

Predatory activity of chlamydospores of the fungus *Pochonia chlamydosporia* on *Toxocara canis* eggs under laboratory conditions

Atividade predatória de clamidósporos do fungo *Pochonia chlamydosporia* sobre ovos de *Toxocara canis* em condições laboratoriais

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Abstract

The objective of this study was to use chlamydospores of the fungus *Pochonia chlamydosporia* (isolates VC1 and VC4) against *Toxocara canis* eggs in a 15-day *in vitro* assay. One thousand *T. canis* eggs were placed in Petri dishes containing 2% water agar medium with different concentrations of chlamydospores (1,000, 10,000 or 100,000) of each fungal isolate of *P. chlamydosporia* (treated groups) and 1,000 eggs in Petri dishes without fungus (control group). Egg counts were performed to determine the ovicidal activity, which was classified as three effect levels: type 1, type 2 and type 3. Significant differences ($P < 0.01$) in egg destruction were found in comparison with the control group. The highest percentage of egg destruction was found in plates containing 100,000 chlamydospores (68.5% for VC1 and 70.5% for VC4). Chlamydospores of *P. chlamydosporia* were effective in destroying *T. canis* eggs and may contribute in the future towards combating the eggs of this parasite.

Keywords: Nematophagous fungi, *Pochonia chlamydosporia*, *Toxocara canis*, nematode eggs, chlamydospores.

Resumo

O objetivo do trabalho foi utilizar clamidósporos do fungo *Pochonia chlamydosporia* (isolados VC1 e VC4) na destruição de ovos de *Toxocara canis*, num ensaio *in vitro*, realizado no intervalo de 15 dias. Em cada placa de Petri com ágar-água 2% foram vertidos 1.000 ovos de *T. canis* em 1.000, 10.000 ou 100.000 clamidósporos de cada isolado do fungo (grupos tratados). Foram realizadas as contagens para verificar a atividade ovicida, classificada em três níveis de efeito: tipo 1, tipo 2 e tipo 3. Os resultados demonstraram que houve diferença significativa ($P < 0,01$) na destruição dos ovos em relação aos ovos observados nas placas do grupo controle. O maior percentual de ovos destruídos foi observado nas placas contendo 100.000 clamidósporos (68,5% para VC1 e 70,5% para VC4). Clamidósporos do fungo *P. chlamydosporia* foram efetivos na destruição dos ovos de *T. canis* podendo contribuir no futuro para o combate aos ovos deste parasito.

Palavras-chave: Fungos nematófagos, *Pochonia chlamydosporia*, *Toxocara canis*, ovos de nematoides, clamidósporos.

Toxocara canis is the causative agent of diseases known as visceral larva migrans and ocular larva migrans in humans (REY, 2008). In addition, close association between humans and domestic animals is considered to be a public health hazard (VASCONCELLOS et al., 2006). Increasing numbers of pets have led to greater contact with humans, thereby increasing the

risk of exposure to zoonosis (GENNARI et al., 1999). These authors also reported that children, especially at preschool ages, are commonly infected through ingestion of embryonated eggs.

Dogs acquire *T. canis* by ingesting eggs that contain L₃ or by preying upon rodents, reptiles and birds that may be infected, and act as paratenic hosts. Transplacental and lactogenic migration also account for infection (ALDAWEK et al., 2002; LEITE et al., 2004; MONTEIRO, 2010; BOWMAN, 2010). Use of anthelmintic drugs is a common approach towards controlling adult parasites in hosts. However, resistance to some drugs used for controlling

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infection in host animals, especially pyrantel, has been mentioned in the literature (KOPP et al., 2008). On the other hand, there are no reports relating to controlling infective forms (larvae and eggs) in the environment (REY, 2008).

Nematophagous fungi have been studied under laboratory conditions as an alternative for controlling *T. canis* eggs and other potentially zoonotic helminths (ARAÚJO et al., 2009; CARVALHO et al., 2010). *Pochonia* is a chlamyospore-producing genus capable of withstanding such conditions. However, there are only a few studies on use of chlamyospore structures for destruction of helminth eggs *in vitro* (BRAGA et al., 2011). These have demonstrated that species of the genus *Pochonia* are inoffensive to animals and humans and are able to destroy helminth eggs in a short time using special pressure organs called appresoria.

