SEBASTIÃO RODRIGO FERREIRA

ATIVIDADE OVICIDA DO FUNGO *Pochonia chlamydosporia* SOBRE OVOS DE *Ascaris suum* E ATIVIDADE PREDATÓRIA DE FUNGOS NEMATÓFAGOS SOBRE FORMAS INFECTANTES DE *Oesophagostomum* spp.

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: 15 de agosto de 2011.

Prof. Leandro Grassi de Freitas
(Co- orientador)

Prof. Antônio Galvão do Nascimento

Prof. Jackson Victor de Araújo
(Orientador)

O futuro pertence àqueles que acreditam na beleza de seus sonhos.

Elleanor Roosevelt

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BIOGRAFIA

Sebastião Rodrigo Ferreira, nasceu em 20 de janeiro de 1984, em Eugenópolis-MG. Filho de Braz Lurde Ferreira e Maria Tereza Ferreira. Concluiu o ensino médio em 2001, na Escola Estadual "Assis Brasil" – Vieiras, MG. No ano de 2004, iniciou o curso de graduação em Ciências Biológicas, na Faculdade de Filosofia Ciências e Letras Santa Marcelina, e graduou-se no ano de 2007. Em agosto de 2009, ingressou no mestrado no programa de Pós-graduação em Medicina Veterinária da Universidade Federal de Viçosa

SUMÁRIO

RESUMO

FERREIRA, Sebastião Rodrigo, M.Sc., Universidade Federal de Viçosa, agosto de 2011. **Atividade ovicida do fungo *Pochonia chlamydosporia* sobre ovos de *Ascaris suum* e atividade predatória de fungos nematófagos sobre formas infectantes de *Oesophagostomum* spp.** Orientador: Jackson Victor de Araújo. Co-orientadores: Leandro Grassi de Freitas e Aloízio Soares Ferreira.

A carne suína é uma das principais fontes de proteína animal consumida no mundo e a forte demanda por carne suína no continente Asiático tem estimulado o desenvolvimento do mercado interno, entretanto, têm se exigido um sistema de produção que vise o bem estar animal e a produção orgânica. Contudo, em dadas situações as parasitoses intestinais continuam a ser um problema para criação destes animais, principalmente quando essa se faz pelo sistema de manejo extensivo. Entre estas parasitoses destacam-se os nematoides, *Ascaris suum* e *Oesophagostomum* spp, cujas formas infectantes encontram-se no ambiente. Dessa forma, os objetivos do presente trabalho foram: avaliar a atividade ovicida do fungo *Pochonia chlamydosporia* sobre ovos de *A. suum* e atividade predatória do fungo *Duddingtonia flagrans*, *Monacrosporium sinense* e *Artrhobotrys robusta* sobre formas infectantes e *Oesophagostomum* spp. Para isso, foram montados quatro ensaios, onde foi avaliada a atividade *in vitro* dos referidos fungos sobre as formas infectantes destes nematoides e um teste *in vivo* onde se avaliou a capacidade de *P. chlamydosporia* e *D. flagrans* suportar a passagem pelo trato gastrointestinal de suínos. Isolados fúngicos de *P. chlamydosporia* destruíram ovos de *A. suum* no teste *in vitro* e foram capazes de suportar a passagem pelo trato gastrointestinal de suínos sem perder sua capacidade de destruição de ovos. *D. flagrans, M. sinense* e *A. robusta* predaram as larvas l_3 de *Oesophagostomum* spp. nos testes i*n vitro. D. flagrans* manteve esta habilidade após a passagem pelo aparelho gastrointestinal dos suínos. Os resultados apresentados no presente trabalho sugerem que os fungos *Pochonia chlamydosporia e Duddingtonia flagrans* podem ser utilizados para auxiliar no controle das formas infectantes dos nematoides *Ascaris suum* e *Oesophagostomum* spp, respectivamente.

ABSTRACT

FERREIRA, Sebastião Rodrigo, M.Sc., Universidade Federal de Viçosa, August of 2011. **Ovicidal activity of the fungus *Pochonia chlamydosporia* on *Ascaris suum* eggs and predatory activity of the nematophagous fungi on *Oesophagostomum* spp. infective forms.** Adviser: Jackson Victor de Araújo. Co-advisers: Leandro Grassi de Freitas and Aloízio Soares Ferreira

Swine meat is one of main sources of animal protein consumed in the world and strong demand for swine meat in the Asian continent has stimulated the development of the internal market, however, has been demanded a production system aimed at animal welfare and organic production. Even though, in given situations intestinal parasites remain a problem for breeding these animals, especially when this is done through the extensive management system. Among these, stands out the parasitic nematodes, *Ascaris suum* and *Oesophagostomum* spp, whose infective forms are present in the environment. By the way, the objectives of this study were to evaluate the ovicidal activity of fungus *Pochonia chlamydosporia* on *A. suum* eggs and predatory activity of the *Duddingtonia flagrans, Monacrosporium sinense* and *Artrhobotrys robusta* on infective forms of *Oesophagostomum* spp. For this purpose, four experimental trials were set up, where it was evaluated (*in vitro*) activity of these fungi on the infectious forms of these nematodes and (*in vivo*) test which evaluated the ability of *P. chlamydosporia* and *D. flagrans* to support the passage through the gastrointestinal tract of pigs. Isolates fungal of *P. chlamydosporia* were able to destroy *A. suum* eggs *in vitro* and capable of supporting the passage through the gastrointestinal tract of pigs without losing its capacity to destroy eggs. *D. flagrans, M. sinense* and *A. robusta* predated *Oesophagostomum* spp L3 *in vitro. D. flagrans* kept its predatory ability after passing through the gastrointestinal tract of pigs. Results presented in this study suggest that the fungi *P. chlamydosporia* and *D. flagrans* can be used to assist in the control of infectious forms of the nematodes *A. suum* and *Oesophagostomum* spp, respectively.

INTRODUÇÃO GERAL

A carne suína é uma das principais fontes de proteína animal consumida no mundo, representando quase a metade do consumo e produção de carnes, com mais de 94 milhões de toneladas. Os maiores consumidores são os países europeus, norte-americanos e a China. O Brasil é o quarto maior produtor e o sexto maior consumidor de carne suína (Faria e Machado, 2006; Dill et al., 2010). Segundo Abipecs (2007), a forte demanda por carne suína no continente Asiático tem estimulado o aumento das exportações brasileiras, promovendo o desenvolvimento do mercado interno. Entretanto, de acordo com Dill et al. (2010), um novo mercado está surgindo com preferências em relação ao sistema de produção destes animais, trata-se de um sistema que visa o bem estar animal e a produção orgânica. Esse sistema, geralmente, permite uma criação extensiva (piquetes abertos) e em dadas situações uma criação semi-extensiva (piquetes abertos intercalados com baias). Esta realidade é uma resposta a uma maior demanda dos consumidores, principalmente os europeus, por estes alimentos, que são considerados mais saudáveis e saborosos, pois são livres do excesso de produtos químicos e são produzidas de maneira sustentável com menor agressão ao meio ambiente e aos animais (Dill et al., 2010). Também são crescentes os investimentos na agricultura familiar, incentivando a criação de animais e agricultura para subsistência e a venda do excedente destes como uma alternativa de renda extra (Aguiar 2009).

Segundo Macrae (1993) e Araújo et al. (2004), as parasitoses intestinais continuam sendo um sério agravante para produção de animais no Brasil. Na criação de suínos este problema torna-se mais relevante quando a criação destes animais é feita pelo sistema de manejo extensivo, uma vez que desta forma os animais estão mais expostos aos agentes parasitários. Entre essas parasitoses, destacam-se as nematodíases, cujos danos à saúde do animal estão relacionados aos agentes etiológicos e susceptibilidade do hospedeiro. Contudo, as nematodíases produzem efeitos deletérios que influenciam na capacidade produtiva, conversão alimentar e taxa de crescimento. Além disso, aumenta os custos com os tratamentos curativos e profiláticos e, em alguns casos, pode levar o animal a óbito. Geralmente os nematoides parasitas intestinais de suínos estão representados pelos seguintes gêneros: *Ascaris*, *Oesophagostomum*, *Strongyloides*, *Trichuris* e *Hyostrongylus* prevalentes em diferentes áreas geográficas (Wagner e Polley, 1997; Eijck e Borgsteede, 2005; Nansen e Roepstorff, 1999).

No gênero *Ascaris*, a espécie *Ascaris suum* é o nematoide parasita intestinal de suínos que apresenta distribuição mundial, geralmente este nematóide é muito comum em animais criados em sistemas extensivos (Wagner e Polley, 1997; Eijck e Borgsteede, 2005). *Ascaris suum* está diretamente relacionado às baixas taxas de crescimento e diminuição no ganho de peso, devido a alterações digestivas e competição por nutrientes. Além de estar associado com dano hepático popularmente conhecido como "manchas do leite", resultado da migração de suas larvas pelo fígado do animal e, frequentemente, esta lesão resulta o descarte no órgão durante o abate (Wagner e Polley, 1997). Os parasitos adultos vivem no intestino delgado dos suínos e seus ovos são eliminados junto às fezes, sendo as fêmeas muito fecundas. Uma fêmea pode produzir mais de 200.000 ovos / dia. No ambiente se as condições de oxigenação, temperatura e umidade forem favoráveis, dentro de alguns dias uma larva irá evoluir dentro do ovo até o estádio infectante (L3) (Urquhart et al., 1998). Esses ovos são bastante resistentes às condições adversas no ambiente, além de possuírem grande capacidade de aderência, pois uma vez aderidos às superfícies não são removidos com facilidade (Massara et al., 2003; Urquhart et al. 1998). O homem é um hospedeiro acidental de *A. suum*, e diversos trabalhos têm atribuído à suas larvas a ocorrência da zoonose Larva Migrans Visceral (LMV) (Nejsum et al., 2005; Arimura et al., 2001; Sakai et al., 2006; Okada et al., 2007)

Oesophagostomum é um dos gêneros de nematoides mais comum em suínos e apresenta distribuição mundial (Talvik et al., 1997). Para os suínos o gênero geralmente é representado por *O. dentatum* e *O. quadrispinulatum*, essas espécies são capazes de causar uma enterite, resultado do comportamento das larvas que penetram pela mucosa do intestino, provocando uma resposta inflamatória e com formação de nódulos. Este fato é mais comum em animais que estiveram expostos a mais de uma infecção por esse nematóide, contudo, mesmo quando não há manifestação clínica da doença os prejuízos devido à baixa produtividade são consideráveis (Urquhart et al., 1998). Este nematóide apresenta ciclo de vida direto, os ovos são liberados junto às fezes para o ambiente e, se em condições ideais, dentro de alguns dias irá eclodir, de onde emergirá uma larva (L_1) que sofrerá duas ecdises dando origem à forma infectante a L_3, o animal irá se infectar pela ingestão dessa, que então sofrerá a ultima muda dando início a penetração na mucosa (Talvik et al., 1997; Aguiar, 2009). Embora o principal meio de infecção seja a ingestão da L_3, Nosal et al. (1998) afirmaram que é possível que infecção também ocorra por via percutânea.

