

Quantifying parasitic nematode larvae on pasture

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ABSTRACT

The aim of this study was to explore sources of paddock and laboratory variability in parasitic third stage larval (L3) tests. Recovery of *Teladorsagia circumcincta* larvae was greater than *Trichostrongylus colubriformis* larvae recovery from water (60 vs 43%; $P < 0.005$), from water contaminated with soil (55 vs 36%; $P < 0.03$) and from water with pasture (27 and 6%; $P < 0.001$). Recoveries following seeding with either *T. circumcincta* or *T. colubriformis* L3s onto pasture were similar (44% vs 56% respectively). Naturally contaminated pasture samples had similar mean larval number (larvae/kg DM) following extraction using either the plastic bag method (566) or Baermanns extraction (533). Pasture plucking to ground level 50, 100, 150 or 300 times had L3s 1,390 (range 406 – 3,846), 1250 (0 – 4,878), 360 (0 – 1,069) and 450 (182 - 742)/kg DM respectively ($P < 0.005$). Plucking or cutting with electric shears had no effect on L3 number which were 680 (range 0 – 2,944), 100 (0 - 355), 2,830 (0 – 6,818) and 9,870 (0 – 22,090)/kg DM in four paddocks. For all methods the coefficients of variation were high, necessitating the need to collect more than eight samples to correctly estimate L3 challenge and to detect significant differences between treatments or paddocks. Pasture L3 challenge can only be used to differentiate high L3 number and only if replicated samples are collected. This makes a simple pasture L3 test impractical for farmers.

Keywords: L3 larvae; measurement; parasites; pasture.

INTRODUCTION

Diagnostic measures for the estimation of gastrointestinal parasitism in ruminant animals largely rely upon faecal egg counts (FEC), a measure of eggs produced by L4 larvae. However, FEC is mainly a reflective rather than a predictive measure of parasite infection. Ideally farmers would like to predict parasitism via quantification of the number of parasitic third stage (L3) larvae on pasture.

Historically parasitologists have used pasture L3 counts in research studies but few commercial tests exist. Obtaining a representative paddock sample is difficult because L3 distribution has a negative binomial distribution, where L3 numbers are high in small non random patches (Donald, 1967). Furthermore L3 development, migration, survival and movement to and from the soil are highly variable, dependent both on moisture and temperature (Al Saqur *et al.*, 1982; Callinan & Westcott 1986).

In the laboratory, L3 are firstly removed from pasture into water (Stage 1 extraction) and secondly separated from debris and then concentrated for identification and counting (Stage 2 extraction). Commercial laboratories in New Zealand would prefer that the Stage 1 extraction be completed before samples are sent to the laboratory because this stage is space demanding.

The aim of this paper was to set up practical Stage 1 extractions and explore sources of variation in pasture L3 tests, firstly in Stages 1 and 2 and finally in combination with paddock sampling. This was done in order to assess the practicality of setting up a pasture L3 test for New Zealand farmers.

MATERIALS AND METHOD

Sample collection

Experiment 1: Ten microscope slides containing samples of L3 *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* grown in a monocultures (Source: L. McMurtry, Wallaceville) were counted at x100 magnification using a light microscope and then flushed into a 20 cm diameter plastic funnel for Stage 2 extraction (see below). The above procedure was repeated ($n = 8$ slides/species) but with inclusion in the funnel of either a 6 mL pre-boiled soil solution or 100 g fresh weight (FW) grass which had been pre-heated at 60°C for 2 h to kill resident L3s.

Experiment 2a: An area of 8 m² of pasture, free from grazing animals for more than two years, was cut to ground level using electric hand shears, and the clippings mixed thoroughly and divided into six 300 g FW subsamples. Three of the samples served as blanks, and three samples were each seeded with approximately 50 *T. colubriformis* or *T. circumcincta* L3 by single aliquot seeding (error measured as 20% coefficient of variation (CV)). The experiment was repeated twice giving six replicates. The L3 were removed for identification and counting using the Stage 1 plastic bag method, followed by the Stage 2 extraction both described below.