The aim of this study was to assess the *in vitro* ovicidal activity of different concentrations of chlamyospores from different fungal isolates of *Pochonia chlamydosporia* on *T. canis* eggs.

T. canis eggs were dissected from adult specimens expelled from naturally infected dogs and morphologically analyzed by means of optical microscopy (10× objective), as described by Urquhart et al. (1998). The eggs were washed in distilled water, centrifuged (1,000 g for 5 minutes) and incubated at 25 °C for 14 days in a solution containing 0.005% streptomycin sulfate and 0.01% chloramphenicol. This procedure was used to obtain embryonated eggs. This assay was based on the protocol described by Araujo et al. (1995).

Chlamyospores were obtained from two *P. chlamydosporia* isolates (VC1 and VC4). This fungus has been maintained under continuous culture at the Parasitology Laboratory of the Department of Veterinary Medicine, Federal University of Viçosa, Minas Gerais, Brazil. The fungal isolates were grown in Petri dishes containing 20 mL of YPSSA medium (4 g of yeast extract; 1 g of K₂HPO₄; 0.5 g of MgSO₄; 20 g of soluble starch; 20 g of agar; and water to complete 1 liter of solution) at 25 °C in the dark for 10 days. The dish surfaces were rinsed (10 mL of distilled water) using a paintbrush. The suspension obtained was sieved into a plastic

container to remove mycelium fragments. Chlamyospores were recovered and quantified using a Neubauer chamber, following the identification methods proposed by Gams and Zare (2001).

The following protocol established by Braga et al. (2011) was used. The experiment comprised six treatments consisting of 1,000 eggs of *T. canis* plated on 9 cm Petri dishes containing 2% water agar (2% WA) medium with the different concentrations of chlamyospores (1,000, 10,000 and 100,000) from each fungal isolate (VC1 or VC4). A control group containing 2% WA, consisting of 1,000 eggs of *T. canis*, was established for each concentration of chlamyospores, with six repetitions.

At the end of the experimental period (15 days), 100 eggs of *T. canis* were removed from each dish (treated and control dishes) and examined for ovicidal activity (LÝSEK et al., 1982): type 1, lytic effect without morphological damage to eggshell, with hyphae adhering to the shell; type 2, lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell; and type 3, lytic effect with morphological alteration of embryo and eggshell, as well as hyphal penetration and internal colonization. Eggs were collected from each plate containing the isolate and from the control without fungus, as described by Araújo et al. (1995), placed on glass slides with a drop of 1% Amam blue and evaluated under a 40x lens. The data were analyzed by means of the Friedman nonparametric test at the 1% probability level (AYRES et al., 2003).

Pochonia chlamydosporia chlamyospores were effective in destroying *T. canis* eggs. There was a significant difference ($P < 0.01$) in egg destruction at the end of the experiment between the treatment and control groups (Figure 1a-f).

For the concentrations of 1,000 chlamyospores, the percentages of egg destruction were 48.0% (VC1) and 50.0% (VC4); for the concentrations of 10,000 chlamyospores, the percentages of egg destruction were 66.0% (VC1) and 69.0% (VC4). The highest percentages of egg destruction were found with the concentration of 100,000 chlamyospores of both isolates (68.5% for VC1 and 70.5% for VC4) at the end of 15 days (Table 1).

Table 1. Percentages and standard deviations for type 1, 2 and 3 effects of ovicidal activity against *Toxocara canis* eggs, caused by *P. chlamydosporia* (isolates VC1 and VC4) at concentrations of 1000, 10000 and 100000 chlamyospores and control group in 2% water agar (2% WA), after 15 days of interaction.

Chlamyospores	Fungi	Effect after 15 days		
		Type 1*	Type 2**	Type 3***
1000	VC1	12.0 ^A ± 18.3	40.0 ^A ± 10.5	48.0 ^A ± 19.8
	VC4	16.0 ^A ± 19.5	34.0 ^A ± 13.4	50.0 ^A ± 25.8
	Control	0 ^B ± 0	0 ^B ± 0	0 ^B ± 0
10000	VC1	0.8 ^A ± 1.7	34.2 ^A ± 17.9	66.0 ^A ± 11.1
	VC4	1.1 ^A ± 2.4	29.9 ^A ± 16.4	69.0 ^A ± 14.6
	Control	0 ^B ± 0	0 ^B ± 0	0 ^B ± 0
100000	VC1	0.5 ^A ± 1.5	31.0 ^A ± 18.9	68.5 ^A ± 19.3
	VC4	0.9 ^A ± 2.8	28.6 ^A ± 18.0	70.5 ^A ± 16.8
	Control	0 ^B ± 0	0 ^B ± 0	0 ^B ± 0

