Rev Argent Microbiol. 2018;50(3):249–254

asociación argentina de microbiología

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram

ORIGINAL ARTICLE

In vitro effect of *Chrysosporium indicum* and *Chrysosporium keratinophylum* on *Toxocara canis* eggs

María V. Bojanich[a], **Juan A. Basualdo**[b], **Gustavo Giusiano**[c,*]

[a] *Universidad Nacional del Nordeste, Facultad de Ciencias Exactas y Naturales y Agrimensura, Cátedra de Microbiología General, Corrientes, Argentina*
[b] *Universidad Nacional de la Plata, Facultad de Ciencias Médicas, Cátedra de Microbiología y Parasitología, Centro Universitario de Estudios Microbiológicos y Parasitológicos, La Plata, Buenos Aires, Argentina*
[c] *Universidad Nacional del Nordeste, CONICET, Facultad de Medicina, Instituto de Medicina Regional, Área de Micología, Av. Las Heras 727, 3500 Resistencia, Argentina*

Received 9 March 2017; accepted 22 August 2017
Available online 6 December 2017

KEYWORDS
Biological control;
Nematophagous fungi;
Ovicidal activity;
Geohelminths

Abstract The degree of antagonism exercised by fungi on geohelminth development varies according to the morphological alterations caused by different fungal species. Saprophytic fungi may exert ovicidal or ovistatic effects. The aim of this study was to apply scanning electron microscopy (SEM) to observe the action of two soil saprophytic species of *Chrysosporium* (*C. indicum* and *C. keratinophylum*) on *Toxocara canis* eggs. The fungal strains to be tested were incubated for 28 days at 28 °C in 2% water agar with a suspension of unembryonated *T. canis* eggs. A suspension of *T. canis* eggs in 2% water agar was used as control group. The assay was done in triplicate for each fungus and the control group. SEM observations were performed on the 4th, 7th, 14th, 21st, and 28th day after inoculation. The effect of the fungi on eggs was evaluated in accordance with the alterations observed on the surface and the changes in the normal characteristics of the eggs. Hyphae around the eggs, *appresoria* penetrating the shell and changes in the typical egg membrane were observed in this assay. Type 3 effect (alterations that occur both in the embryo and the shell, and hyphal penetration of the eggs) was the prevalent effect. SEM allowed us to observe clearly the morphological alterations in *T. canis* eggs due to the effect of *C. indicum* and *C. keratinophylum*. Both saprophytic species of *Chrysosporium* alter the egg structure and alterations increase as exposure increases.

* Corresponding author.
E-mail address: gustavogiusiano@yahoo.com.ar (G. Giusiano).

https://doi.org/10.1016/j.ram.2017.08.001

PALABRAS CLAVE
Control biológico;
Hongos nematófagos;
Actividad ovicida;
Geohelmintos

Efecto *in vitro* de *Chrysosporium indicum* y *Chrysosporium keratinophylum* sobre huevos de *Toxocara canis*

Resumen El grado de antagonismo ejercido por los hongos sobre el desarrollo de los geohelmintos depende de la especie fúngica y las alteraciones morfológicas que causan. Los hongos saprófitos pueden tener efecto ovicida u ovistático sobre los huevos. El objetivo fue aplicar la microscopía electrónica de barrido (MEB) para observar la acción de 2 especies de *Chrysosporium* (*C. indicum* y *C. keratinophylum*) saprófitas de suelos, sobre huevos de *Toxocara canis*. Las especies a ensayar se sembraron en agar agua al 2% con una suspensión de huevos no embrionados de *T. canis* y se incubaron 28 días a 28 °C. Como grupo control se utilizó una suspensión de huevos de *T. canis* en agar agua al 2%. El ensayo se realizó por triplicado para cada hongo y el grupo control. Las observaciones con MEB se realizaron a los 4, 7, 14, 21 y 28 días de incubación. La acción de los hongos se evaluó según las alteraciones en la superficie y los cambios en las características normales de los huevos. En este ensayo se observaron: hifas rodeando los huevos, *appresorios* penetrando la cubierta y cambios en la membrana característica del huevo, prevaleciendo el efecto tipo 3 (alteraciones que se producen tanto en el embrión como en la cubierta y penetración de hifas al interior de los huevos). La aplicación de la MEB permitió observar claramente que las 2 especies de *Chrysosporium* saprófitas de suelos, afectan el normal desarrollo de los huevos de *T. canis*, alteran su estructura y las alteraciones aumentan con el tiempo de exposición.

