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Ovicidal Activity of Chemical Disinfectants and the Nematophagous Fungus *Duddingtonia flagrans and Toxocara canis*

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Abstract

Toxocara canis is a zoonotic canine geohelminth that contaminate the environment with eggs which later hatch the larvae that can infect human beings. Its environmental control is a challenge and require studies focused on establishing efficient strategies for reducing the risks for public health. Thus, this study aimed to evaluate the in vitro ovicidal efficacy of two commercially disinfectants used singly, combined, or in association with the nematophagous fungus *Duddingtonia flagrans* (AC001) against *T. canis* eggs. For this purpose, seven groups were created as follow: G1 (AC001), G2 (sodium hypochlorite 2%), G3 (benzalkonium chloride 15%), G4 (AC001 + sodium hypochlorite 2%), G5 (AC001 + benzalkonium chloride 15%), G6 (sodium hypochlorite 2% + benzalkonium chloride 15%), and G7 (water - control group). There were statistically significant differences ($p < 0.05$) between the treated and control groups, and the egg reductions were G1 (48.2%), G2 (100%), G3 (60.9%), G4 (100%), G5 (26.9%), and G6 (100%). The use of disinfectants alone or combined among them was more effective in destroying eggs than *D. flagrans*. In conclusion, sodium hypochlorite 2% and benzalkonium chloride 15% were effective for destructing *T. canis* eggs in vitro, while *D. flagrans* had a moderate ovicidal action. However, the application of this fungus is promising to be used as a biological and safety strategy for decontaminating the environments where leisure activities occur and exist risks for public health.

Keywords: Toxocariasis; Biological Control; Sodium Hypochlorite 2%; Benzalkonium Chloride 15%

Introduction

The ascarid nematode *Toxocara canis* parasite dogs and is a zoonosis distributed worldwide. *T. canis* larvae can cause a severe disease in humans: the visceral larva migrans (VLM) syndrome OJHA et al. [1,2]. *T. canis* life cycle occurs also in the environment (soil) and consequently has been considered as a "geohelminth" [3]. Generally, helminth infections in domestic animals are controlled through administration of anthelmintic drugs. However, cases of therapeutic failure and drug resistance among canine nematodes have been described [4]. Thus, other strategies focused on controlling this parasite are required. In the case of geohelminths, although disinfectants such as sodium hypochlorite 2% and benzalkonium chloride be considered as effective, their use is limited on impermeable surfaces such as concrete or ceramic floors [5]. Thus, the application of nematophagous fungi may be as a viable control strategie [6], mainly in sandy and permeable soils. In the environment, *Duddingtonia flagrans* produces traps that capture and fixate these nematodes and later destroy their internal structures [7]. In Brazil, the presence of zoonotic parasites in the soil of public squares is an environmental and public health problem, requiring a One Health approach for the control. There is a risk for the infection among children and adults who use these leisure environments [8,9] because the soils

are areas where these parasites are difficult to control, and their occurrence are frequently reported. Thus, the aim of this study was to evaluate the in vitro ovicidal efficacy of two commercial disinfectants and the nematophagous fungus *D. flagrans* against *T. canis* eggs, through singly or combined treatments.

Materials and Methods

This study was submitted to and approved by the Ethics Committee on the Use of Animals (CEUA- Vila Velha University, Process nº 406). The *T. canis* eggs were obtained through dissecting the uterus of adult females that were obtained from two parasitized dogs. The process of recovering and preparing the parasite eggs was performed according to the methodology described by Okul et al. (2010). The Sodium hypochlorite 2% (Água Sanitária Q'Boa®, Anhembi, Brazil) and benzalkonium chloride 15% (Herbalvet T.A.®, Ourofino, Brazil) were used and prepared according to manufactures instructions. The nematophagous fungus *D. flagrans* (AC001) was donated by the Veterinary Parasitology Laboratory of the Federal University of Viçosa. This fungus is maintained with permission at the Experimental Parasitology and Biological Control Laboratory of Vila Velha University.A solution of conidia of AC001 was used in this study. Initially, the fungus was cultured in a Petri dish of 9 cm in diameter containing potato-dextrose-agar 2% (PDA 2%) where the mycelial growth occurred after seven days. Afterwards, 5 mL of distilled water were placed in the Petri dish and the conidia were scraped from the agar surface using a glass slide. Subsequently, the suspension of conidia in water was poured into a 15 mL Falcon tube (Araújo & Maia, 1993).

Seven experimental groups were created in 1.5 mL microtubes, and six replicates were performed for each group (Table 1). The readings were performed on the following days after assembling the tests: day one (24 hours), day seven (168 hours), day 14 (336 hours) and day 21 (504 hours). The quantities of eggs and conidia added to the microtubes was standardized by means of aliquots, such that the concentrations were approximately 120 eggs/18 µl and 120 conidia/10 µl. The volumes of sodium hypochlorite 2%, benzalkonium chloride 15% and distilled water were calculated to obtain a final volume of 100 µl per microtube.