Experiment 2b: L3 contaminated, sheep grazed pasture, from within a 5 m² area, was clipped to ground level using an electric hand piece. The pasture was thoroughly mixed and then sub-sampled by quartering to give 40 sub-samples; two subsamples were then randomly bulked together to

give 20 samples of approximately 300 g FW each. Twenty one days after collection half of the samples were extracted using either the Stage 1 plastic bag extraction or Stage 1 hard funnel extraction both described below. The 10 mL from the bottom of the funnel were mixed by inversion and subsampled down to 2 mL on site before performing a Baermanns extraction described below, L3 count and species identification (Gribbles Laboratories, Palmerston North).

Experiment 3: A transect of approximately 100 × 5 m was set up on the diagonal across a flat paddock historically intensively grazed by sheep. Two operators walked along the transect and each collected five randomised replicates of 50, 100, 150, or 300 herbage plucks at 09:00 and 13:10 hours. The herbage was plucked to ground level using a sharpened teaspoon, stored in plastic bags in a chiller at 4°C for 4 to 5 days before the L3 were extracted using the Stage 1 plastic bag method. The 10 mL sediment was then mixed by inversion and subsampled down to 2 mL on site before performing Baermanns extraction, L3 count and species identification (Gribbles Laboratories, Palmerston North).

Experiment 4: In June 2005 four, small (<0.5 ha), flat, intensively grazed sheep paddocks with pasture covers of 1,200 to 2,400 kg DM/ha, were sampled in triplicate by both randomly plucking to ground level (>300 plucks, single operator, mean pasture weight 343 g FW) and electric hand shears (five 1 m by 6 cm strips, 396 g FW). Pasture samples were stored in a chiller at 4°C for 2 to 3 days before the larvae were extracted using the hard funnel system and then as described in Experiment 3.

Extraction methodologies

Stage 2: Extraction method: L3 in water or sediment were placed in a 20 cm diameter plastic funnel containing 3.5 L water (~20°C) and a submerged nylon sieve (20 µm aperture) which when left overnight for approximately 16 h, allowed L3 to migrate into the funnel base. The bottom 50 mL in the base of the funnel was collected and refrigerated (~4°C). The 50 mL was then siphoned from top-to-bottom with a large hooked syringe down to 5 mL. This 5 mL was then examined for L3 content on 2 × 2.5 microscope slides.

Stage 2: Extraction at Gribbles Laboratory: The 2 mL sub-sample was pipetted onto filter paper which was inverted onto a 20 µm mesh sieve in a Baermann funnel containing luke-warm water. The 50 mL funnel base was collected, refrigerated, centrifuged, and siphoned down to 10 mL. A 2 mL sub-sample of this was removed and counted on a microscope slide.

Stage 1: Plastic bag methodology: Pasture samples at room temperature were placed in a 5 L bucket with water. a non-ionic detergent (Tween)

was added, the grass was agitated and pasture sample then soaked for a minimum of four hours. The sample was sieved from the bucket then rinsed with a further 2 L of warm water into the bucket. The bucket was left to settle for a minimum of 4 h and the supernatant siphoned down to 4 L. The remaining 4 L of sediment was poured through a coarse sieve (1 mm aperture) and placed in a plastic bag heat sealed to form a funnel shape (angle 5 to 1). This was allowed to settle for a minimum of 4 h after which the basal 50 mL of sediment was collected into a Falcon tube and allowed to settle overnight. The supernatant was then siphoned down to a final 10 mL.

Stage 1: Hard funnel system: Pasture samples were placed in fibreglass Baermann funnels with approximately 5 L of warm water with a few drops of Tween detergent added. Funnels were left to settle overnight. The basal 200 mL of water and sediment was then transferred into a small glass Baermanns funnel lined with one layer of pre-wetted tissue paper. After 24 hours, the basal 50 mL of sediment was removed and placed in a Falcon tube. The sediment was left to settle in the Falcon tube overnight at 4°C before siphoning off and discarding 40 mL of supernatant to leave a 10 mL sample.

Statistical analysis

The data were analysed using GLM models of SAS with method as the fixed effect, either in its raw state or when necessary following log_e transformation.

