DOI: 10.5433/1679-0359.2019v40n2p713

Comparison of two methods for the quantification of gastrointestinal nematode infective larvae from pasture

Comparação de dois métodos para quantificação de larvas infectantes de nematódeos gastrintestinais da pastagem

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Abstract

The economic losses caused by gastrointestinal nematodes are one of the biggest obstacles in the small ruminants production. Understanding the population dynamics of the infective larvae (L3) in the pasture is the key point to develop control programs, and reliable results depend on the used methodology to quantify L3 numbers. The use of the sampling directly from the pasture appears as a viable option, since it is not required the use of animals with an esophageal fistula or tracer animals, decreasing the costs involved in the study. Therefore, the present project, which had as objective evaluate the efficiency of two collection methods for quantification of L3 in the pasture, utilized 64 lambs (n = 16) allocated to four integrated crop-livestock systems (treatments) with 12 paddocks each. Pasture samples were collected every nine days. The W method consists in traversing the area in the form of a W and again an inverted W, forage samples being collected every 10 steps, and the Square method, in tossing a 0.16 m^2 square to four random points within the area, the forage within the square being collected after each toss. After the forage samples had been processed, the L3 were recovered and identified. Cohen's Kappa coefficient (k) was determined. The W-transect and Random-plot methods did not differ ($p \geq 0.05$) with respect to the number of L3 recovered from the pasture, and a positive correlation was found between them, suggesting agreement with one another, being that when the number of L3 recovered by the W-transect method increases, the same occurs in the Random-plot technique. The Random-plot method, which is already used to collect samples of forage for chemical analyses, can also be employed to estimate the pasture contamination by L3. The W-transect and Random-plot methods showed to be important in the epidemiological study of gastrointestinal nematodes in sheep. Therefore, the use of both on the same occasions and with different purposes, with one complementing the information that is not provided by the other, may be more effective in the investigation of environmental contamination by L3 of gastrointestinal nematodes.

Key words: Epidemiology. Lambs. Methods of sampling. Nematoda.

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Received: June 14, 2018 - Approved: Oct. 04, 2018

Resumo

As perdas econômicas causadas por nematódeos gastrintestinais são um dos maiores entraves na produção de pequenos ruminantes. Entender a dinâmica populacional das larvas infectantes (L3) no capim é o ponto chave para que se desenvolva programas de controle, e resultados confiáveis dependem das metodologias utilizadas para quantificar o número dessas L3. O uso da amostragem diretamente da pastagem aparece como uma opção viável, já que não requer o uso de animais fistulados ou traçadores, reduzindo os custos envolvidos no estudo. Assim, no presente estudo, que teve como objetivo avaliar a eficácia de dois métodos de coleta para quantificação de L3 na pastagem, foram utilizados 64 cordeiros (n = 16) alocados em quatro sistemas integrados de produção agropecuária (tratamentos) com 12 piquetes cada. As amostras de pasto foram coletadas a cada nove dias. O método W consiste em percorrer a área sob a forma de um W e novamente um W invertido, sendo as amostras de forragem coletadas a cada 10 passos, e o método Quadrado, em lançar um quadrado de 0,16 m² em quatro pontos aleatórios dentro da área, sendo coletada a forragem contida dentro do quadrado após cada lance. Após o processamento das amostras de forragem, as L3 foram recuperadas e identificadas. O coeficiente Kappa de Cohen (k) foi determinado. Os métodos W e Quadrado não diferiram ($P \geq 0,05$), quanto ao número de L3 recuperadas do pasto, sendo que existe uma correlação positiva entre os dois métodos, sugerindo que eles correlacionam positivamente entre si, sendo que quando a quantidade de L3 recuperada no método W aumenta, no método Quadrado também aumenta. O método Quadrado, que já é utilizado para coletar amostras de forragem para análises bromatológicas, pode ser utilizado para estimar a contaminação da pastagem por L3. Os métodos W e Quadrado se mostraram importantes no estudo epidemiológico dos nematódeos gastrintestinais de ovinos. Com isso, o uso de ambos, na mesma ocasião e com diferentes objetivos, de forma que um complemente as informações que o outro não oferece, pode ser mais eficaz no estudo da contaminação ambiental por L3 de nematódeos gastrintestinais.

