INGRID NEY KRAMER DE MELLO

AVALIAÇÃO DE DOIS ISOLADOS DO FUNGO NEMATÓFAGO *Duddingtonia flagrans* NO CONTROLE DE LARVAS INFECTANTES DE *Ancylostoma* spp. DE CÃES

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Magister Scientiae*.

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APROVADA: 18 de fevereiro de 2013.

Silamar Ferraz

Leandro Grassi de Freitas
(Coorientador)

Jackson Victor de Araújo
(Orientador)

A Deus,

Ao meu amado esposo Alexson,

Dedico.

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BIOGRAFIA

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SUMÁRIO

RESUMO

MELLO, Ingrid Ney Kramer de, M.Sc., Universidade Federal de Viçosa, fevereiro de 2013. **Avaliação de dois isolados do fungo nematófago *Duddingtonia flagrans* no controle de larvas infectantes de *Ancylostoma* spp. de cães.** Orientador: Jackson Victor de Araújo. Coorientadores: Leandro Grassi de Freitas e Fabio Ribeiro Braga.

Os nematóides do gênero *Ancylostoma* são endoparasitas de cães e também geohelmintos zoonóticos que podem infectar o ser humano. O controle destes nematóides em estágio adulto é baseado na utilização de anti-helmínticos. No entanto, o uso de agentes biocontroladores pode ser uma medida complementar para reduzir a população em estágios pré-parasitários em desenvolvimento no ambiente. Este estudo objetivou avaliar o fungo predador *Duddingtonia flagrans* no controle da forma larval infectante (L_3) de *Ancylostoma* spp., em areia de praia. Foi avaliada a infectividade *in vitro* de dois isolados do fungo nematófago *D. flagrans* (AC001 e CG768) sobre larvas infectantes (L_3) de *Ancylostoma* spp. de cães. Utilizou-se como inóculos fúngicos estruturas vegetativas (micélio), reprodutivas (conídios) e de sobrevivência (clamidósporos). A interação foi avaliada ao final de 10 dias de incubação em placas de Petri contendo meio ágar-água 2% em temperatura de 25 °C. O antagonismo em condições semi-naturais foi avaliado por meio da utilização de uma produção massal de inóculo fúngico em grãos de milho moído. O fungo foi incorporado à areia, em grãos colonizados de milho moído na concentração de 15.000 clamidósporos/grama de areia. Essa concentração se mostrou a mais efetiva em ensaio *in vitro* preliminar (redução de 59,2%). Os resultados mostraram a eficiência do fungo *D. flagrans* no controle de larvas infectantes de *Ancylostoma* spp. em areia de praia. Isso sugere que isolados desse fungo podem ser utilizados como parte de um programa de controle de *Ancylostoma* spp. no ambiente.

ABSTRACT

MELLO, Ingrid Ney Kramer de, M.Sc., Universidade Federal de Viçosa, February of 2013. **Evaluation of two isolates of the nematophagous fungus *Duddingtonia flagrans* in the control of infective larvae of *Ancylostoma* spp. dogs.** Adviser: Jackson Victor de Araújo. Co-advisers: Leandro Grassi de Freitas and Fabio Ribeiro Braga

The nematodes of the genus *Ancylostoma* are endoparasites of dogs and also zoonotic geohelminths that can infect humans. The control these nematodes in adult stage is based on the use of anthelmintics. However, the use of biocontrol agents may be an additional action to reduce the population in pre-parasitic stages developing in the environment. This study aimed to evaluate the *Duddingtonia flagrans* predator fungus for the control of the larval form (L_3) of *Ancylostoma* spp., in beach sand. We evaluated the in vitro infectivity of two isolates of the nematophagous fungus *D. flagrans* (AC001 and CG768) on infective larvae (L_3) of *Ancylostoma* spp. dogs. Vegetative structures (mycelium), reproductive (conidia) and survival (chlamydospores) were used as fungal inoculum. The interaction was evaluated at the end of 10 days of incubation in Petri dishes containing agar-water 2% medium in a temperature of 25 ° C. The antagonism in semi-natural conditions was assessed by use of a mass production of fungal inoculum in grains of milled maize. The fungus was introduced into the sand in colonized milled maize at the concentration of 15,000 chlamydospores / gram of sand. This concentration was the most effective in the preliminary *in vitro* assay (reduction of 59.2%). The results showed the efficiency of the fungus *D. flagrans* in the control of infective larvae of *Ancylostoma spp*. in beach sand. This suggests that isolates of this fungus may be used as part of a control program of *Ancylostoma* spp. in the environment.

INTRODUÇÃO GERAL

Os cães são hospedeiros de inúmeros parasitas e estão envolvidos na transmissão involuntária de mais de 60 infecções zoonóticas (Macpherson et al., 2005). Dentre elas, as parasitoses gastrintestinais causadas por geohelmintos, estão entre as mais prevalentes e importantes infecções parasitárias destes animais (Oliveira-Sequeira, 2002).

Dentre os nematóides parasitas intestinais que utilizam o cão como hospedeiro definitivo e são zoonóticos, os do gênero *Ancylostoma* têm requerido importante atenção médica e veterinária (Katagiri & Oliveira-Sequeira, 2008; Mulvenna et al. 2009). Este gênero está classificado zoologicamente no filo Nematoda, classe Secementea, ordem Strongylida, superfamilia Ancylostomatoidea, família Ancylostomatidae e subfamilia Ancylostomatinae (Maggenti, 1981). Os membros desta subfamília utilizam apenas carnívoros como hospedeiros definitivos (Lichtenfels, 1980). As espécies *A. braziliense*, *A. caninum* e *A. ceylanicum* são as mais prevalentes nestes animais (Baker et al. 1989; Traub et al. 2005). Segundo Urquhart et al. (1998) os ancilostomídeos são responsáveis por ampla morbidade e mortalidade em cães sendo que a espécie *A. caninum* é a mais patogênica devido à maior espoliação sanguínea.

Além de sua importância em saúde animal este parasito tem se destacado como agente causador da Síndrome da larva migrans cutânea. Esta síndrome ocorre por meio do contato direto da pele humana com a L_3 ativa de *Ancylostoma* spp. presente em solos contaminados por fezes de cães (Robertson e Thompson, 2002). Após a penetração na epiderme, a larva migra no tecido subcutâneo ocasionando reações inflamatórias caracterizadas por intenso prurido e erupções serpiginosas (Mattone-Volpe, 1998). Além disso, adultos imaturos de *A. caninum* podem ser ocasionalmente encontrados no intestino causando enterite eosinofílica (EE), caracterizada por dor abdominal aguda associada a eosinofilia periférica, anorexia, náusea e diarreia (Prociv & Croese, 1996; Croese et al, 1994;. Landmann & Prociv, 2003). O *A. caninum* também foi reportado como agente da neuroretinite subaguda unilateral difusa que é uma doença causada pela presença de uma larva no espaço subretiniano (Casella et al. 2001; Venkatesh et al., 2005; Vedantham et al., 2006).

Epidemiologicamente, os cães errantes são os principais responsáveis pela infestação do solo com ovos de helmintos (Ragozo et al., 2002; Scaini et al., 2003;

Santarém et al., 2004) uma vez que maior prevalência de parasitismo é observada nestes animais em comparação aos domiciliados, em virtude de não receberem tratamento antiparasitário e da facilidade com que circulam por áreas públicas (Palmer et al., 2008). Portanto, o solo de áreas públicas representa um foco potencial de transmissão para a população humana e animal pelo fato de estar sendo freqüentemente infestado por ovos veiculados nas fezes de cães que comumente estão infectados (Robertson et al., 2000; Blazius et al., 2005; Macpherson, 2005). Surtos de larva migrans cutânea (LMC) são relatados e relacionados à atividade em caixas de areia contaminadas com fezes de cães. (Lima et al., 1984; Nunes et al., 2000; Araújo et al., 2000; Santarém et al., 2004). Ainda, diversos autores têm relatado elevada prevalência de ovos e ou larvas de *Ancylostoma* spp. em areias de praças públicas e em canteiros de praias em diferentes regiões do Brasil (Araújo et al., 1999; Scaini et al., 2003; González et al., 2004; Castro et al., 2005; Santos et al., 2006; Silva et al., 2009).