Introduction

Toxocariasis is the clinical presentation of human infection by *Toxocara* spp., a roundworm that lives in the small intestines of domestic dogs and cats. The infection occurs by the accidental ingestion of embryonated *Toxocara* eggs present in contaminated soil or on dirty hands[6,14]. Toxocariasis is present worldwide and is a consequence of the human habit of keeping dogs and cats for company, which favors the persistence of the parasite in the environment and its transmission[13,22]. Despite its extensive geographical distribution, infection is more frequent in tropical and subtropical regions, especially in populations with poor sanitary conditions[20]. Human *T. canis* infection is a public health concern in the Americas, Europe and in all developing countries. However, a full appreciation of the global burden of this disease may be greatly underestimated[18].

Toxocara spp. eggs, as the eggs of other geohelminths, have a high degree of resistance to adverse environmental conditions and diverse chemicals, since they are protected by a thick, complex shell. This shell is made up of three membranes or layers – the outer vitelline layer, a middle chitinous layer, and the inner lipidic layer – that makes eggs resistant to chemicals and temperature changes, allowing them to survive outside the host for long periods[22]. Consequently, soil contamination with infective eggs is a worldwide health issue, and that is why the interest in finding biological control agents to reduce this contamination has increased in the past decades[4].

Fungal parasitism on nematode eggs is a natural biological phenomenon that can be used for biological control of geohelminth eggs in the environment, since they are the most resistant stage in the life cycle of nematodes[4,5]. The penetration process of the hypha through the egg shell has not been completely elucidated yet. In 1995, Bonants et al.[8] were the first to mention that the fungi colonization mechanism may be mechanical and/or enzymatic. Special penetration organs (''*appresoria*'') formed from the hypha help the fungus apply pressure on the egg shell (mechanical effect). Other investigations suggest the involvement of exoenzymes such as proteases and chitinases breaking up egg shells (enzymatic mechanism)[4].

The degree of antagonism exercised by fungi on geohelminth development varies according to the morphological alterations caused by the different fungal species. Thus, a saprophytic fungus may exert ovicidal or ovistatic effects, where the ovistatic ability is shown by the delay in embryo development or inhibition with no morphological damage to the egg shell[12]. Therefore, several researchers have assayed the *in vitro* effect of different fungi on geohelminth eggs. Knowledge about the effect of the genus *Chrysosporium* is scarce, since there is no register of studies on the subject except for the study carried out by Ciarmela et al.[10], who characterized *Chrysosporium merdarium* species as having very high ovicidal activity on *Toxocara canis* eggs, along with other soil saprophytic fungi.

The purpose of this study was to apply scanning electron microscopy (SEM) to observe the action of two soil saprophytic species of *Chrysosporium*, *Chrysosporium indicum* and *Chrysosporium keratinophylum*, on *T. canis* eggs.

Materials and methods

Fungal strains

Strains IMR-MF-816 *C. indicum* and IMR-MF-40 *C. keratinophylum* deposited in the culture collection at the Mycology Department, Instituto de Medicina Regional, Universidad Nacional del Nordeste, Argentina, were assayed. Both

strains were obtained from soils of parks of Corrientes city, Argentina[23].

C. indicum and *C. keratinophylum* were selected for being the most commonly isolated strains from soils of parks in the area where the assay was conducted[23] and, also because of the high ovicidal activity described for the *C. merdarium* species[10].

Source of *T. canis* eggs

Adult female worms of *T. canis* were obtained after deworming naturally-infected puppies. Eggs were extracted from the uterus of the female nematode, treated with 0.1% (v/v) NaClO and washed repeatedly with sterile distilled water. Eggs were resuspended in sterile distilled water at a final concentration of 1×10^3 eggs/ml. Microscopic observation revealed that most eggs were unembryonated[17].