Palavras-chave: Cordeiros. Epidemiologia. Métodos de amostragem. Nematódeos.

Introduction

One of the major obstacles in small ruminant production are infections caused by gastrointestinal nematodes, which culminate in economic losses due to animal mortality and/or decreased production rates (BURKE et al., 2016). The emergence of parasite populations featuring multiple resistance to commercial anthelmintics has prompted a search for alternative control methods (VAN ZYL et al., 2017).

Sheep are infected by these parasites upon consuming pasture contaminated with infective larvae (L3). Understanding the population dynamics of L3 should be the starting point for the development of sustainable worm-control programs (HECKLER; BORGES, 2016). Reliable results depend on the use of methodologies that allow for a precise quantification of the number of L3 in the pasture. Several techniques exist for the recovery of L3 from the pasture, as the use of animals with an esophageal fistula (GETTINBY et al., 1985), tracer animals (MARTIN et al., 1990), and sampling directly from the pasture (TAYLOR, 1939).

Because there is a correlation between the number of L3 in the pasture and the parasitic load of tracer animals (MARTIN et al., 1990; AMARANTE; BARBOSA, 1998), in addition to providing rapid results, sampling directly from the pasture does not require the use of animals, reducing the costs involved in the study. Many researchers have thus adopted methodologies whereby they sample contamination by gastrointestinal nematodes directly from the pasture. The most widely used methodology is that described by Taylor (1939), which consists of tracing W- and inverted-W-shaped transects in a given area.

Methods aimed at determining the total dry matter availability per hectare, the chemical composition, and the fresh matter availability per hectare are employed in the study of forage crops (MORAES et al., 2005). In this case, the pasture is collected near the soil level in plots bounded by a 0.5 × 0.5-m metal square frame that are chosen at random in the paddock under evaluation (MCMENIMAN, 1997). With a view to optimizing these procedures, an interesting idea would be to use the same sample, harvested for the chemical analysis, to quantify infective larvae. Furthermore, this approach would make it possible to estimate the total number of larvae present in the pasture of a paddock of known area. This methodology (Random-plot sampling) was adapted by Verschave et al. (2015), in Belgium, to estimate the contamination of the pasture by L3. The method consists of throwing a 0.16 m^2 square four times over the area at random. All the pasture within the frame is then harvested near the soil surface. In comparing the Random-Plot with the W-transect method, the authors obtained similar results; however, collection was faster by the former method.

Despite the advantages of measuring the number of L3 directly from the pasture, this methodology must be improved to become less laborious and more precise. On this basis, the present study was developed to determine whether samples harvested for chemical analysis by the Random-plot method can also be used to evaluate the pasture contamination by infective larvae.

Materials and Methods

Experimental area

The experiment was carried out at the Lageado Experimental Farm, belonging to the Faculty of Veterinary Medicine and Animal Science (FMVZ/UNESP) in Botucatu - SP, Brazil (22º51’01” S, 48º25’28” W, 777 m asl), during the crop year of 2014-2015, under integrated crop-livestock system (ICLS). The soil was a clayey, kaolinitic, thermic Typic Haplorthox (FAO, 2006) with 630, 90 and 280 g kg^{-1} of clay, silt and sand, respectively. According to the Köppen classification system, the prevailing climate in the region is the Cwa type (subtropical humid), characterized by hot, rainy summers and dry winters (ALVARES et al., 2013). The average accumulated monthly precipitation is highest (260.7 mm) in January and lowest (38.2 mm) in August. February is the hottest month and July is the coldest, with respective average monthly temperatures of 23.2 to 17.1 °C (ESCOBEDO et al., 2011).