Percentages followed by same letter in the same column are not significantly different ($P > 0.01$) according to the Friedman test. *Physiological and biochemical effect, without morphological damage to the eggshell, with hyphae adhering to the shell. **Lytic effect with morphological change to the eggshell and the embryo, without hyphal penetration through the shell. ***Lytic effect with morphological change to the shell and the embryo, with hyphal penetration and internal colonization of the egg.

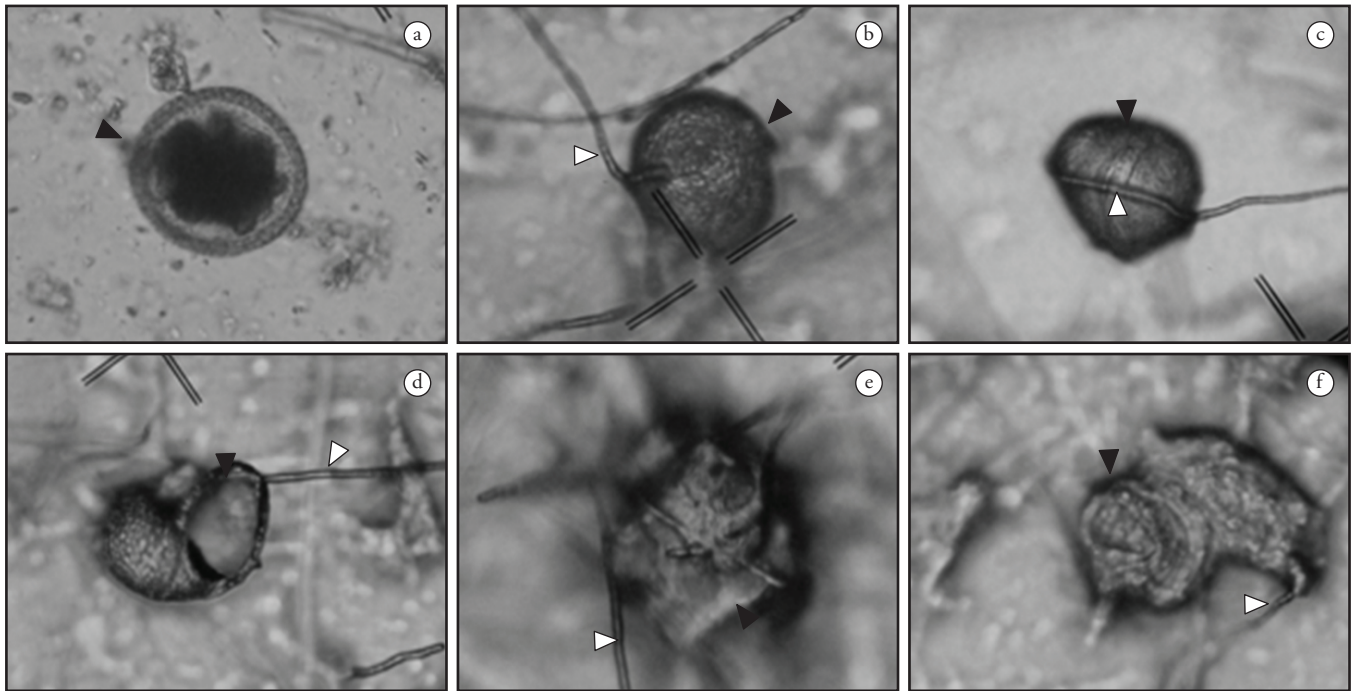


Figure 1. (a) *Toxocara canis* eggs (black arrow), control. (b) Hyphae of the fungus *Pochonia chlamydosporia* (white arrow) attached to the eggshell, a type 1 effect. (c) *P. chlamydosporia* hyphae of the fungus (white arrow) causing deformity in the *T. canis* egg (black arrow), a type 2 effect. (d) to (f) *T. canis* eggs (black arrow) and hyphae of *P. chlamydosporia* destroying the eggs (white arrow), a type 3 effect.