Interaction assays

Interaction assays were conducted according to the technique described by Basualdo et al.[3], with the following modifications: from a culture of *C. indicum* and *C. keratinophylum* in potato dextrose agar, a piece of agar (4 mm in diameter) of each fungal strain was placed onto Petri dishes containing 2% water agar and they were incubated at 28 °C for 4 days to obtain a considerable fungus growth. Subsequently, a suspension of approximately 1×10^3 per ml immature or non-embryonated *T. canis* eggs was added to each dish[7,17]. Once the dishes were inoculated with the egg suspension, they were incubated for 28 days at 28 °C. A suspension of *T. canis* eggs in 2% water agar was used as control group. The assay was done in triplicate for each fungus and the control group.

Scanning electron microscopy (SEM)

After inoculating the dishes with the *T. canis* egg suspension, observations with SEM were conducted on the 4th, 7th, 14th, 21st and 28th day under a Joel 5800 LV (Tokyo, Japan) scanning electron microscope at Servicio de Microscopia Electrónica (Universidad Nacional del Nordeste, Argentina).

Fixation, dehydration, critical point drying, setup, metallization and observation steps were carried out following the technique outlined by Sarmiento et al.[24] with the following modifications. Fixation of the material was done for 48 h in a 2% v/v freshly prepared formaldehyde solution. After 48 h, dehydration was carried out through consecutive passages in ethyl alcohol in increasing concentrations (10%, 30%, 50%, and 70% v/v), and the material was left for 15 min in each alcohol concentration.

At the Microscopy Service, the material was dehydrated again *in situ*, through passages into 70%, 85%, and 100% acetone. Then, critical point drying with CO_2 was done, followed by setup of the dry material over a metal plate which was subjected to gold plating for 3 min, prior to observation. Observations were conducted at different magnifications (220×–2200×).

Evaluation of the fungal effect on eggs

The effect of the fungi on eggs was evaluated in accordance with the alterations observed on the surface and the changes in the normal characteristics of 100 eggs, according to Lysek and Sterba[19] and classified into: Type 1 effect, or lithic effect with no morphological damage to the shell or hyphal penetration through it; Type 2 effect, or lithic effect with morphological alteration in the embryo and shell but no penetration of the shell; and Type 3 effect, or lithic effect with morphological alteration of the embryo, penetration and internal colonization.

Statistical analysis

The statistical significance of the values obtained was evaluated using the Student's *t*-test. A probable value of $p < 0.01$ was considered significant.

Results

The effect of fungi on the eggs was evaluated according to the alterations observed in the surface and the changes in the normal characteristics of the eggs. Figure 1A and B shows normally developed *T. canis* eggs of the control group after 14 days of incubation, with no morphological alterations and intact shells, exhibiting their typical surface.

In contrast to the control group, *T. canis* eggs submitted to the interaction assays with both *Chrysosporium* were observed submerged into a hyphal network as from the 7th day of incubation and penetration (''appresoria'') organs that finally managed to penetrate the shell appeared. Figures 2 and 3B show hyphae surrounding the egg. *Appresoria* penetrating the shell can be observed in Figure 3A and B. hyphae around the eggs make the shell softer and thinner. Changes in the typical egg membrane can be observed in Figures 2 and 4.

The highest percentage of affected eggs was observed between days 7 and 14. After 7 days of incubation, 46% and 69% of the eggs were altered by *C. indicum* and *C. keratinophylum*, respectively. On day 14, this percentage had increased 68.7% with *C. indicum* and 74% *C. keratinophylum*. From day 14 onwards, no major changes were detected.

Non-significant differences were obtained between both *Chrysosporium* species.

Several structural changes of the egg shell occur when fungal hyphae contact the egg, consequently affecting the embryo. This observation of hyphae inside the egg can be possible only if, when assembled for the SEM, the shell breaks and allows to see what happens inside (Fig. 2), otherwise these observations are not possible by this technique.

According to Lysek and Sterba[19], the effect of the fungi classified as Type 3 prevails in our study. This effect includes hypha penetration into the eggs and alterations occurring both in the shell and the embryo.