O aparecimento de relatos de resistência às drogas anti-helmínticas levou pesquisadores de todo o mundo a buscar medidas alternativas para o controle de endoparasitoses de animais domésticos, visando à diminuição do emprego de quimioterápicos (Mota et al., 2003). Dentre as propostas que tem sido trabalhadas com o intuito de melhorar esse controle, sugere-se o controle biológico, como uma alternativa viável e promissora que reduz as infecções causadas por helmintos parasitos gastrintestinais, e cuja ação se dá por meio de organismos vivos como os fungos nematófagos que atuam como antagonistas naturais no ambiente (Araújo et al., 2004). Pesquisas a campo ou sob condições experimentais mostram que espécies de fungos nematófagos são bons agentes de controle biológico de nematóides de animais (Larsen, 1999).

Os fungos nematófagos estão catalogados com mais de 150 espécies (Barron, 1977). Eles são divididos em três grupos e podem ser isolados do solo, fezes, esterco e outros ambientes, ou materiais pelo método de espalhamento do solo de Duddington (1955). A maioria das espécies está classificada como fungos predadores de nematóides. Estes fungos produzem um extenso sistema de hifas vegetativas ao longo das quais ocorre diferenciação morfológica em estruturas funcionais denominadas armadilhas que capturam e retém nematóides vivos (Barron, 1977) O aprisionamento à armadilha é seguido pela penetração das hifas na cutícula do nematóide. Dentro do nematóide, ocorre o crescimento das hifas e a digestão dos conteúdos internos (Nordbring-Hertz,

2006). Segundo Gray (1987) este grupo diferencia suas hifas vegetativas em seis estruturas de captura (armadilhas): hifas adesivas não diferenciadas; ramificações de hifas que sofrem anastomose, formando redes adesivas tridimensionais; ramificações adesivas, onde em algumas vezes podem se unir formando redes adesivas simples bidimensionais; nódulos adesivos; anéis constritores e anéis não constritores. Entretanto o tipo de armadilha mais encontrado em fungos predadores são as redes adesivas (Mota et al., 2003).

O segundo grupo, denominados fungos endoparasitos, é capaz de infectar os nematóides através de esporos, que uma vez ingeridos desenvolvem hifas responsáveis pela absorção do conteúdo interno do nematóide. Estes fungos não produzem hifas vegetativas fora do corpo do hospedeiro, mas somente hifas férteis ou conidióforos contendo esporos. O terceiro grupo de fungos é denominado oportunistas, parasitos de ovos (Araújo et al., 1995). As hifas penetram a casca do ovo, através dos pequenos poros existentes na camada vitelínica, causando alteração na permeabilidade da casca e expandindo seu volume. A hifa aumenta de tamanho ao passar pela camada vitelínica e atravessa a camada adjacente quitínica e lipídica. Como conseqüência do processo, a camada vitelínica se divide, a camada de quitina se torna vacuolizada e a camada de lipídios se torna dispersa. Estes tipos de fungos colonizam o conteúdo do ovo, ou ainda a larva em desenvolvimento no seu interior (Morgan-Jones & Rodríguez-Kábana, 1988).

Os fungos nematófagos podem apresentar esporos bastante diversificados no tamanho, coloração, forma e resistência no ambiente. A maioria dos fungos nematófagos apresenta esporos secos, emergindo de estruturas de frutificação, denominadas conidióforos, essenciais na dispersão aérea dos conídios. Os conidióforos crescem verticalmente, em direção perpendicular ao substrato o qual o isolado foi cultivado. Algumas espécies produzem conidióforos contendo apenas um conídio em sua extremidade, outras espécies apresentam cachos de conídios em toda a estrutura do conidióforo. Estruturas denominadas clamidósporos também podem ser produzidas. Estes são esporos de parede espessa, diferenciada a partir das hifas, aparecem em condições de estresse extremo e podem dar origem a hifas, conidióforos e conídios (Barron, 1977).

Grandes partes dos fungos nematófagos estão incluídos dentro do grupo dos predadores. Este grupo de fungos é o mais utilizado no controle biológico de

nematóides que parasitam animais domésticos, reduzindo de forma efetiva a sua população tanto em condições laboratoriais quanto em condições a campo, além disso, possuem a vantagem de apresentar maior potencial de industrialização (Larsen, 1999).

No grupo de fungos predadores, a espécie *Duddingtonia flagrans* tem sido a mais estudada, havendo características que propiciam o seu uso como agente de controle biológico de parasitos de animais, destacando-se a produção de grande número de clamidósporos que resistem as condições adversas (Sanyal et al. 2008). O potencial desta espécie como biocontrolador está consolidado e tem sido relatado por vários autores em experimentos *in vitro* (Araújo et al., 2004; Araújo et al., 2006;) e em condições *in vivo* (Mendoza-de-Gives et al., 1998; Dias et al., 2007).

Devido à capacidade dos clamidósporos de *D. flagrans* suportarem condições adversas, eles têm sido administrados na alimentação de bovinos, ovinos. caprinos, eqüinos e suínos por resistirem à passagem através do trato gastrintestinal destes animais e posteriormente germinarem nas fezes, formando armadilhas (Campos et al., 2009; Silva et al., 2010; Braga et al., 2010; Ferreira et al., 2011).

Em cães, a administração oral de fungos biocontroladores não é uma opção prática devido ao elevado número de cães errantes os quais tem grande importância na disseminação de helmintos no ambiente devido à maior freqüência de parasitismo em conseqüência do abandono (Labruna et al., 2006). Isto justifica a utilização de uma abordagem em que o fungo seja incorporado à areia, o que deve ser feito juntamente com um substrato de crescimento fúngico para favorecer seu crescimento e estabelecimento.

O controle biológico de parasitas de cães no ambiente por fungos nematófagos é promissor. Entretanto ele deve ser visto como parte de um programa integrado em complementação ao controle químico de endoparasitas em cães domiciliados e ao controle da população de cães errantes, uma vez que reduz satisfatoriamente as formas pré-parasitarias no ambiente (Waler & Larsen, 1993).

Capítulo 1

Different structures of the nematophagous fungus *Duddingtonia flagrans* in the control of infective larvae of *Ancylostoma* spp.

Article submitted to Experimental Parasitology (impact factor 2.122)

Abstract

The infectivity of two isolates of the nematophagous fungus *Duddingtonia flagrans* (AC001 and CG768) on infective larvae (L_3) of *Ancylostoma* spp. was evaluated *in vitro* using mycelium, conidia and chlamydospores as fungal inocula. Evaluation of the isolates was performed at the end of 10 days of interaction between the fungus and nematodes in Petri dishes containing water-agar 2% at 25°C. The control without fungus contained only 1 x 10^3 L_3/Petri dish, while the other treatments contained the same concentration of L3 in mycelial discs of 5 mm in diameter, and 1 x 10^3 conidia or 1 x 10^3 chlamydospores/Petri dish. Both isolates had a significant effect ($p < 0.05$) on reducing the average of L_3 recovered. The treatment with chlamydospores of the isolate CG768 showed the highest predatory activity by reducing the number of L_3 of *Ancylostoma* spp. recovered by 80.7% when compared to the control treatment, without fungus. Moreover, the predatory activity of the isolate AC001 exceeded that of CG768 when conidia were used as an inoculum source, reducing the recovery by 73.58% and 52.93%, respectively. The *D. flagrans* fungus may be used as an alternative for biological control of L_3 form *Ancylostoma* spp.

