Counting infective-stage nematode larvae in rumen fluid as an indicator of parasite challenge in grazing sheep

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ABSTRACT

The aim of this study was to evaluate rumen fluid sampling as a novel method for quantifying nematode challenge encountered by grazing sheep. In Experiment 1, rumen fluid samples were seeded with known numbers of third stage infective larvae (L3) of *Teladorsagia circumcincta, Trichostrongylus colubriformis* and *Cooperia curticei*. Immediate recovery rates averaged 92% (Coefficient of variation, 13%), 53% (15%) and 50% (30%) for each species respectively. Recovery rates from rumen fluid stored at 7°C decreased to 44% (29%), 33% (18%) and 0% (0%) after 72 hours. In Experiment 2, 15 ewe hoggets maintained indoors were dosed with 5,000 L3 *T. circumcincta* and 5,000 L3 *T. colubriformis*. At 1, 2 and 12 hours after dosing the numbers of L3 recovered from the rumen fluid were 0.2 (125%), 0.16 (145%) and 0.01 L3/mL (300%) respectively. In Experiment 3, rumen fluid samples were taken from small groups of lambs grazing four paddocks with variable herbage infestation rates of mixed species of L3. Species composition of L3 on the herbage and in the rumen fluid was *T. circumcincta* 8% and 0%; *T. colubriformis* 27% and 19%; *C. curticei* 0% and 5%; *Nematodirus spp.* 63% and 69% and *Haemonchus contortus* 2% and 9%. A significant number of technical challenges remain before the method has a practical application.

Keywords: gastrointestinal nematodes; sheep; measurement; pasture; rumen fluid; infective larvae.

INTRODUCTION

Gastrointestinal nematode parasites are a major cause of lost productivity in grazing sheep in New Zealand. While the epidemiology and population dynamics of the several species involved varies widely, it is reasonable to assume that the numbers of infective third-stage larvae (L3) ingested with pasture is a major driver of the severity of parasitism. Unfortunately, there is no method available which can quickly and accurately determine the numbers of L3 on pasture. The existing method, in which 300 to 400 plucks of grass are taken across a paddock, equating to approximately 0.1 kg dry matter (DM), followed by sedimentation of larvae, is slow and has large inherent sampling and extraction efficiency errors (Litherland & Deighton, 2008). It is also difficult to replicate the ingestion of herbage by grazing animals using this method (Hutchings *et al.*, 2001). By comparison, a grazing lamb takes approximately 30,000 bites per day of pasture to consume approximately 1.5 kg DM.

An alternative method could involve direct sampling of rumen fluid to determine the numbers of L3 in individual animals. Rumen fluid is often sampled using stomach tubes in nutritional studies and the present study was designed to determine the applicability of this approach to quantifying L3 encountered by grazing sheep.

MATERIALS AND METHOD

All experiments were approved by the AgResearch Grasslands Animal Ethics committee. In Experiment 1 it was determined if L3 could be removed from rumen fluid in a state clean enough to count. In Experiment 2, following L3 dosing of parasite free animals, it was determined if repeatable rumen concentrations could be collected over time. In the final experiment it was examined if the rumen fluid L3 concentration would reflect varying pasture L3 contaminations in grazing sheep.

Experiment 1: Rumen fluid was collected from rumen-fistulated wethers grazing pasture, strained through cheese cloth, boiled to kill all larvae and stored at 7°C pending use. Ten samples each containing approximately 50 L3 of *Teladorsagia circumcincta* and 50 *Trichostrongylus colubriformis* L3 were seeded separately into either 50 mL of water or rumen fluid and then immediately extracted using the method described below. A further 10 replicates each of both species were placed in rumen fluid and then stored at 7°C for 24, 48 or 72 h before undergoing extraction. In addition, twenty 1 mL samples containing approximately 70 L3s of *Cooperia curticei* were pipetted from a stock suspension into rumen fluid and then immediately extracted.