Discussion

In order to contribute to the biological control of geohelminths, many studies in Latin America have assessed

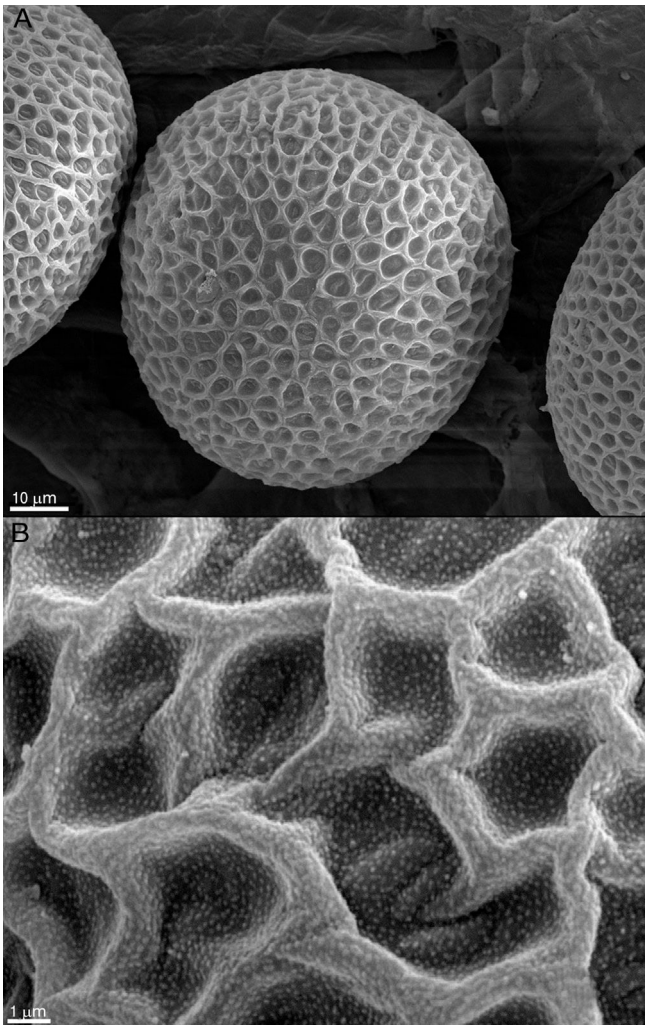

Figure 1 SEM observation of *T. canis eggs* of the control group, incubated for 14 days. (A) Structure of the shell and shape of the egg preserved without any alterations (1100×). (B) Magnification of the egg shell (8000×).

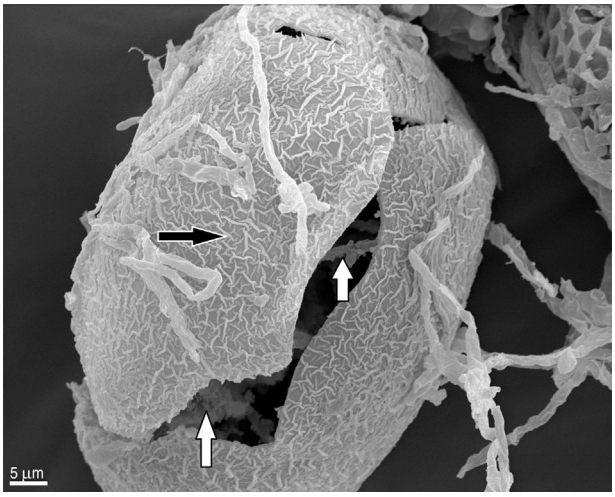

Figure 2 SEM observation of *T. canis eggs* + *C. keratinophylum* incubated for 14 days. Alterations in the shell structure (black arrow), presence of mycelia inside the egg and egg rupture (white arrow) (1400×). Type 3 effect.