Table 1: Experimental groups (G1 to G7) designed to evaluate the in vitro efficacy of commercial disinfectants (sodium hypochlorite 2% and benzalkonium chloride 15%) and the fungus *D. flagrans* (AC001), singly and in combination with each other, against *T. canis* eggs during 1, 7, 14 and 2.

Groups	Experimental design
G1	18 µl of eggs + 10 µl of conidia AC001
G2	18 µl of eggs + 82 µl of sodium hypochlorite 2%
G3	18 µl of eggs + 82 µl of benzalkonium chloride 15%
G4	18 µl of eggs + 10 µl of conidia AC001 + 72 µl of sodium hypochlorite 2%
G5	18 µl of eggs + 10 µl of conidia AC001 + 72 µl of benzalkonium chloride 15%
G6	18 µl of eggs + 41 µl of sodium hypochlorite 2% + 41 µl of benzalkonium chloride 15%
G7	18 µl of eggs + 82 µl of distilled water

The aliquots were homogenized, and then the content of the tubes was pipetted and deposited on glass slides under coverslips. The readings occurred under an optical microscope at 40x and 100x magnification. The remaining eggs were counted with the using a statistical manual counter and were assessed visually considering their general appearance, color, shell integrity, thickness, content appearance and other conditions that differed from the initial state. Only the eggs with visual characteristics like the initial ones were counted as surviving eggs. The results from the experiment were evaluated by means of analysis of variance (ANOVA) and Tukey's test at 5% probability, using the BioEstat 5.0 software [10]. The reduction percentages were calculated through the equation (MENDOZA-DE-GIVES & VASQUEZ-PRATES, 1994):

% Reduction= (Mean number of eggs in the control group -Mean number of eggs in the treated group)/ (Mean number of eggs in the control group) x 100

Results and Discussion

Table 2 presents the results of the efficacy of both disinfectants and *D. flagrans* against *T. canis* eggs. The egg reductions were G1 (48.2%), G2 (100%), G3 (60.9%), G4 (100%), G5 (26.9%), and G6 (100%) after 21 days. The highest ovicidal reductions were observed for groups G2 and G6, containing sodium hypochlorite 2-2.5% and sodium hypochlorite 2% + benzalkonium chloride 15% without combination to *D. flagrans*. The ovicidal activity of *D. flagrans* (G1), even lower, is important. Previously, the action of this fungus on helminth eggs had already been suggested by [11]. The authors evaluated the ovicidal capacity of AC001 on Ascaris lumbricoides and demonstrated that this fungus adhered to the eggs, suggesting an ovicidal capacity. In the present study, AC001 was able to promote a lytic effect without morphological damage to the eggshell, where hyphae and chlamydospores were adhered (Figure 1). Considering the same parasite used in this study, [12]

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established a reduction of 63% on *T. canis* larvae (L2), after 24 hours of applying AC001. This approach is very interesting due the risks that this parasite can cause for public and environmental health.

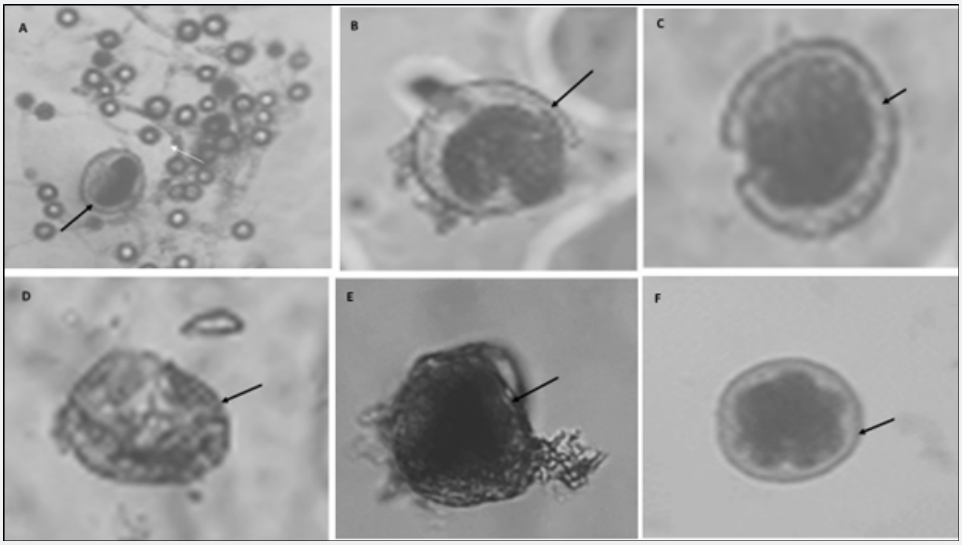

Figure 1: *Toxocara canis* eggs (black narrows). (A) Interaction of *D. flgrans* (white narrow) on *T. canis* eggs (G1). (B, C and D) – destroyed eggs (G2 and G4). (E) destroyed egg (G5). (F) viable egg (G6).

Table 2: Mean results and percentage reductions obtained from groups G1 to G7 regarding the number of *T. canis* eggs on days 1, 7, 14 and 21 of the experiment.