Keywords: Nematophagous fungi, *Duddingtonia flagrans*, *Ancylostoma* spp., larva migrans, biological control, dogs.

Introduction

The syndrome of cutaneous larva migrans is an important public health problem in most countries around the world. This syndrome is caused by infective larvae (L_3) in the third stage of some geohelminths, mainly *Ancylostoma caninum* and *Ancylostoma braziliensis* species, where the dog is a definitive host and human beings are eventually infected (Acha and Szyfres, 2003). The risk of infection is higher in children because of their frequent contact with soil (Capuano and Rocha, 2006).

This syndrome occurs through direct contact of human skin with L_3 of *Ancylostoma* spp. present in soil contaminated with dog feces (Robertson and Thompson, 2002). After penetration in the epidermis, the larva migrates into the subcutaneous tissue causing inflammatory reactions characterized by intense itching and serpiginous rashes (Mattone-Volpe, 1998).

The emergence of reports on resistance to anti-helminthic drugs has led to the search of alternative sustainable strategies for control of animal-parasitic nematodes, highlighting the use of nematophagous fungi (Waller and Larsen, 1993). Field studies have shown that nematophagous fungi species are good biological control agents of animal-parasite nematodes (Larsen, 1999). These fungi control the parasites by destroying their pre-parasitic stages free living in the environment, which limits exposure of hosts to the agent of the disease and prevents new infections. In this context, predatory nematophagous fungi such as the genera *Duddingtonia*, *Arthrobotrys* and *Monacrosporium* have been considered promising (Mota et al., 2003).

Potential use of the species *Duddingtonia flagrans* as a biocontrol agent of animal-parasitic helminthes is established and has been reported by several authors in *in vitro* experiments (Araújo et al., 2004; Araújo et al., 2006; Braga et al., 2010) and *in vivo* conditions and in the field (Mendoza-de-Gives et al., 1998; Dias et al., 2007). In these *in vitro* studies, conidia have been commonly used as the inoculum source. Although other inoculum such as chlamydospores have been previously used in *in vivo* studies for the control of *Haemonchus contortus* in sheep (Campos et al., 2009) and mycelia in the control of *Ancylostoma* spp. in dogs (Carvalho et al., 2009), it is not known if the type of inoculum influences predatory ability.

This study aimed to compare the *in vitro* infectivity of two isolates of the predatory nematophagous fungus *Duddingtonia flagrans* (AC001 and CG768) on L_3 of *Ancylostoma* spp. and to evaluate which inoculum type (chlamydospores, mycelia or conidia) provides a higher nematophagous activity of this fungus.

Material and Methods

Fungus

Two isolates of the nematophagous fungus *D. flagrans* (CG768 and AC001) obtained from samples of Brazilian soils and animal feces were used in this experiment. They were isolated using the soil-sprinkling method of Duddington (1955), modified by Santos et al. (1991). The isolate AC001 was stored in the dark at 4ºC in test tubes containing 2% corn-meal-agar (2% CMA). The isolate CG768 was stored in same conditions described above, but on integral rice grains in 5 mL BD Vacutainer® glass tubes (Becton Dickinson, Brazil) containing blue silica gel.

Mycelia production

Culture disks of the isolate AC001 (5 mm in diameter) and one rice grain colonized by isolate CG768 were transferred to 50 mm × 10 mm Petri dishes containing 2% water-agar (2% WA) culture medium. These plates were incubated for 7 days at 26 °C in a dark room. After mycelial growth, a 5 mm diameter culture disk of one isolate was transferred to a 90 mm x 15 mm Petri dish containing a 2% CMA. Next a 5 mm diameter culture disk of this new culture was transferred to 50 mm × 10 mm polypropylene Petri dishes containing 2% WA and incubated for 10 days as previously described.

Conidia and chlamydospore production

Culture disks of the isolate AC001 (5 mm in diameter) and one rice grain colonized by the isolate CG768 were transferred to 50 mm × 10 mm Petri dishes containing 2% WA culture medium. These plates were incubated for 7 days at 26 ºC in a dark room. After mycelial growth, a 5 mm diameter culture disk was transferred to a 90 mm x 15 mm Petri dish containing a 2% potato dextrose agar. These plates were incubated for 10 and 20 days at 26 ºC in a dark room, to obtain conidia and chlamydospores, respectively.

At the end of this period, the surface of the fungal colony was gently scraped with a fine sterile brush and washed with 10 mL sterile ultrapure water. The fungal suspension was filtered through gauze (two layers) to eliminate mycelial fragments, according to Maciel *et al.* (2009). Next two aliquots of the conidia suspension were counted in a Neubauer chamber and the suspension was adjusted to the desired concentration in the experiment. The same procedure described above was used to obtain the chlamydospore concentration from fungal colonies with 20 days of incubation.

Ancylostoma spp. L3

The *Ancylostoma* spp. L3 was obtained from fresh feces of naturally infected stray dogs, by vermiculite coproculture for 10 days at 26 ºC in a BOD. After this period, active larvae were harvested from the culture using a Baermann funnel, which is based

on positive thermo/hydrotropism of the larvae and gravity sedimentation. After 12 hours the sediment containing L_3 was transferred to centrifuge tubes and washed in sterile ultrapure water 5 times followed by centrifugation at 1000 rpm for 5 min. The L_3 was filtered, decontaminated for 10 minutes with 10 mL of 0.5% (v/v) sodium hypochlorite solution, as described by Barçante et al. (2003), and then rewashed five times in sterile ultrapure water. The L_3 suspension was homogenized and 3 aliquots of 10 µL were placed on a microscope glass slide marked with longitudinal lines to facilitate counting. Each aliquot was covered with a glass coverslip after adding 10 µL of lugol's solution to kill L_3. They were then counted using a light microscope (40x magnification) and the concentration of the suspension was adjusted to 1,000 L_3/10 µL. The viability of L_3 was verified by microscopic examination before inoculating the dishes.

Experimental assay

The interaction experiment between *D. flagrans* isolates and *Ancylostoma* spp. L_3 consisted of seven treatments as follows:

(1) Colony of the isolate CG768 with 10 days of grown and 1 x 10^3 L_3;

(2) Colony of the isolate AC001 with 10 days of grown and 1 x 10^3 L_3;

(3) 1 x 10^3 conidia of the isolate CG768 and 1 x 10^3 L_3;

(4) 1 x 10^3 conidia of the isolate AC001 and 1 x 10^3 L_3;

(5) 1 x 10^3 chlamydospores of the isolate CG768 and 1 x 10^3 L_3;

(6) 1 x 10^3 chlamydospores of the isolate AC001 and 1 x 10^3 L_3;

(7) Control without fungus and 1 x 10^3 L_3.

The assay was performed in 50 mm x 10 mm polypropylene Petri dishes containing 2% WA. Plates were sealed with polyvinyl chloride (PVC) transparent film and incubated for 10 days at 26 °C in a dark room. At the end of the interaction period, the non-predated L_3 were harvested from the 2% WA medium by the Baermann funnel technique, as described before. After 12 hours of larval concentration under gravity in 5 mL vacutainer-like glass tubes connected to a funnel, 3 mL of water without larvae were discarded and a drop of Lugol`s solution was added to the remaining volume to kill *Ancylostoma* spp. L_3. Then, the larvae were counted in a Peter's counting slide (Peters 1952) using a stereomicroscope (40x magnification).

Statistical method

The experiment was arranged in a completely randomized design with 10 replications per treatment, where each experimental plot consisted of a Petri dish. After the data were analyzed by ANOVA, the Tukey's test was used for comparison among the averages of the treatments and the Dunnett's test for comparison among these and the fungus-free control, both at 5% significance level.

Results

The two isolates (CG768 and AC001) of *D. flagrans* fungus produced significantly reduced ($p<0.05$) in the reduction of the average number of L3 of *Ancylostoma* spp., but they presented different efficiency in the predation of *in vitro* tests (**Table 1)**. The reduction in the average number of L3 ranged from 30.5% to 80.7%, according to the type of inoculum (conidia, chlamydospores or mycelium) and isolate fungal used.