A principal medida de controle para estes nematoides é uso regular de anti-helmínticos (Roepstorff e Nansen, 1994 appud Wagner and Polley, 1999; Gerwert et al., 2002). Entretanto, geralmente, esta medida é muito impactante para o ambiente, além de só remover parte da população dos nematoides, pois a grande maioria está presente no ambiente e nas diversas formas do ciclo biológico: ovos e estádios larvais, dependendo da espécie de nematóide (Wagner e Polley, 1999). Além disso, existe preocupação crescente sobre possível emergência de resistência às drogas, uma vez que esta pode tornar-se um empecilho para criação animal (Larsen, 2006; Geerts et al., 1997).

Considerando que parte do ciclo de vida destes nematoides se passa no ambiente, medidas alternativas que possam auxiliar no controle das formas infectantes destes nematoides são constante objeto de estudo de pesquisadores em todo mundo, dentre essas medidas merece destaque o controle biológico realizado com nematófagos.

Os fungos nematófagos são organismos saprófitas mundialmente estudados no controle de formas infectantes (ovos e ou larvas) de helmintos. Sua atividade predatória é direcionada para o ambiente fecal, onde estão presentes os ovos e larvas, por meio da formação de armadilhas ao longo de suas hifas (Lopez-Llorca e Robertson, 1992; Larsen, 1999; Araújo et al., 2008; Braga et al., 2010; Braga et al., 2009). Esses fungos se comportam como antagonistas naturais, promovendo a captura e a destruição do parasito e, de acordo com seu modo de ação, são classificados em endoparasitas, predadores e oportunistas (parasitas de ovos). Os grupos dos predadores de larvas e destruidores de ovos (oportunistas) têm sido amplamente estudados, com sucesso, no controle biológico dos helmintos gastrintestinais, em condições laboratoriais e a campo. (Araújo et al., 1995; Braga et al., 2009; Braga et al., 2010). Esses fungos obedecem à premissa do controle biológico de produção de clamidósporos, estruturas resistentes que permitem a passagem de fungos nematófagos pelo aparelho gastrintestinal de animais domésticos e o que torna mais viável sua dispersão.

De acordo com Sanyal et al. (2008) e Skipp et al. (2011), uma das características que contribui para o sucesso de fungo predador *D. Flagrans* no controle biológico é a grande produção de clamidósporos, estruturas que são altamente resistentes a situações adversas, formados principalmente em condições de crescimento desfavoráveis. *D. flagrans* produz vários conídios nas extremidades dos conidióforos. Esses conídios apresentam parede espessa, podendo variar de elíptico a ovoide e com tamanho, aproximadamente, de 25-50µm de comprimento por 10-15µm de largura; seus

clamidósporos são intercalados e com paredes grossas, e suas hifas formam redes adesivas relativamente curtas (Braga, 2008; Skipp et al., 2011). Este fungo se destaca pela rapidez que ataca e destrói as larvas dos nematoides (Cruz et al., 2011). Diversos trabalhos, em nível mundial, têm relatado a aplicabilidade deste fungo como agente controlador biológico de nematodioses de diversos animais domésticos (Sanyal et al., 2008; Campos et al., 2008; Epe et al., 2008; Peart, 2002; Braga et al., 2009; Skipp et al., 2011). Entretanto, pouquíssimos são os estudos que relatam sua aplicabilidade para nematoides de suínos.

O fungo *Monacrosporium sinense* e *Artrhobotrys robusta* também têm apresentado resultados promissores no controle de helmintos parasitos de cães e bovinos (Araújo et al., 2006; Carvalho et al 2010). *M. sinense* produz conídios hialinos, septados e fusiformes, esses conídios tem um tamanho de aproximadamente 25-30µm de comprimento por 15-18 µm de largura, já seus clamidósporos variam de 20-24µm de comprimento por 17-27µm de largura (Braga, 2008). *Artrhobotrys robusta* apresenta conídios com 18-27µm de comprimento por 8 -12 µm de largura, sendo esses conídios hialinos, em formato ovoide e septados na região mediana (Mauad, 2008).

A associação entre *P. chlamydosporia* e ovos de nematoides foi reportada pela primeira vez por Willcox e Tribe (1974) (appud Giaretta, 2008) após ter sido isolado de ovos de *Heterodera schachtii* (um fitonematóide). *P. chlamydosporia* produz colônias com crescimento rápido, hifas delgadas, conídios pequenos e hialinos, geralmente em forma de bastão e com uma estrutura globular na extremidade superior; clamidósporos com paredes espessas e morfologia tridimensional. Este fungo tem sido testado, com sucesso, contra os ovos de geohelmintos e alguns autores relatam que a destruição desses ovos ocorre por meio de ações mecânica e enzimática (Araújo et al., 2008; Braga et al., 2010a). Segundo Sergers et al. (1996) *P. chlamydosporia* produz enzimas extracelulares que participam do processo de infecção dos ovos causando sua destruição (efeito do tipo 3). Embora vários trabalhos reportem a capacidade e aplicabilidade deste fungo como agente controlador biológico de diversos nematoides dos animais domésticos, nenhum deles aborda a sua capacidade de suportar a passagem pelo trato gastrointestinal de suínos e a manutenção de sua habilidade de destruição de ovos.

Capítulo 1

Ovicidal activity of seven *Pochonia chlamydosporia* fungal isolates on *Ascaris suum* eggs

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Abstract

The ovicidal effect of the nematophagous fungus *Pochonia chlamydosporia* on eggs of *Ascaris suum* was tested under laboratory conditions. *A. suum* eggs were plated on 2% water-agar with seven fungal isolates (Isol.5, Isol.31, Isol.1, VC1, Isol.12, Isol.22 and VC4) and control without fungus. After 5, 7, 10, 14, 15 and 21 days of incubation, approximately one hundred eggs were removed from the plates and classified according to the following parameters: type 1, biochemical and physiological effect without morphological damage to the eggshell, type 2, lytic effect with morphological alteration of the eggshell and embryo and type 3, lytic effect with morphological alteration of eggshell and embryo showing hyphal penetration and internal egg colonization. The isolates effectively destroyed *A. suum* eggs and all types of effects were observed during the experiment. There was no variation in ovicidal capacity (type 3 effect) among the isolates ($P > 0.05$) throughout the experiment. After 21 days, Isolate 5 showed the highest percentages of type 3 effect (58.33%). The results indicated that *P. chlamydosporia* (Isol.5, Isol.31, Isol.1, VC1, Isol.12, Isol. 22 and VC4) can destroy *A. suum* eggs and is, therefore, a potential biological control agent of nematodes.

Keywords: Nematophagous Fungi, *Pochonia chlamydosporia*, *Ascaris suum*, Biological Control.

1. Introduction

Swine production involves a great diversity of management practices, which interferes in the verminosis spectrum and infection intensity. Nematode parasitism is still a worldwide problem in technological production systems and very often infection is not detected, persisting at subclinical levels for long periods and frequently causing animal death (Roepstorff and Nansen, 1994).

The most prevalent gastrointestinal nematodes in pigs are *Ascaris*, *Oesophagostomum*, *Trichuris* and *Strongyloides* (Roepstorff et al. 1998). *Ascaris suum* is common in pigs raised through extensive management systems worldwide (Wagner and Polley, 1997). This parasite is associated with liver damage ("milk spot" lesions caused by migrating larvae), resulting in convictions in court slaughter. Infection with this helminth was found to negatively affect various production parameters such as rate of gain weight and feed efficiency (Hurnik and Dohoo, 1995). Eggs of *A. suum* stand out for their

resistance in the environment, remaining viable for long periods (Urquhart et al. 1998). Eggs of *A. suum* and other ascarids contaminate soil, water and foods (Gupta et al. 2009). Once these eggs are present in foods they become extremely difficult to be removed because of their ability to adhere to food surfaces (Massara et al. 2003). Thus, the knowledge of the biology of free-living stages of this and other gastrointestinal nematodes can help in developing appropriate control programs.

Current research has shown progress in the control of gastrointestinal nematodes of domestic animals, in particular the biological control with nematophagous fungi. Nematophagous fungi are classified as endoparasites, predators and parasites of nematode eggs (Araújo et al. 2004, 2008). *Pochonia chlamydosporia* colonizes and destroys helminth eggs using appressoria developed from undifferentiated hyphae (Braga et al. 2007; Braga et al. 2008a, b; Araújo et al. 2008).

This study evaluated the *in vitro* ovicidal effect of seven *P. chlamydosporia* isolates on eggs of *A. suum*.

2. Material and methods

Seven isolates of nematophagous fungus *P. chlamydosporia* (Isol.5, Isol.31, Isol.1, VC1, Isol.12, Isol.22 and VC4) were kept in test tubes containing 2% corn meal-agar, in the dark, at 4^{o} C for 10 days. Culture disks, 4 mm in diameter, were transferred to 9-cm diameter Petri dishes containing 20 mL of 2% water-agar (2% WA) and incubated at 25° C, in the dark, for 10 days. The isolates were obtained from the Phytopathology Department of the Federal University of Viçosa and are been kept at Veterinary Department of the Federal University of Viçosa, Minas Gerais, Brazil.

After growth of the isolates, new culture discs, 4 mm in diameter, were transferred to 9.0 cm diameter Petri dishes containing 20 ml of 2% water-agar (2% WA) for 10 days.

2.1. Obtaining of *Ascaris suum* eggs

The eggs of *A. suum* were recovered from the dissection of a mature female worm obtained from a swine which died of natural causes. Eggs were morphologically analyzed for their integrity under a light microscope (10 x objectives) according to Urquhart et al.

