Original Article

In Vitro Ovicidal Activity of Nematophagous Fungus *Paecilomyces lilacinus* on the Eggs of Parasitic Helminths

Faezeh Najafi[1], Saied Reza Naddaf[2], Sasan Rezaie[1], Eshrat Beigom Kia[1], Gholamreza Mowlavi[1, 3*]

[1]Department of Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; [2]Department of Parasitology, Research Center for Emerging and Reemerging Diseases, Pasteur Institute of Iran, Tehran, Iran; [3]Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran

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Introduction: The nematophagous fungi have been suggested as an alternative way to eliminate the zoonotic helminths eggs from the environment. In the present study, we evaluated the ovicidal activity of a fungus, *Paecilomyces lilacinus*, recovered from a compost soil, on the eggs of parasitic helminths under *in vitro* condition. **Methods:** Water suspension of the soil samples collected from different areas of Iran were transferred to 2% water-agar culture media baited with *Rhabditis* sp. larvae. The nematode larvae trapped by fungi were transferred to potato dextrose agar (PDA) media. The sequencing of a beta-tubulin gene identified the nematophagous fungi as *P. lilacinus*. Eggs of three helminth species, *Syphacia obvelata, Hymenolepis diminuta* and *Echinococcus granulosus* were exposed to one isolate of the recovered fungi, and the ovicidal activity was monitored for up to 21 days. **Results:** Out of 300 samples, only three compost soils, contained the nematophagous fungus *P. lilacinus*. Microscopical examinations revealed invasion of the helminths eggs by the *P. lilacinus*. The eggs of *H. diminuta* were more vulnerable to this fungus invasion while *E. granulosus* eggs were the least affected ones ($P<0.05$). **Conclusion:** *Paecilomyces lilacinus* can grow on fresh feces and attack the eggs of the parasitic helminths. Therefore, a combination of its hyphae with feces or administration of the spores in the animals' food may reduce the helminths eggs in the environment and consequently intervene with the transmission of parasitic helminthes in the animal houses. *J Med Microbiol Infec Dis, 2016, 4 (3-4): 52-56.*

Keywords: Nematophagous, *Paecilomyces lilacinus, Syphacia obvelata, Hymenolepis diminuta, Echinococcus granulosus.*

INTRODUCTION

Parasitic helminths eggs released into the environment through defecation of definitive hosts are affected by various environmental factors [1]. It is believed that these eggs are also invaded and eliminated by different biological agents including fungi [2]. Nematophagous fungi are potential agents for biocontrol of helminths eggs; these fungi, divided into predators, endoparasites, and ovicidals, can survive in soil as saprophytes and colonize the helminths eggs [3]. More than 150 species are known to interfere with the development of helminths eggs in soil [4], among these *Paecilomyces lilacinus* (Thom) Samson from the class hyphomycetes has received more attention. Previously, this species demonestrated a high *in vitro* ovicidal activity on *Toxocara canis* eggs and was recommended as a potential agent for biological control of the zoonotic helminths in outdoors [5]. Defecation of animals is not only the primary transmission route of helminths eggs in outdoors, but also in animal houses leading to infection of experimental animals and the laboratory staff as well [6]. Adopting non-chemical approaches, *e.g.*, using fungi as biological agents for decreasing the helminths eggs in animal husbandries may intervene with transmission cycle of these parasites. These agents may as well prevent the unfavorable effects of chemical agents on the experimental animals, which may adversely affect the results of tests. The species, *Syphacia obvelata, Hymenolepis diminuta* [7], and *Echinococcus granulosus* are the known causative agents of zoonotic helminth infections in Iran [8]. *Syphacia obvelata* commonly infects laboratory animals, *e.g.*, mice and rats [9]; the short and the direct life cycle of *S. obvelata* can result in a high prevalence of this helminth infection among laboratory mice [10]. *Hymenolepis diminuta* is more common in wild rodents and rarely affects laboratory animals [9], which may infect technicians and researchers as well [11]. *Echinococcus granulosus* the causative agent of echinococcosis not only causes health problem, but also results in economic losses and animal productivity losses [12].

In the present study, we searched for nematophagous fungi in the soils collected from different areas of Iran and evaluated the ovicidal activity of the fungus *P. lilacinus* on the eggs of three above mentioned zoonotic helminths.

***Correspondence:** Gholamreza Mowlavi

Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Science, PurSina St., Keshavarz Blvd., Tehran, Iran, 141556446.

Email: molavig@yahoo.com

Tel: +98 (21) 88941400 **Fax:** +98 (21) 66462267

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MATERIAL AND METHODS

Soil samples and isolation of nematophagous fungi. Three hundred soil samples were collected from different localities including parks, husbandries, and gardens in 11 different counties of Pakdasht, Damavand, Farahzad, Vanak village and Kan in Tehran province, Marivan and Hamadan in the west, Lahijan and Sisangan jungle along Caspian Sea littoral in the north, and Qom and Isfahan province in central Iran (Fig. 1). Amounts of 50 gr of soil, from the surface to the depth of 1.5 cm, were collected in plastic bags. The information about the soils was recorded, and the samples were stored at room temperature. From the soil samples, 15 gr was poured into falcon tubes, mixed with 50 ml distilled sterile water, vortexed vigorously and then centrifuged at 4000 rpm for 5 min. From the supernatants, 200 µl were transferred on 2% water-agar culture medium, with two replicates for each sample. The culture media were baited with one ml of distilled water containing 400 *Rhabditis* sp. larvae and monitored for two months at 25ºC [13]. The nematophagous fungus, *Arthrobotrys oligospora*, (CBS 251.82) was cultured by the same method as a control.



