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Original article

Trichoderma virens as a biocontrol of *Toxocara canis*: *In vivo* evaluation

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ABSTRACT

Background: Microorganisms have been widely studied as biological control agents of parasites of medical and veterinary importance. Coprophagous arthropods, bacteria and fungi are among the different organisms evaluated as potential biological control agents. Nematophagous fungi capture and digest the free forms of nematodes in the soil. Due to its zoonotic potential, *Toxocara canis* have been brought to the attention of researchers.
Aims: The aim of the present study was to determine whether the administration of embryonated *T. canis* eggs exposed to the nematophagous fungus *Trichoderma virens* reduces parasite infection in experimental animals.
Methods: Embryonated *T. canis* eggs were exposed to *T. virens* mycelium for 15 days at 25 °C. Subsequently, 100 fungus-exposed eggs were orally administered to 20 Swiss mice. As a positive control, another 20 mice received 100 embryonated eggs that were not exposed to the fungus. After 48 h, the animals were killed, and heart, lungs and liver were harvested for the recovery of larvae.
Results: The organs of the animals that received embryonated *T. canis* eggs exposed to the fungus showed a lower mean larval recovery when compared with the animals that received embryonated eggs without fungus exposure ($p < 0.05$).
Conclusions: The exposure of *T. canis* eggs to *T. virens* reduces the experimental infection, demonstrating the potential of this nematophagous fungus as a biocontrol agent.

Trichoderma virens como control biológico de *Toxocara canis*: evaluación *in vivo*

Palabras clave:
Control biológico
Hongos nematófagos
Helmintos
Modelo experimental animal

RESUMEN

Antecedentes: Algunos microorganismos han sido ampliamente estudiados como agentes de control biológico de parásitos de importancia médica y veterinaria. Los artrópodos coprófagos, las bacterias y los hongos están entre los diferentes organismos que sirven como agentes para el control con potencial biológico. Los hongos nematófagos capturan y digieren las formas libres de nematodos en el suelo. *Toxocara canis*, debido a su potencial zoonótico, ha captado la atención de los investigadores en estos estudios.
Objetivos: El objetivo del presente estudio fue evaluar si la exposición de huevos embrionados de *T. canis* al hongo nematófago *Trichoderma virens* reduce la infección parasítica en un modelo experimental animal.
Métodos: Los huevos embrionados de *T. canis* fueron expuestos al micelio de *T. virens* durante 15 días a 25 °C. Posteriormente, 100 huevos de *T. canis* expuestos al hongo fueron administrados por vía oral a un grupo de 20 ratones Swiss. Como control positivo se usó otro grupo de 20 ratones que recibieron 100 huevos embrionados no expuestos al hongo. Después de 48 h, los animales fueron sacrificados y corazón, pulmones e hígado fueron extraídos para la posterior obtención de larvas.

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Resultados: El número de larvas obtenidas en los diferentes órganos fue menor en el grupo de animales que fueron infectados con los huevos embrionados de *T. canis* expuestos al hongo en comparación con el grupo de animales que recibieron huevos embrionados sin la exposición al hongo ($p < 0{,}05$).
Conclusiones: La exposición de los huevos de *T. canis* a *T. virens* reduce la infección experimental, lo que demuestra el potencial de este hongo nematófago como agente para el control biológico.

Toxocariasis is a disease caused by the nematode *Toxocara canis* and different clinical forms have been observed, including visceral larva *migrans*, ocular larva *migrans* and occult or subclinical toxocariasis.[18] Infections in paratenic hosts primarily occur through the accidental ingestion of the embryonated eggs of the parasite, most frequently affecting children of up to five years of age due to increased contact with contaminated soil,[12] geophagy habit[21] and onychophagy.[2]

The use of microorganisms as biological agents acting on eggs and larvae of nematodes has been widely used in recent years as an alternative control method for nematodes. Thus, the nematophagous fungi are the microorganisms most studied for this purpose. These fungi live in the soil organic matter, develop parasitic or predatory relationships with nematodes, and are classified as ovicides, endoparasites and predators.[14]

Due to the problems caused by chemical control, mainly the prejudicial effects on human health and environment, the development of alternative control methods has become increasingly important.[21] Thus, biological control is a natural tool and an ecological alternative for the control of parasites of medical and veterinary importance. According to Araújo et al.,[4] biological control reduces infections caused by gastrointestinal helminth parasites, reflecting the use of living organisms as natural antagonists in the environment. Coprophagous arthropods, bacteria and fungi are among the different organisms evaluated as potential biological control agents. Nematophagous fungi capture and digest the free forms of nematodes in the soil.[20]