The experimental area was used in the crop years of 2010/2011 and 2011/2012 with maize (*Zea mays*) silage intercropped with palisade grass (*Urochloa brizantha* cv. Marandu and Piatã) in the summer/autumn, and yellow oat (*Avena byzantina* cv. São Carlos) was overseeded in rows (with a seeder-fertilizer), with grazing by lambs in the winter/spring (PARIZ et al., 2017a). In the crop year of 2012/2013, soybean [*Glycine max* (L.) Merr.] silage was intercropped with guinea grass (*Panicum maximum* cv. Aruãna) in the summer/autumn, and the pasture was cut in the winter/spring (PARIZ et al., 2016, 2017b). In the crop years of 2013/2014, 2014/2015 and 2015/2016, maize silage was intercropped with palisade grass and pigeon pea (*Cajanus cajan*) in the summer/autumn, and black oat (*Avena strigose*) was overseeded in rows (with a seeder-fertilizer) or by broadcast seeding (manually), with grazing by lambs in the winter/spring (ALMEIDA et al., 2018). Thus, in the present study, the experimental area was divided into four ICLSs: maize + palisadegrass + pigeon pea + black oat rows-sown; maize + palisade grass + pigeon pea + black oat broadcast; maize + palisade grass + black oat rows-sown; and maize + palisade grass + black oat broadcast.

The maize was sown with palisadegrass and/or pigeonpea in December 2014 and mechanically harvested for ensiling in April 2015. Subsequently, in the same month, the black oat was oversown by two sowing methods: row sowing (seeder-fertilizer machine) and broadcasting (manually), followed by incorporation through light harrowing. Each ICLS was divided into twelve 225-m^2 paddocks to be grazed by sheep from July to September 2015.

Handling of experimental animals

Sixteen male Poll Dorset × Corriedale crossbred lambs at approximately eight months of age, with a body weight of 25 kg, were used per treatment, totaling 64 animals. Lambs were naturally infected by gastrointestinal nematodes with an average count of 6748 eggs per gram of feces (EPG). The animals were identified and vaccinated against clostridial diseases (Sintoxan Polivalente T®, Merial SA) and allotted to the treatments according to their body weight.

Lambs were rotated across the 12 paddocks, with three days of occupation and 33 days of rest. The experimental period was 72 days, with two grazing cycles in each period per treatment.

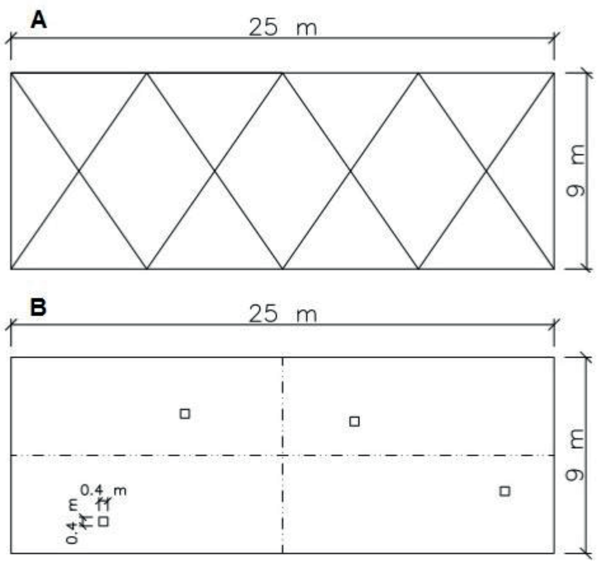
Paddocks were surrounded by a six-wire electrified fence, and the animals had free access to water. The area was covered with shade nets to provide greater thermal comfort to the animals. Lambs were allocated to their respective paddocks at 06h00 and moved after 16h00 to a covered shed where they would receive silage + concentrate, totaling 10 h of grazing activity.

This study was approved by and conducted in accordance with the experimental protocol approved by the local Ethics Committee (approval no. 01/2016-CEUA).

Pasture sample collection

To evaluate the two harvesting methods W-transect and Random plots (Figure 1), pasture samples were collected throughout the experimental period at every nine days. Collections took place in the morning, after the animals had entered the area, in paddocks 1, 6, and 11 of each treatment, totaling eight collections per paddock. Paddocks were chosen based on their distribution, aiming to obtain a homogeneous sample of the area. The total number of collections was 96 (8 collections/paddock, 3 paddocks/treatment, 4 treatments).