Table 1

Average values, standard deviation (±) and reduction percentage (%) of *Ancylostoma* spp. dog infective larvae recovered from 2% water-agar culture medium by the Baerman method after 10 days of interaction in Petri dishes containing fungal isolates *Duddingtonia flagrans* (isolates CG768 and AC001) at three fungal inocula structures (conidia, chlamydospores and mycelium) in comparasion to fungus-free control treatment.

Fungal isolates	Treatments		
	Conidia	Chlamydospores	Mycelium
CG768	a195,2B ± 73,79 (52,93)	b79,8C ± 11,37 (80,76)	a183,9C ± 28,89 (55,7)
AC001	c109,6C ± 41,37 (73,58)	b201,5B ± 62,43 (51,5)	a288,4B ± 38,63 (30,5)
Control		414,7A ± 66,74	

Means followed by the same capital letter in the column and small letter in the row were not significantly different at 5% probability level by the Tukey's test. Inside the parentheses: Reduction percentage = 100 – (treatment average × 100/ control group average).

Colonies of the CG768 isolate, developed from the chlamydospore inoculum, showed the most predatory activity and resulted in a reduction of L3 of *Ancylostoma* spp. of 80.7%. On the other hand, the predation activity of AC001 isolated surpassed that of the CG768 isolated, when conidia was used as the inoculum.

The lowest percentages of reduction in the average number of L3 of *Ancylostoma* spp. were observed when the fungi had mycelium as the inoculum. This lower efficiency was observed for both isolated of *D. flagrans.*

Discussion

Results showed the potential use of two isolates of *D. flagrans* for the control of L_3 of *Ancylostoma* spp. These results are consistent with those encountered by Maciel et al. (2006) and Carvalho et al. (2009), in which the species *D. flagrans* showed high predatory activity against *Ancylostoma* spp. when comparing this fungus species with two others (*Arthrobotrys robusta* and *Monacrosporium thaumasium*).

The predatory activity of isolates of *D. flagrans* on *Ancylostoma* spp. larvae *in vitro* is related to the formation of traps by the fungus. Previous studies report the presence of traps after 24 hours of exposure of larvae to fungal isolates (Maciel et al., 2006; Carvalho et al., 2009). This strategy of predatory nematophagous fungi was also observed by Araújo et al. (2006), who used these microorganisms to control *Cooperia* and *Oesophagostomum* in cattle. Traps are formed in response to the presence of nematodes and may occur as a result of limited nutritional conditions and/or lack of water (Scholler and Rubbner, 1994; Gronvold et al., 1996). The mobility of nematode acts as a stimulus to the formation of these structures (Nansen et al., 1988). Thus, in this study, the presence of L_3 of *Ancylostoma* spp. in dishes containing a poor nutritional environment (2% WA) was essential for the formation of traps, as analyzed by Maciel et al. (2006).

In this study, although the isolate CG768 showed to be effective in reducing the average of L3 larvae of *Ancylostoma* spp., an average of 50% were recovered. This percentage was lower than that reported in similar studies conducted by Maciel et al. (2006), in which this isolate demonstrated high efficiency in the capture of *Ancylostoma* spp. (reduction percentage of 89.89%). In relation to the isolate AC001, the results presented for treatment with conidia were similar to those observed in the study

conducted by Carvalho et al. (2009), in which the same isolate demonstrated high efficiency in the predation of L_3 of *Ancylostoma* spp. (reduction percentage of 87.02%) when treated with conidia.

Campos et al. (2009) evaluated the dynamics conidia transition, chlamydospores and mycelium of the fungus *D. flagrans* (isolated CG768) in the digestive duct in goats and the effect of different structures on larval development of *Haemonchus contortus* in coprocultures, and found a higher reduction percentage of larval development in the group treated with chlamydospores (61.23%).

The use of chlamydospores, in this experiment *in vitro*, indicated a better performance of the isolate CG768 in the reduction of L_3, while the activity of the isolate AC001 was more favored when using conidia. Works have shown that intra-specific variation in the predatory activity of fungi are common (Araújo et al., 1993, 1994; Mendoza-de-Gives et al., 1994; Larsen, 2000), regardless of the species of fungus or nematode used in this experiment. These variations may be related to different factors, such as experimental design, loss of viability during storage in the laboratory (Gronvold et al., 1996) and antigenic variation present in different isolates of the same fungal species (Mendoza-de-Gives et al., 1999). Furthermore, while some isolates need only a few conidia to achieve the desired effect, others require large amounts of inoculum to achieve the same effect (Maciel et al., 2009).

In *in vitro* studies, fungi are not exposed to adverse factors of the environment (competition with other organisms and pH and temperature changes), which may help to explain the higher reduction percentage of L_3 when using conidia inoculum of isolate AC 001, compared to the use of chlamydospores. It is possible that the greater strength of the chlamydospore structure exceeds that of conidia and mycelia in field conditions. Moreover, studies subjected to *in vitro* conditions are essential to select fungal isolates with greatest potential for application in biological control programs. In this sense, the results of the present study show that the isolate CG768 had its better activity when using chlamydospores as a source of inoculum. However, the isolated AC001 showed to be more efficient for predation of L_3 of *Ancylostoma* spp. when conidia served as the source of inoculum. *D. flagrans* fungi can be used as an alternative for biological control of L_3 of *Ancylostoma* from dogs.

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Capítulo 2

Biological Control of infective larvae of *Ancylostoma* spp. in beach sand
Article submitted to Revista Iberoamericana de Micología (impact factor 1.156)

Abstract

Background: Geohelminths are parasites that stand out for their prevalence and wide distribution, depending on the soil for their transmission. *Aims:* The aim of this study was to evaluate the predatory capacity of the fungal isolate of the genus *Duddingtonia* (CG768) on third stage larvae (L_3) of *Ancylostoma* spp. in beach sand under laboratory conditions. *Methods:* In the first assay, five treatments were formed with the fungus and one treatment was the control without the fungus. The treatments contained 5000, 10000, 15000, 20000 or 25000 chlamydospores of the fungal isolate and 1000 *Ancylostoma* spp. L_3 in pots containing 30 g of sand, and the control treatment contained only 1000 *Ancylostoma* spp. L_3 and distilled water in pots with 30 g of sand. *Results:* Evidence of predatory activity was observed at the end of 15 days and the percentages of reduction for the five treatments were, respectively, 4.5 %, 24.5%, 59.2%, 58.8% and 63% the number of L_3. Statistical difference was observed ($p < 0.01$) only at concentrations 15000, 20000 and 25000 in relation to control treatment. The second assay consisted of two treatments, in Petri dishes of 9 cm in diameter containing agar water 2% medium. In the first treatment, each Petri dish contained 500 *Ancylostoma* spp. L_3 and 5 g of sand containing the isolate CG 768 at a concentration of 25,000 chlamydospores /g of sand, and the second treatment the control without fungus, contained only 500 L_3. After 7 days non-predated L_3 were recovered from the Petri dishes by the method of Baermann. The use of the fungus reduced ($p < 0.01$) the average number of *Ancylostoma* spp., L_3 in 84%. *Conclusions*: The results of this study confirm earlier work on the efficiency of the *Duddingtonia* genus in control of *Ancylostoma* spp. infective larvae.

Keywords: Nematophagous fungi, *Duddingtonia flagrans, Ancylostoma* spp., sand.

Introduction

Geohelminths are parasites that stand out for their prevalence and wide distribution, and depend on the soil for their transmission. According to Silva et al.[1] some factors are important to evaluate the context of the transmission of these parasites and, among these stand out: (1) the presence of infected animals, in this case, stray dogs, (2) the favoritism of the environmental conditions, (3) the presence of eggs and/or larvae in the environment and (4) faecal contamination of the soil.