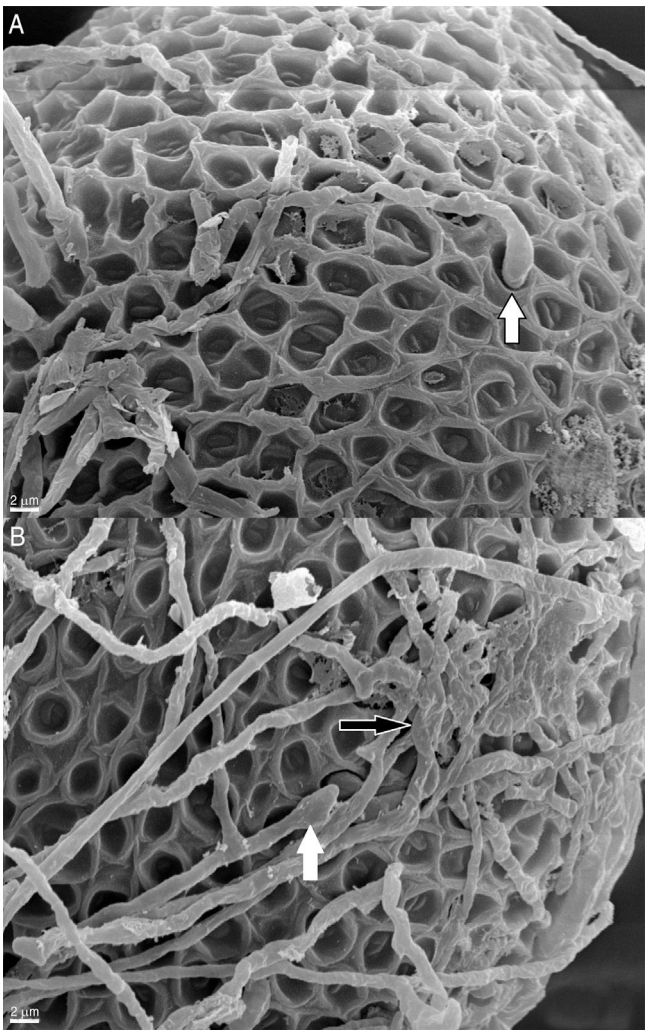

Figure 3 SEM observations of *T. canis eggs* + *C. keratinophylum*, incubated for 7 days. (A, B) Appresoria structures (white arrow) (2500×) and the network of mycelia formed by hyphae around the egg (black arrow) (B)

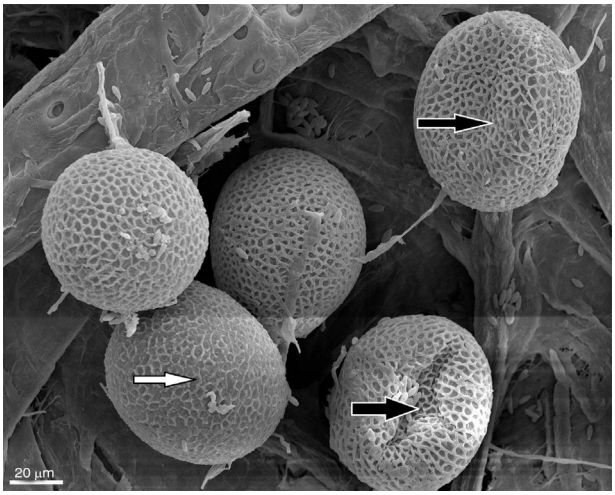

Figure 4 SEM observations of *T. canis eggs* + *C. indicum* incubated for 14 days. Egg deformation due to ''consumption or utilization'' of the embryo (black arrow) and alterations in the shell structure (white arrow) (500×).

the effect of different fungi on *T. canis* eggs in Argentina[3,10,11,16,17] and Brazil[1,2,9,12,15]. In Europe, the contribution of Mazurkiewicz-Zapalowicz et al.[21] regarding the effects of several species of soil saprophytic fungi on *T. canis* deserves to be mentioned.

Chrysosporium is a filamentous keratinophilic fungus, commonly isolated from soils, vegetal material, manure and birds. It lives among the remains of hair and feathers on the soil. Besides being a common contaminant, it is occasionally isolated from human infections. This genus adapts to hot weather areas, is constant and dominant throughout northern Argentina and is cosmopolitan as regards its distribution[23].

The use of SEM allowed us to observe clearly the interaction fungi-eggs and how *C. indicum* and *C. keratinophylum* affect the normal development and alter the structure of *T. canis* eggs. We also noted that these alterations increased depending on the length of exposure. A similar action was reported for only one *Chrysosporium* species by Ciarmela et al.[10] in their studies on *C. merdarium*. These authors report a high *in vitro* ovicidal activity of *C. merdarium* and describe that the fungus growth and the egg alteration was visible after 21 days of incubation. Unlike our results, we already observed an active interaction as from the 7th day. At this time, a hyphal network surrounding the eggs with appressorium formation and the thinning and smoothness of the shell maintained throughout the days of exposure were observed with both *Chrysosporium*. As it was documented for *C. merdarium*, we observed that *T. canis* eggs had smooth shells since the 14th day post-incubation[10].

