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Original Article

Control of sheep gastrointestinal nematodes using the combination of *Duddingtonia flagrans* and Levamisole Hydrochloride 5%

Controle de nematódeos gastrintestinais de ovinos utilizando a combinação de *Duddingtonia flagrans* e Cloridrato de Levamisole 5%

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Abstract

The objective was to evaluate the action of *D. flagrans* pellets in association with Levamisole Hydrochloride 5% for controlling sheep gastrointestinal nematodes in the northeastern Brazil. Three groups of six sheep each were formed: group 1 received 3 g of the pellets (0.6 g of *D. flagrans* mycelium) for each 10 kg b.w., twice a week for six months, and deworming with Levamisole Hydrochloride 5% when EPG ≥ 1500; group 2 received a dosage of Levamisole Hydrochloride 5% when EPG ≥ 1500; and group 3 received 3 g of pellets without fungi for each 10 kg b.w., twice a week for six months. EPG counts, larval cultures, packed cell volume (PCV) and weighing were performed every 15 days; monthly, samples of grass from each paddock were collected. The mean EPG of the groups began to statistically differ from day 30 ($p < 0.05$). Group 1 required less deworming with Levamisole Hydrochloride 5% and showed superiority of PCV values throughout the experiment ($p < 0.05$). There was a significant reduction ($p < 0.05$) in L3 recovery in the group 1 paddock from day 30 onwards. The use of *D. flagrans* pellets in association with Levamisole Hydrochloride 5% was effective for controlling gastrointestinal nematodes.

Keywords: Integrated control, nematophagous fungi, sheep-farming, *Haemonchus* sp.

Resumo

O objetivo foi avaliar a ação de péletes de *Duddingtonia flagrans* em associação ao Cloridrato de Levamisole 5% no controle de nematódeos gastrintestinais de ovinos no Nordeste do Brasil. Foram formados três grupos de seis animais cada: grupo 1 recebeu 3 g de péletes (0,6 g de micélio de *D. flagrans*) para cada 10 kg p.v., duaz vezes por semana durante seis meses, e vermifugações com Cloridrato de Levamisole 5% quando OPG > 1500; grupo 2 recebeu uma dosagem de Cloridrato de Levamisole 5% quando OPG ≥ 1500; e grupo 3 recebeu 3 g de péletes sem fungos para cada 10 kg de p.v., duas vezes por semana durante seis meses. Contagens de OPG, coproculturas, de volumes globulares (VG) e pesagens foram realizadas a cada 15 dias. Mensalmente, amostras de pasto de cada piquete eram coletadasa. A média de OPG dos grupos começou a diferir estatisticamente a partir do dia 30 ($p < 0,05$). O grupo 1 necessitou de menos vermifugações com Cloridrato de Levamisole 5% e demonstrou superioridade nos valores de VG durante todo o experimento ($p < 0,05$). Houve redução significativa ($p < 0,05$) nas L3 recuperadas no piquete do grupo 1 a partir do dia 30. Em conclusão, a utilização de péletes de *D. flagrans* em associação ao Cloridrato de Levamisole 5% foi eficaz no controle de nematódeos gastrintestinais de ovinos.

Palavras-chave: Controle integrado, fungos nematófagos, criação de ovinos, *Haemonchus* sp.

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Introduction

Research on application of the nematophagous fungus *Duddingtonia flagrans* for treating gastrointestinal nematodiasis in sheep has demonstrated its potential as a biological control agent against the free-living stages of these nematodes under *in vivo* experimental conditions (SILVA et al., 2009, 2010).

For animals, nematophagous fungi are usually administered in formulations based on a vehicle of sodium alginate. These formulations have been evaluated experimentally for controlling nematodes that parasitize animals and have provided good results under laboratory and field conditions, especially those with *D. flagrans*, which is considered to be the most promising species (BRAGA & ARAÚJO, 2014).

Studies have focused on evaluating the efficacy of various species of fungus in different animal species, with different doses and in different environments (ARAÚJO et al., 2007; BRAGA et al., 2009; TAVELA et al., 2013; VILELA et al., 2013, 2016). Studies evaluating the effectiveness of nematophagous fungi in integrated nematodiasis control systems in association with use of chemical compounds are scarce.

Use of biological control is based on the premise that treatment with nematophagous fungi cannot replace chemical control. According to Sanyal et al. (2004), while parasite control regimens that involve use of chemical anthelmintics cannot be implemented in organic livestock-rearing systems, combinations of anthelmintics and biological control can be implemented in most conventional animal production systems.