(1998). The eggs were washed ten times in distilled water and centrifuged at 1000 rpm for 5 minutes in each time. The supernatant was discarded at the end of each centrifugation cycle. The eggs were then incubated at 25º C for 21 days with a solution containing 0.005% streptomycin sulphate and 0.01% chloramphenicol as described by Araújo et al. (1995)

2.1.2. Experimental assay

Eggs were inoculated on 9.0 cm diameter Petri dishes with 2% WA with fungal isolates grown for 10 days and a control plate without fungus, with 6 repetitions per group. Each plate contained one thousand eggs of *A. suum* with only one fungal isolate. In intervals of 5, 7, 10, 14, 15 and 21 days, approximately one hundred eggs were removed from each plate containing the isolates and from the control, as described by Araújo et al. (1995), placed on glass slides with a drop of 1% Amam-blue and examined in 40x objective according to parameters set by Lysek et al. (1982): type 1, biochemical and physiological effect without morphological damage to the eggshell; type 2, lytic effect with morphological alteration of the eggshell and embryo; and type 3, lytic effect with morphological alteration of eggshell and embryo with hyphal penetration and internal egg colonization.

2.1.3. Statistical Analysis

Data from each interval were analyzed by the Friedman's non parametric test at 5% probability level (Ayres et al. 2003).

3. Results

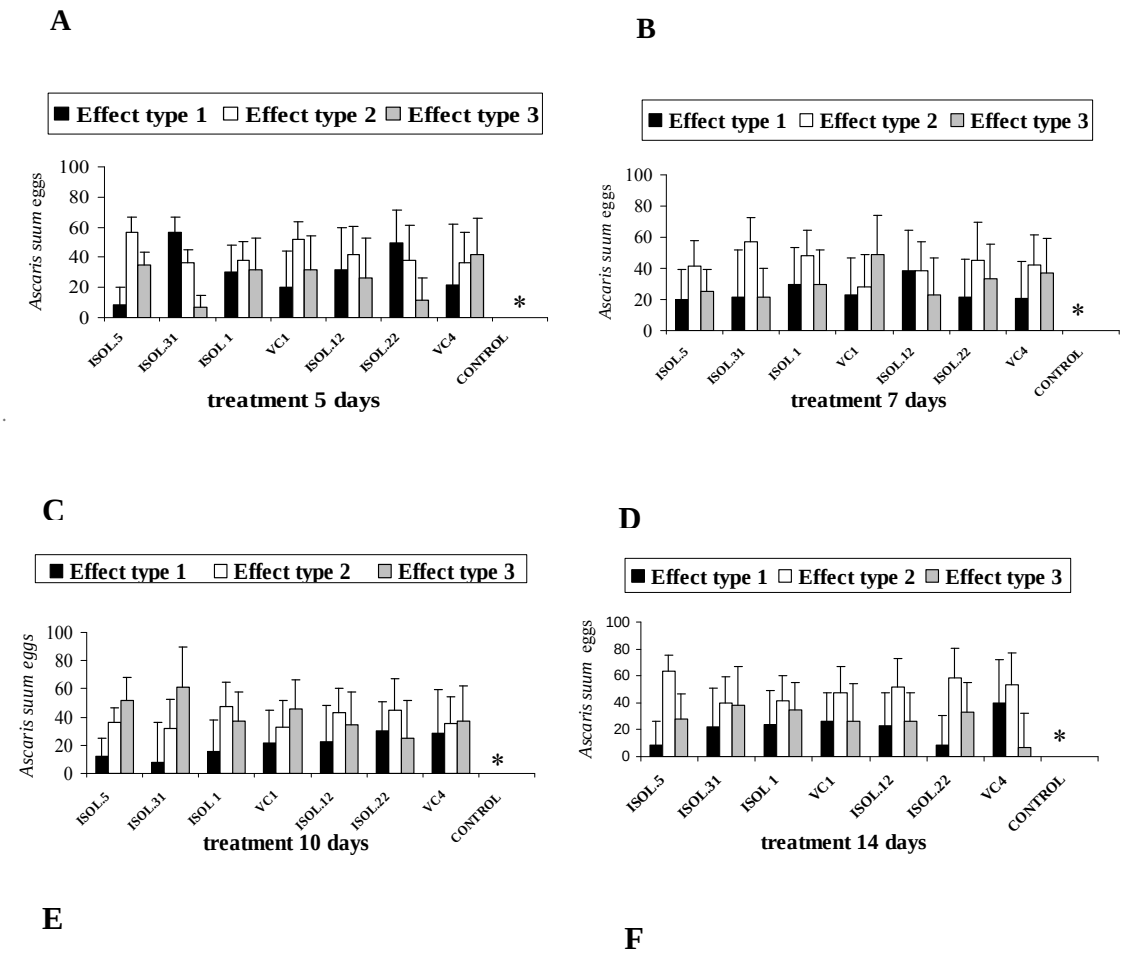
At figure 1 (A-F) are demonstrated the results of the interaction between *P. chlamydosporia* isolates and *A. suum* eggs during the experiment.

All *P. chlamydosporia* isolates (Isol.5, Isol.31, Isol.1, VC1, Isol.12, Isol.22 and VC4) showed the three types of effects (1, 2 and 3) at 5, 7, 10, 14, 15 and 21 days, but there was not variation in ovicidal capacity (type 3 effect) among the isolates ($P > 0.05$) throughout the experiment.

The following percentage results for type 3 effect were found for the isolates: isolate 5 (35.0%, 25.0%, 52%, 28.32%, 52.34% and 58.33%), isolate 31 (6.68% 21.69% 60.89% 38.32% 46.34% and 58.32%), isolate 1 (31.67%, 30.0%, 36.68%, 35.0%, 32.16%

and 32.17%), isolate VC1 (31.69%, 48.83%, 45.9%, 26.68%, 56.67% and 57.67%), isolate 12 (26.67%, 23.33%, 34.2%, 26.0%, 30.0%, 48.0%), isolate 22 (11.66%, 33.34%, 25.0%, 33.33%, 39.83% and 53.17%) and isolate VC4 (41.67% 36.68% 36.67% 6.68% 48.33% and 55.33%), at 5, 7, 10, 14, 15 and 21 days, respectively.

Light microscopy showed vegetative structures (hyphae) from *P. chlamydosporia* colonizing eggs of *A. sum.* The subsequent egg rupture characterizes effect type 3. Chlamydospore production by *P. chlamydosporia* was also observed in the Petri dishes treated with the isolates.



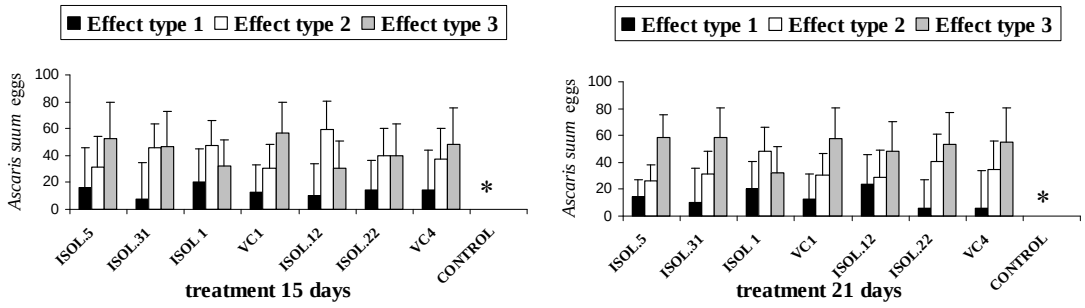


Fig.1(A-F) - Percentages and standard deviation (bar) for the effects at type 1■$^{+}$, 2□$^{++}$ and 3 ▣$^{+++}$ levels of the nematophagous fungus *Pochonia chlamydosporia* isolates (ISOL.5, ISOL.31, ISOL.1, VC1, ISOL.12, ISOL.22 and VC4) compared with the control group (without fungi) on *Ascaris suum* eggs at 5, 7, 10, 14, 15 and 21 interaction days. $^{+}$Physiological and biochemical effects without morphological damage to the eggshell, with hyphae adhered to the shell. $^{++}$Lytic effects with morphological alterations of the embryo and eggshell, without hyphal penetration. $^{+++}$Lytic effect with morphological alteration of the embryo and eggshell, in addition to hyphal penetration and internal colonization. *All effects for all isolates significantly different from the controls ($P<0.05$).

4. Discussion

A fungus must be capable of internal egg colonization and destruction to be considered ovicidal (Lysek et al. 1982). In the present work, all *P. chlamydosporia* isolates showed type 3 effect, with hyphae penetrating *A. suum* eggs and destroying them, characterizing the ovicidal activity.

P. chlamydosporia has proven action against eggs of parasitic nematodes of animals and plants (Lopes et al. 2007; Araújo et al. 2008). Recent studies, under laboratory conditions, have shown that it can also destroy eggs of trematodes, cestodes and nematodes parasitizing domestic animals (Braga et al. 2007, 2008a, c; Araújo et al. 2008).

Isolates VC1 and VC4 were effective against *A. suum* eggs after 21 days of incubation, with percentage results of 56.67% and 55.33%, respectively. Braga et al. (2007) demonstrated the effectiveness of the *P. chlamydosporia* isolates VC1 and VC4 against eggs of *Ascaris lumbricoides*, a human gastrointestinal nematode, in intervals of 7, 10 and 14 days. At the end of the experiment they reported ovicidal percentages above 26% for both isolates.

Araújo et al. (2008) evaluated the effects of *P. chlamydosporia* isolates in laboratory conditions and reported ovicidal activity ($p < 0.01$) of VC1 and VC4 on *A. suum* eggs with percentages of 13.0% and 17.3%, 13.9% and 17.7% and 19.0% and 20.0% at 7, 14 and 21 days respectively. In the present work, the isolates VC1 (48.83%, 26.68%, 56.67%) and VC4 (36.68%, 6.68%, 55.33%) showed better results for the same time intervals. The other fungal isolates (Isol. 5, 31, 1, 12 and 22) were included to evaluate the variability.

