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In vitro predatory activity of conidia of fungal isolates of the *Duddingtonia flagrans* on *Angiostrongylus vasorum* first-stage larvae

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ABSTRACT

Introduction: *Angiostrongylus vasorum* is a nematode that parasitizes molluscs, dogs, and even man. **Methods:** The objective was to evaluate the predatory activity of the conidia of two fungal isolates of *Duddingtonia flagrans* (AC001 and CG722) on first-stage larvae (L_1) of *A. vasorum* in laboratory conditions. **Results:** At the end of the experiment, there were significant reductions ($p<0.01$) of 74.5% and 63.2%, on average, in the A. vasorum L_1 recovered in the AC001 and CG722 treatment conditions, respectively. **Conclusions:** The two isolates of fungi were efficient in the capture and destruction of *A. vasorum* L_1.

Keywords: *Nematophagous fungi*. *Duddingtonia flagrans*. *Angiostrongylus vasorum*.

Parasites of the genus *Angiostrongylus* (*A. vasorum*, *A. cantonensis*, and *A. costaricensis*) infect aquatic and terrestrial molluscs, which are the intermediate hosts. The life cycle of *Angiostrongylus*, although not understood completely, demonstrates a complexity of situations in which man may appear as a potential host[1,2]. According to Saeed et al.[1], *A. vasorum* is a protostrongylid nematode with a biological cycle of the heteroxenic type, occurring in several regions where they were previously considered harmless[3]. Molluscs (intermediate hosts) become infected by ingesting first-stage larvae (L_1) of the parasite left in the feces of infected definitive hosts (dogs and wild canids). The larvae go through two changes in the mollusc until the third evolutionary stage (L_3), when it becomes infectious to the definitive host[4]. Dogs infected with *A. vasorum* may have a variety of symptoms, including neurological ones[5], that can have severe consequences. In addition, due to close contact of humans with pets, especially dogs, there is a possibility of human contamination with *A. vasorum*. Because of the medical importance of angiostrongyliasis in humans and animals, studies have been carried out to determine the efficacy of treatment with albendazole[6]. However, there are no officially approved drugs for the treatment of dogs with angiostrongyliasis[7].

The use of nematophagous fungi and ovicidal predators can help in the environmental decontamination of infective forms (or eggs and larvae) of potentially zoonotic parasites and therefore reduce the recurrence of helminth infections[8]. *Duddingtonia flagrans* is considered the most promising nematode-trapping species in the control of nematodiasis in domestic animals due to its large chlamydospore production. In laboratory conditions, different fungal isolates of *D. flagrans* have previously been successfully utilized to control gastrointestinal nematodes in domestic animals, especially the isolates AC001 and CG722[9,10]. However, this is the first report comparing the *in vitro* predatory activity of the conidia of different isolates of the fungus *D. flagrans* on first-stage (L_1) larvae of *A. vasorum*.

The objective of the present study was to evaluate the predatory activity of the conidia of two fungal isolates of *D. flagrans* (AC001 and CG722) on first-stage larvae (L_1) of *A. vasorum* in laboratory conditions.

Two isolates of the nematophagous fungus *D. flagrans* (AC001 and CG722) were used. These isolates were obtained from Brazilian agricultural soil. After growth of the isolates in 2% cornmeal agar, new culture disks 4mm in diameter were transferred to 9cm-diameter Petri dishes containing 20ml of 2% water agar (2% WA). Then, for a period of 21 days, 1ml of distilled water containing 1,000 larvae of *Panagrellus* sp. was added daily to induce fungal conidia formation. When complete fungal development was observed, 5ml of distilled water were added to each Petri dish, and the conidial and mycelial fragments were removed using the technique described by Araújo et al.[11]. The suspension present in the plates was screened through a sieve attached to a plastic container to remove the mycelium fragments.

The strain used in the assays was originally isolated from feces of two naturally infected dogs from the Brazilian City of Caratinga in the State of Minas Gerais[12]. This strain had been maintained by successive passages in dogs. Feces of infected dogs were collected and placed in a modified Baermann funnel for 12h for L_1 recovery[13]. After this period, the tube was removed and centrifuged at 200 × *g* for 2min. The supernatant

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was discarded, and the pellet containing *A. vasorum* L_1 was resuspended in 5ml of 0.85% saline solution. The content was homogenized, and three 10µl samples were removed and distributed in 7.5 × 2.5cm glass slides. A count of the larvae was carried out under a stereomicroscope (25×). The total larval number was estimated by a simple rule of three.