Among several genera of fungi evaluated for the biological control of gastrointestinal nematodes, *Pochonia chlamydosporia* and *Paecilomyces lilacinus* have exhibited ovicidal activity on *T. canis*.[6,11] Nevertheless, the genus *Trichoderma* has also shown ovicidal activity *in vitro* in *T. canis* eggs.[7,8,16] Additionally, this genus has been extensively studied and promising results, *in vitro* and *in vivo*, have been observed in the biological control of plant parasitic nematodes.[10,22]

The aim of the present study was to verify whether the exposition of embryonated *T. canis* eggs to the nematophagous fungus *T. virens* reduces the infection by this parasite in experimental animals.

Materials and methods

Fungal isolate

The fungal isolate used in the present study was obtained from the Mycology Laboratory of the Department of Microbiology and Parasitology at Universidade Federal de Pelotas (UFPel), Brazil. This fungus is an autochthonous isolate previously identified as *T. virens* based on morphological and molecular characteristics.

Obtention of T. canis *eggs*

T. canis eggs were obtained through hysterectomies performed on parasite females according to Maia Filho et al.[16] Subsequently, the eggs were maintained in a formalin solution (2%) containing streptomycin sulfate (0.05%) and chloramphenicol (0.01%). The eggs were embryonated after incubation at 25 °C/15 days with daily aeration.

Exposition of T. canis *eggs to* T. virens

One 4 mm-disk of fungal culture was transferred to Erlenmeyer flask containing 150 ml of modified minimal culture medium [NH_4NO_3 (0.4 g/l); $MgSO_4{\cdot}7H_2O$ (0.12 g/l); $Na_2HPO_4{\cdot}7H_2O$ (3.18 g/l), KH_2PO_4 (0.26 g/l), and yeast extract (0.3 g/l)]. A total of 5 flasks were inoculated with *T. virens* and incubated at 25 °C under gentle manual stirring twice a day during 15 days. On the 15th day, 500 embryonated *T. canis* eggs were added to each flask with the *T. virens* mycelium, and returned to incubation in the same conditions for further 15 days. Additionally, in the same day, 500 embryonated *T. canis* eggs were added to 5 flasks containing 150 ml of modified minimal culture medium, without *T. virens*, and were incubated in the same conditions previously described. Subsequently, the culture medium was centrifuged at 2000 rpm/5 min. The supernatant was discarded, and the pellet was suspended in 1 ml of 0.01 M phosphate buffer solution, pH 7.4 (PBS). To count and evaluate the eggs and viability, 10 μl of this solution were placed onto a slide, coverslipped and examined under a 40× objective. The eggs were considered viable when there was larvae inside, as described by Rey.[19]

Inoculation of experimental animals

Forty *Swiss* mice females (*Mus musculus*) of 4 weeks old were acquired from the animal facility at UFPel. The animals were maintained in appropriate cages at 25 °C, with water and food *ad libitum*. The animals were divided into two groups of 20 animals each: in group 1 (control) animals were infected by gavage feeding with 0.2 ml PBS containing 100 *T. canis* eggs, and in group 2 (fungus-exposed eggs) mice were infected by gavage feeding with 0.2 ml PBS containing 100 *T. canis* eggs exposed to *T. virens*. Forty-eight hours after the infection, the mice were killed by cervical dislocation. The liver, lungs and heart were harvested to recover the larvae. The organs were macerated and digested overnight in 50 ml of 1% hydrochloric acid solution and 1% pepsin at 37 °C with constant shaking at 120 rpm. Subsequently, the digested organs were centrifuged at 2000 rpm/5 min. The supernatant was discarded, and the total sediment of each organ was evaluated on glass slides using optical microscopy (10× and 40× lens) to count the larvae.[24]

All animal procedures were approved by the Ethics Committee on Animal Experimentation/UFPel.

Statistical analysis

The data for larval counting from the digested organs in both groups (group 1 and group 2) were submitted to a normality test using Shapiro–Wilk, Kolmogorov–Smirnov, Cramer–von Mises and Anderson–Darling tests. As the response variable did not show normality, data were subjected to the non-parametric chi-square test. In addition, data were submitted to analysis of measures of position

and frequency distribution. The analysis was performed using SAS statistical software (version 9.4), with a 5% significance level.