Braga et al. (2010) reported percentages of (31.5%, 39.4%) and (35.3%, 57.9%) for type 3 effect of *P. chlamydosporia* (isolates 5 and 12) on *Toxocara vitulorum,* a bovine gastrointestinal nematode, in laboratory conditions at intervals of 10 and 15 days respectively. In this work, the same isolates were also effective against *A. suum* eggs. Isolate 5 showed higher percentages for effect type 3 (52.0%, 52.34%) in the same intervals. Braga et al. (2008c) stated that the penetration mechanism of ovicidal fungi in parasitized eggs is still not completely elucidated and differences in the ovicidal activity have been reported. Some authors have demonstrated that the enzymatic activity is one of the main mechanisms used by fungi for of attack and penetration through egg shells (Araujo et al. 2009; Braga et al. 2010). This may explain the differences in the percentage results for effect type 3 between the two studies.

The first mode of mechanical action mentioned for an ovicidal fungus is the appressorium, which is the structure used to penetrate the helminth eggs (Lysek, 1978). Mizobutsi et al. (2000) evaluated the effect of 64 fungal isolates on *Meloidogyne javanica* eggs and found that *P. chlamydosporia* showed the highest ovicidal activity, confirming its importance as an ovicidal fungus.

The tested isolates (Isolates 31, Isol.1 and Isol.22) also showed ovicidal activity, this is first study that demonstrated their action against gastrointestinal nematode parasites of domestic animals.

Several field works aimed at controlling agricultural pests have been carried out and demonstrated that *P. chlamydosporia* is effective in reducing nematode populations (Kerry, 2001; Tobin et al. 2008). However, there is a scarcity of studies on the use of this fungus to control helminthiasis in domestic animals.

Hansen and Perry (1994) pointed out serious concerns with the continued use of antihelminthics. Parasite resistance to one or many anithelminthics is now widespread, particularly in domestic animals, leaving many countries without means of controlling

gastrointestinal nematode parasitism. Therefore, the biological control of gastrointestinal nematodiosis using ovicidal nematophagous fungi can be an important tool combined with the chemical control.

The results of this work demonstrate that *P. chlamydosporia* (Isol.5, Isol.31, Isol.1, VC1, Isol.12, Isol.22 and VC4) could be a potential biological control agent against *A. suum*.

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Capítulo 2

Biological control of *Ascaris suum* eggs by *Pochonia chlamydosporia* fungus

Veterinary Research Communications, in press (impact factor 1.05)

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Abstract

Ascaris suum is a gastrointestinal nematode parasite of swines. The aim of this study was to observe *Pochonia chlamydosporia* fungus on biological control of *A. suum* eggs after fungus passage through swines gastrointestinal tract. Eighteen pigs, previously dewormed, were randomly divided into three groups: group 1, treated with the fungus isolate VC4; group 2, treated with the fungus isolate VC1 and group 3 did not receive fungus (control). In the treated groups, each animal received a 9g single dose of mycelium mass containing *P. chlamydosporia* (VC1 or VC4). Thereafter, animal fecal samples were collected at the following intervals: 8, 12, 24, 36, 48, 72 and 96 hours after treatment beginning and these were poured in Petri dishes containing 2% water-agar culture medium. Then, one thousand *A. suum* eggs were poured into each dish and kept in an incubator at 26^{o}C and in the dark for 30 days. After this period, approximately one hundred eggs were removed from each Petri dish and morphologically analyzed under light microscopy following the ovicidal activity parameters. The higher percentage observed for isolated VC4 eggs destruction was 57.5% (36 hours) after fungus administration and for isolate VC1 this percentage was 45.8% (24 hours and 72 hours) ($p>0.01$). *P. chlamydosporia* remained viable after passing through the gastrointestinal tract of swines, maintaining its ability of destroying *A. suum* eggs.

Keywords: *Ascaris suum*, *Pochonia chlamydosporia*, Nematophagous fungi, Biological control

Introduction

Swines are affected by many parasites species resulting in considerable production losses, as a result of the low feed conversion rate and animal growth, increased susceptibility to other diseases and organ condemnation at slaughter (Wagner and Polley 1999). Among the parasites, stand out the gastrointestinal helminths, represented by nematodes of the genera: *Ascaris*, *Oesophagostomum*, *Strongyloides*, *Trichuris* and

Hyostrongylus prevalent in different geographic areas (Roepstorff et al. 1998; D'Alencar et al. 2006).

Ascaris suum is common among swines raised in extensive management systems in the world (Nansen and Roepstorff 1999). This parasite is usually associated with liver damages ("milk spots" caused by larvae migration), resulting in organ condemnation. *A. suum eggs* stand out for their resistance in the environment, remaining viable for long periods (Urquhart et al. 1998). Like other ascarids, it eggs can contaminate soil, water and food (Paulino et al. 2001; Gupta et al. 2009), once present in the food they are not easily removed, as they have a great capacity of adhering to surfaces (Massara et al. 2003). The changes in breeding system can decrease this nematode infection rates, however, the infection agents can persist even in farms with good management practices (Roepstorff and Nansen 1994).

Among the natural antagonists of gastrointestinal helminthes, for eggs and larvae present in environment, nematophagous fungi stand out (Gronvold et al. 1996; Larsen 1999). Several works have reported the success of these fungi in biological control under laboratory conditions (grown fungus on solid medium, in vitro) and in natural conditions, passage through domestic animals gastrointestinal tract, with germination and growth in feces (Braga et al. 2010a; Tavela et al. 2010). Among the fungi species used, *P. chlamydosporia* has been successfully tested *in vitro* (Ferreira et al. 2010) and after passing through the gastrointestinal tract of domestic animals (Braga et al. 2010a; Singh et al. 2010), however, there is no report about its passage through gastrointestinal tract of swines with subsequent germination and growth.

The aim of this study was to observe *P. chlamydosporia* fungus on biological control of *A. suum* eggs after fungus passage through swines gastrointestinal tract.

Materials and methods

Study area

The experiment was conducted at the Swine experimental sector of the Federal University of Viçosa, located in the city of Viçosa, Minas Gerais, Brazil, latitude 20° 45'20 "and longitude 42° 52'40.

Fungi strains

Two fungal isolates (VC4 and VC1) of *P. chlamydosporia*, originated from Brazilian soil, were tested for their ability passing through gastrointestinal tract of swines. Disc cultures of isolates (VC1 and VC4) kept in test tubes containing the culture medium 2% water-agar (2% WA) were transferred to Erlenmeyer flasks containing 150ml of liquid medium GPY (glucose, sodium peptone and yeast extract), and these were kept shaking at 120 rpm in the dark at a temperature of 26ºC for 10 days. After this period, the mycelia were removed, filtered and weighed on analytical balance (Braga et al 2010).

Obtaining of *A. suum* eggs

The eggs of *A. suum* were recovered from the dissection of a mature female worm obtained from a swine which died of natural causes. Then, these eggs were morphologically analyzed under light microscopy, objective lens 10x, according to the description of Urquhart et al. (1998). Thereafter, the eggs were washed by centrifugation 10 times in distilled water and centrifuged at 1,000 rpm for 5 minutes each time. The supernatant was discarded at the end of each centrifugation cycle. Eggs were then incubated to embryonate at 25ºC for 21 days with a solution containing 0.005% streptomycin sulphate and 0.01% chloramphenicol as described by Araújo et al. (1995).

In vivo essay

It was used eighteen crossbred swines, male, with average weight of 32.08 kg (± 1.83), kept in masonry bays, previously dewormed with swines anthelmintic, febemdazole 4% (Intervet®) orally, using a dose of 0.12 mg per kilogram of body weight, 14 days before receiving the fungal treatment, according to the methodology described by Braga et al. (2010a). Then, the animals were divided into three groups, with 6 animals each. (1) group treated with the fungus (VC4), (2) group treated with the fungus (VC1) and (3) group that did not receive fungus (control). In the treated groups (1 and 2) each animal received 9g of mycelium fungi mass in a single dose, containing *P. chlamydosporia* isolates (VC4 or VC1) mixed in 100 grams of swine commercial food (cottonseed meal, sodium chloride, Corn gluten meal 21, soybean meal, wheat bran, flour of meat and bones of cattle, dicalcium phosphate, corn germ, ground whole corn, Mineral premix and vitamin premix) according to the methodology described by Braga et al. (2010a).

Fecal samples from each animal were collected at 8, 12, 24, 36, 48, 72 and 96 hours interval after the fungus administration. Samples were homogenized; and feces (2 g) were

removed of each sample and placed in Petri dishes, 9 cm diameter, containing 2% WA, for each interval collected there was 18 dishes, six dishes for each group. One thousand embryonated eggs of *A. suum* were placed in each dish of treated and control groups, and incubated at 26° C in the dark for 30 days (Braga et al 2010). Petri dishes of treated and control groups were examined daily to assess *P. chlamydosporia* (VC1 or VC4) characteristic structures, such as conidia, conidiophores and chlamydospores, which were analyzed according to the classification proposed by Zare et al. (2001).

After thirty days, one hundred eggs were removed from each Petri dish of treated and control groups according to the technique described by Araújo et al. (1995) and placed on glass slides with a drop of Aman blue 1%. Eggs were examined under a light microscope (40X objective lens) using the parameters established by Lysek et al. (1976): effect of type 1; type 2 and type 3 (eggs destruction).

Statistical analysis

Data were submitted to Friedman at 1% probability non parametric statistical test (Ayres et al. 2007).

Results

At Table 1 are demonstrated the results of *P.chlamydosporia,* isolates VC4 or VC1, ovicidal activity on *Ascaris suum* eggs in different intervals (8, 12, 24, 36, 48, 72 and 96 hours). The first characteristic structures of *P.chlamydosporia* to be observed in dishes of treated groups with VC1 and VC4 were the chlamydospores. Later, during the study, it was also possible to verify *P. chlamydosporia* typical hyphae and conidia presence on the dishes of the treated groups. *P. chlamydosporia* showed ovicidal activity against *A. suum* eggs after passing through swines gastrointestinal tract, and this activity remained at all intervals. In the dishes of the control group (without fungus) was not observed the presence of chlamydospores, hyphae and conidia of *P. chlamydosporia*.

Although isolate VC4 showed a higher percentage of type 3 effect, observed most of the time, there was not statistical difference between the isolates tested ($p > 0.01$) referring to ovicidal activity.