In this context, the hookworm have a cosmopolitan distribution and are more prevalent in tropical and subtropical regions where the soil presents temperature and moisture conditions suitable for the development of pre-parasitic forms (egg and/or larvae) [2,3]. On the other hand, the literature has reported that larvae and eggs of these gastrointestinal parasites are commonly found in samples of sand. Moreover, this environment serves not only as a source of leisure but also presents risks to animal and human health [4,5]. Also in relation to this fact, Guimarães et al.[6] reported that in several cities of the country a considerable canine population circulates through the streets and public squares, where often their habits of defecation contaminate the soil with various types and potentially zoonotic parasitic forms. These authors observed the occurrence of *Toxocara* spp., and eggs or larvae of *Ancylostoma* spp. in 69.6% of soil samples collected in public squares. In this context these same authors showed that infected samples of sand from schools or kindergartens were positive only with larvae of *Ancylostoma* spp. In recent work Silva et al.[7] demonstrated contamination of the sand on the beaches of southeastern of Pernambuco state by larvae of *Ancylostoma* spp.

Regarding the control of these parasites, the same is based on the use of antihelminthic drugs, however, there are problems with parasitic resistance already installed in production animals, and that in the future may represent a problem for the combat of parasitic nematodes of dogs and cats. High resistance of *Ancylostoma caninum* to anthelmintic-therapy with pyrantel was demonstrated by Kopp et al.[8]. Thus, much has been researched about the use of biological control in environmental decontamination of eggs and larvae of the genera *Ancylostoma* and *Toxocara* spp.[9] However, it should be emphasized that biological control by nematophagous fungi is of the type "classic", ie, an environmental flood of organisms antagonists present in the environment and whose action is concentrated in the faecal environment by reducing the amount of pre-parasitic forms of helminths[10,11]. Thus, stands out the fungus *Duddingtonia flagrans* extensively studied as biocontroller organism under laboratory and natural conditions[12,13]. However, there are no reports regarding the biological control using nematophagous fungi on beach sand, representing a new approach.

The aim of this study was to evaluate the effect of different concentrations of chlamydospores of the fungus *D. flagrans* (isolate CG 768) on the destruction of *Ancylostoma* spp. L_3 on beach sand under laboratory conditions.

Methods

Organism

The predator nematophagous fungus *Duddingtonia flagrans* (CG768) obtained from samples of Brazilian soil and of animal feces was used in this experiment. The isolate was stored on integral rice grains inside 5 mL BD Vacutainer® glass tubes (Becton Dickinson, Brazil) containing blue silica gel, based on the conservation technique described by Smith and Onions[14]. The species was chosen for this study due to the success in the destruction of *Ancylostoma* spp. third stage larvae observed in previous studies and its great capacity for production of chlamydospores.

Production of chlamydospores

The chlamydospore production was performed in 2.5 L plastic bags with biological filter for aeration. Milled maize was used as substrate. Bags containing 300 g of substrate and 110 ml of distilled water were closed and sterilized for 30 minutes at 121 ◦ C. The sterilized substrate in each bag was inoculated with six mycelium discs of about 5 mm diameter taken from the edges of culture *D. flagrans* after growth on plates containing agar-water medium 2% during 7 days at 25 °C. The bags were then closed to allow growth of *D. flagrans* on the substrate for 15 days in the dark at 25 ◦ C. The substrate was agitated every five days until the end of the incubation period to ensure homogeneous mycelial growth. After the incubation period, six samples of 1 g of colonized substrate were transferred of each bag to an Erlenmeyer flask containing 10 mL of distilled water and 0.2% (v / v) dispersing polysorbate (Tween ® 80) and shaken by two minutes for dispersion of chlamydospores. Then two aliquots of 10µL were placed in a Neubauer chamber to estimate the number of chlamydospores per gram of substrate.

Ancylostoma spp. L_3

Ancylostoma spp. L_3 was obtained from fresh feces of naturally infected stray dogs, by vermiculite coproculture kept for 10 days at 26 ºC in a BOD. After this period, active larvae were harvested from culture using the Baermann funnel technique The L_3 suspension was homogenized and 3 aliquots of 10 µL were placed on a microscope glass slide. Each aliquot was covered with a glass coverslip after adding 10 µL of

lugol's solution to kill L_3. Then, they were counted using a light microscope (40x magnification) and the concentration of the suspension was adjusted for 1,000 L_3/10 µL.

Collection and preparation of sand samples

Throughout the test sand samples were collected in the resort city of Guarapari, Espírito Santo state and submitted to the particle size and chemical by the methodology of Ruiz[15] (coarse sand: 76%; fine sand: 22%; silt 0%; clay: 2%; sodium: 342 mg/dm^3; phosphorus: 34,15 mg/dm^3; potassium: 20 mg/dm^3; calcium: 0,46 cmol/ dm^3; magnesium: 0,32 cmol/ dm^3; aluminum: 0 cmol/ dm^3; ph: 8,2). The beaches of this resort are much visited by tourists throughout the year, and in this context we chose to perform this work. Samples were collected at a depth of 0 to 20 cm and stored in plastic bags. The samples were autoclaved for 1 hour in order to eliminate possible pre-parasitic forms.

Experimental assay

Assay A

Five treatment groups and one control group were formed in accordance with the following description: Group 1 (5000 chlamydospores of the fungus *D. flagrans* per gram of sand and 1000 *Ancylostoma* spp., L_3); Group 2 (10000 chlamydospores of the fungus *D. flagrans* per gram of sand and 1000 *Ancylostoma* spp., L_3); Group 3 (15000 chlamydospores of the fungus *D. flagrans* per gram of sand and 1000 *Ancylostoma* spp. L_3); Group 4 (20000 chlamydospores of the fungus *D. flagrans* per gram of sand and 1000 *Ancylostoma* spp. L_3); Group 5 (25000 chlamydospores of the fungus *D. flagrans* per gram of sand and 1000 *Ancylostoma* spp. L_3) e group 6 (control) containing only distilled water and 1000 L_3.

The present assay consisted of 30 g of sand inside of transparent polypropylene pots (PP size). The sand in each jar was artificially infected with 1000 *Ancylostoma* spp. L_3. Then the pots were closed with their own plastic lids and incubated for 15 days in the dark at 25 °C. Every three days the pots were shaken to promote interaction between microorganisms. At the end of the incubation period the larvae were recovered from the sand by the method of Baermann and counting as previously described.

Assay B

Two groups were formed into Petri dishes of 9cm in diameter containing 2% agar-water medium, with 6 replicates for each group. In the treated group, each Petri dish contained 500 *Ancylostoma* spp. L_3 and 5 g of sand containing the isolated CG 768 at a concentration of 25 000 chlamydospores/g of sand, and the control group (without fungus) contained only 500 L_3 in the plates with WA 2%. The plates were maintained for 7 days in BOD in the dark at 25 °C. At the end of this period, were recovered the non-predated L_3 from the content of Petri dishes by the method of Baermann.

Statistical analysis

The data obtained in the assays A and B were statistically interpreted by analysis of variance in levels of significance of 1% probability[16]. The predation efficiency of L_3 compared to control was evaluated by Tukey's test at 1% probability. Subsequently the percent reduction from the average of L_3 was calculated according to the following formula:

$$\% \text{ Reduction} = \frac{\text{Average of } L_3 \text{ recovered from control} - \text{Average of } L_3 \text{ recovered from treatment}}{\text{Average of } L_3 \text{ recovered from control}} \times 100$$

Results

The tested fungal isolate *D. flagrans* (CG768) was able to prey on the *Ancylostoma* spp. L_3 in both *in vitro* experimental assays. In the assay A, the proof of predatory activity was observed at the end of the experiment (fifteen days), where we observed the following percentages of reduction of L_3 for each concentration of chlamydospores used were observed 4,5 %, 24,5%, 59,2%, 58,8% and 63% for the concentrations of 5,000, 10,000, 15,000, 20,000 e 25,000 respectively, however, a statistical difference ($p < 0.01$) was noted only at concentrations 15,000, 20,000 and 25,000 in relation to control group (Figure 1). Since there was no statistical differences among the means from the treatments with 15,000, 20,000 and 25,000, the indicated dosis for the nematode control in the beach sand used in the experiment is 15,000 chlamydospores/ g of sand.