Experiment 2: Fourteen parasite free ewe hoggets and a mean live weight of 38.9 kg, were moved indoors and fed a diet calculated as 1.5 x maintenance which consisted of a 1:2 ratio of lucerne pellets and oaten chaff. One hour after feeding, five lambs were dosed with water and nine

dosed with 10,000 L3 (50% *T. circumcincta* and 50% *T. colubriformis*) at ambient temperature. The L3 were administered directly into the rumen using a 10 mm stomach tube which was flushed twice post-administration with 60 mL water. At 1, 2 and 12 hours after dosing, 50 mL samples of rumen fluid were collected via a stomach tube fitted with a metal sieve (pore size 2 mm) and suction applied with a 20 mL syringe. The rumen fluid was immediately placed on ice and L3 extracted within 3 hours of collection using the method described below. Larvae were then identified and recorded as either sheathed or exsheathed.

For Experiment 1 and 2 rumen fluid samples were centrifuged at 1,200 rpm for 5 minutes, refrigerated for 1 hour and siphoned down to 5 mL. This was then pipetted onto a nylon sieve (20 µm pore size) which was placed on top of a small Baermann funnel containing water maintained at 20°C and left overnight. L3 migrated to the base of the funnel. The base 50 mL of water was collected, centrifuged at 1,200 rpm for 5 minutes and refrigerated for 1 hour before being siphoned down to 5 mL for counting and identification.

Experiment 3: Six 350 g pasture samples were plucked or cut with an electric hand piece to ground level from each of 4 small flat paddocks previously grazed by sheep. Each sample was placed on a Baermann funnel, after which L3 were concentrated and counted (Litherland & Deighton, 2008). Rumen fluid samples were then collected from 10 lambs grazing the same pastures.

Following collection, the rumen fluid samples were immediately stored at 4°C. Within 24 hours, a 2.5 mL sub-sample of rumen fluid from each lamb was removed and sent to a commercial laboratory for identification of larvae to species level. A Baermann extraction to clean the samples was undertaken by the laboratory before counting began. This involved pipetting each 2.5 mL sample onto a filter paper which was then inverted onto a mesh sieve (20 µm pore size) in a Baermann funnel containing luke-warm water. The resulting L3 were then collected from the funnel base, refrigerated, centrifuged, and siphoned down to a volume of 5 mL. A 2.5 mL sub-sample of this was removed and L3 counted and identified on a microscope slide.

Statistical analysis

Data were analysed using GLM models of SAS.

RESULTS

Experiment 1: The mean recovery of *T. circumcincta* and *T. colubriformis* L3 from water samples was 82%, with a low variability, which was similar for both species (Table 1). Recovery of L3 from rumen fluid was lower (P <0.01) than from water, at 73% (P <0.01) with a higher coefficient of variation (30%) largely due to the lower recovery of *T. colubriformis* and *C. curticei* (species*fluid P <0.001) (Table 1).

TABLE 1: Experiment 1 - Percentage recovery (coefficient of variation) of seeded larvae in water or rumen fluid after storage at 7°C for varying intervals. *Tel = Teladorsagia circumcincta; Trich = Trichostrongylus colubriformis; Coop = Cooperia curticei.*

Fluid	Storage time (h)	Recovery (%)			P value Species effect
		Tel.	Trich.	Coop.	
Water	0	83 (13)	81 (10)		0.62
Rumen fluid	0	92^{a} (8)	53^{ab} (23)	47 (40)	<0.001
	24	78^{b} (15)	14^{c} (30)		<0.001
	48	87^{ab} (10)	34^{b} (26)		<0.001
	72	44^{c} (29)	33^{b} (18)		0.03
P value Storage time effect		<0.001	<0.001		

Superscripts down the columns that differ are significantly different (P <0.05)

Storage in rumen fluid at 7°C reduced recovery of *T. circumcincta* and *T. colubriformis* L3s over time (Table 1), with the reduction in recovery rate being greater for *T. circumcincta* than for *T. colubriformis* (Species*storage P<0.001).