Results

The microscopic analyses of the eggs without fungal exposure (used in the control group) showed that these eggs were embryonated and maintained their structural integrity. *T. canis* eggs incubated with *T. virens* were also embryonated but showed colonization with fungal hyphae on the surface (Fig. 1).

The larvae were recovered from different organs (heart, lungs and liver) in both groups. The organs of the animals that received embryonated *T. canis* eggs exposed to the fungus showed a lower mean larval recovery (46.3) ($p < 0.05$) compared with the organs of animals infected with embryonated eggs not exposed to the fungus (80.6). Moreover, the reduction of larvae in the group that received embryonated *T. canis* eggs exposed to the fungus was 57.4%. The frequency distribution analysis demonstrated difference between the groups evaluated ($p < 0.05$). It was observed that 100% of the animals' organs in the control group (group 1) showed larvae count above 70. On the other hand, in the fungus-exposed eggs group (group 2), 85% of the organs evaluated showed larvae count below 50. The median values for group 1 and 2 were 80.5 and 46, respectively (Fig. 2).

Discussion

Nematophagous fungi have been widely used for biological control, reflecting an ability to capture and infect nematodes. *In vitro* studies evaluating the use of ovicidal nematophagous fungi including *Pochonia chlamydosporia*, *Paecilomyces lilacinus* and *Fusarium pallidoroseum* suggest that the use of these biocontrol agents in *T. canis* is an ecological and viable alternative, representing a natural tool for biocontrol.[3,6–8,11,13] In contrast with previous *in vitro* studies, the present study evaluated the recovery of *T. canis* larvae from mice experimentally infected with embryonated *T. canis* eggs previously exposed to the nematophagous fungi *T. virens*. The recovery of larvae from the tissues of mice infected with *T. canis* eggs previously exposed to the fungus *T. virens* was significantly lower ($p < 0.05$) than that in the animals infected with embryonated eggs without fungus exposure. These results suggest that the eggs colonized by the fungus could exhibit reduced viability due to structural damages on the eggs and/or damage on larvae development.

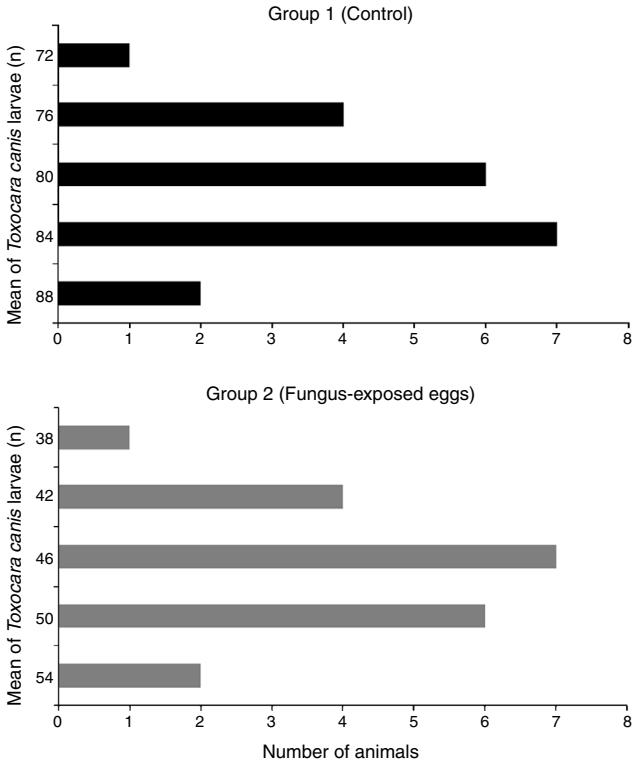

Fig. 2. Frequency distribution of *Toxocara canis* larvae in the organs (heart, lungs and liver) of the animals in group 1 (control – infected with *T. canis* eggs) and group 2 (infected with *T. canis* fungus-exposed eggs).

According to Lysek,[15] the ovicidal activity of nematophagous fungi occurs by the destruction of the egg's layers, leading to the exposure and death of the embryo or by the fungal damage on the embryo's development avoiding a successful infection.