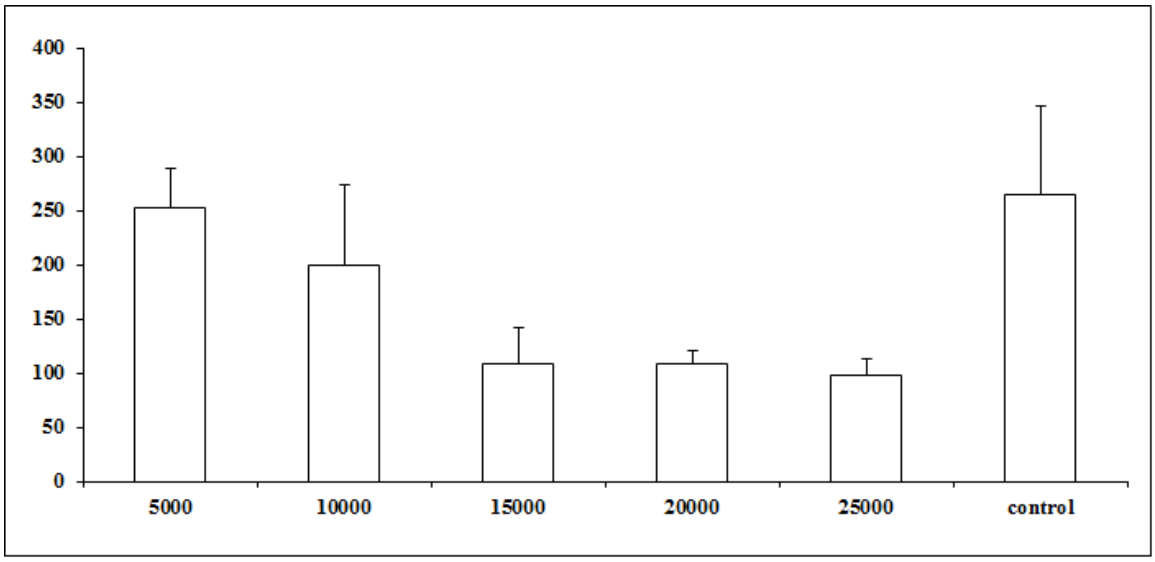

Figure 1. Average number of non-preyed infective larvae of *Ancylostoma* spp. recovered of sand by Baermann method on the fifteenth day of treatment after interaction with the fungus *Duddingtonia flagrans* (CG768) and control (without fungus). Bars represent the standard deviation. Asterisk denotes difference ($p < 0.01$) of the treated groups compared to control group.

In the assay B, a significant difference ($p < 0.01$) was observed between the treatment with the fungus and the control treatment, reducing in 84% the average number of *Ancylostoma* spp. L_3. This fact proves that the fungus was able to establish itself in the sand from the substrate used, since, after 15 days mixed with sand, the inoculum was still viable and infective. The plates of treated group were examined under the microscope during the first 24 hours and formation of traps and predated larvae were observed (Figure 2).

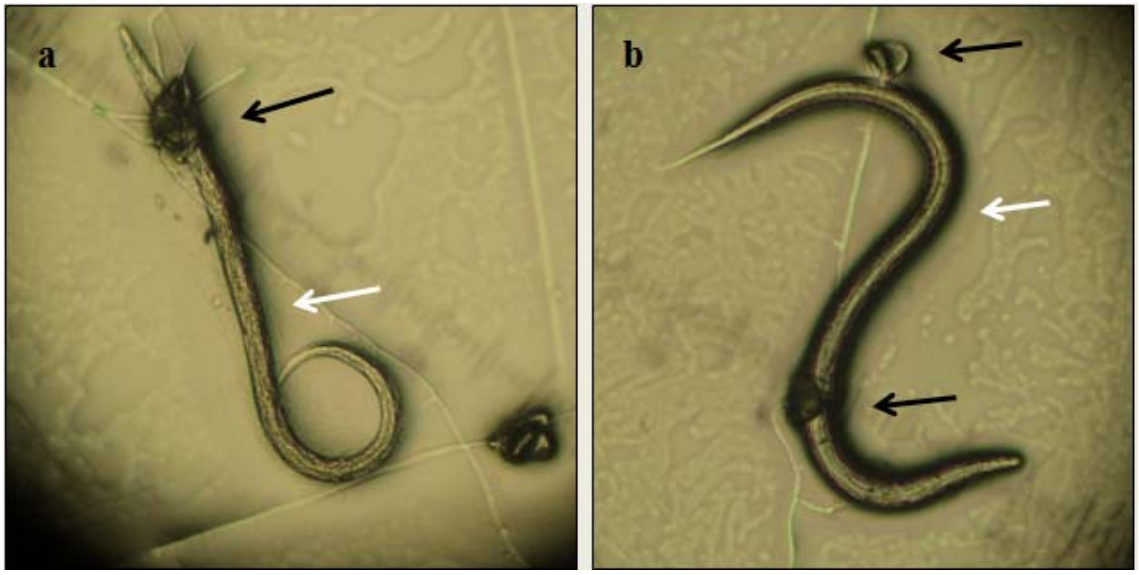

Figure 2 (a-b). Formation of trap (black arrow) by the tested fungal isolate of *Duddingtonia flagrans* (CG768) and third stage larvae of *Ancylostoma* spp. preyed (white arrow).

Discussion

Sand of public areas represent a potential source of transmission to human and animal population because it is frequently infested by zoonotic parasite eggs such as *Ancylostoma* spp. in the faeces of dogs that are commonly infected[17,18]. The predation and population reduction of this nematode in the beach sand, as observed in this work, justify this approach as an important tool to decontaminate the environment.

The are some reports about the of success use of nematophagous fungi in the control of potential zoonotic geohelminths, especially in an experimental model with dogs[9,19]. On the other hand, most studies are carried out under laboratorial conditions and even partially natural conditions, since it is observed the viability of tested nematophagous fungi after passage through the gastrointestinal tract. Carvalho et al.[9] demonstrated that the fungus *D. flagrans* (AC001) was able to withstand the passage through the gastrointestinal tract of dogs and was viable in predation on *Ancylostoma* spp L_3 under laboratory conditions. In that work, using another approach, those authors have observed that the administration of 0.5 g/10 kg of mycelial mass containing the fungus *D. flagrans* (isolate AC001) was effective in reducing egg counts per gram of faeces and in the recovery of *A. caninum* larvae in treated animals compared to the control group. In connection with this, in recent work Araujo et al.[19] demonstrated that

one isolate of nematophagous fungus was able to pass through the gastrointestinal tract of dogs and show its predation on *Toxocara canis* eggs an *in vitro* assay.

Nematophagous fungi should be applied in the environment (soil or sand) together with a fungal growth substrate, in order to promote their growth and therefore promote their establishment. However, until now there are no studies about the biological control of hookworm in beach sand, serving as a constant source of contamination. In the constitution of the sand used in this work were shown only minerals, without evidence of organic matter that could help in the proliferation of the fungus. Therefore, the main nutrients for the fungus were the substrate (corn meal) and the nematode.

Moreover, there is a lack of studies on the use of different concentrations of chlamydospores. In recent work, Maciel et al.[20] tried to determine the best dose of the fungus *D. flagrans* comparing different concentrations of the fungus, but no differences were observed between the concentrations of 10,000 to 25,000 chlamydospores per gram of soil in microcosm setups, but the control of the nematode ranged from 72,0% to 79,4% at the concentration of 10,000 chlamydospores/g of soil.