Experiment 2: No larvae were found in samples of the feed. Rumen fluid samples of 50 mL were readily collected from lambs in approximately one to three minutes per animal. Mean concentrations of L3 in the rumen fluid of dosed animals were very low, and ranged from 2 to 72 larvae/sheep, giving final numbers of 0.2 (Coefficient of variation 125%), 0.16 (145%) and 0.01 (300%) L3/mL for 1, 2 and 12 hours after dosing respectively. In individual dosed animals the number of L3 present one and two hours after dosing were very similar (R^2 = 0.93) (Figure 1). There was no difference between the concentration of *T. circumcincta* and *T. colubriformis* L3 at one

FIGURE 1: Experiment 2 - Concentration of L3s in rumen fluid in individual sheep 1, 2 and 12 hours after dosing with 10,000 L3 if extraction recovery was assumed to be 100%.

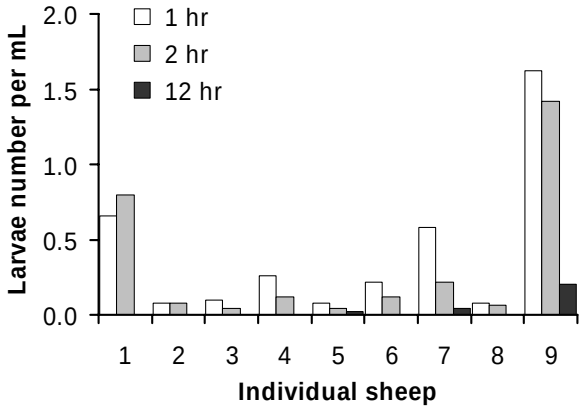

TABLE 2: Experiment 3 - Mean number of L3 and coefficient of variation on pasture (per kg DM) or in rumen fluid (per mL) and species composition of larvae (%) in four groups of lambs grazing different paddocks.

Parameter	Paddock							
	Pasture				Rumen fluid			
Number of L3	100	676	2,834	9,866	0	0	0.039	0.017
Coefficient of variation (%)	159	172	103	73	0	0	186	314
Trichostrongylus colubriformis	50	18	20	34	0	0	7	30
Nematodirus spp.	50	20	72	0	0	0	67	70
Teladorsagia circumcincta	0	33	4	12	0	0	0	0
Cooperia curticei	0	29	0	0	0	0	10	0
Haemonchus contortus	0	0	4	0	0	0	17	0

hour1 (0.21 vs. 0.20 L3/mL), 2 hours (0.16 vs. 0.16 L3/mL) and 12 hours (0.015 vs. 0.013 L3/mL) after dosing. No larvae were exsheathed at one and two hours but all larvae of both species were at 12 hours.

Experiment 3: Rumen fluid samples were much more difficult to obtain from grass-fed lambs than from those animals used in Experiment 2, primarily due to ingesta blocking the metal sieve at the end of the stomach tube. On average, 27, 39, 61 and 21 mL of rumen fluid per lamb was collected for Paddocks 1 to 4 respectively, and took 6.0, 5.4, 4.5 and 5.2 minutes per lamb on each occasion. Pasture cover was 1,400, 1,250, 2,400, 1,650 kg DM/ha for paddocks 1 to 4 respectively, while mean numbers of L3 on herbage, as assessed by conventional methods, were 9,900, 2,834, 680 or 100 L3/kgDM respectively. The mean numbers of L3 in rumen fluid, including animals with no larvae, were 0.017, 0.039, 0 and 0 L3/mL respectively (Table 2). Larvae were only recovered from a small number of animals in Paddocks 1 and 2. Variability in numbers of larvae in the rumen fluid was greater than that found using conventional methods, while variability between split samples was extremely high.