The aim of the present study was to evaluate the action of formulations of *D. flagrans* pelletized in a vehicle of sodium alginate in association with strategic anthelmintic treatment with Levamisole Hydrochloride 5% for controlling sheep gastrointestinal nematodiasis.

Materials and Methods

Fungi and production of mycelial mass

An isolate of *Duddingtonia flagrans* (AC001) was maintained in darkness at 4 °C, in tubes containing 2% cornmeal agar (CMA). This isolate came from soils in the region of Viçosa, Minas Gerais, and had been obtained by means of the method described by Duddington (1955), as modified by Santos et al. (1991).

Fungal mycelium was obtained by transferring culture discs (approximately 5 mm in diameter) of isolates of 2% CMA to 250 mL Erlenmeyer flasks containing 150 mL of potato-dextrose liquid medium (Difco), at pH 6.5. These were then incubated under agitation at 120 x g in darkness at 26 °C, for 10 days. After this period, the mycelium was removed, filtered and weighed on a precision analytical balance. All the procedures followed the methodology of Araújo et al. (2010).

Experimental trial and animals

This experiment was conducted in the municipality of Patos, Paraíba, northeastern Brazil (latitude 7°1'28" S; longitude 37°16'48" W), from Abril to September 2013.

An area of 0.6 hectares of pasture sown with Tifton grass (*Cynodon dactylon*) that was irrigated every day was divided into three paddocks. Each paddock was then infested over a 30-day period through the grazing actions of four five-month-old male Dorper sheep that presented a mean egg per gram of feces (EPG) count of 4870 ± 990, of which 70% was *Haemonchus* sp., 22% *Trichostrongylus* spp., 6% *Strongyloides* sp. and 2% *Oesophagostomum* sp.

The previous experiment was performed to choose the anthelmintic to be used, 24 sheep from the same farm were divided into four groups and underwent the fecal egg count reduction test, as described by Coles et al. (1992). The anthelmintics tested were Moxidectin 0.2%, Albendazole 5%, Ivermectin 0.08% and Levamisole Hydrochloride 5%. This last agent produced the largest reduction (95%) and was therefore chosen for this study.

Eighteen female Dorper sheep aged between 24 and 36 months, with a mean weight of 50 kg, were used. Fifteen days before the experiment started, they were dewormed through oral administration of Levamisole Hydrochloride (5 mg/kg of live weight), on three consecutive days. Seven days after the first deworming, EPG counts were performed by means of the technique described by Gordon & Whitlock (1939). Three examinations were performed on each sample, and all the animals were found to be negative.

Three groups of six sheep each were formed. In group 1, each animal received 3 g of the pellets (0.6 g of *D. flagrans* fungal mycelium) for each 10 kg of live weight, as part of their feed, twice a week for six months. Whenever analyses showed that the animals presented EPG ≥ 1500, they received a dosage of Levamisole Hydrochloride 5% (fungus + chemical group). In group 2, each animal that presented EPG ≥ 1500 received a dosage of Levamisole Hydrochloride 5% (chemical group). In group 3, each animal received 3 g of pellets without fungi for each 10 kg of body weight, as part of their feed, twice a week for six months (control group). Each group remained in one different paddock, at a stocking density of 1.5 animal units per hectare. To prevent deaths, rescue anthelmintic treatments were administered individually when the animals presented packed cell volume (PCV) less than 16%. Every day, all the animals received supplementation with protein-energy concentrate at the proportion of 0.75% of live weight, along with balanced mineral salt and water *ad libitum*.

Feces and blood were collected from all the animals every 15 days, in order to analyze EPG counts, fecal cultures (ROBERTS & O'SULLIVAN, 1950) and PCV measurements (FERREIRA et al., 1981). Also every 15 days, the animals were weighed to ascertain whether they were maintaining their weight.