Figure 1. W-transect (A) and Random-plot (B) methods.



Pasture collection by the W-transect method followed the recommendations of Taylor (1939), which consist of collecting grass samples at every ten footsteps, tracing the area in W- and inverted-W-shaped transects. By this method, approximately 11 points of the area were sampled per collection.

The other tested method, named ‘Random plots’, described by Verschave et al. (2015), consisted of throwing a 0.16-m^2 square frame four times at random over the area and collecting all the grass within it.

The grass samples were harvested near the soil level with pruning shears and packed in identified plastic bags that were taken immediately to the laboratory to be processed. At the start of the experiment, any grass at a distance smaller than 30 cm from the sheep feces would not be collected so as not to overestimate the L3 contamination of the pasture sample. As time passed, because of the high stocking rate (59 animals/ha), the feces became scattered across the entire area and it was not possible to maintain this 30-cm distance for sampling.

Processing consisted of separating the samples in buckets previously identified with the number of the paddock, treatment, and collection, where the pasture samples remained immersed in 4 L of water with 0.5 mL of neutral detergent (to facilitate separation of the larvae from the grass) for four hours. After this period, each sample was transferred to another bucket that was also identified, containing 4 L of water and 0.5 mL of neutral detergent, and left at rest for another three hours, totaling seven hours of immersion in water (NIEZEN et al., 1998).

After this 7-h period, the grass samples were removed from the buckets, packed in paper bags, and dried in an oven at 60 ºC for 72 h to determine the dry matter content. The water from both buckets was mixed and kept at rest for 24 h. Afterwards, the supernatant was discarded and the sediment was transferred to a sedimentation cone. The L3 were recovered and quantified in accordance with Carneiro and Amarante (2008). Aliquots of 20% of the recovered larvae were killed, lugol-stained, and identified following Ueno and Gonçalves (1998).

The time in minutes required to collect the pasture in each of the two methods was measured in the first collection.

Statistical analysis

Data from the same collection, paddock, and treatment were compared in each of the evaluated methods. The treatments were not considered in the statistical analysis, as it was previously observed that they did not differ statistically from each other ($p \geq 0.05$).

Data normality was checked using PAST software version 2.17c. Because of the non-normality of data, comparisons were achieved by Mann-Whitney’s non-parametric test and interpreted using Minitab software version 16.2.4 (Minitab Inc., State College, PA, USA). P values lower than or equal to 0.05 were considered statistically significant. Figures were built in Graphpad Prism 5 software, containing the median values (minimum value - maximum value).

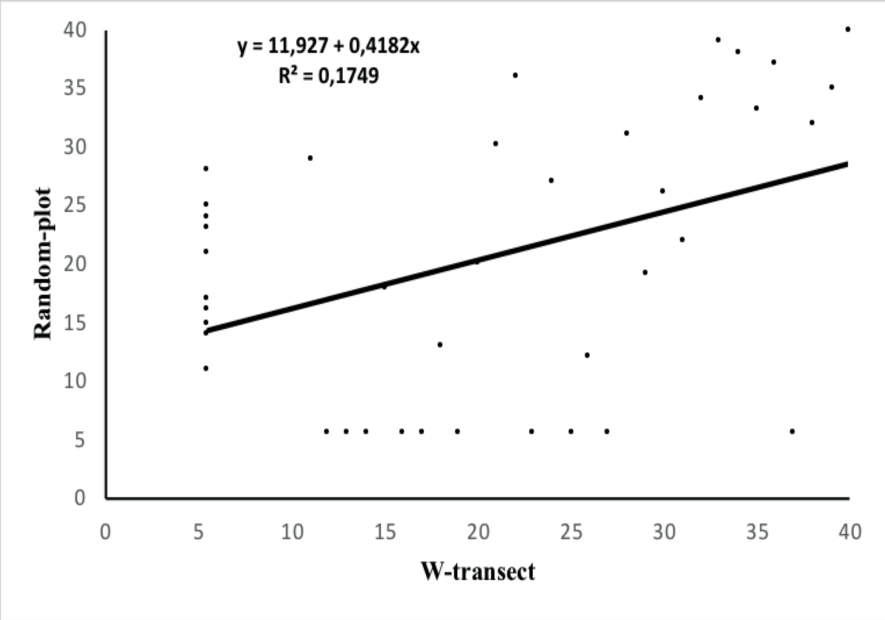
Cohen’s Kappa coefficient (k) was determined. This coefficient is a measure of agreement between two tests, where 1 represents perfect agreement between them, and values near and even below 0 indicate no agreement or that the agreement was precisely that expected by chance (COHEN, 1960).