For paddocks in which lambs were found to contain L3 in the rumen, the composition of the L3 populations present on pasture compared to those present in rumen fluid was *T. circumcincta* 8% and 0%; *T. colubriformis* 27% and 19%; *C. curticei* 0% and 5%; *Nematodirus spp.* 63% and 69% and *Haemonchus contortus* 2% and 9%. (Table 2). In these cases, 87% of the variation in species composition of L3s in rumen fluid was explained by species composition on pasture.

DISCUSSION

The present study was designed to assess the use of counting parasitic L3 in rumen fluid to provide a measure of pasture contamination. A number of experiments compared results from conventional pasture pluck methodology with rumen sampling, and the impact of storage conditions and extraction techniques on the likely success of the procedure.

Higher and more consistent recoveries of L3 were obtained from seeded rumen fluid than was achieved from a previous study using seeded pasture (Litherland & Deighton, 2008). However, there are a number of significant concerns with the use of rumen sampling. First, it is important to recognise that only a portion of those L3 ingested over the course of a day will be in the rumen at one time. Dakkak *et al.* (1981) found that L3 pass through the rumen at half the rate of marked liquid, which implies a passage time comparable with that of fibre (Bartocci *et al.,* 1997). As the expected degradation rate of consumed DM on summer and autumn pastures is around 8% per hour, this suggests that the majority of L3 are likely to have passed through the rumen by 12 hours after ingestion.

The use of sampling rumen fluid to provide an indication of pasture L3 contamination is also hindered by technical and logistical problems. For example, the storage of L3 in previously boiled, cooled rumen fluid reduced recovery in both species; this could be due to both increased mortality and a reduction in motility through a Baermann apparatus. The extent of the effect of storage on *T. colubriformis* was unexpected because, as a small intestine parasite, it should in theory be relatively inert to rumen fluid. By contrast, the adverse effect of storage on the recovery of *T. circumcincta* was not unexpected, as for this species rumen fluid triggers exsheathment, and, with sustained contact with albeit warm rumen fluid, mortality is increased (Hertzberg *et al.*, 2002; DeRosa *et al.*, 2005).

Exsheathment of L3s normally occurs in the proximal organ to their desired location (Hertzberg *et a*l., 2002). Thus nematodes such as *T. colubriformis* and *Nematodirus spp*., which inhabit the small intestine, usually do not exsheath in the rumen although a proportion of C. curticei may do (Hertzberg *et al*., 2002). On the other hand, abomasal nematodes, such as *T. circumcincta, Trichostrongylus axei* and *Haemonchus contortus*, will exsheath within 1 to 2 hours of entering the rumen (Hertzberg *et al.,* 2002; DeRosa *et al*., 2005). As the outer cuticle may assist in the microscopic identification of L3, even brief storage of the abomasal species in rumen fluid after collection is problematic as the bicarbonate/carbonic acid buffer system stimulates exsheathment (Hertzberg *et al.,* 2002; DeRosa *et al*., 2005). It is possible that the development of rapid and accurate DNA analysis could negate the need for visual identification, should the need be identified (Zarlenga *et al*., 2001).

Extracting clean L3 from rumen contents for counting is difficult. The total number of L3 in rumen fluid samples will generally be very low, as only around 50 mL of rumen fluid can be removed easily from a weaned lamb (A.J. Litherland, Unpublished data.). Recovery of as many of those L3 present is therefore essential. In addition, due to the low numbers of L3, the extraction system should not involve any dilution steps to minimise further sampling error. Unfortunately in Experiment 3 a dilution step was unexpectedly introduced by the commercial laboratory. As a consequence few conclusions can be drawn in Experiment 3 about the effectiveness of rumen fluid technique in the field.

In conclusion, rumen sampling potentially offers a rapid measure of L3 in the grazing horizon of pasture, however a significant number of technical challenges would have to be overcome in sample preparation and counting before this method could be further developed.

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