Research Note

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In vitro biological control of infective larvae of *Ancylostoma ceylanicum*

Controle biológico *in vitro* de larvas infectantes de *Ancylostoma ceylanicum*

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Abstract

The aim of this study was to evaluate the predatory activity of the fungus *Duddingtonia flagrans* (AC001) on infective larvae of *Ancylostoma ceylanicum* after gastrointestinal transit in hamsters. Twenty animals were used in the experiment, divided into two groups: a treated group (10 animals) and a control group (10 animals). In the group treated with *D. flagrans*, each animal received mycelium from the AC001 isolate, at an oral dose of 5 mg/25 g of live weight. To evaluate the predatory activity of the fungus, fecal samples were collected from the animals in both groups, at the times of 6, 8, 12, 24 and 36 hours after the treatment. Then, subsamples of 2 g of feces were placed in Petri dishes containing 2% water-agar (2% WA) culture medium and 1000 L_3 of *A. ceylanicum*. Over the study period, the following percentage reductions were observed: 43.2% (6 hours), 30.8% (8 hours), 25.8% (12 hours), 30% (24 hours) and 11% (36 hours). The fungus *D. flagrans* presented predatory activity on the L_3 of *A. ceylanicum*, after passing through the hamsters' gastrointestinal tract. It was therefore concluded that the fungus *D. flagrans* may be an alternative for biological control of the L3 of *A. ceylanicum*.

Keywords: Nematophagous fungi, *Ancylostoma ceylanicum*, *Duddingtonia flagrans*, biological control.

Resumo

O objetivo deste trabalho foi avaliar a atividade predatória do fungo *Duddingtonia flagrans* (AC001) sobre larvas infectantes de *Ancylostoma ceylanicum* após o trânsito gastrintestinal em hamsters. Foram utilizados vinte animais no experimento, divididos em dois grupos: um grupo tratado (10 animais) e um grupo controle (10 animais). No grupo tratado com *D. flagrans*, cada animal recebeu 5mg/25g de peso vivo de micélio do isolado AC001, por via oral. Para avaliar a atividade predatória do fungo, amostras fecais foram coletadas de ambos os grupos de animais nos horários de: 6, 8, 12, 24 e 36 após o tratamento. A seguir, 2g de fezes foram colocadas em placas de Petri contendo o meio de cultura ágar-água 2% (AA2%) e 1000 L_3 de *A. ceylanicum*. Ao longo dos horários estudados os seguintes percentuais de redução foram observados: 43,2% (6 horas); 30,8% (8 horas); 25,8% (12 horas); 30% (24 horas) e 11% (36 horas). O fungo *D. flagrans* (AC001) apresentou atividade predatória sobre as L_3 de *A. ceylanicum* após o trânsito pelo trato gastrintestinal de hamsters. Além disso, foi observada uma diferença significativa nos percentuais obtidos de cada horário em relação ao numero de L_3 recuperadas ($P < 0,01$). Conclui-se, portanto, que o fungo *D. flagrans* pode ser uma alternativa de controle biológico das L_3 de *A. ceylanicum*.

Palavras-chave: Fungos Nematófagos, *Ancylostoma ceylanicum*, *Duddingtonia flagrans*, controle biológico.

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The role of pet animals (dogs and cats) as reservoirs of potentially zoonotic nematodes has been recognized as a worldwide public health problem (FAIRFAX et al., 2009). Prominent among these nematodes is the genus *Ancylostoma*, which includes several species (*A. caninum, A. braziliense* and *A. ceylanicum*). The hookworms of dogs and cats are important from the viewpoint of veterinarians and also from a public health perspective (LANDMANN; PROCIV, 2003; TRAUB et al., 2008). In relation to using hamsters as an experimental model, Garside and Behnke (1989) and Bungiro Junior (2003) mentioned that when these animals were infected with *A. ceylanicum*, L_3 presented clinical features observed in children, in other words, anemia and growth delay, thus serving as a good experimental model. Thus, some studies have aimed towards using nematophagous fungi as biological controls to assist in environmental decontamination relating to infectious forms (eggs and/or larvae) of gastrointestinal parasites (ARAÚJO et al., 2004; BRAGA et al., 2010a; FERREIRA et al., 2011).