Spearman’s non-parametric correlation coefficient was also determined between the collection times using Graphpad Prism 5 software. This coefficient takes into consideration the order of the data rather than their intrinsic value and serves to check the inter-relationship among the variables.

Results and Discussion

Of the 96 collections, L3 were only recovered in 40. On various occasions (56 collections), no L3 were recovered by either technique. The level of agreement (Cohen’s kappa coefficient (k ± standard error)) between the two methods was 0.51 ± 0.09, classified as moderate. A positive Spearman correlation was observed between the methods (r = 0.42; p = 0.0072), indicating that the rankings of the two variables follow approximately the same pattern (Figure 2).

Figure 2. Representation of the Linear Regression between the W-transect and Random-plot methods.



Infective larvae of the genera *Haemonchus*, *Trichostrongylus*, and *Oesophagostomum* were recovered from the herbage samples. *Haemonchus* spp. was the genus found at largest quantity, corresponding to 87.23% of the total larvae obtained by the W-transect method and to 74.22% of the total by the Random-plot method. The second most abundant genus in the samplings was *Trichostrongylus* spp. (11.77% in the W-transect method and 23.28% in the Random-plot method). The genus *Oesophagostomum* spp. was the most infrequent, with 1 and 2.5% of the total larvae recovered by the W-transect and Random-plot methods, respectively.

Considering only the 40 collections in which L3 were recovered, no significant statistical differences were observed between the W-transect and Random-plot methods in the analysis of the genera *Haemonchus* spp. (p = 0.31), *Trichostrongylus* spp. (p = 0.33), and *Oesophagostomum* spp. (p = 0.22). The medians (minimum value - maximum value) of *Haemonchus* spp., *Trichostrongylus* spp., and *Oesophagostomum* spp larvae recovered by the W-transect method were 633.28 (0 - 50,925.93), 231.12 (0 - 3,867.64), and 137.32 (0 - 393.4) L3/kg DM. By the Random-plot method, in turn, the respective medians were 345.43 (0 - 4.136.5), 121.15 (0 - 1,496.26), and 0 (0 - 327.01) L3/kg DM (Figure 3).

The median of the total larvae recovered from the pasture using the W-transect and Random-plot methods were 368.7 (0 - 53,240.75) and 253.7 (0 - 4,395.05) L3/kg DM, respectively. Despite the high discrepancy between the numbers of larvae found with both strategies (Figure 4), there was no significant statistical difference between them (p = 0.29).

Figure 3. Boxplot of number of infective larvae per genus per kilogram of dry matter (L3/kg DM), by the W-transect and Random-plot methods, showing the median, quartiles, and maximum-value bar.

