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COMPARISON OF MODIFIED FLOTAC AND BAERMANN TECHNIQUES FOR QUANTIFYING LUNGWORM LARVAE IN FREE-RANGING BIGHORN SHEEP (*OVIS CANADENSIS*) FECES, MONTANA, USA

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ABSTRACT: Lungworms are important parasites of wildlife and host infection status is often evaluated using coprologic techniques, most commonly the Baermann method. Recently, the FLOTAC® has emerged as a new tool for diagnosing lungworm infections, and methodologic comparison studies in domestic species suggest that this method outperforms many other established techniques. We compared a modified FLOTAC with the beaker-modified (bm)–Baermann to evaluate the relative performance of the two techniques for counting lungworm larvae in bighorn sheep (*Ovis canadensis*) feces. Both methods generated equivalent larval counts and both were highly repeatable. The major difference between the two methods was that the FLOTAC was poorer at detecting mixed infections. The ultimate choice between using the FLOTAC and bm-Baermann methods for quantifying lungworm larvae in wildlife studies may depend on the specific nature of the research questions being addressed, balanced by practical constraints.

Key words: Coprologic diagnosis, nematode, *Ovis canadensis*, parasite.

INTRODUCTION

Lungworms are widespread parasites of domestic and wild animals. Lungworms typically reside in the respiratory tract of their hosts, where they lay eggs. Eggs or larvae are coughed up and swallowed and larvae are passed into the environment in host feces. Given this life cycle, coprologic techniques are commonly used for diagnosis of lungworm infections (Foreyt 1989; Bowman 1999). The presence of larvae in feces indicates active infection, and morphologic or molecular identification of larvae can facilitate specific diagnosis in live hosts. Using coprologic techniques, researchers have examined relationships between lungworm infection and host physiology (Goldstein et al. 2005; Ezenwa et al. 2012), condition (Irvine et al. 2006; Vicente et al. 2007), behavior (Pelletier and Festa-Bianchet 2004; Corlatti et al. 2012), reproduction (Pelletier et al. 2005; Santiago-Moreno et al. 2010), and susceptibility to secondary infections (Jenkins et al. 2007).

The most widely used method for coprologic investigation of lungworm infections is the Baermann technique (Foreyt 1989; Eysker 1997; Bowman 1999). A classical Baermann protocol involves submerging a known amount of feces in water for 8–24 h in a funnel (Bowman 1999; Foreyt 2001). The submerged feces are typically wrapped in a porous material that traps large particles but allows larvae to pass through. Larvae that emerge from the feces are pulled by gravity into the neck of the funnel and can be collected for quantification. The classic Baermann method has been modified several times to maximize larval recovery. For example, a beaker modification of the standard technique that involves breaking apart pelleted feces and replacing the funnel with a beaker was shown to improve larval recovery from ungulate fecal samples up to sevenfold (Forrester and Lankester 1997a, b).

Recently, the FLOTAC system emerged as a tool for diagnosis and quantification of parasites in fecal samples. FLOTAC is a flotation-based technique that makes use of a wide range of flotation solutions to separate parasitic elements from fecal

debris, followed by visualization and quantification of these elements in a specialized chamber (Cringoli et al. 2010). The FLOTAC method has been validated for a range of parasite types, including eggs of gastrointestinal nematodes, protozoan oocysts, and lungworm larvae (Cringoli 2006; Cringoli et al. 2010). For quantifying lungworm larvae, the FLOTAC method is fast, requiring <1 h for sample processing, and is flexible enough to accommodate fresh or preserved fecal samples (Cringoli et al. 2010), unlike the Baermann method, which relies on fresh feces and takes up to 24 h. Recent comparisons of the FLOTAC to the classic Baermann suggest that FLOTAC frequently detects a greater number of infected hosts and produces significantly higher larval counts per host (Rinaldi et al. 2007, 2010; Gaglio et al. 2008).

Most studies using the FLOTAC for quantifying lungworm larvae have focused on domesticated animals. Because patterns of infection often differ between domestic and wild species, we tested how the FLOTAC performs in comparison to the beaker-modified (bm)–Baermann method for quantifying lungworms in free-ranging bighorn sheep (*Ovis canadensis*) in Montana, US. Bighorn sheep in western North America are often infected with lungworms of the genera *Protostrongylus* and *Muellerius* (Uhazy et al. 1973; Kistner et al. 1977; Pybus and Shave 1984; Ezenwa et al. 2010). In many populations, lungworm prevalence is high and infections are caused by more than one species (Forrester and Senger 1964; Goldstein et al. 2005; Ezenwa et al. 2010). Lungworms may have important fitness consequences for free-ranging sheep (Festa-Bianchet 1991; Pelletier and Festa-Bianchet 2004; Pelletier et al. 2005) and fast and effective methods of quantifying larvae shed in feces and determining the taxonomic composition of these larvae can facilitate studies on the impact these parasites have on bighorn ecology and conservation. We investigated whether the FLOTAC and bm-Baermann methods produce comparable results in terms of larval counts, larval taxonomic composition, and within-assay repeatability.