To determine the levels of environmental infestation with L3, five samples of 200 g of leaf mass were collected every month, from different parts of each paddock, thus totaling approximately 1000 g of leaf mass 1000 g (RAYNAUD & GRUNER, 1982). These samples were placed separately in plastic buckets of capacity 10 liters, which were then filled with water at a temperature of 37 °C. The immersed samples were left to rest for four hours so that larvae would be released. The leaf mass was then removed carefully so that the larvae would not enter into suspension, and the supernatant was discarded. The precipitate was strained through a 200 µm sieve and was then decanted into a Hoffman flask. The sediment was examined under an optical microscope

and the larvae were quantified and identified. Subsequently, the arithmetic mean of the total numbers of larvae recovered from each of the five samples from each paddock was calculated. The leaf mass that had earlier been removed from the plastic buckets was placed in metal containers that were put into a forced-ventilation chamber and kept there at 45 °C for 48 h. The dry matter was then weighed to determine values for L3/kg of dry matter.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) and the Tukey's test at the 5% probability. Were performed tests to verify the presuppositions of ANOVA. The EPG values were analyzed using the logarithmic transformation log (x + 1). Nevertheless, they are presented in the figures as arithmetic means of the non-transformed values. The analyses were performed using the BioEstat 5.0 software (AYRES et al., 2007).

Results

The mean EPG values of the groups started to differ statistically ($p < 0.05$) from the 30th day onwards (Figure 1A). In the group treated with *D. flagrans* and Levamisole Hydrochloride 5% (fungus + chemical group), the EPG count on the 30th day of the experiment was 250, and it remained at low levels throughout the experiment, reaching day 180 with a mean of 480. There was a statistical difference between the chemical and control groups over most of the period of the experiment (days 30, 120, 150 and 180), but both of these groups had high EPG values throughout the experiment, except on days 60 and 90. At the end of the experiment, the mean EPG count for the chemical group was 1320 and for the control group, 2340.

Regarding the number of treatments with Levamisole Hydrochloride 5%, i.e. when EPG ≥ 1500, a statistically significant difference was observed ($p < 0.05$) between the fungus + chemical and the chemical group (Table 1). The group, which received an association of *D. flagrans* and chemical treatment, only presented eight occurrences of EPG ≥ 1500 over the course of the experiment, all of these were by the 120 day. On the other hand, in the chemical group, 17 occurrences of EPG ≥ 1500 were observed, which required 17 deworming treatments distributed between the start and end of the experiment.

The fungus + chemical group presented the highest PCV values throughout the experiment ($p < 0.05$) and differed from the control group from the 30th day onwards (Figure 2). The PCV values of the chemical group remained at intermediate levels, compared with the other two groups, and differed from the control group only on days 30 and 60.

In the fungus + chemical group, due low PCV, none of the animals required rescue deworming over the course of the experiment. However, in the chemical group, two sheep received rescue deworming: one on day 90 and the other on day 150. In the control group, seven rescue deworming treatments were needed: one sheep received three doses, on days 90, 150 and 180; and two sheep received two doses, both on days 90 and 120.

A high rate of environmental infestation in the paddocks was observed at the start of the experiment (mean of 4500 L3/kg of dry matter in each group). However, the mean numbers of L3 recovered from the pasture presented statistically significant differences ($p < 0.05$) from the 30th day onwards, when the fungus + chemical group started to show a gradual reduction in the numbers of L3 in the pasture, reaching a mean of 800 L3/kg of dry matter on

Table 1. Numbers of deworming treatments applied in the fungus + chemical group (*D. flagrans* concentration of 0.6 g/10 kg of live weight, twice a week, and Levamisole Hydrochloride 5% when EPG ≥ 1500) and chemical group (Levamisole Hydrochloride 5% when EPG ≥ 1500), in the northeastern Brazil over a 180-day period.

Groups	Days						Total
	30	60	90	120	150	180	
Fungus + chemical	2	3	2	1	-	-	8*
Chemical	3	4	2	2	3	3	17
						P value	0.01

*Represents a statistically significant different ($p < 0.05$) according to the F test at 5% significance.