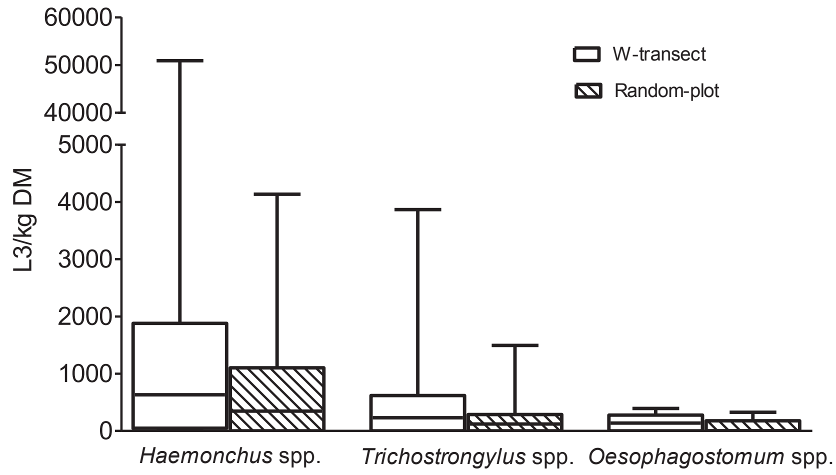
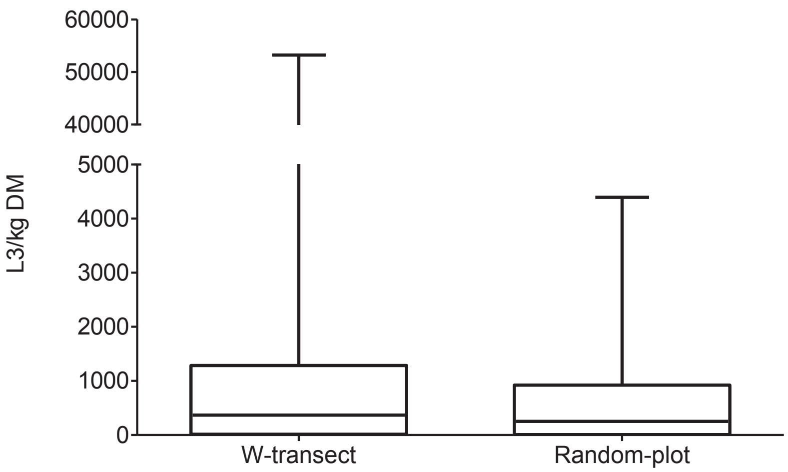


Figure 4. Boxplot of total infective larvae per kilogram of dry matter (L3/ kg DM), by the W-transect and Random-plot methods, showing the median, quartiles, and maximum-value bar.

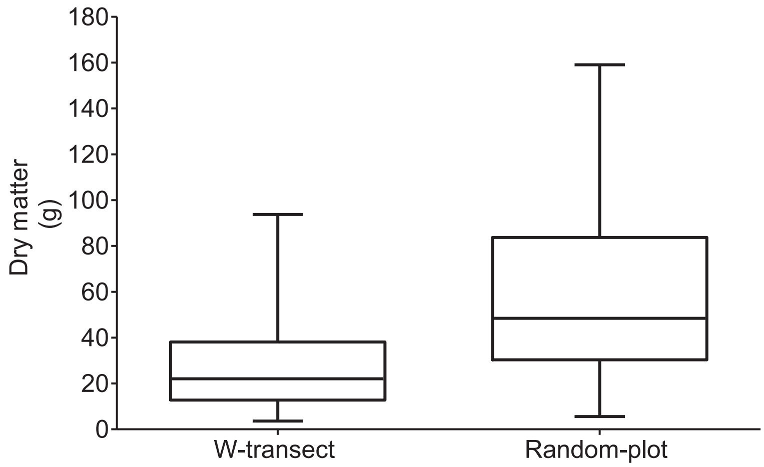


In terms of quantity of pasture sample harvested, there was a statistical difference between the two methods ($p < 0.05$). The median of the amount of herbage dry matter was higher by the Random-plot method (48.39 g (5.57 - 159.07)) as compared with the W-transect method (22.07 g (3.59 - 93.76)) (Figure 5).

The average time necessary for the collection of pasture by the W-transect method was 44 s, whereas the Random-plot method took 6 min and 3 s.