It is worth considering that the eggs used in this study to conduct the *in vitro* test were immature or unembryonated. Lysek and Sterba[19] mentioned other investigations in which *Paecilomyces lilacinus* (current name *Purpureocillium lilacinum*) colonizes *Globodera pallida* eggs more readily when they are in the early stages of development. Depending on their maturity, these authors reported that eggs have different degrees of resistance to being invaded. For most fungi, when eggs are in a more advanced stage or there is larval development inside them, the ovicidal activity and the ability to colonize eggs are reduced[19].

Due to their ovicidal nature, *P. lilacinum* and *Pochonia chlamydosporia* are the most studied fungi *in vitro*. Only *P. lilacinum* has been isolated from soils in northeastern Argentina[23], but its *in vitro* activity has been shown widely enough, and therefore was not considered for this study. *P. lilacinus* has been studied by Basualdo et al.[3], who proved that *T. canis* eggs colonized by this fungus do not fully develop and the fungus produces special penetration organs, causing mechanical damage to the egg. Likewise, it is important to observe that both species of *Chrysosporium* tested in this study have formed hyphal networks around the eggs with attack-specialized mycelium (*appresoria*), which favored the penetration of this ovicidal fungus through resistant egg-shells inside the eggs, with the same effect.

Carvalho et al.[9] also studied the interaction of *P. lilacinum* and *P. chlamydosporia* on *T. canis* eggs. These researchers believe that both fungi can destroy the eggs under laboratory conditions, and claim that the longer the contact between fungus and egg, the more efficient the ovicidal activity will be. In our study, SEM observations allowed us to reach similar conclusions on the effect of *C. indicum* and *C. keratinophylum*, with a high activity of these fungi from 7th day, being able to alter and penetrate the eggs from the 14th day.

Despite their slow growth, Ciarmela et al.[10] show, their mechanically and enzymatically-driven ovicidal activity for the species *C. merdarium*, since, by the 14th day post-incubation, eggs with a smooth shell were observed, similarly to what we have reported in our assay (Fig. 2). Despite their high antagonic activity, Ciarmela considers it a control agent only for limited use because it may affect human beings[10]; however, the presence of these *Chrysosporium* species in soils can play a very important role as natural biological control agents.

This is the first assay conducted in northeast Argentina. We can conclude that both *C. indicum* and *C. keratinophylum* have, *in vitro*, a high capacity to destroy *T. canis* eggs, which can be well observed and described under SEM. We should deepen our studies to complete the knowledge about the mechanisms used by these fungi to, fully or partially, destroy *T. canis* eggs, as well as, to learn about the influence of the type of soil, humidity, temperature, and presence of other organisms on the *in vivo* action of these fungi on geohelminth eggs.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors wish to thank to: Secretaría de Ciencia y Técnica, Universidad Nacional del Nordeste, for their financial support; Biochemist María Mercedes Sarmiento, for isolating fungal strains and to Ms. Liliana Alegre for assisting in the preparation of materials. We also wish to acknowledge Laura Cipolla for her translation of the manuscript.

References

1. Araujo JM, Araújo JV, Braga FR, Ferreira SR, Oliveira Tavela A. Predatory activity of chlamydospores of the fungus *Pochonia chlamydosporia* on *Toxocara canis* eggs under laboratory conditions. Rev Bras Parasitol Vet. 2013;22:171–4.
2. Araujo JV, Santos MA, Ferraz S. Ovicidal effect of nematofagous fungi on embryonate eggs of *Toxocara canis*. Arq Bras Med Vet Zootec. 1995;47:37–42.