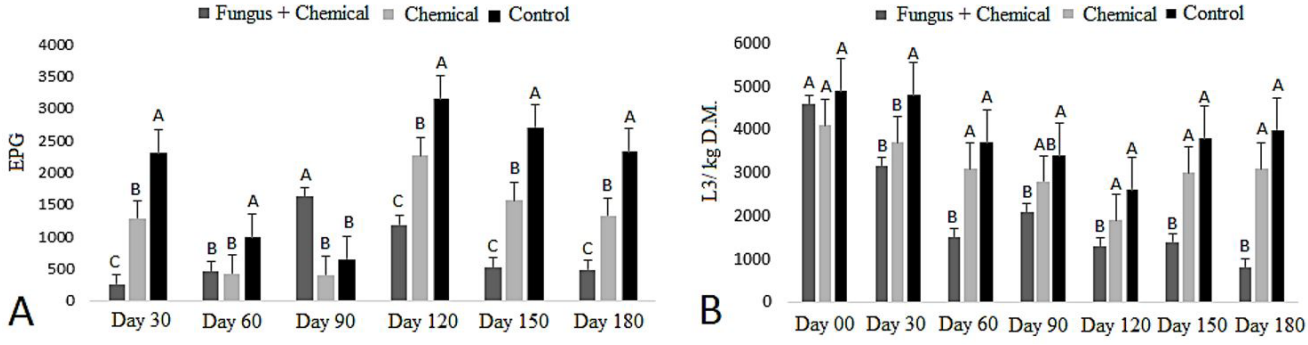

Figure 1. (A) Monthly means and standard deviations of egg per gram of feces (EPG) counts; (B) monthly means and standard deviations of the numbers of infective larvae per kilogram of dry matter (L3/kg D.M.). Sheep groups: fungus + chemical (D. flagrans concentration of 0.6 g/10 kg of live weight, twice a week, and Levamisole Hydrochloride 5% when EPG ≥ 1500), chemical group (Levamisole Hydrochloride 5% when EPG ≥ 1500) and control group. Values followed by the same letter are statistically similar (p > 0.05, according to Tukey test at 5%).

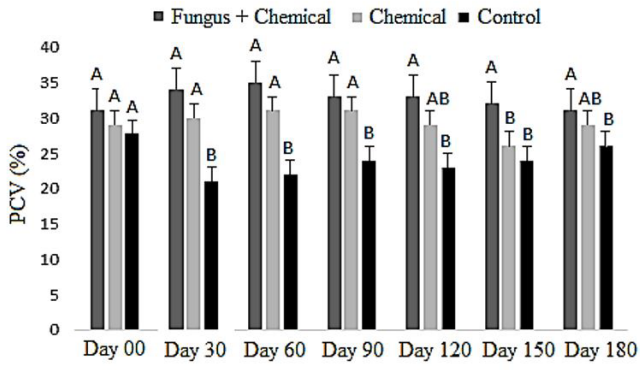

Figure 2. Monthly means and standard deviations of packed cell volume (PCV) among sheep in the fungus + chemical group (D. flagrans concentration of 0.6 g/10 kg of live weight, twice a week, and Levamisole Hydrochloride 5% when EPG ≥ 1500), chemical group (Levamisole Hydrochloride 5% when EPG ≥ 1500) and control group, in the northeastern Brazil over a 180-day period. Values followed by the same letter are statistically similar (p > 0.05, according to Tukey test at 5%).

day 180. The chemical group only differed statistically ($p < 0.05$) from the control group on day 30 (Figure 1B).

In both groups was observed that *Haemonchus* sp. was the most prevalent genus in all the fecal cultures, followed by *Trichostrongylus* spp., *Strongyloides* sp. and *Oesophagostomum* sp.

There was no statistically significant difference ($p > 0.05$) in the weights of the animals in the different groups, and a mean weight of 50 kg was maintained throughout the experiment.

Discussion

Starting at day 30, there was a statistical difference ($p < 0.05$) between the fungus + chemical group and the other groups. In this group, on day 180, the fecal egg count reduction was 80%, compared with the control group. Vilela et al. (2016) coadministered pellets of *D. flagrans* and *Monacrosporium thaumasium* to sheep in the semiarid region of Paraíba under the same grazing pressure. They observed that statistical differences in EPG counts ($p < 0.05$) only started at the 60th day of the experiment, with a fecal egg count reduction on day 180 that was similar to what was seen in the present study (76% in adults and 83% in juveniles). Other studies have also demonstrated the ability of *D. flagrans* to survive after passage through the gastrointestinal tract of animals, making it a strong ally in combating free-living stages of the gastrointestinal nematodes (WALLER, 2006; PAZ-SILVA et al., 2011; SAGÜÉS et al., 2011; FITZ-ARANDA et al., 2015; ARIAS et al., 2013; SILVA et al., 2014). Strategic use of chemical treatment aided in this, by enabling accelerated reduction of the parasite load in the animals.

There was a statistical difference of the EPG between the chemical and control groups over most of the experiment (days 30, 120, 150 and 180). However, the chemical group continued to present high EPG counts throughout the experiment. At the end, the fecal egg count reduction of this group in comparison with the control group was 44%. Although the chemical used presented good anthelmintic efficacy on this herd (95%), the high grazing pressure together with the high environmental infestation with L3 of the gastrointestinal nematodes led to high rates of reinfection of the animals. Graminha et al. (2005) observed low rates of helminthiasis in sheep kept at low grazing pressure, even those animals that did not receive anthelmintic treatment.