RESULTS

Experiment 1

The mean recovery of counted L3 (seeded larvae in water) was 52% (CV 29%). *Teladorsagia* had a higher (P <0.005) recovery 60% (CV 24%) than *Trichostrongylus* 43% (CV 23%). The species difference occurred in all treatments (Table 1). There was a small non significant (P = 0.16) drop (52% to 45%) in recovery rates associated with soil contamination. The inclusion of pasture in the funnel greatly (P <0.001) reduced recovery rate (Table 1).

Experiment 2a

Mean recovery of seeded larvae was 44% (CV 102%) and 56% (55%) for, *T. circumcincta* and *T. colubriformis* respectively.

Experiment 2b

Both the plastic bag (566 (81%) larvae/kg DM) and hard funnel systems (533 (106%) larvae/kg DM) gave similar mean L3 numbers and species composition, though the variability between individual pasture samples were disturbingly high with two of the 10 samples using both methods having no larvae.

TABLE 1: Mean recovery rates (coefficient of variation) for *Teladorsagia circumcincta* (*Tel.*) and *Trichostrongylus colubriformis* (*Trich.*) L3 larvae using the basic Stage 2 L3 extraction procedure with either soil or grass added to the funnel.

Treatment	Species		P value Species
	<i>Tel.</i>	<i>Trich.</i>	
Basic	60 (24)	43 (23)	0.005
Added soil	52 (27)	45 (27)	0.03
Added grass	27 (53)	6 (74)	<0.001
P value Ttreatment	0.0002	0.0002	

TABLE 2: Effect of number of plucks on the mean, range, coefficient of variation (CV (%)) and standard error of mean (SEM) of L3 larvae/kg DM of pasture.

Parameter	Number of plucks			
	50	100	150	300
Mean	1,390	1,255	361	448
Range	406 - 3,846	0 - 4,878	0 - 1,069	182 - 742
CV	77	112	96	49
SEM	339	444	346	69

TABLE 3: Effect of collection method on the mean, range, coefficient of variation (CV (%)) of L3 larvae number/kg DM of pasture across four paddocks.

Method	Paddock number				P value Paddock
	1	2	3	4	
Plucked					
Mean	12,830 ^a	1,959 ^b	1,183 ^b	118 ^b	0.02
Range	7,002 - 22,100	0 - 5,880	0 - 2,994	0 - 355	
CV	63	173	134	173	
Electric					
Mean	6,905	3,707	168	81	0.10
Range	0 - 11,439	1,675 - 6,818	0 - 505	0 - 242	
CV	88	73	173	173	
P value Collection	0.36	0.52	0.33	0.80	

Superscripts that differ in a row are significantly different (P<0.05)

Experiment 3

There was no effect of operator, or pluck number by operator interaction on L3/kg DM. As pluck number increased L3/kg DM was reduced (P = 0.005) (Table 2).

Experiment 4

Plucking versus cutting had no effect on L3 larval number (4021 vs 2,715 L3/kg DM) or variability (CV 163% vs 151%) (Table 3). Pasture larval challenge was 100, 676, 2,833 and 9,865 L3/kg DM in the four paddocks (Table 3).

The variability within the individual samples was such that statistical difference was only detectable between the paddock with 9,865 L3/kg DM and the other paddocks (Table 3). Using a modest standard deviation of 2,000 L3/kg DM, in a power analysis (80%) eight pasture samples would be required to detect a difference of 3,000 L3/kg.

DISCUSSION

Following seeding of L3 into water, mean recovery was low at 52%. This is probably because the stored larvae had to actively migrate through a sieve. Recovery levels fall by 20% when L3 have been stored 19 days (Fine *et al.*, 1993). Later when fresher, although still stored, L3 were seeded onto pasture and underwent two extraction processes recovery rates were similar. These recoveries from L3 seeded onto pasture fall well within the 30 to 60% range cited for similar extraction methods (Lancaster, 1970; Couvillion, 1993; Fine *et al.*, 1993). However, recoveries of L3 seeded onto pasture may be different from naturally infected pasture (Donald, 1967). In our study, the number of L3 extracted from naturally infected pasture was similar for both the plastic bad and hard funnel extraction methods.