MATERIALS AND METHODS

Study site and sample collection

Fecal samples were collected at the National Bison Range (NBR), Moiese, Montana, US (47°20′N, 114°15′W), from individually identifiable bighorn sheep ($n=17$) on 13 August 2013 within 1 h of an observed defecation. Samples were placed in a cooler in the field and stored at approximately 4 C until processed 6–10 d later. Lungworm larvae were quantified using the bm-Baermann (Forrester and Lankester 1997a, b) and the FLOTAC (Cringoli et al. 2010) modified as below. For each sample, both protocols were implemented on the same day.

Beaker-modified Baermann

Ten grams of fecal pellets was broken up, wrapped in a Kimwipe and screen packet, and submerged in a beaker with 200 mL of water for approximately 20 h. Once the sample packet was removed from the beaker and discarded, fluid was siphoned off to the 30-mL mark. The 30-mL concentrate was mixed and evenly divided into two 15-mL centrifuge tubes. The tubes were centrifuged for 5 min at ~300 × G and supernatant was siphoned off to ~1 mL. The pellet from each tube (two per sample) was examined with a compound microscope at 100× and 400×. The number of lungworm larvae per gram of feces (LPG) was estimated as the sum of the count from both tubes divided by 10. For each sample, we recorded tube counts separately to determine the repeatability of LPG estimates derived from each of the two tubes examined per sample.

FLOTAC

This procedure was implemented with a FLOTAC 400 device, initially following the basic protocol of Cringoli et al (2010). The FLOTAC device consists of two 5-mL wells, into which homogenized feces and flotation solution are loaded and then centrifuged and examined using a microscope. Ten grams of feces was homogenized in 100 mL of water and the solution passed through a 7.6-cm stainless steel strainer. After briefly mixing the strained liquid, an 11-mL aliquot was centrifuged for 3 min at ~300 × G. The resulting pellet was resuspended in a zinc sulfate flotation solution (specific gravity=1.2) to 11 mL. After a thorough mixing, 5 mL of the solution was loaded into each of two wells of

the FLOTAC slide. The FLOTAC slide was centrifuged at ~130 × G for 5 min and examined using a compound microscope at 100× and 400×. The number of larvae observed represents the estimated LPG. We examined the repeatability of FLOTAC LPG estimates by counting two 11-mL aliquots of each sample.

In our preliminary FLOTAC trials, the majority of lungworm larvae were curled and dead, precluding morphologic identification. To compensate, we modified the first step of the standard FLOTAC protocol outlined above. Specifically, we homogenized feces in a 100-mL NaCl solution in place of water, and allowed the solution to soak for 40 min prior to filtration and centrifugation. This pretreatment reduced larval curling, allowing for consistent identification of ~80% of larvae. All FLOTAC results reported are with the salt solution pretreatment.

Larval identification

For both the bm-Baermann and FLOTAC procedures, we identified larvae during the counting step using morphologic features, particularly tail shape (Foreyt 2001). Identifications were performed at 400× magnification. Our previous work in the NBR bighorn population indicated that dorsal-spined larvae (DSL; specifically *Muellerius capillaris*) dominate the lungworm larva community, whereas larvae lacking dorsal spines (*Protostrongylus* spp.) are less prevalent (Ezenwa et al. 2010). Thus, we classified larvae as DSL or non-DSL during counting. Larvae were recorded as unidentifiable if the tail was not visible (e.g., a larva was curled or folded in the FLOTAC chamber or obscured by debris).

Statistical analysis

To compare the results of the bm-Baermann and FLOTAC methods we tested for a correlation between the LPG estimates using a Spearman rank test. Then, we tested for differences in LPG using a Wilcoxon matched-pairs signed rank test. Third, we evaluated whether there were differences in detectability of less prevalent *Protostrongylus* larvae between the two methods using a chi-square test. Finally, we evaluated the repeatability of both methods by examining the correlation between LPG estimates derived from duplicate counts with Spearman rank tests.