Predatory fungi have been studied as alternative controls under laboratory and natural conditions mostly in relation to combating the eggs and/or larvae of nematodes (NORDBRING-HERTZ et al., 2002; ARAÚJO et al., 2010). In this context, *Duddingtonia flagrans*, a fungus in the predator group, has been the species most studied. Reductions in the quantities of infective larvae of nematode parasites have been successfully demonstrated using this species in tests under laboratory and environmental conditions. Moreover, recent studies have demonstrated its potential for use in environmental decontamination relating to geohelminths (CARVALHO et al., 2009; BRAGA et al., 2010b). Only one study in the literature has suggested that *D. flagrans* would act on *A. ceylanicum* L_3, but it did not focus on this fungus and therefore did not provide much demonstration of the viability of *D. flagrans* after passing through the gastrointestinal tract of laboratory animals (hamsters) or of its predatory activity on *A. ceylanicum* (BRAGA et al., 2010a).

The objective of the present study was to evaluate the predatory activity of the fungus *D. flagrans* (AC001) on infective larvae of *A. ceylanicum* after gastrointestinal transit in *Mesocricetus auratus*.

An isolate of the fungus *D. flagrans* (AC001) that had been kept in test tubes containing 2% corn meal agar (2% CMA), at 4 °C and in the dark, was used. This fungus was grown in YPG liquid medium (yeast extract, sodium peptone and glucose) under stirring at 120 rpm, in accordance with the methodology described by Carvalho et al. (2009).

Infective larvae (L_3) of *A. ceylanicum* were obtained from coprocultures performed using contaminated feces from hamsters that had previously been infected with 100 larvae of *A. ceylanicum* per animal. After this period, the L_3 were recovered by means of the Baermann apparatus, in accordance with the methodology described by Araújo et al. (1993). The larvae were then identified using the parameters established by Bowman et al. (2006).

Twenty hamsters (males and females) of approximate weight 90 to 110 g and age 6-8 weeks were subjected previously to oral treatment with 4 mg/kg of ivermectin (Chemitec Agro, Brazil) over seven consecutive days, followed by a 20-day wait to cover the grace period of the drug. After treatment, a parasitological examination was performed on the feces to confirm that the animals were negative. Subsequently, these animals were randomly divided into two groups, each group containing 10 animals. The groups of animals were formed in separate cages that were appropriate and suitable for housing them. During the experiment the animals received water ad libitum and commercial feed for hamsters (Labina Cargill Nutrição Animal, Brazil). In the *in vivo* assay, each animal in the treated group received fresh mycelium from the isolate (AC001), at an oral dose of 5 mg/25 g of live weight, by means of a gavage needle (BD-10 stainless steel needle). The control group animals only received water, as described by Braga et al. (2009). Next, fecal samples from the animals in the treated and control groups were collected 6, 8, 12, 24 and 36 hours after administration of fungal treatment (CARVALHO et al., 2009). The samples were homogenized in a "pool", and subsamples of 2 g of feces were removed and placed in Petri dishes with 4.5 cm in diameter containing 2% water-agar (2% WA). In each Petri dish of the treated and control groups, 1000 L_3 of *A. ceylanicum* were shed, and these were placed in an incubator at 25 °C in the dark for 10 days. At each scheduled time, six replicates per group of animals were performed. Every day, the Petri dishes were observed to detect any structures of *D. flagrans* (AC001), and conidia, conidiophores and chlamydospores, in accordance with the classification key of Van Oorschot (1985). On the tenth day, the L_3 that had not been depredated were recovered from the Petri dishes by means of the Baermann method, and the percentage reduction was calculated. The results relating to the numbers of larvae recovered at different times were subjected to variance analysis (ANOVA). Next, the means of the L_3 counts in the treated and control groups were compared using the Tukey test at the 1% probability level (AYRES et al., 2003).

The mean numbers of *A. ceylanicum* L_3 recovered after 10 days of infection, at the different times of the fecal sampling (6, 8, 12, 24 and 36 hours), from the Petri dishes are shown in Figure 1. This was also observed in relation to the fecal samples collected after 12 hours of infection, but with a significant difference ($p < 0.01$) at all the times studied in relation to the control group. It was observed that from the fifth day after administration of the fungus, conidia and chlamydospores of *D. flagrans* were present in the Petri dishes of the treated group, thus confirming that the fungus had passed through the gastrointestinal tract of the hamsters.