The chlamydospores of the isolates VC1 and VC4 used at the three concentrations (1,000, 10,000 and 100,000) destroyed parasite eggs effectively. Use of chlamydospores against gastrointestinal helminth parasites has been reported for many years with the positive result of effective destruction (LARSEN, 1999). There is, however, a lack of studies concerning the ovicidal activity of chlamydospores under controlled conditions (BRAGA et al., 2011). Use of nematophagous fungi as biological control agents is promising, although contamination during chlamydospore production is still a serious obstacle (ARAÚJO et al., 2004).

Comparing the results found here with those reported by Braga et al. (2011), it can be affirmed that: (1) both studies used increasing concentrations of chlamydospores that effectively destroyed eggs *in vitro*; (2) in the present study, eggs of an ascarid parasite were used and were morphologically different from the eggs of Cestoda used by Braga et al. (2011), which provides justification for further studies to identify the possible obstacles to use of *P. chlamydosporia*, given that geohelminths are present in large quantities in the environment; (3) the 2% WA medium used in the *in vitro* tests of both studies showed that *P. chlamydosporia* has low nutritional requirements, although, as Costa et al. (2001) pointed out, this does not reflect the natural environmental conditions; and finally, (4) we confirmed that the ovicidal activity of *P. chlamydosporia* continues over long time periods, as reported by Braga et al. (2011). This last point is an important finding, because helminth eggs generally have completely different developmental stages. In the present study, the decision to use only one time interval (15 days) was based on the prepatent period of 15 to 21 days for *T. canis*. The results

suggest that the *P. chlamydosporia* isolates VC1 and VC4 can be used for biological control of ascarid eggs.

T. canis eggs were most effectively destroyed at the highest concentration of chlamydospores. This result was already expected, since Braga et al. (2011) also reported that eggs of *Taenia taeniaeformis* were most effectively destroyed at the concentration of 20,000 chlamydospores. On the other hand, in a richer culture medium such as 2% cornmeal agar (BRAGA et al., 2011), chlamydospore production may be better observed, thus perhaps suggesting that there is greater ovicidal activity.

Several reports have shown the efficacy of the isolates VC1 and VC4, with no difference in egg destruction. There are, however, few studies on the effectiveness of chlamydospores from these fungal isolates. Carvalho et al. (2010) showed that *P. chlamydosporia* isolates grown in 2% WA were effective in destroying *T. canis* eggs using time intervals of 7, 14 and 21 days, with ovicidal activity of 20.3% (VC1) and 21.7% (VC4) at the end of the experiment. Frassy et al. (2010) used the same isolates of *P. chlamydosporia* fungus on *T. canis* eggs and registered percentage values for type 3 effect of 43.3% and 47.3%, respectively, after 15 days.

It should be noted that the above studies were conducted using fungal isolates grown in Petri dishes containing the 2% WA medium. However, in the present work, the fungal isolates (VC1 and VC4) were evaluated in the form of chlamydospores, which are resistant vegetative structures. These germinated on the plates and destroyed *T. canis* eggs. Hence, it can be suggested that use of chlamydospores of *P. chlamydosporia* fungus might present better results with regard to biological control of this nematode.

This is the first report on using different concentrations of chlamyospores of *P. chlamydosporia* for destruction of *T. canis* eggs. All the concentrations of chlamyospores of the isolates VC1 and VC4 were efficient in destroying the eggs, but further studies are required for future use of this fungus for biological control of *T. canis* eggs.