RESULTS

Lungworm larvae were detected in all samples using both methods. Larval counts derived from the FLOTAC and bm-Baermann methods were significantly and positively correlated (Spearman correlation: rho=0.56, $P=0.019$; Fig. 1A). Mean LPG was slightly higher for the bm-Baermann method compared with the FLOTAC method ($n=17$, mean±SD: bm-Baermann= 52.34±27.18, FLOTAC=45.61±22.93; Table 1), but this difference was not statistically significant (Wilcoxon test: S=−11.50, $P=0.611$; Fig. 1B). *Muellerius* larvae were detected in all samples irrespective of the method. By contrast, *Protostrongylus* larvae were detected in 6 of the 17 (35%) samples using the bm-Baermann method, and only 1 of 17 (6%) samples using the FLOTAC method. However, the difference in detection of *Protostrongylus* larvae was not statistically significant (Pearson's $\chi^2=1.95$, $P=0.163$).

Both methods appeared to be highly repeatable. For the bm-Baermann, larval counts derived from the two tubes collected per sample were highly correlated (Spearman correlation: rho=0.95, $P<0.001$), suggesting that counting a subset of the concentrate (one of two tubes) is sufficient for accurate LPG estimation. Likewise, counts derived from duplicate FLOTAC slides were highly correlated (rho=0.91, $P<0.001$).

DISCUSSION

The bm-Baermann and FLOTAC methods performed comparably in terms of quantifying the number of lungworm larvae per gram of bighorn sheep feces, but the basic FLOTAC technique required modification to allow larval identification and evaluation of parasite taxonomic composition. Both methods were highly repeatable, suggesting that either can be used to derive accurate estimates of the number of larvae in a sample. Based on our observations, decisions to use the bm-Baermann vs. FLOTAC for studying lungworms in wildlife fecal samples should depend on the specific study questions being addressed and practical constraints.

Variation in the estimate of taxonomic composition was the main difference we

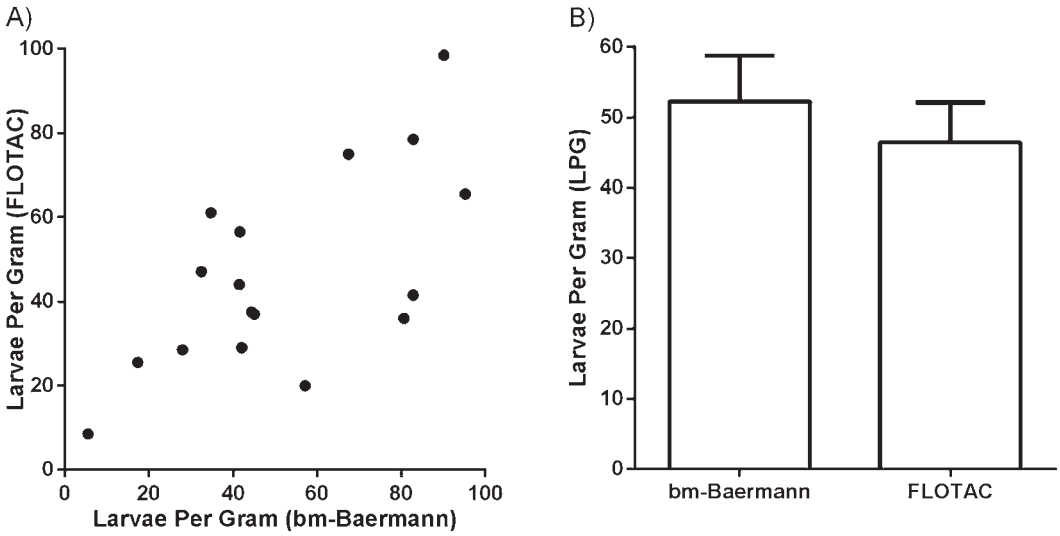

FIGURE 1. (A) Correlation between larval counts using the beaker-modified (bm)–Baermann and FLOTAC methods. Each point is a single fecal sample ($n=17$) assayed using both methods. (B) Comparison of mean total larvae per gram estimates based on the bm-Baermann versus FLOTAC.

observed between the two techniques. Our previous work on bighorn sheep at NBR using the bm-Baermann method showed that the relative prevalence of *Protostrongylus* to *Muellerius* can range from 0% to 40%, suggesting that mixed lungworm infections are common (Ezenwa et al. 2010). Although mixed lungworm infections in bighorns have not been examined in detail, correctly identifying the species causing infections is critical because different lungworm species have different consequences for host health. For example, *Muellerius* infections are rarely associated with disease in bighorns, whereas *Protostrongylus* has been implicated in seasonal die-offs (Miller et al. 2012). Given this, the identifiability of parasites should be taken into account when choosing a detection method.

