sources of variation.

In conclusion, we believe that the newly proposed method of submerging fecal pellets in a beaker of water greatly reduces the variability in larval recovery due to technique and provides a new tool to more accurately assess variation in larval counts between seasons, between animals, among samples from the same animal, and between pellets within a group. Being more efficient at estimating larval counts, it also would be a more sensitive test, improving the detectability of larvae at low densities (see Mason, 1989; Welch et al., 1991). Therefore, we recommend the following method for collecting protostrongylid larvae from ungulate feces:

(1) Weigh a sample of 10 to 15 fecal pellets to be examined and 5 additional pellets as a subsample to be dried in order to provide a dry-weight conversion factor. Numbers of larvae collected should be expressed on a dry weight basis since the moisture content of fecal material collected in the wild will vary. The number of larvae/gm of dried feces = the number of larvae collected from the sample ÷ [fresh weight of the sample x (dried weight of the subsample ÷ initial weight of the subsample)]; (2) Place the sample of 10 to 15 pellets on a piece of fiber glass window screening (12 × 12 cm) and fold and staple the edges of the screen-

ing to form an envelope in which the pellets are uniformly distributed side-by-side in a monolayer (wrapping the screen envelope in a single layer of tissue will reduce the amount of debris sedimenting from some pellets and produce a cleaner sample of larvae, but the proportion of total larvae, and therefore sensitivity, also are reduced); (3) Submerge the envelope in a 250 ml beaker filled with tap water at room temperature (to prevent it from sinking, the envelope may have to be held in place by clamps such as clothes pins and for samples requiring larger numbers of pellets or for species of ungulates producing large fecal pellets, a larger piece of screening and larger beaker can be used); (4) After 24 hr, gently remove and discard the screen envelope containing feces and let the solution settle for an additional 1 hr (the slowest larvae sink at a rate of about 11 cm/hr); (5) Decant and discard all but 50 ml of solution by gentle vacuum suction (aspirator), and pour the remaining 50 ml into a 9 cm diameter Petri dish with a grid etched on the bottom and examine the solution for larvae at 16 to 25× (depending on experience of the observer and the amount of debris present), but if a beaker larger than 250 ml is used, decant all but 100 to 150 ml of solution and centrifuge at 1,100 r.p.m. for 10 min (Samuel and Gray, 1982) to concentrate the larvae into ≤50 ml of solution so it can be examined in an etched 9 cm Petri dish; (6) Wash glassware with hot soapy water, then rinse with 95% ethanol (Whitlaw and Lankester 1995).

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