Days	G1	G2	G3	G4	G5	G6	G7
1	94	93	49*	87.83	64.83	83.67	124.8
7	69.33	0*	62	51.17	58.5	0*	86.17
14	45.5	0*	56.67	0*	38.67	0*	69.67
21	33.33	0*	25.17	0*	47	0*	64.33
% Reduction	48.2	100	60.9	100	26.9	100	-

Additionally, this fungus can adhere to Schistosoma mansoni eggs and may have affect its development [13]. These finds have provided evidence of an ovicidal effect due the production of proteolytic enzymes from AC001. Morgan Jones et al. (1993) stated that fungi's adhesion to parasite eggs can result on nematodes be unfeasible. Geohelminth eggshell are composed of protein and lipidic structures (Wharton, 1980) and the application of fungus can support on destructing them due enzymatic action [7]. Thus, our data contribute for a better understanding of an integrated and strategic control because the groups G4 and G5 (those containing *D. flagrans*) had an ovicidal effect, however, evidence of antagonism effects of chemical disinfectants can be observed for some groups.

The groups G2, G4 and G6, which contained 2-25% sodium hypochlorite, had morphological changes in the eggs, mainly referring to the shell, and they progressed during the time. At day one, the eggshells had thinner thicknesses and some broken eggs were observed, exposing their content, while at days 7, 14 and 21 an irregularity in the shell was observed, with smaller diameter and deterioration of the content. Additionally, it was not possible to fully visualize them, leaving only fragments of eggs and dispersed contents at days 14 and 21. [14] evaluated in vitro the hypochlorite solution of 2-2.5%, benzalkonium chloride 15% (Herbalvet T.A.®) and others of commercial genesis on the evolution of embryogenic of *T. canis* during 36 days. These authors observed that the sodium hypochlorite promotes the degradation of the out layer of eggs. Additionally, [5] established that this same solution was able to degenerate 50% of *T. canis* eggs after 24 days. In this study, eggs degeneration occurred after 21 days and agree with these studies.

The groups G3 and G5, which contained 15% benzalkonium chloride, showed lower ovicidal activity, after G1. There are reports in the literature of 15% benzalkonium chloride presenting ovicidal and larvicidal potential on geohelminths [15]. In the

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present work, the ovicidal efficacy of this disinfectant was also proven throughout the experiment, and after 21 days the reduction was 44.2%. The remaining eggs at 21 days showed slightly altered morphology, but many had their contents intact. On the other hand, there are studies demonstrating that the ovicidal potential of 15% benzalkonium chloride is significantly lower compared to the effect of 2-2.5% sodium hypochlorite and 70% alcohol [5,14,16]. Therefore, further comparative studies are needed, as well as associating chemical disinfectants and biological agents, to optimize the ovicidal and larvicidal potential of these agents. Thus, more updated studies are required for a better comprehension of their effects and compare chemical disinfectants and biological strategies to optimize their effects against eggs and larvae.

The G6 group had the highest percentage of ovicidal reduction. The activity of both disinfectants used (sodium hypochlorite + benzalkonium chloride) was potentiated and presented a 75.7% of ovicidal activity. This is an interesting practical result considering that many persons use two or more associated chemical products as household disinfectants, mainly in dog's kennels and cat shelters. Ribeiro (2004) reported the importance of kennel and soil hygiene to avoid maintaining eggs and larvae in a direct transmission cycle and to prevent the infection of intermediate and paratenic hosts. However, Prats et al. (2005) highlighted that the resistance of gastrointestinal parasitic nematode eggs makes any disinfectant-based prophylaxis in soil impossible. Thus, studies performed using a soil matrice must be done for a better practical comprehension of their effects, mainly associated with nematophagous fungus *D. flagrans*.

Regarding the number of days used in this study, we aimed to mimic the life cycle time and the pre-patent period (21 days) of *T. canis* (Urqhuart et al. 1998). Since the first day of the experiment (day 1) a reduction in the number of eggs was clearly noted, mainly by the action of the chemical compounds (disinfectants) used. Ascarids have eggs with thick shells capable of resisting desiccation and temperature variations, remaining for a long time in the environment, making their elimination difficult (Tavares, 2011). According to [17-31], it is important that prevention and control of parasites be implemented to reduce environmental contamination by the infective eggs and larvae. These authors highlighted the importance of using methods to prevent eggs in the environment through disinfectants routinely used at the environments frequented by dogs and cats and our results may be extrapolated to them, contributing for reducing the possible risks for public health. However, further studies are required to improve the ovicidal percentage of these products, mainly trying to potentiate the activity when associated or with a biological control method, such as the nematophagous fungi.

Conclusion

Sodium hypochlorite 2% and benzalkonium chloride 15% were effective for destructing *T. canis* eggs in vitro, while *D. flagrans* had a moderate ovicidal action. The application of this fungus is promising to be used as a biological and safety strategy for decontaminating the environments where leisure activities occur and exist risks for public health. However, further studies using soil matrices are required to prove this practical application.

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