The W-transect and Random-plot methods did not differ ($p \geq 0.05$) with respect to the number of L3 recovered from the pasture, and a positive correlation was found between them, suggesting agreement with one another. In other words, when the number of L3 recovered by the W-transect method increases, the same occurs in the Random-plot technique. These results are confirmed by the Kappa test, which suggests moderate agreement between the methods. The low R^2 in the linear regression analysis indicates little association between the results obtained by both methods. This is assumed to have been because the pasture contamination was not uniform. Verschave et al. (2015) assessed the same pasture-collection methods for quantifying L3 of bovine gastrointestinal nematodes in Belgium and also did not find significant statistical differences between the methods.

Figure 5. Boxplot of weights (g) of samples collected using the W-transect and Random-plot methods, showing the median, quartiles, and maximum/minimum-value bars.



Because of the preset measures of the frame, in addition to quantifying the number of L3 in the pasture, the Random-plot method can also be used to quantify the herbage availability in the area and thus estimate the amount of L3 ingested by the animal. Thus, if we consider the recovery of 1,609 L3 in one of the collections in 74.56 g of pasture harvested within 0.64 m^2 (four frame throws), by extrapolating this value to 225 m² (area of each paddock), a herbage availability of 26.2 kg DM and an approximate contamination number in the area of 565,000 L3 are obtained.

Considering the 16 lambs used in each paddock of the experiment, with an average of 6,748 EPG, with 25 kg live weight, and defecating around 5% of their live weight per day (AMARANTE et al., 2007), it can be estimated that approximately 404,880,000 eggs were deposited during the three occupation days. Therefore, the L3 recovery rate in this study was 0.14%, which is a low number despite being higher than those reported by other authors performing this evaluation.

In a trial conducted in Botucatu - SP, Brazil, by Carneiro and Amarante (2008), the authors reported 0.02% L3 as the highest average recovery from the grass 28 days after contamination. In the same municipality, Santos et al. (2012) obtained similar results, with an average L3 recovery of 0.04% in the summer, 0.02% in the fall, and 0.03% in the winter. Exposure of the larvae to ultraviolet radiation increases their mortality rate, reducing the pasture contamination (VAN DIJK et al., 2009), which may explain their low recovery.

In addition to determining the herbage availability in the area, with the data obtained by the Random-plot method it is possible to estimate the number of L3 an animal ingests per day. Using the previous example of 1,609 L3 recovered in 74.56 g of DM, a 25-kg animal consuming around 3% of its live weight (DM) in black oat (BERNARDES et al., 2015) is estimated to consume 16,185 L3 daily. It is not possible to infer whether this L3 intake value might compromise the animal production, as there are no literature reports of a limit value after which animal performance would start declining. Research providing such results is necessary and of paramount importance in the study of gastrointestinal nematodes.

By the W-transect method, the most common of the two, it is not possible to know the herbage availability in the area under analysis, although more points are sampled (11) than in the Random-plot method (4). Nevertheless, the W-transect method was faster to be executed, taking half the time necessary for collection to be concluded by the Random-plot technique. This may be due to the small area used in the experiment (225 m²), which made it easier for the researcher to trace the path designated by the W-transect method and caused this approach to be easier to undertake than pasture collection.

However, Verschave et al. (2015) reported that the Random-plot method was faster, and the larger area (1 ha) was possibly responsible for this difference between both studies. According to those authors, larger areas require that the sampler spend more time to obtain the sample by the W-transect method.

Conclusions

The Random-plot method, which is already used to collect samples for chemical analyses of the grass, can also be employed to estimate the pasture contamination by L3. The W-transect and Random-plot methods showed to be important in the epidemiological study of gastrointestinal nematodes in sheep. Therefore, the use of both on the same occasions and with different purposes — with one complementing the information that is not provided by the other — may be more effective in the investigation of environmental contamination by L3 of gastrointestinal nematodes.

Acknowledgments

The first author received a scholarship from São Paulo Research Foundation (FAPESP), grant #2015/25413-1. The authors thank the FAPESP (grant #2013/13702-3, #2013/23853-9 and #2015/25718-7), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grant #458225/2014-2), and the Fundação Agrisus (Project 1378/14) for financial support. The authors would also like to thank the CNPq for an award for excellence in research to the fourth and fifth authors.

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