Microscopic identification of lungworm larvae relies on key morphologic features, such as tail shape (Foreyt 2001). Using the standard FLOTAC protocol in our preliminary work, all larvae were curled and unidentifiable. By using a saline modification of the standard method we were able to identify up to 80% of larvae. A 40-min exposure of the fecal sample to salt produced the most consistent results. Shorter exposures were less effective and the longest exposure times resulted in morphologic deformations in the larvae. We did not test whether our salt solution pretreatment increased the identifiability of larvae at the cost of reducing total larval counts. However, we observed no signs of broken or deformed larvae, suggesting that the pretreatment did not influence final LPG estimates. Even with modification, there was an approximately 30% difference in the *Protostrongylus* identification rate of the FLOTAC vs. the bm-Baermann. Although the difference was not statistically significant, this drawback of the FLOTAC needs to be considered when study goals involve distinguishing lungworm larvae by morphology. It is possible, however, that additional modifications to the basic protocol or the use of alternative flotation solutions could overcome this shortcoming of the FLOTAC.

In our study the bm-Baermann performed as well as the FLOTAC for estimating LPG, unlike most other FLOTAC-Baermann comparison studies. However, three of four previous studies used the classic Baermann procedure (Rinaldi et al. 2007, 2010; Gaglio et al. 2008).

TABLE 1. Lungworm larval counts by method for 17 bighorn sheep (*Ovis canadensis*) fecal samples collected in Montana, USA. Total larvae per gram (LPG) refers to combined *Muellerius* and *Protostrongylus* counts. LPG estimates are raw larval counts for the FLOTAC method and raw counts divided by 10 for the beaker-modified Baermann method. Numbers represent mean±SD.

	Total LPG	*Muellerius* LPG	*Protostrongylus* LPG	Unidentifiable LPG
FLOTAC	45.61±22.93	36.97±19.89	0.03±0.17	8.61±5.44
Beaker-modified Baermann	52.34±27.18	52.24±26.64	0.94±1.82	0

One study that found the two methods to be equivalent also used a modified Baermann protocol (Bauer et al. 2010). The bm-Baermann method improves the performance of the classic Baermann by exposing a greater surface area of feces and by preventing losses associated with larvae sticking to the walls of a Baermann funnel (Forrester and Lankester 1997a, b). Thus the bm-Baermann is widely used in bighorn sheep lungworm studies (Pelletier and Festa-Bianchet 2004; Goldstein et al. 2005; Pelletier et al. 2005; Rogerson et al. 2008). Our results suggest that the bm-Baermann performs as well as the FLOTAC for estimating overall larval counts in bighorn sheep fecal samples.

A potential advantage of the FLOTAC method over the bm-Baermann is the slightly faster active processing time (modified FLOTAC 40–60 min per sample, each sample duplicated; bm-Baermann 50–70 min per sample). However, the high within-assay repeatability for both methods suggests that processing times can be cut nearly in half by counting either a single FLOTAC slide per sample or a single centrifuge tube for the bm-Baermann. With this modification, both methods would have processing times <40 min per sample, minimizing time as a constraint on sample processing. In addition, experienced observers may be able to process a sample by the bm-Baermann method in much less than 40 min, again making any time difference between the two methods inconsequential. In terms of total processing time (i.e., including soaking steps), the bm-Baermann requires a relatively long 12–24-h soak, whereas the unmodified FLOTAC has no soaking step. However, our saline pretreatment added a 40-min soaking step to the FLOTAC protocol. These time issues also need to be considered in the context of potential disadvantages of the FLOTAC over the bm-Baermann. For example, the FLOTAC requires specialized equipment (i.e., FLOTAC slide) and the use of a relatively precise flotation solution, whereas the bm-Baermann can be performed with general lab supplies and tap water.

In conclusion, the bm-Baermann and a modified FLOTAC method were equally effective at quantifying lungworm larvae in bighorn sheep fecal samples, but some key trade-offs emerged. Issues with larval identification are a major drawback of the FLOTAC method, although the procedure can be completed faster than the bm-Baermann method. On the other hand, whereas the bm-Baermann allows for effective morphologic identification of larvae, it involves longer preparatory and processing times. Thus the slight improvement in time provided by the FLOTAC may be countered by the objective of larval identification (55% of recent bighorn sheep studies assessed morphology of lungworm larvae). However, there may be a place for the FLOTAC in studies where total counts, and not taxonomic identity, are the primary objectives. Future work is also needed to assess whether the FLOTAC method can be further modified to be more amenable to the study of mixed lungworm infections in bighorn sheep.

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