Table 1-Percentages and standard desviations of ovicidal activity of the isolates (VC1 and VC4) *Pochonia chlamydosporia* and control against *Ascaris suum* eggs referring to the collection of 8, 12, 24, 36, 48, 72 and 96 hours, after 30 days of interaction.

Groups	Effect at 8 hours		
	effect type 1*	effect type 2**	effect type 3***
VC4	49.8^{A}±8.1	29.3 ^{A} ±2.1	20.8 ^{A} ±6.8
VC1	47.5 ^{A} ±8.8	27.5 ^{A} ±6.8	26.6 ^{A} ±4.0
Control	0 ^{B} ±0	0 ^{B} ±0	0 ^{B} ±0
	Effect at 12 hours		
VC4	31.6 ^{A} ±11.2	33.3 ^{A} ±9.8	35.0 ^{A} ±10.9
VC1	30.0 ^{A} ±15.1	33.3 ^{A} ±8.7	36.6 ^{A} ±10.3
Control	0 ^{B} ±0	0 ^{B} ±0	0 ^{B} ±0
	Effect at 24 hours		
VC4	15.0 ^{A} ±6.3	32.5 ^{A} ±6.1	52.5 ^{A} ±6.1
VC1	16.6 ^{A} ±8.7	38.3 ^{A} ±6.8	45.8 ^{A} ±9.9
Control	0 ^{B} ±0	0 ^{B} ±0	0 ^{B} ±0
	Effect at 36 hours		
VC4	15.0 ^{A} ±4.4	27.5 ^{A} ±7.5	57.5 ^{A} ±11.2
VC1	19.1 ^{A} ±8.0	30.8 ^{A} ±8.1	50.0 ^{A} ±6.3
Control	0 ^{B} ±0	0 ^{B} ±0	0 ^{B} ±0
	Effect at 48 hours		
VC4	18.3 ^{A} ±10.3	31.6 ^{A} ±5.1	50.0 ^{A} ±8.3
VC1	38.1 ^{A} ±5.2	32.1 ^{A} ±5.2	29.6 ^{A} ±1.8
Control	0 ^{B} ±0	0 ^{B} ±0	0 ^{B} ±0
	Effect at 72 hours		
VC4	15.0 ^{A} ±8.3	28.3 ^{A} ±5.1	56.6 ^{A} ±7.5
VC1	19.2 ^{A} ±8.0	35.0 ^{A} ±6.3	45.8 ^{A} ±4.9
Control	0 ^{B} ±0	0 ^{B} ±0	0 ^{B} ±0
	Effect at 96 hours		
VC4	18.3 ^{A} ±8.7	40.8 ^{A} ±6.6	40.8 ^{A} ±5.8
VC1	22.5 ^{A} ±11.2	32.5 ^{A} ±4.1	45.0 ^{A} ±8.9
Control	0 ^{B} ±0	0 ^{B} ±0	0 ^{B} ±0

Percentages followed by the same letter, in the line, are not significantly different (p>0.01). *Physiological and biochemical effects without morphological damage to the eggshell, with hyphae adhered to the shell. **Lytic effects with morphological alterations of the embryo and eggshell, without hyphal penetration. ****Lytic effect with morphological alteration of the embryo and eggshell, in addition to hyphal penetration and internal colonization.

Discussion

Fungus utilization in intestinal parasites helminths eggs and larvae biological control must pass through domestic animals gastrointestinal tract, grow in feces and predate nematode eggs (Larsen, 1999). One of the factors that can favor this passage is chlamydospores production (resistance structures). In the present work, chlamydospores production in Petri dishes of groups treated with *P. chlamydosporia* (VC1 and VC4) was observed, demonstrating that the fungus might be able to survive after passing through swines gastrointestinal tract. Still, for chlamydospores production is important to stand out viability this structure for its use in environmental biological control (Larsen 1999; Terril et al. 2004)

The present study showed *A. suum* eggs destruction (type 3 effect) by *P. chlamydosporia* isolates (VC4 and VC1) after a long period of interaction. These results are in agreement with Ferreira et al. (2010) and Araújo et al. (2008), that also reported the destruction of *A. suum* eggs after twenty one days of interaction *in vitro*, with *P. chlamydosporia* (VC4 and VC1) grown in 2%WA medium. However, these studies have been conducted only in laboratory conditions, the fungus was already grown. In this study the fungus *P. chlamydosporia* passed through the swines gastrointestinal tract, germinated from their feces and destroyed the *A. suum* eggs at the end of thirty days of interaction. Still in comparisons to the work of Ferreira et al. (2011) and Araújo et al. (2008), the obtained destruction percentages for VC4 and VC1 were the following: (57.67% and 55.3%) and (19% and 20%), respectively, at the end of study. The results in the present study were similar (Table 1), at little different conditions. Costa et al. (2001) argued that 2%WA medium did not reflect the environmental conditions and so little the habitat in which nematophagous fungi (natural antagonists) are present. In that way, studies that might mimic these fungi environment, in this case the fecal environment, are important. Moreover, according to Araújo et al. (2010) predatory and ovicidal nematophagous fungi are generally saprophytic and therefore should be routinely tested under experimental conditions in order to prove its predatory activity in adverse conditions.

Present results obtained can be compared to what is already known in the literature, being that: Lysek (1976) reported that fungi may be considered as ovicidal if it presents type 3 effect-destruction of eggs. De et al. (2008) and Braga et al. (2010a) investigated *P. chlamydosporia* ovicidal activity against trematode and nematode eggs and noted that this

fungus was effective destructing eggs. The present study is in accordance with the literatures.

According to Braga et al. (2010b) it is important that the fungus have quick ovicidal action and that it remains for long time, thus, it can be to used in control of helminth eggs with short and long hatching period. The results presented and discussed in this study suggest that *P. chlamydosporia* could be used in biological control of *A. suum* eggs present in the environment.

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Capítulo 3

Nematophagous fungi against infective larvae *Oesophagostomum* spp of pigs

Article submitted to Veterinarni Medicina (impact factor 0.644)

Abstract

Pigs are affected by many species of gastrointestinal nematodes of the genera *Ascaris* and *Oesophagostomum*. Two experimental assays (A and B) evaluated the action of conidia of the nematophagous fungi *Duddingtonia flagrans* (AC001), *Monacrosporium sinense* (SF53) and *Artrhobotrys robusta* (I-31) against infective larvae (L_3) of *Oesophagostomum* spp in 2% water-agar (2%WA) medium and coprocultures. The first assay consisted of three groups of 1000 *Oesophagostomum* L_3 treated with 1000 conidia of isolates AC001, SF53 and I-31 and control without fungus plated in 2% WA. In the second assay, 1000 conidia of the same isolates were added to 20g of feces and incubated at 26°C for 8 days. The L_3 were recovered after this period. Fungal treatments in the first assay were efficient in the capture of *Oesophagostomum* spp L_3 ($p<0.01$) when compared to the control treatment(without fungi). There was no variation in the predatory capacity among the tested fungal isolates ($p>0.01$) during the experimental period of seven days. There were significant reductions ($p<0.01$) of 94.4%, 92.9% , and 88.3% in the means of *Oesophagostomum* L_3 recovered from the treatments with isolates AC001, SF53 and I-31, respectively, when compared to the control treatment. Significant difference ($p<0.01$) was found between the number of L_3 recovered from the treated groups and the control group at the end of assay A. Assay B also showed statistical differences ($p<0.01$) between the means of recovered L_3 in the treated groups and the control: 75.3% (AC001), 63.7% (SF53) 63.3% (I-31). In this study, the three isolates of predatory fungi *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) were efficient in the *in vitro* capture and destruction of *Oesophagostomum* L_3 and are potential biological control agents of this nematode.

Keywords: Biological control, nematophagous fungi, *Oesophagostomum*, pigs.

1. Introduction

Helminths that infect pigs are widely varied in size, life cycle and pathogenicity. Diseases caused by internal and external parasites are reported in pig farms worldwide. The infections are not always apparent and persist at subclinical levels for extended periods, leading to death of animals (Sobestiansk et al., 1998). The genus *Oesophagostomum,* with worldwide distribution, it is among the main intestinal helminths

infecting pigs. The major species are *O. dentatum* and *O. quadrispinulatum* (Stewart and Gasbarre, 1989). *Oesophagostomum* infection occurs through the ingestion of infective larvae (L_3) present in the environment. Roepstorff and Jorsal (1989) reported the occurrence of helminths in 66 pig herds in Denmark, with 58% of *Oesophagostomum* spp.

Roepstorff and Nansen (1994) pointed out that the expanding technology in production systems improves sanitary conditions by creating monitoring systems that reduce chances of parasite transmission. However, pigs raised extensively on pasture demand reformulated control measures against parasites, with special attention to the control of free-living infective stages. In this case, the control with nematophagous fungi is suggested because their action is concentrated in the fecal environment and directed against the free-living forms (Larsen, 1999).

Nematophagous fungi are classified into predators, opportunists and endoparasites. The genera *Duddingtonia*, *Monacrosporium* and *Arthrobotrys* of the predator group are the most studied for the control of nematodiosis in domestic animals (Braga et al., 2010a). *D. flagrans* is considered the most promising because of the production of large amounts of chlamydospores. *M. thaumasium* and *A. robusta* have been successfully used in the control of infective larvae of gastrointestinal nematodes of domestic animals in laboratory and field conditions (Carvalho et al., 2010; Silva et al., 2010; Silva et al., 2010). However, there are not reports on the action of nematophagous fungi in the control of gastrointestinal nematodes of pigs.

This study evaluated the *in vitro* predatory activity of the fungi *D. flagrans*, *M. sinense* and *A. robusta* against larvae of *Oesophagostomum* spp in two experimental assays.

2. Material and Methods

2.1. Fungal culture

Isolates of *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) were obtained from a Brazilian soil, in Viçosa, Zona da Mata region, State of Minas Gerais, between 20° 45'20"S and 42 ° 52'40" W longitude, 649 m altitude. The isolates were kept in test tubes with 2% corn-meal-agar (2% CMA), at 4°C in the laboratory of Parasitology of the Federal Universidad of Viçosa.