There was a statistically significant difference ($p < 0.05$) regarding the numbers of treatments with Levamisole Hydrochloride 5% when EPG ≥ 1500. In the fungus + chemical group, only eight deworming treatments were administered, mainly in the early part of the experiment. However, in the chemical group, 17 deworming treatments were necessary, and these were evenly distributed until the end of the experiment. Also in the fungus + chemical group, none of the animals required rescue deworming at any time during the experiment. In the chemical group, two sheep received rescue deworming. In the control group, seven rescue deworming treatments were needed. According to Araújo et al. (1998), clinical parasitism does not occur when nematophagous fungi are administered, because of the diminished numbers of larvae in the pasture, which reduces the reinfection rate among the animals and leaves them capable of developing natural immunity against nematodes.

There was a gradual reduction in the numbers of L3 in the pasture of paddock occupied by the fungus and chemical group from the 30th day onwards. By the end of the experiment, levels 80% lower than those encountered in the paddock occupied by the control group were attained. The chemical group differed statistically from the control group only on day 30, and reached the end of the experiment with levels of pasture infestation due to L3 that were only 22.5% lower than those of the control group. Pasture irrigation may have favored fungal development. However, it may also have increased the challenge, as it also favors a better development of nematodes. Dias et al. (2007) also observed reductions in the numbers of L3/kg of dry matter, of 85%, after six months of administering pellets of *D. flagrans* to cattle in southeaster Brazil. According to these authors, use of nematophagous fungi enabled reduction of the numbers of infective larvae in the pasture after one month. However, Larsen et al. (1998) only observed a statistical difference ($p < 0.05$) in the numbers of L3/kg of dry matter among horses treated with *D. flagrans*, in relation to the control group, in the last two months of their experiment.

Haemonchus sp. was the most prevalent helminth genus in all the fecal cultures. High prevalence of this genus was also observed in goat herds in the Sertão mesoregion of Paraíba, northeastern Brazil, by Vieira et al. (2014). This genus accounted for 79.9% the parasite load among these animals. In this region, other researches too showed that that *Haemonchus* sp. was the most frequent helminth of sheep and goats (LIMA et al., 2010; VILELA et al., 2012b; MELO et al., 2013).

The fungus + chemical group presented the highest PCV values throughout the experiment ($p < 0.05$) and differed from the control group from the 30th day onwards. Vilela et al. (2012a) also observed that the PCV values were better among goats in the semiarid region of northeastern Brazil that received pellets of *D. flagrans* for six months, than among those in the control group. However, Silva et al. (2010) did not observe any statistically

significant difference ($p > 0.05$) in PCV among sheep in southeastern Brazil that received pellets of *D. flagrans*.

There was no statistically significant difference ($p > 0.05$) in the weights of the animals in the different groups. Chandrawathani et al. (2004) observed that the sheep greater weight gain in Malaysia occurred in the group receiving *D. flagrans*. Silva et al. (2009) also did not observe any significant changes to the weights of sheep that underwent treatment with pellets containing *D. flagrans* and *Monacrosporium thaumasium* in southeastern Brazil, probably because these were adult animals that were receiving a weight maintenance diet. In a study testing the efficacy of pellets of *D. flagrans* and *M. thaumasium* that were coadministered to juvenile sheep, Vilela et al. (2016) observed a statistical difference from the 90th day of the experiment onwards, with a mean weight gain of 18.7 kg at day 180, while the control had a gain of 8.1 kg.

In the present study, use of *D. flagrans* to control environmental infestation, together with use of chemical treatment consisting of Levamisole Hydrochloride 5% when the animals' EPG count exceeded acceptable levels, resulted in an effective anthelmintic treatment strategy. Thus, the animals in the fungus + chemical group maintained low parasite loads throughout the experiment, which was thus reflected in lower numbers of deworming treatments, better weight gains, better PCV indices and lower ratios of L3/kg of dry matter.

Conclusion

It can be concluded that use of pellets of *D. flagrans* in association with strategic anthelmintic treatment using Levamisole Hydrochloride 5% was effective for controlling gastrointestinal nematodiasis among sheep that were maintained on irrigated pasture in the semiarid region of Brazil.

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