The predation test was conducted on the surface of the Petri dishes according to a modified technique previously described by Braga et al.[8]. Three groups were formed on 9cm-diameter Petri dishes containing 20ml of 2% WA: two treatment groups (AC001 and CG722) and one control group (without fungi). Six repetitions were made for each group. The Petri dishes were previously marked into 4mm-diameter fields. In the treated groups, each Petri dish contained *A. vasorum* L_1 and 500 conidia of the fungal isolate AC001 or CG722 in 2% WA. Each Petri dish in the control group contained only 500 L_1 in 2% WA. Every 24 h for 7 days, ten 4mm-diameter random fields on each plate in the treated and control groups were observed under an optical microscope at 10× magnification; the number of L_1 that had not been preyed on was counted on each plate. At the end of the 7-day period, the non-predated L_1 were recovered from the Petri dishes using the Baermann apparatus with water at 42ºC.

The data obtained were examined by analysis of variance at 1 and 5% probability levels using the BioEstat 3.0 software (Manuel Ayres, Brazil). The efficiency of the predation activity was evaluated using Tukey's test at the 1% probability level. The percentage reduction in the mean larval recovery was calculated by the following equation:

$$\text{Reduction (\%)} = \frac{(\text{mean } L_1 \text{ recovery from control} - \text{mean } L_1 \text{ recovery from treatment}) \times 100.}{\text{Mean } L_1 \text{ recovery from control}}$$

In the present study, even with the administration of conidia, the fungus produced optimum results as early as in the first 24 hours of reading **(Table 1)**. The mean number of *A. vasorum* L_1 not preyed on per 4mm-diameter field in the control condition was significantly different ($p<0.01$) from that on the plates treated with the fungus *D. flagrans* (AC001 and CG722) throughout the experiment. In addition, there were typical fungal structures (conidia and traps) on the boards of the treated groups during the test. At the end of the experiment, the following *A. vasorum* L_1 percentage reductions were observed: 74.5% for AC001 and 63.2% for CG722 **(Figure 1)**.

The linear regression coefficients calculated by the analysis of the mean number of *A. vasorum* L_1 per 4mm-diameter field in the treated and control groups were: 0.21 (AC001), 0.17 (CG722), and 0.67 (control) **(Figure 2)**.

It is estimated that close to 1 billion people are currently infected by geohelminths, mainly due to contact with common soil, indicating that this is an important route of human infection, which itself is associated with varied grave health consequences if left untreated. In addition, studies on parasites infesting domestic animals have provoked increasing interest due to the intimate relationship that exists between man and these animals, which may be a public health concern[14].

TABLE 1 - Daily means and standard deviations of non-predated first-stage larvae (L_1) of* Angiostrongylus vasorum *per 4mm-diameter field in 2% water agar for the group treated for seven days with the fungus* Duddingtonia flagrans *(AC001 and CG722) and for the control group (without fungi).

	Treatments (means of non-predated L_1)		
Time (days)	**AC001**	**CG722**	**Control**
1	$2.6^a \pm 3.03$	$2.5^a \pm 2.1$	$7.0^b \pm 2.0$
2	$1.2^a \pm 1.6$	$1.3^a \pm 1.7$	$4.6^b \pm 4.0$
3	$2.1^a \pm 2.8$	$1.4^a \pm 1.7$	$3.9^b \pm 3.0$
4	$1.3^a \pm 1.8$	$1.1^a \pm 1.6$	$3.0^b \pm 3.1$
5	$1.2^a \pm 1.1$	$1.5^a \pm 2.0$	$2.5^b \pm 3.2$
6	$1.2^a \pm 1.3$	$1.4^a \pm 2.1$	$2.8^b \pm 2.5$
7	$0.9^a \pm 1.2$	$0.8^a \pm 1.1$	$2.4^b \pm 2.2$

AC001: *Duddingtonia flagrans;* **CG722:** *Duddingtonia flagrans.*

Means with a superscript letter indicate that the lines are not statistically different ($p>0.01$), Tukey's test.

The genera *Ancylostoma* and *Toxocara*, which are parasites of dogs and cats, and possibly the species *A. vasorum* stand out among helminths with zoonotic potential. Besides showing medical–veterinary importance, *A. vasorum* as a cardiopulmonary parasite of domestic and wild dogs requires special attention and investigation also because it can infect humans as well[6,14]. Few studies have mentioned the *in vitro* predatory activity of different nematophagous fungi on larvae of nematode parasites of dogs.