In the present work, differences could not be observed ($p < 0.01$) in the concentrations from 15000 to 25000 of chlamydospores/g of soil, the highest nematode L_3 predation. However, these differences are interesting from the biological point of view and even when dealing with sand and not soil as described above. Furthermore, the authors of the present study suggest that possibly the environment of "soil" may be rich in organic matter and can directly influence the development of fungal isolates. Thus, further studies using the material "beach sand" should be conducted in order to observe the predatory activity of the fungus *D. flagrans*.

The results of this study confirm earlier work on the efficiency of the *Duddingtonia* genus in control of *Ancylostoma* spp. infective larvae. However, this is the first report of the use of this fungus in beach sand, suggesting that this fungal isolate may become part of an alternative and complement program to the control of this nematode on the environment, reducing the use of chemical control agents.

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Capítulo 3

Effect of the fungus *Duddingtonia flagrans* on infective larvae *Ancylostoma* spp. of dogs under semi-natural conditions

Abstract

Nematophagous fungi have performed their role in the capture and destruction of geohelminths. The objective of this study was to evaluate the effect of nematophagous fungus *Duddingtonia flagrans* on infective larvae of *Ancylostoma* spp. of dogs in semi-natural conditions. The experiment consisted of two treatments, one with fungus at 15,000 chlamydospores/g sand and other without the fungus, both in 100 g of sand, infested with 2,000 L_3 of *Ancylostoma* spp. Sand samples from each treatment were collected right after the incorporation of chlamydospores into the sand and at the end of assay for determination of fungus population. For isolation of *D. flagrans* was applied the technique of serial dilution and plating on the semi-selective culture medium. The fungal isolate (CG768) showed predatory activity on the *Ancylotoma* spp. L_3 and reduced in of 47.9% the number of nematodes recovered when compared to the control group ($p < 0.05$). The biological control with nematophagous fungi may become a tool in combating geohelminths of dogs, working directly in the free life stages of *Ancylostoma* spp.

Keywords: Biological control, Nematophagous fungi, *Duddingtonia flagrans*, *Ancylostoma* spp., sand.

Introduction

The nematophagous fungi are widely distributed geographically around the world, inhabiting all types of soil, especially those rich in organic matter (NORDBRING-HERTZ et al., 2006; SANYAL et al., 2008). These organisms are nutritionally versatile and their good saprophytic capacity allows them to survive in the soil for long periods in the absence of nematodes (GRAY, 1987). Most species of nematophagous fungi is predatory (LARSEN, 1999) and is characterized by developing an extensive system of vegetative hyphae, along which morphological differentiation occurs in structures called "traps" that capture and retain living nematodes to nourish themselves of nematode internal contents (BARRON, 1977). This change from a saprophytic phase to a parasitic phase with morphological differentiation in traps and use of nematodes as food, influenced by biotic and abiotic factors, provides a nutritional advantage for these microorganisms in soil (NORDBRING-HERTZ et al., 2006).

Nematophagous fungi have done their role in capturing destruction of geohelminths, gastrointestinal parasites potentially zoonotic, and on those parasites that

conduct part of their life cycle in the soil. In this context, the *Duddingtonia flagrans* species has been tested on various conditions (natural and laboratory) (PAZ-SILVA et al., 2011), however, this is the first report of the study on sand.

The survival of these parasites is influenced not only by physical and chemical factors such as temperature, pH, moisture and aeration (CARVALHO et al., 2009; BRAGA et al., 2011; ARAUJO et al., 2012). In another context, the survival of a nematode in the external environment may be influenced by the presence or absence of known agents as biocontrollers and in this sense, stand out particularly: bacteria, fungi, nematodes, insects, mites and protozoa (GRONVOLD et al., 1996; STROMBERG, 1997; KERRY, 1987). However, the main biocontrollers of nematodes are the nematophagous fungi (NORDBRING-HERTZ et al., 1988).

Thus, considering this fact, parasites of the genus *Ancylostoma* have part of their life cycle in the soil (SOULSBY, 1982) and these fungi could become part of an effective and complementary alternative in its control (CARVALHO et al., 2010; LARSEN, 2000).

The objective of this study was to evaluate the effect of nematophagous fungus *Duddingtonia flagrans* on infective larvae of *Ancylostoma* spp. of dogs in semi-natural conditions.

Materials and Methods

Fungal inoculum

In present assay, it was used the isolate CG768 of the nematophagous fungus *Duddingtonia flagrans* obtained from samples of Brazilian soil and of animal feces were used in this experiment. The isolate was stored on integral rice grains inside 5 mL BD Vacutainer® glass tubes (Becton Dickinson, Brazil) containing blue silica gel, based on the conservation technique described by Smith and Onions (1983).

Production of chlamydospores

The chlamydospore production was performed in 2.5 L plastic bags with biological filter. Milled maize was used as substrate. Bags containing 300 g of substrate and 110 ml of distilled water were closed and sterilized for 30 minutes at 121 °C. The sterilized substrate in each bag was inoculated with six mycelium discs of about 5 mm diameter removed from the edges of *D. flagrans* culture, after growth on plates

containing agar-water medium at 2% during 7 days at 25 °C. The bags were then closed to allow growth of *D. flagrans* on the substrate for 15 days in the dark at 25 ° C. The substrate was agitated every five days until the end of the incubation period to ensure homogeneous mycelial growth. After the incubation period, six samples of 1 g of colonized substrate of each bag were transferred to an Erlenmeyer flask containing 10 mL of distilled water and 0.2% (v/v) dispersing polysorbate (Tween ® 80) and shaken by two minutes for dispersion of chlamydospores. Then two aliquots of 10µL were placed in a Neubauer chamber to estimate the number of chlamydospores per gram of substrate.

Ancylostoma spp. L_3

The *Ancylostoma* spp. L_3 was obtained from fresh feces of naturally infected stray dogs, by vermiculite coproculture kept for 10 days at 26 ºC in a BOD. After this period, active larvae were harvested from culture using the Baermann funnel technique The L_3 suspension was homogenized and 3 aliquots of 10 µL were placed on a microscope glass slide. Each aliquot was covered with a glass coverslip after adding 10 µL of lugol's solution to kill L_3. Thus, they were counted and identified under a light microscope with the magnification of 40X, allowing the estimation of the total number of larvae in the suspension. The motility of *Ancylostoma* spp. was checked by microscopical examination, before they were used in the experiment.

Experimental procedure

Sand samples were used (coarse sand: 76%; fine sand: 22%; silt 0%; clay: 2%; sodium: 342 mg/dm^3; phosphorus: 34,15 mg/dm^3; potassium: 20 mg/dm^3; calcium: 0,46 cmol/ dm^3; magnesium: 0,32 cmol/ dm^3; aluminum: 0 cmol/ dm^3; ph: 8,2). These samples were derived from the resort city of Guarapari, state of Espírito Santo and were submitted regarding the particle size analysis and chemical analysis by methodology of Ruiz (2005). Samples were collected at a depth of 0 to 20 cm and stored in plastic bags. The minimum and maximum temperatures were measured daily by a mercury column thermometer.

The present assay was conducted within polypropylene pots of 145 cm ^{3} transparent containing 100g of sand. The sand in each pot was artificially infested by means of a standardized suspension of 171 µL of distilled water containing about 2000 *Ancylostoma* spp. L_3. The fungal suspension was 0.46 g of milled maize per pot, in order to result in a concentration of 15.000 chlamydospores per gram of sand. A control

group was formed, containing infested sand with *Ancylostoma* spp., without the fungus. Next, the opened pots were placed in black polypropylene pots of 350 cm ³ and kept inside a greenhouse protected from direct sunlight for a period of 15 days. The sand of the pots was daily irrigated to prevent dehydration of the larvae and to allow germination of chlamydospores. At the end of the incubation period the larvae were recovered from the sand by the Baermann's funnel technique (Baermann, 1917) and counting was performed as described above.