2.2. Conidia Collection

Culture disks (4 mm in diameter) of fungal isolates in 2% CMA were removed from the test tubes and transferred to 9.0 cm Petri dishes, containing 20 ml of 2% potato dextrose agar and kept at 25 °C in the dark for 10 days. After growth, new culture disks (4 mm in diameter) were transferred to 9.0 cm diameter Petri dishes containing 20 ml of 2% water-agar (2% WA). Distilled water (1 ml) containing 1000 larvae of *Panagrellus* sp was daily added to the plates to induce conidial formation for 21 days. After complete fungal development, 5 mL of distilled water were added to each plate and conidia and mycelial fragments were removed using technique described by Carvalho et al. (2009).

2.3. *Oesophagostomum* spp L_3

Infective larvae (L_3) of *Oesophagostomum* spp. were recovered from feces of naturally infected pigs using vermiculite coproculture for 15 days. At the end of this period, third stage larvae (L_3) were obtained by the Baermann method, with water at 42-45^{o}C and 12h decantation time. Larvae were identified according to Ueno and Gonçalves (1998).

2.4. Assays

Two *in vitro* assays (A and B) were carried out with an interval of eight days. Assay A evaluated fungal activity of conidia of *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) on *Oesophagostomum* L_3 in 2% WA. Assay B evaluated the action of conidia of isolates AC001, SF53 and I-31 in coprocultures containing *Oesophagostomum* larvae.

2.5. Assay A

Assay A consisted of four treatments of fungal isolates (AC001, SF53 and I-31) and a control without fungus plated in 9.0cm Petri dishes containing 20 ml of 2%WA, with six repetitions each. Petri dishes were marked into 4mm fields. A thousand *Oesophagostomum* L_3 were plated with 1000 conidia of the fungal isolates. The control contained 1000 L_3 without fungus, according to the methodology used by Braga et al. (2010a). Ten random fields (4mm diameter) were examined per plate of the treated and control groups, using an optical microscope (10 X objectives) for L_3 counts, every 24 hours, for seven days. After 7

days, the non-predated L_3 were recovered from the Petri dishes using the Baermann method, with water at 42 ºC (Araújo et al., 1993).

2.6. Assay B

Fresh feces with positive EPG were used for preparing coprocultures, which were mixed with autoclaved and moistened vermiculite. Treatments consisted of four groups of fungal isolates (AC001, SF53 and I-31) and a control without fungus, with six repetitions each. Each replicate received 1000 fungal conidia. The control group contained fecal culture only. Coprocultures, from treated and control groups, were incubated at 26 °C and for 8 days. At the end of this period, L_3 were recovered by the Baermann method, which were identified and quantified according to the criteria described by Ueno and Gonçalves (1988) under optical microscope (10 x objectives).

2.7. Statistical analysis

Means of *Oesophagostomum* L_3 recovered from tests A and B were examined by analysis of variance at 1% probability level (Ayres et al. 2007). Predation efficiency of L_3 relative to the control group was assessed by the Tukey's test at 1% probability level. The percent reduction in means of recovered L_3 was calculated by the following equation:

$$\text{Reduction \%} = \frac{(\text{Mean of } L_3 \text{ recovered from control group} - \text{Mean of } L_3 \text{ recovered from treated group}) \times 100}{\text{Mean } L_3 \text{ recovered from control group}}$$

3. Results and Discussion

Isolates of predatory fungi *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) were capable to prey *Oesophagostomum* L_3 in assay A.

No significant difference ($p>0.01$) was found in the comparison of capture and destruction of *Oesophagostomum* L_3 among plates of the groups treated with isolates AC001, SF53 and I-31 during the experimental assay (Table 1). However, there was difference ($p<0.01$) between the means of non-predated *Oesophagostomum* L_3 per 4mm diameter field in the plates of the control group and means of L_3 recorded in the fungi-treated groups. The recorded percent reduction in *Oesophagostomum* L_3 were: 94.4% (AC001), 92.9% (SF53) and 88.3% (I-31).

Table 1- Daily means and standard deviation (±) of infective larvae (L_3) not predated of *Oesophagostomum* spp by field 4 mm diameter in 2% water-agar during the period of seven days, for treatments with the fungal isolates *Duddingtonia flagrans* (AC001), *Monacrosporium sinense* (SF53), *Arthrobotrys robusta* (I-31) and in control without fungus.

Time (days)	**Treatments** (Means of non-predated *Oesophagostomum* L_3 per 4mm)			
	AC001	**SF53**	**I31**	**Control**
1	$3.86^{a\,b} \pm 2.4$	$4.98^{a} \pm 2.8$	$1.78^{b} \pm 1.0$	$11.8^{c} \pm 7.6$
2	$1.21^{a} \pm 1.26$	$1.41^{a} \pm 1.02$	$1.43^{a} \pm 0.72$	$4.15^{b} \pm 5.15$
3	$1.36^{a} \pm 1.17$	$1.76^{a} \pm 1.35$	$1.5^{a} \pm 1.2$	$2.53^{b} \pm 1.56$
4	$1.4^{a} \pm 1.2$	$1.8^{a} \pm 1.3$	$1.4^{a} \pm 1.3$	$2.5^{b} \pm 1.5$
5	$1.5^{a} \pm 1.08$	$1.68^{a} \pm 0.9$	$1.18^{a} \pm 0.8$	$5.01^{b} \pm 4.6$
6	$0.73^{a} \pm 0.8$	$0.71^{a} \pm 0.9$	$0.91^{a} \pm 0.9$	$4.81^{b} \pm 3.6$
7	$1.08^{a} \pm 1.0$	$1.8^{a} \pm 1.4$	$2.96^{a} \pm 2.7$	$11.98^{b} \pm 7.7$

Means followed by at least one common letter, in the lines, are not significantly different by the Tukey's test at a 1% probability level.

Nematophagous fungi were not observed in plates of the control group during the experiment, so this shown variation in the percentage of larvae observed in the table, probably, it is due to migration of larvae to regions of the plates where there was more humidity. The presence of *Oesophagostomum* L_3 was essential for trap formation in plates containing nutrient-poor medium such as 2% WA. Evidence of predation was confirmed by the means of recovered L_3 using the Baermann method 7 days post-plating, at the end of the assay A (Fig. 1). Difference ($p<0.01$) was found between the number of recovered L_3 in the treated groups and the control without fungi.

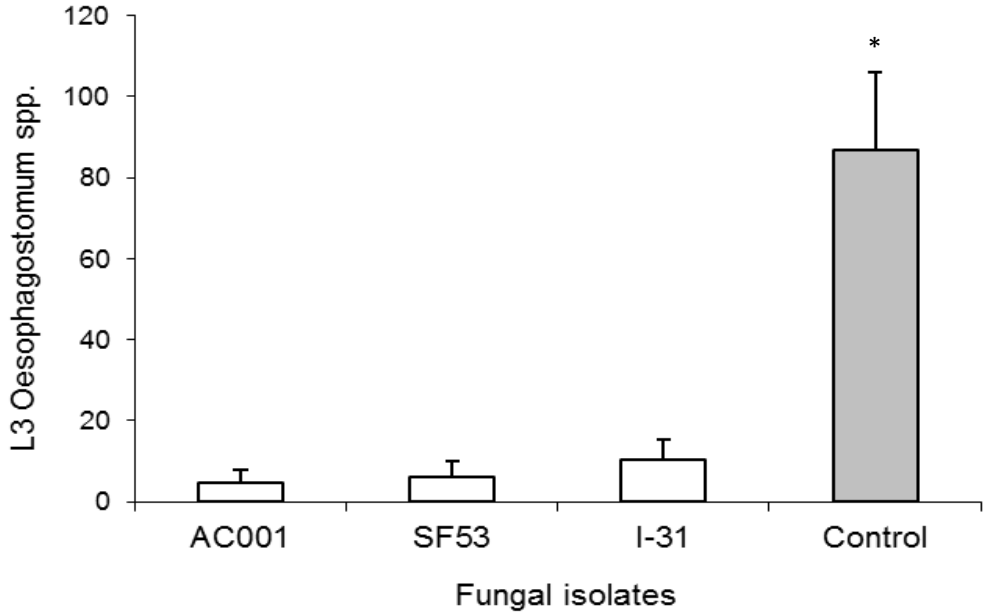

Fig. 1 -Means and standard deviation (bars) of infective non-predated *Oesophagostomum* spp. larvae recovered from 2% water-agar plates by the Baermann method on the seventh day of treatment with the following fungal isolates: *Duddingtonia flagrans* (AC001), *Monacrosporium sinense* (SF53), *Arthrobotrys robusta* (I-31) and a control group (without fungi). Asterisk denotes significant difference ($p<0.01$) between the fungus-treated group and the control - Tukey's test at a 1% probability level.

At the end of 8 days, means of recovered larvae in assay B showed that fungal conidia of *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) were effective in reducing *Oesophagostomum* L_3 (Fig. 2). Significant difference ($p<0.01$) was found between the means of larvae recovered from the treated groups and the control group with the following percent reductions: 75.3% (AC001), 63.7% (SF53) and 63.3% (I-31).

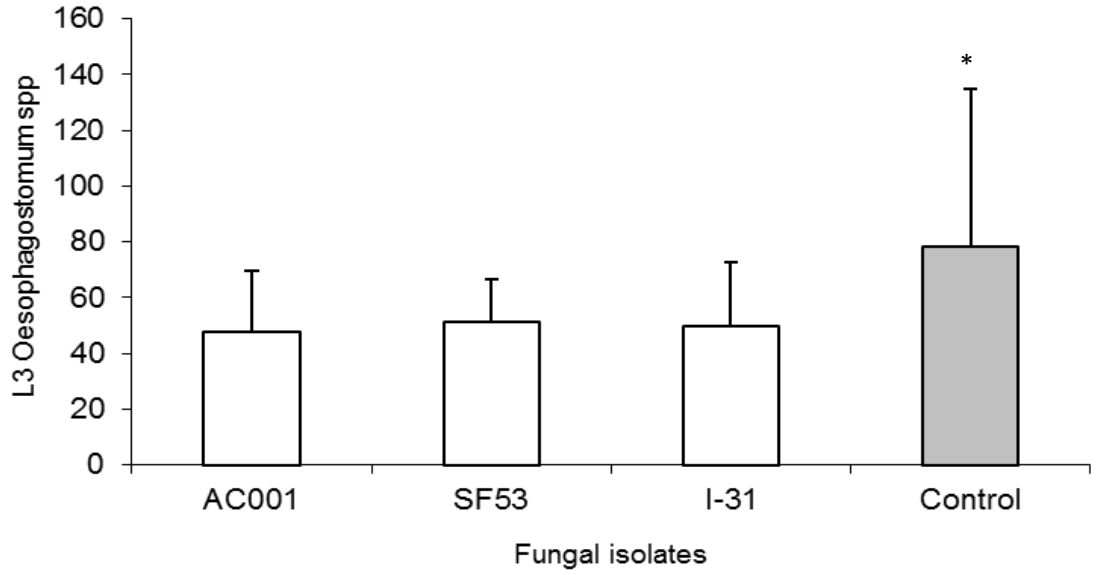

Fig. 2 - Means and standard deviation (bars) of infective non-predated *Oesophagostomum* spp. larvae recovered from coproculture by the Baermann method on the seventh day of treatment with the following fungal isolates: *Duddingtonia flagrans* (AC001), *Monacrosporium sinense* (SF53), *Arthrobotrys robusta* (I-31) and a control group (without fungi). Asterisk denotes significant difference ($p<0.01$) between the fungus-treated group and the control - Tukey's test at a 1% probability level.