In the present study, the isolate CG722 demonstrated a mean value of 0.8 in relation to viable L_1 in the treated Petri dishes at the end of the 7-day period. This result is concordant with Campos et al.[9], who, using the same isolate (CG722) preying on L_3 of *Strongiloydes papillosus*, demonstrated a mean of 10.41 *in vitro* at the end of 7 days. However, the changes observed here may have occurred as a result of various factors. According to Mendoza De Gives et al.[15], the composition of the cuticle of nematodes can be a determining factor in the ability of fungi to prey on the nematodes. Also, antigenic variations in different nematodes or in the fungal isolate used may

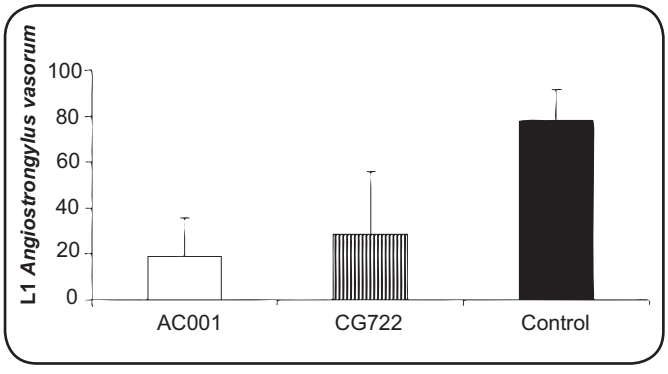

FIGURE 1 - Means and standard deviations (bars) of infective non-predated* Angiostrongylus vasorum *larvae recovered from 2% water-agar plates by the Baermann method on the seventh day of treatment with the following fungal isolates:* Duddingtonia flagrans *(AC001 and CG722) and control group (without fungi).

The asterisk denotes a significant difference ($p<0.05$) between the fungus-treated group and the control; Tukey's test at 1% probability level.

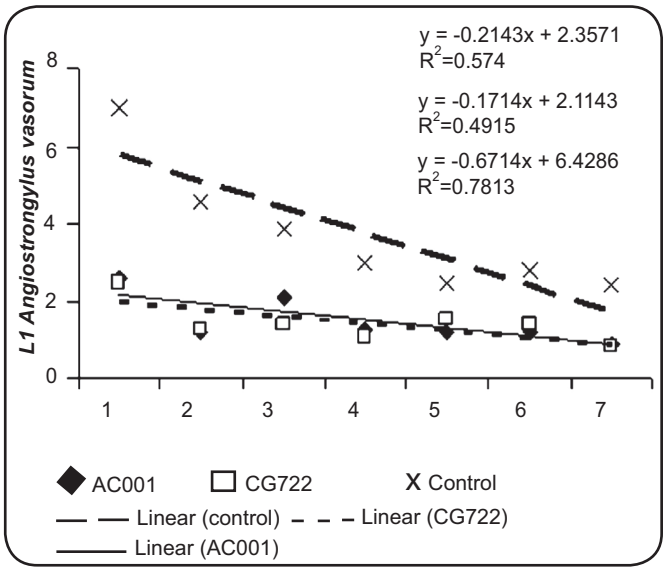

FIGURE 2 - Linear regression curves calculated using the mean* Angiostrongylus vasorum *larvae (L_1) per 4mm-diameter field for the group treated with the fungus* Duddingtonia flagrans *(AC001 and CG722) and the control group (without fungi) as a function of time (1 to 7 days).

influence the rate of predation. Braga et al.[10] demonstrated that AC001 grown in Petri dishes containing a solid culture (2% WA) did prey on and consequently destroyed 80.3% of *A. vasorum* L_1 at the end of 7 days. However, comparing this predatory activity with the observation in the present study, it can be noted that the efficiency (74.5%) in this same strain was similar since the predation of larvae occurred after 24h. In this study, there was no statistically significant difference in the predatory ability of isolates of *D. flagrans*, as evidenced by the number of *A. vasorum* larvae recovered from the plates with the strains CG722 and AC001. However, differences in the inter- and intraspecific activity of predatory nematophagous fungi are common and have been observed in experiments with other fungal isolates[11].

The negative coefficients of correlation indicate a downward behavior of the regression curves for the treatments with the fungal isolates AC001 (0.21) and CG722 (0.17). This was caused by the reduction in the mean numbers of non-predated *A. vasorum* L_1 per 4mm-diameter field during the experimental assay, mainly due to the capture of L_1 in fungal traps. The reduction in the number of L_1 per 4mm-diameter field in the control group during the study, however, was caused by the migration of larvae to the periphery of the Petri dishes, where the moisture level was higher. This finding was also reported by Araújo et al.[15], who carried out *in vitro* tests in Petri dishes. The results of this study confirm previous works on the efficiency of *D. flagrans* in the control of larvae of nematode parasites of dogs. As the larvae are free in the environment, there exists the possibility of human infection since other parasites of the genus *Angiostrongylus* have been proven to be zoonotic[2]. In this context, we suggest the application of nematophagous fungi, especially any of the isolates of the species *D. flagrans* tested (AC001, CG722, and CG768), that are capable of destroying L_3 of potentially zoonotic gastrointestinal nematode parasites.

In conclusion, the fungal conidia of *D. flagrans* (AC001 and CG722) have predatory activity on first-stage larvae of *A. vasorum* and could be used as a possible alternative method of biological control of *A. vasorum* larvae.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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