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References

- Aldawek AM, Levkut M, Revajová V, Kolodzievski L, Seveiková Z, Dubinsky P. Larval toxocarosis in sheep: the immunohistochemical characterization of lesions in some affected organs. *Vet Parasitol* 2002; 105(3): 207-214. [http://dx.doi.org/10.1016/S0304-4017\(02\)00019-5](http://dx.doi.org/10.1016/S0304-4017(02)00019-5)
- Araújo JM, Araújo JV, Braga FR, Carvalho RO, Silva AR, Campos AK. Interaction and ovicidal activity of nematophagous fungus *Pochonia chlamydosporia* on *Taenia saginata* eggs. *Exp Parasitol* 2009; 121(4): 338-341. PMID:19141298. <http://dx.doi.org/10.1016/j.exppara.2008.12.011>
- Araújo JV, Mota MA, Campos AK. Controle biológico de helmintos parasitos de animais por fungos nematófagos. *Rev Bras Parasitol Vet* 2004; 13(S1): 165-170.
- Araújo JV, Santos MA, Ferraz S. Efeito ovicida de fungos nematófagos sobre ovos embrionados de *Toxocara canis*. *Arq Bras Med Vet Zootec* 1995; 47(1): 37-42.
- Ayres M, Ayres JM, Ayres DL, Santos AS. *Aplicações estatísticas nas áreas de ciências biomédicas*. Belém: Sociedade Civil Maniraua; 2003.
- Braga FR, Silva AR, Carvalho RO, Araújo JV, Pinto PSA. Ovicidal activity of different concentrations of *Pochonia chlamydosporia* chlamyospores on *Taenia taeniaeformis* eggs. *J Helminthol* 2011; 85(1): 7-11. PMID:20338078. <http://dx.doi.org/10.1017/S0022149X10000179>
- Bowman DD. *Parasitologia Veterinária*. 9. ed. Elsevier; 2010. 448 p.
- Carvalho RO, Araújo JV, Braga FR, Araujo JM, Alves CDF. Ovicidal activity of *Pochonia chlamydosporia* and *Paecilomyces lilacinus* on *Toxocara canis* eggs. *Vet Parasitol* 2010; 169(1-2): 123-127. PMID:20097478. <http://dx.doi.org/10.1016/j.vetpar.2009.12.037>
- Costa MJN, Campos VP, Pfenning LH, Oliveira DF. Toxicidade de filtrados fúngicos a *Meloidogyne incognita*. *Fitopatol Bras* 2001; 26(4): 749-755. <http://dx.doi.org/10.1590/S0100-41582001000400011>
- 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(1): 102-104. PMID:20305981. <http://dx.doi.org/10.1590/S0037-86822010000100024>
- Gams W, Zare R. A revision of *Verticillium* sect. *Prostrata*. III. Generic classification. *Nova Hedwig* 2001; 73(3-4): 329-337.
- Gennari SM, Kasai N, Pena HFJ, Cortez A. Ocorrência de protozoários e helmintos em amostras de fezes de cães e gatos da cidade de São Paulo. *Braz J Vet Res Anim Sci* 1999; 36(2): 87-91. <http://dx.doi.org/10.1590/S1413-95961999000200006>
- Kopp SR, Coleman GT, McCarthy JS, Kotze AC. Application of *in vitro* anthelmintic sensitivity assays to canine parasitology: Detecting resistance to pyrantel in *Ancylostoma caninum*. *Vet Parasitol* 2008; 152(3-4): 284-293. PMID:18242867. <http://dx.doi.org/10.1016/j.vetpar.2007.12.020>
- Larsen M. Biological control of helminthes. *Int J Parasitol* 1999; 29(1): 139-146. [http://dx.doi.org/10.1016/S0020-7519\(98\)00185-4](http://dx.doi.org/10.1016/S0020-7519(98)00185-4)
- Leite LC, Cirion SM, Diniz JMF, Marinoni LP, Silva AW, Luz E, et al. Contaminação por ovos de *Toxocara* spp. em praças públicas e parques recreacionais (jardinetes) de Curitiba – Paraná – Brasil. *Rev Acad*. 2004; 2(2): 59-64.
- Lýsek H, Fassatióvá O, Pineda NC, Hernández NL. Ovicidal fungi in soils of Cuba. *Folia Parasitol* 1982; 29(3): 265-270.
- Monteiro SG. *Parasitologia na Medicina Veterinária*. São Paulo: Roca; 2010. 356 p.
- Rey L. *Parasitologia: parasitos e doenças parasitárias do homem nos trópicos ocidentais*. 4. ed. Rio de Janeiro: Guanabara Koogan; 2008. 883 p.
- Urquhart GM, Armour J, Duncan JL, Dunn AM, Jennings FW. *Parasitologia Veterinária*. Rio de Janeiro: Editora Guanabara Koogan S.A.; 1998. 273 p.
- Vasconcellos MV, Barros JSL, Oliveira CS. Parasitas gastrointestinais em cães institucionalizados no Rio de Janeiro, RJ. *Rev Saúde Public* 2006; 40(2): 321-323. PMID:16583045. <http://dx.doi.org/10.1590/S0034-89102006000200020>