The present study showed that conidia of predatory fungi *D. flagrans*, *M. sinense* and *A. robusta* were capable of preying ($p>0.01$) *Oesophagostomum* spp. L_3 at the end of the experimental assay A. Isolate AC001 showed efficiency in the capture and destruction of L_3, with percent reduction of 94.4% L_3 recovered from the plates after 7 days. These results are consistent with reports by Braga et al. (2010a) that isolate AC001 was more efficient in preying *Ancylostoma ceylanicum* L_3, a geohelminth that affects dogs. Braga et al. (2010b) also showed that isolate AC001 stored in silica-gel for seven years was efficient in the capture and destruction of *Haemonchus contortus* L_3. These findings confirm the efficiency and applicability of *D. flagrans* (AC001).

M. sinense (SF53) and *A. robusta* (I-31) were also shown efficient in the capture and destruction of *Oesophagostomum* spp L_3 with percent reductions of 92.9% and 88.3%, 7 days post-plating. Araujo et al. (2010) reported recently that *M. thaumasium* (NF34) and *A. robusta* (I-31) effectively captured and destroyed *Strongyloides westeri* L_3. These

results show that the nematophagous fungi can be used to control a wide range of larval-stage nematodes.

According to Nansen et al. (1988), a greater mobility of nematodes increases the stimulus for trap-formation and predation of nematophagous fungi. This was also found in our study, as we observed predation of L_3 from the first day, after 24 hours of interaction.

In assay B, conidia of isolates AC001, SF53 and I-31 were effective in reducing ($p>0.01$) *Oesophagostomum* L_3 of coprocultures. However, at 8 days post-plating, isolate AC001 showed the highest percent reduction (75.3%). In a recent study, Silva et al. (2010) reported that in the concentration of 1000 conidia, AC001 was effective in reducing *H. contortus* L_3 after eight days of coproculture, providing for an 87.5% percent reduction. Braga et al. (2009) showed that AC001 was effective in reducing cyathostomin L_3 recovered from coprocultures of horses with positive EPG. In the present study, conidia of isolates *M. sinense* (63.7%) and *A. robusta* (63.3%) in the same concentrations were also effective in reducing the *Oesophagostomum* L_3 in the same interval. Araújo et al. (2006) found no difference ($p>0.05$) between the associations and the various fungal isolates, including *M. sinense* and *A. robusta,* used to prey *Cooperia* sp. and *Oesophagostomum* sp., which agrees with our findings, indicating that nematophagous fungi can be successfully used to reduce recurrent infections.

The difference between percent reduction of larvae of the tests A and B, (AC001), 92.9% (SF53), 88.3% (I-31) and 75.3% (AC001), 63.7% (SF53), 63.3% (I-31), respectively, it is probably related to the fact that the environment of the coprocultures there is greater amount of organic matter and as fungi are saprophyte, they had part of ther action was not directed to the larvae but to for organic matter.

Choosing a nematophagous fungus that produces effective results in nematode control should consider both *in vitro* and field tests. Our results suggest the need for future field studies using AC001, SF53 and I-31 against gastrointestinal nematodes in pigs.

Here, we report that conidia of *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) were efficient in the capture and destruction of L_3 of *Oesophagostomum* spp. The fungi caused reduction in the number of L_3 recovered from the coprocultures. The main route of infection of *Oesophagostomum* spp. is the ingestion of infective L_3 present in the environment. Thus, the findings of this study show that these fungi can help in the control of this nematode.

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Capítulo 4

In vitro predatory activity of nematophagous fungi *Duddingtonia flagrans* on infective larvae of *Oesophagostomum* spp. after passing through gastrointestinal tract of pigs

Tropical Animal Health and Production (impact factor 0.95). Article in press (2011) DOI: 10.1007/s11250-011-9848-7

Abstract

One isolate of predator fungi *Duddingtonia flagrans* (AC001) was evaluated *in vitro* regarding to the capacity of supporting the passage through pigs gastrointestinal tract without losing the ability to prey infective larvae *Oesophagostomum* spp. The fungal isolate survived the passage and was efficient in preying L_3 since the first 8h of collection ($p<0.01$) in relation to the control group (without fungus). Compared to control, there was a significant decrease ($p<0.01$) of 59.6% (8 h), 71.7% (12 h), 76.8% (24 h), 81.0% (36 h), 78.0% (48 h), 76.1% (72 h) e 82.7% (96 h) in the means of infective larvae *Oesophagostomum* spp. recovered from treatments with the isolate AC001. Linear regression coefficients of L_3 of recovered *Oesophagostomum* spp. regarding the collections due to time were -0.621 for control, -1.40 for AC001.The fungus *D. flagrans* (AC001) had demonstrated to be promising for use in the biological control of pig parasite *Oesophagostomum* spp.

Key words: Nematophagous fungi; *Duddingtonia flagrans*; *Oesophagostomum*; pigs

1. Introduction

Pigs reared are susceptible to a wide range of parasitic infections which result in considerable financial losses in pigs' production. Such losses are incurred as a result of: low growth rates, compromised alimentary conversion the animals, increased susceptibility to secondary infections and increased quantity of condemned flesh at time of slaughter (Haugegaard 2010; Lai et al. 2011). Among these parasites, gastrointestinal helminthes are far much prevalent than any other group, being represented by the genera *Ascaris*, *Oesophagostomum*, *Strongyloides*, *Trichuris* and *Metastrongylus* spp, with each having a specific geographical area of prevalence (Boes et al. 2000; Haugegaard 2009; Lai et al. 2011). Nematodes of the genus *Oesophagostomum* have a worldwide distribution, with principal etiological species infecting pigs being *O. dentatum* and *O. quadrispinulatum* (Lin et al. 2008). In Denmark, Haugegaard (2010) reported that a study conducted on 79 farms, 15% were infected with *Oesophagostomun* ssp. and some farms the prevalence rate for this nematode has reached 50%. The mode of infection is principally characterized by oral ingestion of infectious larvae (L_3) found in contaminated environment.

In recent years there has been a growing emphasis on the need to develop new alternatives for additional the control of nematode parasites of domestic animals, two of the reasons are anthelmintic resistance in populations of nematodes and consumers choose for foods with less chemical waste produced in a sustainable way in relation to the environment (Blair 2007; Sanyal 2001)

In this context, it is suggested that biological control using nematophagous fungi that prey on the infectious forms of selected specific nematodes is a viable way of naturally discontaminating the rearing environment (Araújo et al., 2008). Prior studies in laboratory, as well as in open field conditions have shown that nematophagous fungi are capable of controlling infectious forms (L_3) of a wide variety of gastrointestinal parasites in domestic animals with success; with special distinction to the species *Duddingtonia flagrans* (Sagüés et al. 2011; Santurio et al. 2011). However, for a fungus species to be considered effective in controlling a range of specified parasites, it is important that it conserves its predative properties after a complete gastrointestinal transit, besides successfully capturing the aforementioned prey (Waller et al., 1994). The viability of the fungus species *D. flagrans* (AC001) has been widely discussed in literature with respect to studied populations of ruminants, equines and domestic dogs (Braga et al. 2009; Carvalho et al. 2009; Santurio et al. 2011), where the fungus maintained its predative characteristic on L3 of nematodes, after passage by tract gastrointestinal those animal. Contrastingly, there is few literature discusses on the applicability of this fungus in pigs nematodes. The objective of this work was to evaluate the predatory capacity of the fungus *D. flagrans* (AC001) on infective larvae (L_3) of *Oesophagostomum* spp. after passing through gastrointestinal tract of pigs.

2. Material and methods

2.1. Fungal cultures

One isolate of nematophagous fungi *D. flagrans* (AC001) was used. The isolate was stored in test tubes containing 2% corn-meal-agar (2% CMA), in the darkness, at 4 ºC for 10 days**.** Such isolate was obtained from Brazilian agricultural soil.

Fungal mycelia were obtained by transferring culture disks (approximately 5 mm in diameter) of fungal isolates in 2% CMA to 250 mL Erlenmeyer flasks with 150 mL liquid potato-dextrose medium (Difco), pH 6.5, and incubated under agitation (120 rpm), in the

dark at 26° C, for 10 days. After this period, the mycelia were extracted, filtered and weighed using an analytic balance.

2.1.2. Obtaining of infective larvae (L_3) *Oesophagostomum* spp.

L_3 of *Oesophagostomum* spp. were obtained from feces of naturally infected pigs through stool culture in vermiculite, for 15 days, and posterior use of Baermann technique, with water at 42–45°C and decantation time of 12 h identified according to Ueno and Gonçalves (1998).

2.2. Experimental test: Efficacy test about L_3 of *Oesophagostomum* spp. after passage through pigs gastrointestinal tract.

The experiment was conducted at the swino experimental sector of the Federal University of Viçosa, Viçosa, MG-Brazil, latitude 20°45′20″S, longitude 42°52′40″W. Twelve male pigs, average weight of 32.08 kg (±1.33), which were kept in concrete pig-sty, were previously dewormed with Febemdazole 4% 0.12µg live weight (Intervet®). Fourteen days after the anthelmintic treatment, the male were randomly separated into two groups of six animals each (fungus-treated and control) and kept in separate stalls.