3. Basualdo JA, Ciarmela ML, Sarmiento PL, Minvielle MC. Biological activity of *Paecilomyces* genus against *Toxocara canis* eggs. Parasitol Res. 2000;86:854–9.
4. Blaszkowska J, Kurnatowski P, Wojcik A, Goralska K, Szwabe K. *In vitro* evaluation of the ovistatic and ovicidal effect of the cosmopolitan filamentous fungi from soil on *Ascaris suum* eggs. Vet Parasitol. 2014;199:165–71.
5. Blaszkowska J, Wojcik A, Kurnatowski P, Szwabe K. Biological interactions between soil saprotrophic fungi and *Ascaris suum* eggs. Vet Parasitol. 2013;196:401–8.
6. Bojanich MV, Marino G, López MA, Alonso JM. An evaluation of the dot-Elisa procedure as a diagnostic test in an area with a high prevalence of human *Toxocara canis* infection. Mem Inst Oswaldo Cruz (Rio de Janeiro). 2012;107:194–7.
7. Bojanich MVI. Efecto de los Epítopes Glucídicos en la Especificidad de los Enzimoinmunoensayos para Toxocariosis. In: Inmunodiagnóstico de la Toxocariosis humana. Mannheim, Alemania: Académica Española; 2012. p. 41.
8. Bonants PJM, Fitters PFL, Thijs H, den Belder E, Waalwijk C, Henfling JW. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology. 1995;141:775–84.
9. 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;169:123–7.
10. Ciarmela ML, Arambarri AM, Basualdo JA, Minvielle MC. Effect of saprotrophic soil fungi on *Toxocara canis* eggs. Malaysian J Microbiol. 2010;6:75–80.
11. Ciarmela ML, Minvielle MC, Lori G, Basualdo JA. Biological interaction between soil fungi and *Toxocara canis* eggs. Vet Parasitol. 2002;103:251–7.
12. De Souza Maia Filho F, Nunes Vieira J, Aires Berne ME, Stoll FE, Da Silva Nascente P, Pötter L, Brayer Pereira DI. Fungal ovicidal activity on *Toxocara canis* eggs. Rev Iberoam Micol. 2013;30:226–30.
13. Delgado O, Rodríguez-Morales AJ. Aspectos clínico-epidemiológicos de la toxocariasis: una enfermedad desatendida en Venezuela y América Latina. Boletín de Malariología Y Salud Ambiental. 2009;XLIX:1–33.
14. Despommier D. Toxocariasis: clinical, epidemiology, medical ecology and molecular aspects. Clin Microbiol Rev. 2003;16:265–72.
15. Frassy LN, Ribeiro Braga F, e Silva AR, de Araujo JV, Ferreira SR, Grassi de Freitas L. Destruição de ovos de *Toxocara canis* pelo fungo nematófago *Pochonia chlamydosporia*. Rev Soc Bras Med Trop. 2010;43:102–4.
16. Gortari C, Cazau C, Hours R. Hongos nematófogos de huevos de *Toxocara canis* en un paseo público de La Plata, Argentina. Rev Iberoam Micol. 2007;24:24–8.
17. Gortari MC, Galarza BC, Cazau MC, Hours RA. Comparison of the biological properties of two strains of *Paecilomyces lilacinus* (Thom) Samson associated to their antagonistic effect onto *Toxocara canis* eggs. Mal J Microbiol. 2008;4:35–41.
18. Hotez PJ, Wilkins PP. Toxocariasis: America's most common neglected infection of poverty and a helminthiasis of global importance? Plos Negl Trop Dis. 2009;3:e400.
19. Lýsek H, Sterba J. Colonization of *Ascaris lumbricoides* eggs by the fungus *Verticillum chlamydosporium* Goddard. Folia Parasitol. 1991;38:255–9.
20. Magnaval JK, Glickman L, Dorchies P, Morassin B. Highlights of human toxocariasis. Korean J Parasitol. 2001;39:1–11.
21. Mazurkiewicz-Zapalowicz K, Jaborowska-Jarmoluk M, Kolodziejczyk L, Kuzna- Grygiel W. Comparison of the effect of the chosen species of saprotrophic fungi on the development of *Toxocara canis* and *Ascaris suum* eggs. Ann Parasitol. 2014;60:215–20.
22. Mizgajska H. Eggs of *Toxocara* spp. in the environment and their public health implications. J Helminthol. 2001;75:147–51.
23. Sarmiento MM, Mangiaterra M, Bojanich MV, Basualdo JA, Giusiano G. Hongos queratinofílico-líticos en suelos de parques de la ciudad de Corrientes, Argentina. Rev Iberoam Micol. 2016;33:7–12.
24. Sarmiento PL, Ciarmela ML, Sanchez Thevenet P, Minvielle MC, Basualdo JA. Comparison of preparation techniques of mixed samples (fungi-helminth eggs) for scanning electron microscopy by critical point drying. Parasitol Res. 2006;99:455–8.