A recovery rate greater than 50% is considered ideal for a commercial test. The literature highlights multiple opportunities for improving recovery rates but all increase time and labour input. Multiple or extended soakings of grass in higher volumes of water can improve recoveries by 15 to 20%, as can longer sedimentation times (Lancaster, 1970; Raynaud & Gruner, 1981). It is clearly the grass:water ratio in Stage 1, that is the most powerful determinant of recovery (Persson, 1974). This was seen by the 30% drop in recovery of seeded larvae when 100 g pasture was added to 3.5 L of water in our Stage 2 extraction. Chiejina (1982) using a 3.2 L Baermanns extraction reduced recovery by almost 4% per 10 g of pasture over the range 50 to 200 g FW. In both Stage 1 extraction systems we used only 300 g FW:5 L water; a ratio likely to impede recovery. This was a compromise considered acceptable for a practical test. The hard funnel method also had the advantage of requiring minimal labour input.

The consistency of recovery is potentially more important than the magnitude of recovery, yet this is rarely discussed in the literature. All our extraction methods had highly variable recoveries as reported by other workers (Raynaud & Gruner, 1981). The only extraction systems in the literature to achieve consistent recoveries greater than 75% were those where salt gradients and slow centrifugation were used to separate the L3 from debris prior to counting giving a 96% recovery with a CV of 7%. (Chiejina, 1982; Martin *et al.*, 1990). In the early stages of our research, we examined this methodology with commercial providers in New Zealand. Unfortunately they found L3 identification more difficult because of distortion of the larval sheath.

This issue was not mentioned by Martin *et al.*, (1990) or any of the papers using the salt or sugar floatation methods.

It has been established that the distribution of L3 on pasture is driven from faecal pat distribution, giving a binomial distribution where the variance of the L3 count is similar in magnitude to the mean (Donald, 1967). In very small, flat, intensively grazed sheep paddocks we found that 300 plucks gave the most consistent estimates of L3 level. However, the greater sample size originating from the greater number of plucks compromised this analysis. The highly recommended, but largely unjustified, method of Taylor (1939) is to sample a paddock with two operators walking in a W shape. Both operators, although often only one, make around 100 stops, and collect 3 to 4 multiple plucks at each stop, thereby collecting between 250 and 500 g of fresh weight pasture (Couvillion, 1993). It is difficult to see farmers spending the time to collect such samples, especially when replicated samples may be required to achieve the desired accuracy. Pasture samples could however be collected rapidly using an electric hand piece. In contrast to Raynaud & Gruner, (1981), our results concur with those of (Martin *et al.*, 1990) who found no difference in number of L3 resulting from plucking or cutting to ground level when similar quantities of pasture were collected.

The interpretation of a pasture L3 test is also problematic. It has been well established that L3 move into the soil, especially in adverse conditions (Al Saqur *et al.*, 1982). In fact, up to 80% of L3 can be found in the soil at one time (Callinan & Westcott, 1986). These L3 can rapidly return to the herbage given mild temperatures, high humidity and free water on pasture (Krecek & Murrell, 1988). Indeed as part of our sampling, the replicated pasture L3 count changed from 170 to 4,000 L3/kg DM 14 days after 50 mL of rain at a mean temperature of 16°C (A.J. Litherland, Unpublished data). Arguably, potential L3 challenge can only be measured if both soil and pasture reservoirs are measured and then interpreted in association with climatic conditions. Even if a potential L3 challenge can be quantified, actual ingestion also needs to be predicted and the susceptibility of the grazing animal estimated (Gruner & Cabaret, 1985) before a prediction of future parasitism is possible. This is a somewhat daunting prospect.

In conclusion, the current methods for quantifying L3 on pasture give highly variable results. High variability is associated with each step

of the measurement process. Currently this methodology can only be considered suitable for differentiating high pasture L3 challenge and then only if replicate samples are collected. We conclude that this is not a practical test for farmers to use.

ACKNOWLEDGEMENTS

This research was funded by Meat and Wool New Zealand. We thank Helen Van den Ende, Coby Hoogendoorn, Don McDonald, Lawrie McMurtry, Alex Vlassoff, Dave Leathwick and Trevor Cook for all their valued assistance.

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