The animals received water ad libitum and were daily fed with concentrated and balanced ration for pigs. Each animal of the treated group received 9 g mycelium, as a single dose, containing *D. flagrans* mixed in 100 g of ration. Animals of the control received only ration without mycelium.

Fecal samples from animals were collect at 8, 12, 24, 36, 48, 72 and 96 h after administration of fungi. From each sample 2 g of feces was removed, homogenized and placed in Petri dishes, 9-cm diameter with 2%WA and incubated in the dark, at 25^{o}C, in the dark. In these Petri dishes 200 L_3 of *Oesophagostomum* spp. was spread. From each established time, six repetitions were performed for fungus-treated and for control. The Petri dishes from the fungus-treated and control groups were daily observed to assess the characteristic structures of *D. flagrans* (AC001), such as conidia, conidiophores and chlamydospores, which were analyzed according to the classification keys proposed by (Van Oorschot 1985). On the 15th day, L_3 not preyed were recovered from the Petri dishes by Baermann technique, obtaining the mean of not preyed larvae per plate.

2.3. Statistical analysis

Obtained data were submitted for variance and regression analysis. Means were compared using the Tukey test in levels of 1% probability (Ayres et al. 2003). The percent reduction in means of recovered L_3 was calculated by the following equation:

$$\text{Reduction \%} = \frac{(\text{Mean of } L_3 \text{ recovered from control group} - \text{Mean of } L_3 \text{ recovered from treated group}) \times 100}{\text{Mean } L_3 \text{ recovered from control group}}$$

3. Results

The fungus *D. flagrans* (AC001) had the capacity of preying infective larvae of pigs *Oesophagostomum* spp. after the passage through gastrointestinal tract, not losing its viability, and were efficient in preying L_3. Regarding the decrease percentage, results were 59.6% (8 h), 71.7% (12 h), 76.8% (24 h), 81.0% (36 h), 78.0% (48 h), 71.1% (72 h) and 82.7% (96 h) of L_3 ($p<0.01$), when compared to the control group. In the Petri dishes of the control group, the presence of nematophagous fungi was not detected. Throughout the experiment, the mean number of L_3 of the control group was larger in relation to that of treated ($p<0.01$). Mean values of *Oesophagostomum* spp. (L_3), recovered of Petri dishes incubated with 2 g of feces from animals of the treated group (with isolate AC001) and animals of the control group (without fungus) are represented in Table 1.

Table 1 - Mean values of infective larvae (L_3) number of *Oesophagostomum* spp. recovered from Petri dishes with swine faeces, sampled in timelines 8, 12, 18, 24, 36, 48 72, 96 h after the treatment with isolates *Duddingtonia flagrans* (AC001) and control (without fungus)

Groups	**Time (h)**						
	8 h	12 h	24 h	36h	48 h	72 h	96 h
AC001	16,23[a]	17,61[a]	14,25[a]	17,9[a]	15,1[a]	10,2[a]	7,81[a]
Control	40,1[b]	62,2[b]	62,6[b]	94,7[b]	68,2[b]	42,9[b]	45,3[b]

Means followed by the same letter, in the column, are not significantly different ($p>0.01$)

The growth of saprophytic fungal, not predator, species made visualization of conidia difficult, which could only be identified on the 10th day of observation in plates

referring to collections of 12 and 48 h for tested isolate. In plates referring to collections of 24 and 72 h, conidia were visualized from the 11th day on. It was observed that the 96-h period was that in which isolate (AC001) had a lesser mean number of recovered L_3, meaning a higher predatory activity.

Coefficients of linear regression curves of L_3 of *Oesophagostomum* spp., recovered from Petri dishes regarding the collections due to time were -0.621 for control and −1.40 for *D. flagrans* (AC001), (Fig. 1).

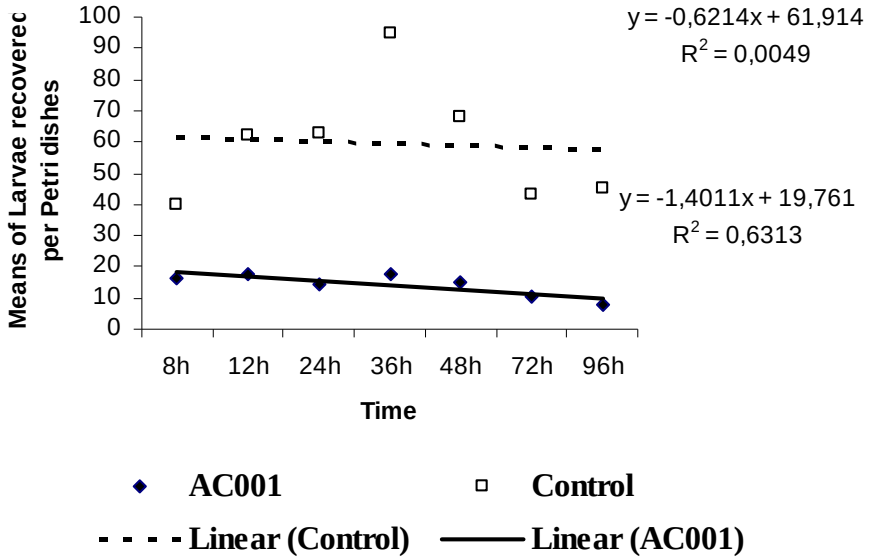

Fig. 1 - Linear regression curves of infective larvae (L3) of Oesophagostomum spp. recovered from Petri dishes regarding collections (8, 12, 24, 36, 48, 72 and 96 h) in treatments with the isolate Duddingtonia flagrans (AC001) and control (without fungus) as a function of time.

4. Discussion

Our experiment show that the fungus *D. flagrans* (AC001) was efficient in destroying the *Oesophagostomum* spp. L_3 even after passing by gastrointestinal tract of pigs. Our results corroborate with those published in other sources which show evidence on the predatory capacity of *D. flagrans* (AC001) on the gastrointestinal nematodes which infect ruminants, domestic dogs and equines, *in vivo* as well as *in vitro* (Braga et al. 2009; Carvalho et al. 2009; Braga et al. 2010; Santurio et al. 2011). However, Araújo et al.

(2006), who worked with various nematophagic fungi including *D. flagrans* (CG768), proved that this isolate was not efficient in controlling *Oesophagostomum* spp. and *Cooperia* spp., *in vitro,* more than either *Arthrobotrys robusta, A. cladodes, Monacrosporium appendiculatum, M. sinense* or *M. thaumasium.* The authors though, did not report any significant difference ($p>0.01$) among the tested isolates. Differently, Campos et al. (2009) observed that the fungus-isolate *D. flagrans* (CG722) was capable of preying upon and subsequently destroying the L_3 of *Strongiloydes pappilosus* at the end of their experiments. However, differences in the inter and intra-specific activity of predatory nematophagous fungi are common and have been observed in experiments with other fungal isolates (Araújo et al. 1993).

According to Mota et al. (2003), this fungus executes its function at optimum conditions constituted by fresh fecal matter. This way, an ideal characteristic of a potential biocontrol of infectious larvae forms should have a considerable capacity to transit the gastrointestinal conditions of domestic animals, as well as able to successfully germinate in excreted fecal matter (Larsen et al. 1999; Singh et al. 2010). Furthermore, the capacity to produce chlamidospores is an important requirement for a fungus to be able to support passage through the gastrointestinal tract of domestic animals (Terril et al. 2004). In the present work, *D. flagrans* (AC001) has satisfied the conditions stipulated by the successful transit of the gastrointestinal tract of pigs.

According to Scholler and Rubner (1994) the traps are formed in response to the presence of nematodes or substances derived from them, may also occur as a result of nutritional limiting conditions and / or water scarcity. Still, according to Nansen et al. (1988), the greater mobility of the nematodes increasing the stimulus to the fungus for the production of traps. The results of this study are consistent with the work mentioned above, since the presence of L3 Oesophagostomum spp was essential for the formation of traps by fungal isolate, because the plates of 2%WA is the environment poor in nutrients.

In other experiments, Petkevicius et al. (1998) demonstrated that different isolates of the nematode-destroying fungus *D. flagrans* were equally able to reduce significantly the number of *O. dentatum* infective larvae *in vitro*. These results are also consistent with the present work. Moreover, Nansen et al. (1996) demonstrated the efficiency this fungus in natural conditions on gastrointestinal nematodes of pigs.

In the experimental test, results of regression curves, represented in Fig.1, demonstrated that the tested isolate (AC001) had negative linear correlation coefficient.

According to Carvalho et al. (2009), the negative value indicates the existence of reverse correlation between variables, which proves the viability of the predatory capacity of fungus isolate after passage through the gastrointestinal tract of domestic animals. In relation to the inverse correlation function in the control group, we infer this finding to the fact that the larvae migrated to the periphens of the Petri dishes which made it difficult to carry out the collection process using the Baermann technique, fact observed too, by Araújo et al. (2006) and Araujo et al. (2010). Above all, the authors suggest on the importance of carrying out further studies to explore the interaction between nematophagous fungi and nematode parasites colonizing the gastrointestinal tract of pigs. This would certainly contribute to employing the fungi in biological control.

The tested isolate (AC001) had *in vitro* action on of *Oesophagostomum* spp L_3 after the passage through gastrointestinal tract of pigs without loss of its viability.

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CONCLUSÕES

- Os sete isolados de *Pochonia chlamydosporia* (Isol.5, Isol.31, Isol.1, VC1, Isol.12, Isol.22 e VC4) tem potencial para serem utilizados no controle biológico de *Ascaris suum*.

- Os fungos *P. chlamydosporia* (Isolados VC1 e VC4) e *Duddingtonia flagrans* suportaram a passagem pelo trato gastrointestinal dos suínos, sem perder sua capacidade ovicida e predatória, respectivamente, portanto essa pode ser uma forma de dispersão destes fungos no ambiente.

- Não houve diferença entre os fungos *D. flagrans, Monacrosporium sinense* e *Arthrobotrys robusta,* quanto à capacidade de predar larvas de *Oesophagostomum* spp, contudo os isolados *M. sinense* e *A. robusta* devem ser testados quanto a sua capacidade de suportar a passagem pelo trato gastrointestinal de suínos.

- Os fungos *Pochonia chlamydosporia e Duddingtonia flagrans* podem ser utilizados para auxiliar no controle das formas infectantes dos nematoides *Ascaris suum* e *Oesophagostomum ssp,* respectivamente.

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