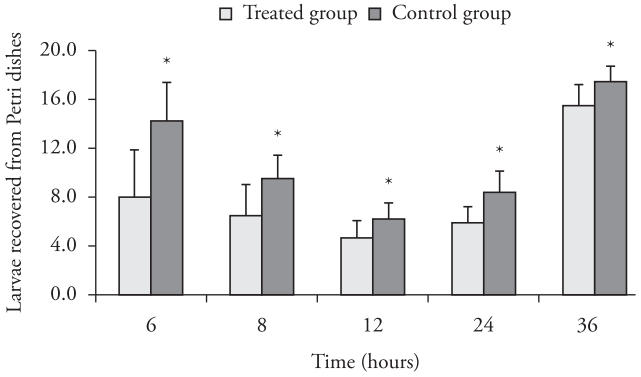

Figure 1. Means and standard deviations of the number of infective larvae (L_3) of *Ancylostoma ceylanicum* recovered from the Petri dishes by means of the Baermann method at different times of fecal sample collection, 10 days after infection with the fungal isolate *Duddingtonia flagrans* (AC001) and control (no fungus). Asterisk denotes a significant difference ($p < 0.01$), using the Tukey test.

In relation to predatory activity, the fungus *D. flagrans* (AC001) showed a capacity to prey on the L_3 of *A. ceylanicum*, thus proving its viability. Over the time period studied the following percentage reductions were observed: 43.2% (6 hours); 30.8% (8 hours); 25.8% (12 hours); 30% (24 hours); and 11% (36 hours). There were significant differences ($p < 0.01$) in the numbers of L3 recovered from the plates of the treated group, in comparison with the control group, at each time studied. Likewise, in relation to the percentage reduction of L_3, the result demonstrated in the present study was significant at all times (6 to 36 hours) ($p < 0.01$), in comparison with the control group. In the present study, *D. flagrans* (AC001) germinated in feces after passage through the gastrointestinal tract of hamsters. According to Bungiro Junior et al. (2001), an animal model that reproduces important clinical sequelae of hookworm infection types seen in humans theoretically constitutes the best way to characterize the main pathological features of hookworm disease. These criteria are quite satisfactorily met through using Syrian golden hamsters. On the other hand, this information is important from the viewpoint that Syrian hamsters may become anemic and present retarded growth after infection with *A. ceylanicum* L_3 (BUNGIRO JUNIOR et al., 2003).

Furthermore, chlamydospore production was observed in all the Petri dishes of the treated group, with consequent reductions in the numbers of L_3 recovered. These results are consistent with the observations made by Maciel et al. (2009) and Carvalho et al. (2009), who examined the effect of *D. flagrans* on the L_3 of gastrointestinal nematodes of potentially zoonotic domestic animals.

Thus, we believe that the results obtained may serve as orientation for future research. Moreover, according Braga et al. (2010a, b), the predatory capacity of the species *D. flagrans* (AC001) has already been widely discussed and proven with regard to L_3 control among geohelminths. However, this capacity had never previously been tested subsequent to gastrointestinal transit in hamsters. Some other studies using the fungus *D. flagrans* (AC001) have been performed under laboratory and natural conditions (MOTA et al., 2003; DIAS et al., 2007; BRAGA et al., 2009, 2010a).

In the literature, it is mentioned that the main premise for using nematophagous fungi for biological control is that this must obey the rule of passage and subsequent germination in the feces (LARSEN, 1999). The results presented in this study are in accordance with this premise, demonstrating once again that *D. flagrans* has wide-ranging predatory activity. Moreover, chlamydospore production is also an important requirement for a fungus to be able to cause an effective reduction in the free life stages of gastrointestinal parasitic nematodes in domestic animals (CAMPOS et al., 2008). This is also consistent with the results presented here, since chlamydospores were seen to be present in the Petri dishes relating to animals of the groups treated with *D. flagrans* (AC001). On the other hand, in the present study, the presence of *A. ceylanicum* L_3 in Petri dishes containing 2% WA was essential for the formation of traps by the AC001 isolate. This information is in agreement with Scholler and Rubner (1994), who reported that nematophagous fungi do not have nutritional requirements, but that the presence of nematodes under laboratory assay conditions leads to higher production of traps.

Greater predatory action by the larvae was directly related to the period of highest fungal elimination by the animals. This may also be influenced by the type and quantity of food provided during the experiment, as well as the dose of fungus tested (ARAÚJO; RIBEIRO, 2003). The highest percentage reduction was observed at the time of 6 hours (43.2%). However, we emphasize that none of the studies in the literature mentions the transit time of *D. flagrans* through the digestive system of hamsters. This information could be useful for future comparisons regarding the times and doses of fungi to be administered to these animals. *D. flagrans* (AC001) showed predatory activity on *A. ceylanicum* L_3 after transit through the gastrointestinal tract of hamsters. It was concluded therefore that the fungus *D. flagrans* may be an alternative for biological control of *A. ceylanicum* L_3.

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