Sand samples from each experimental group were collected right after the incorporation of chlamydospores to sand and the end of assay for determination of population of the fungus. For isolation of *D. flagrans*, 1 g samples of sand were put into test tubes containing 9 ml of sterile water and shaken for 3 minutes at stirrer. Subsequently, the serial dilution technique was employed to $1x10^{-2}$, and an aliquot of 1 mL was spread on Petri dishes containing semi-selective culture medium, with the aid of a Drigalsky strap as Gaspard et al. (1990), with three replicates per treatment. The plates were stored at 25 ºC until the growth of colony forming units (CFU).

The colonies were then pricked out to medium plates containing potato dextrose agar (PDA) and incubated at 25 ºC. After seven days, the fungal colonies were identified according to morphological characteristics of the fungus.

Statistical analysis

The data obtained in the test were interpreted by analysis of variance at a significance level of 1% probability (AYRES et al., 2003). The destruction efficiency of L_3 compared to control was assessed by Tukey's test at 1% probability. Thereafter, the percent reduction from the average of L_3 was calculated according to the following formula:

$$\% \text{ Reduction} = \frac{\text{Average } L_3 \text{ recovered from control} - \text{Average } L_3 \text{ recovered from treatment}}{\text{Average } L_3 \text{ recovered from control}} \times 100$$

Results

In the presente work, the fungal isolate used (CG768) showed predatory activity on the *Ancylotoma* spp. L_3 and it was observed the percentage reduction of 47.9% in the treated group compared to the control group ($p < 0.05$). The observed average temperature inside the greenhouse was 31 ºC.

The isolate evaluated has established itself in the sand throughout the trial period (Table 1). This fact was confirmed by plating sand in semi-selective culture medium and by observation of colony forming units (CFU) of *D. flagrans.* Even after 7 days of plating were observed vegetative and resistance structures, proving that the fungus was able to establish itself in the sand from the substrate used (Figure 1).

Table 1. Population of the fungus *Duddingtonia flagrans* after sand infestation with 15.000 chlamydospores/g of sand and after 15 days. Averages of three replications.

Experimental group	Log CFU/g of sand	
	Test setup	After 15 days
Treated	5,38	3,95
Control	0	0

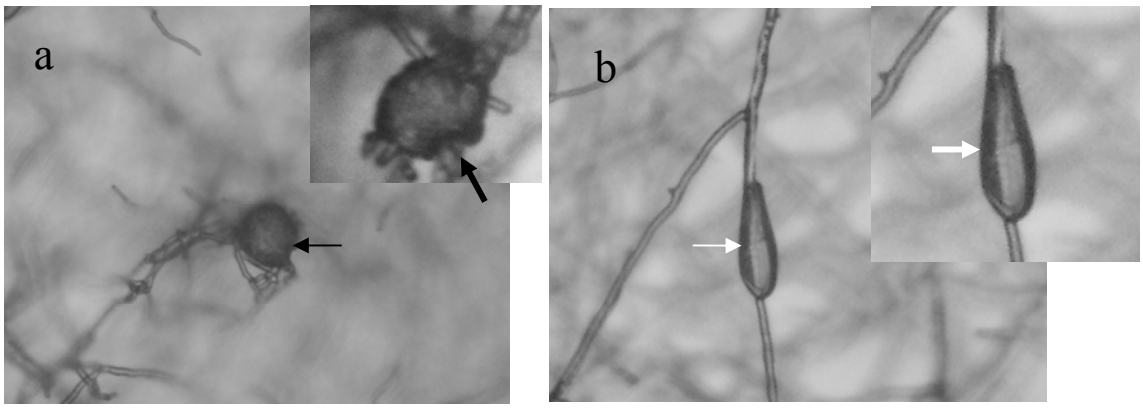

Figure 1. Chlamydospores and conidia of the fungus *Duddingtonia flagrans* observed after 7 days after plating in semi-selective medium. (a) Chlamydospore in germination phase (black arrow) and (b) conidia (white arrow).

Discussion

The results of this study revealed that the fungus *D. flagrans* was able to survive in the sand even in non-favorable conditions (absence of organic matter). The

incorporation of the fungus in the sand along with milled maize was crucial for its establishment and nematophagous activity. This is due to the fact of nematophagous fungi are saprophytic and because they feed on other sources of nutrients in the absence of nematodes in order to ensure their survival in the environment (NORDBRING-HERTZ, 1988)

Environmental temperature directly affects the growth and production of traps by the fungus. According Gronvold et al. (1996) and Morgan et al. (1997), the optimal growth temperature for *D. flagrans* is between 25-33 ºC. In present assay, it was observed that the average temperature was 31ºC. These results are in agreement with previous studies mentioned above.

Regarding the results in respect of the fungus population tested (CG768) at the end of the test, it was observed that after 15 days it was still viable. Also according to Dallemole-Giaretta (2008), the population of nematophagous fungi in soil by means of CFU counts of isolates tested, in this specific case CG768, strongly suggests the establishment of the fungus on soil. Thus, in the present work, even using a poor soil in organic matter, certain types of nematophagous fungi can come to settle. In this sense, the authors call attention to the abundant production of chlamydospores (resistant structures) that are peculiar to their nature (ARAÚJO et al., 2004).

On the other hand, the authors agree that the use of certain sources of N: C is important for the proper establishment of the fungus *D. flagrans* in poor soil. In this context, Braga et al. (2011) reported that the species *D. flagrans* might require a lot of sources of N: C, which on the one hand, suggests that this fungus has not a nutritional demanding character, being this an interesting premise from an economic point of view.

Regarding the use of *Ancylostoma* spp. L_3, the authors suggest that more and more bioassays involving potentially zoonotic nematodes may become an important tool of control in the future, since the nematophagous fungi are natural organisms and antagonists of these parasites in the soil (ARAUJO et al., 2012; CIARMELA et al., 2008). On the other hand, no one knows the real interaction of predatory nematophagous fungi in a soil poor in organic matter on zoonotic nematode L_3, and this is the first report.

Accordingly Soulsby (1982) and Bowman (2009) the survival of *Ancylostoma* spp. L_3 is influenced by several factors, including: (1) the temperature between 23-30 °

C, (2) moisture, which is understood in the literature as "moderate," and (3) the physical and chemical characteristics of the soil.

Moreover, the porosity of the soil, represented by spaces occupied by air or water, constitutes one of the main factors which can allow the physical development of larvae. The eggs and or larvae of hookworms are susceptible to desiccation and therefore require a permanent film of water around them (SOULSBY, 1982; BOWMAN, 2009). Sandy soils possess a higher proportion of pores with diameters larger than 50 μm, called macrospores, which allow the free movement of air and water of percolation (BUCKMAN; BRADY, 1969).

Finally, biological control with nematophagous fungi could be a tool in combating geohelminths of dogs (CARVALHO et al., 2009). However, it should be mentioned that the chemical control integrated with the biological control must be a premise to be followed, since it can act directly on the cycles of parasites and life free geohelminths of dogs, especially here *Ancylostoma* spp.

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CONCLUSÕES

- Apesar dos testes in vitro não representarem as condições ambientais, são importantes no estudo da capacidade predatória de fungos nematófagos, na seleção de isolados promissores e na busca das concentrações mais efetivas.
- A incorporação do fungo na areia em um substrato (milho moído) pode proporcionar suporte nutricional e com isso viabilizar seu estabelecimento no ambiente.
- O isolado CG 768 de *D. flagrans* é um promissor biocontrolador de Ancilostomídeos de cães, uma vez que reduziu o número de larvas infectantes destes nematoides, recuperadas de areia de praia sob condições semi-naturais.
- A espécie *D. flagrans* tem potencial para reduzir a infestação ambiental por formas larvais de *Ancylostoma* spp. de cães. No entanto, são necessários mais estudos em areia sob condições naturais.

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