Trichoderma has been used as bionematicide for the control of plant-parasitic nematodes, particularly of the *Meloidogyne* genus, with excellent results, both *in vitro* and *in vivo*.[1,10,17,22] Ciarmela et al.[7,8] and Maia Filho et al.[16] also demonstrated the ovicidal activity *in vitro* of these fungi against *T. canis*. Elgorban et al.[9] suggested that the probable mechanisms of *Trichoderma* spp. in nematode control involved the direct parasitism of eggs and larvae, and also increased proteolytic and chitinolytic enzyme

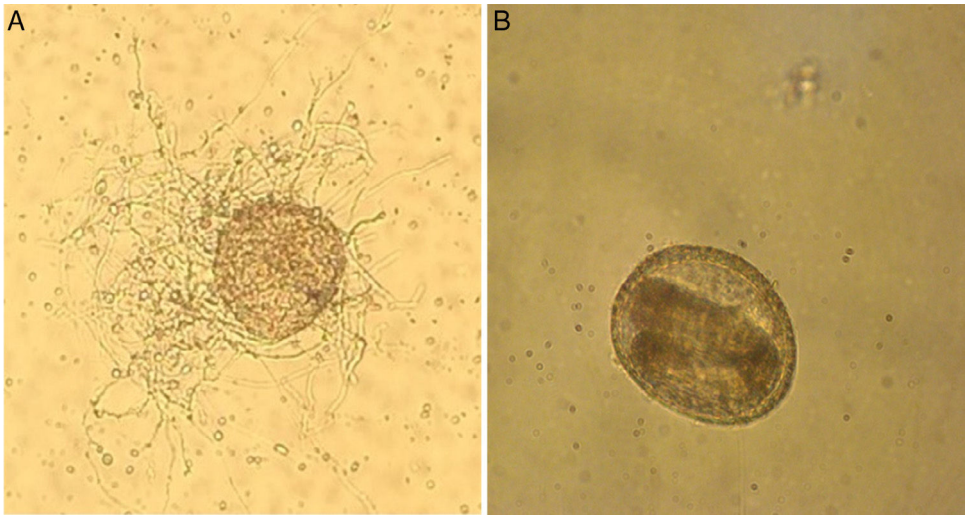

Fig. 1. *Toxocara canis* eggs before administration to the experimental animals. (A) *T. canis* egg colonized by *Trichoderma virens* hyphae after culturing in minimal medium at 25 °C/15 days. (B) Non-fungus exposed embryonated *T. canis* egg (40×).

activity. Additionally, Srivastava et al.[23] reported that the synthesis of proteases, chitinases, glucanases, tubulin, cell adhesion proteins, as well as stress tolerance genes are important mechanisms involved in the biological control effect by *Trichoderma*. We believe that similar biocontrol mechanisms are occurring on *T. canis* eggs; however, additional studies are needed to verify this hypothesis.

The present study is pioneering since there are no reports evaluating the ovicidal activity of nematophagous fungi on *T. canis* eggs in animal models. On the other hand, only studies with predatory nematophagous fungi including the genera *Duddingtonia*, *Monacrosporium* and *Arthrobotrys* have been used to evaluate the experimental passage of fungi through the gastrointestinal tract of domestic animals.[5]

Conclusions

Experimental animals that received embryonated *T. canis* eggs exposed to the fungus *T. virens* showed a lower mean of larval recovery. This result evidences the ovicidal activity of *T. virens* and suggests that this fungus is a potential candidate for the biological control of *T. canis*. However, further studies are needed to evaluate biocontrol mechanisms, as well as the interaction of biotic and abiotic factors of *T. virens* in environmental conditions.

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Conflict of interest

None of the authors of this manuscript has a financial or personal relationship with individuals or organizations that could inappropriately influence the content of this work.

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References

1. Al-Shammari TA, Bahkali AH, Elgorban AM, El-Kahky MT, Al-Sum BA. The use of *Trichoderma longibrachiatum* and *Mortierella alpina* against root-knot nematode, *Meloidogyne javanica* on tomato. J Pure Appl Microbiol. 2013;7:199–207.
2. Alderete J, Jacob C, Pastorino A, Elefant G, Castro A, Fomin A, et al. Prevalence of *Toxocara* infection in schoolchildren from the Butanta region, São Paulo, Brazil. Mem Inst Oswaldo Cruz. 2003;98:593–7.
3. Araújo JV, Santos MA, Ferraz S. Efeito ovicida de fungos nematófagos sobre ovos embrionados de *Toxocara canis*. Arq Bras Med Vet Zootec. 1995;47:37–42.
4. Araújo JV, Mota MA, Campos AK. Controle de helmintos de animais por fungos nematófagos. Rev Bras Parasit Vet. 2004;13:165–9.
5. Braga FR, Araújo JV. Nematophagous fungi for biological control of gastrointestinal nematodes in domestic animals. Appl Microbiol Biotechnol. 2014;98:71–82.
6. Carvalho RO, Araújo JV, Braga FR, Araújo JM, Alves CD. Ovicidal activity of *Pochonia chlamydosporia* and *Paecilomyces lilacinus* on *Toxocara canis* eggs. Vet Parasitol. 2010;169:123–7.
7. Ciarmela ML, Minvielle MC, Lori G, Basualdo JA. Biological interaction between soil fungi and *Toxocara canis* eggs. Vet Parasitol. 2002;103:251–7.
8. Ciarmela ML, Arambarri AM, Basualdo JA, Minvielle MC. Effect of saprotrophic soil fungi on *Toxocara canis* eggs. Malays J Microbiol. 2010;6:75–80.
9. Elgorban AM, Abdel-Wahab MA, Bahkali AH, Al-Sum BA. Biocontrol of *Meloidogyne javanica* on tomato plants by *Hypocrea lixii* (the teleomorph of *Trichoderma harzianum*). Clean – Soil Air Water. 2014;42:1464–9.
10. Ferreira PA, Ferraz S, Lopes EA, Freitas LG. Parasitismo de ovos de *Meloidogyne exigua* por fungos nematófagos e estudo da compatibilidade entre os isolados fúngicos. Rev Trop Cien Agrar e Biol. 2008;2:15–21.
11. Frassy LN, Braga FR, Silva AR, Araújo JV, Ferreira SR, Freitas LG. Destruição de ovos de *Toxocara canis* pelo fungo nematófago *Pochonia chlamydosporia*. Rev Soc Bras Med Trop. 2010;43:102–4.
12. Glickman LT, Schantz PM, Cypess RH. Canine and human toxocariasis: review of transmission, pathogenesis, and clinical disease. J Am Vet Med Assoc. 1979;175:1265–9.
13. Gortari C, Cazau C, Hours R. Hongos nematófagos de huevos de *Toxocara canis* en un paseo público de La Plata, Argentina. Rev Iberoam Micol. 2007;24:24–8.
14. Graminha EBN, Monteiro AC, Silva HC, Oliveira GP, Costa AJ. Controle de nematoides parasitos gastrintestinais por *Arthrobotrys musiformis* em ovinos naturalmente infestados mantidos em pastagens. Pesq Agropec Bras. 2005;40:927–33.
15. Lysek H. A scanning electron microscope study of the effect of an ovicidal fungus on the eggs of *Ascaris lumbricoides*. Parasitology. 1978;77:139–41.
16. Maia Filho FS, Vieira JN, Berne MEA, Stoll FE, Nascente PS, Pötter L, et al. Fungal ovicidal activity on *Toxocara canis* eggs. Rev Iberoam Micol. 2013;30:226–30.
17. Mendoza GAT, Wilson JH, Colina JC. Efecto de *Trichoderma atroviride*, *Trichoderma harzianum* y *Trichoderma viride* sobre huevos de *Meloidogyne* sp. en condiciones de laboratório. Rev Cient Estud. 2013;1:65.
18. Overgaauw PAM, Van Knapen F. Veterinary and public health aspects of *Toxocara* spp. Vet Parasitol. 2013;193:398–403.
19. Rey L. Bases da Parasitologia Médica. 2nd ed. Rio de Janeiro: Editora Guanabara Koogan; 1992. p. 186–93.
20. Sagués MF, Sagués LA, Fusé AS, Fenández LE, Iglesias FC, Moreno CA. Efficacy of an energy block containing *Duddingtonia flagrans* in the control of gastrointestinal nematodes of sheep. Parasitol Res. 2011;109:707–13.
21. Sahebani N, Hadavi N. Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Soil Biol Biochem. 2008;40:2016–20.
22. Sharon E, Chet I, Viterbo A, Bar-Eyal M, Nagan H, Samuels GJ. Parasitism of *Trichoderma* on *Meloidogyne javanica* and role of the gelatinous matrix. Eur J Plant Pathol. 2007;118:247–58.
23. Srivastava M, Shahid M, Pandey S, Singh A, Kumar V, Gupta S, et al. *Trichoderma* genome to genomics: a review. J Data Min Genom Proteomics. 2014;5:162.
24. Wang GX, Luo ZJ. A novel method for the recovery of *Toxocara canis* in mice. J Helminthol. 1998;72:183–4.