Fig. 1. The provinces (*) and counties (●) from which the soil samples were collected. 1, Tehran; 2, Marivan; 3, Hamedan; 4, Lahijan; 5, Sisangan jungle; 6, Qom; 7, Isfahan.

Identification of fungi colonies. The nematode larvae trapped by fungi were transferred to potato dextrose agar (PDA) media. The grown saprophyte and nematophagous fungi colonies were diagnosed using slide culture method as described elsewhere [14]. The isolated nematophagous fungi were subcultured on PDA medium at 25°C for five days.

DNA extraction. DNA extraction was performed as described by elsewhere [15]. Simply, ≈ 4mg of harvested mycelia were subjected to equal amounts (300 µl) of DNA lysis buffer (100mM Tris-HCl, pH 8.0, 2% Triton X-100, 1% sodium dodecyl sulfate, 1mM EDTA), and phenol/chloroform/isoamyl alcohol mixture (25:24:1). The suspension was vortexed for 30 s, centrifuged at 5,000 rpm for 5 min, and the supernatant was recovered. The previous step was repeated with chloroform alone to ensure purity of DNA. Finally, 250 µl of ethanol and 25 µl of 3M sodium acetate (pH 5.2) were added to the supernatant, incubated for 10 min at -20°C and centrifuged at 12,000 rpm for 12 min. The resultant pellet was resuspended in 100 µl distilled water and stored at -20°C until used [15].

PCR and sequencing. The identity of the fungus *P. lilacinus* was confirmed using PCR followed by sequencing. A partial sequence of the beta-tubulin gene was amplified using the primers Bt2a (5′-GGT AAC CAA ATC GGT GCT TTC-3′) and Bt2b (5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′) designed by others [16]. The 25 µl reaction mixture contained 2.5 µl of 10× buffer (SinaClon Company, Iran), 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 µM dNTPs, 1.25 U of *Taq* polymerase and 2 µl of DNA. All amplifications were programmed for an initial denaturation step for 5 min at 95ºC, followed by 35 cycles of 94ºC for 45 s, 60ºC for 45 s and 72ºC for 1 min, with a final step of 6 min at 72ºC. Amplicons from two samples belonging to Kan and Farahzad were sequenced by the same primers used for amplification (Pishgam Company, Iran) in both directions and compared with similar sequences from GenBank database. The generated data were deposited in GenBank database under accession numbers LC278944 and LC278945.

Helminths eggs. Eggs of *E. granulosus* and *H. diminuta* were obtained by dissecting the mature proglottids from road-killed dogs and laboratory rats, respectively. The eggs of *S. obvelata* were recovered by crushing the adult female worms recovered through dissection of laboratory mice. The Eggs were washed 3 times with distilled sterile water, and one ml suspensions containing 500 eggs were prepared.

***In vitro* ovicidal activity of *P. lilacinus*.** The Kan isolate was grown on PDA medium at 25ºC. After 10 days, suspensions with distilled sterile water from the grown colonies were prepared and transferred onto the plates containing 2% water-agar medium and incubated at the same temperature. After 10 days, 500 eggs from each helminth species, *S. obvelata*, *H. diminuta*, and *E. granulosus* were added to the plates as described by others [17]. Eighteen plates were allocated to the eggs from each species along with 18 control plates with no fungus. At days 7, 14, and 21, one hundred eggs from each plate were removed and examined under a microscope with a magnification of 40X and the invaded eggs were counted according to the damages including penetration of the hyphae into the eggs and morphological alterations of the eggshells.

Satistical analysis. Data were analyzed by three-way ANOVA software at the 5% levels of significance.

RESULTS

Isolation of nematophagous fungi. Out of 300 soil samples, only three, which were compost, contained *Paecilomyces* fungus; two from Kan and one from Farahzad counties in the northwestern of Tehran. However, saprophyte fungi other than *Paecilomyces* were recovered from the rest of the soil samples. On PDA medium, the *Paecilomyces* colonies were white at first and then turned to violet when sporulated (Fig. 2). On microscopic

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examination, the *Paecilomyces* isolates had rough wall conidiophores, arising from aerial hyphae, with flask-shaped phialides bearing numerous ellipsoid or fusiform conidia (Fig. 2).

PCR and sequencing. Amplification from three samples yielded the expected ≈ 350 bp bands. The two generated 355 and 342 bp sequences from Farahzad and Kan counties were identical and showed 100% homology with sequences from *Purpureocillium lilacinum* isolate Kw3411 (acc. No. HE648327) and *P. lilacinus* isolate B3A (acc. No. HM242265), and 99% with sequences from other isolates and strains, *e.g., P. lilacinum* isolate 114B beta-tubulin (acc. No. KY488511) and *Paecilomyces* sp. KJ-2012 strain GZU-BCECGX102-9 (acc. No. JQ965115).

Ovicidal activity of *P. lilacinus.* We observed invasion of eggs of three helminth species by the fungus *P. lilacinus*. The invasion defined as attachment of hyphae to the eggs resulting in eggshell damage. Table 1 lists the percentages of invaded eggs by this fungus. The number of eggs affected at days 7, 14 and 21 differed significantly, and ovicidal activity increased with time ($P<0.05$). The eggs of *H. diminuta* showed to be more vulnerable to the fungus invasion than those of two other species ($P<0.05$) and *E. granulosus* eggs were the least affected ones. The damaged eggs by the fungus observed under the optical microscope in comparison to intact ones are depicted in figure 3.

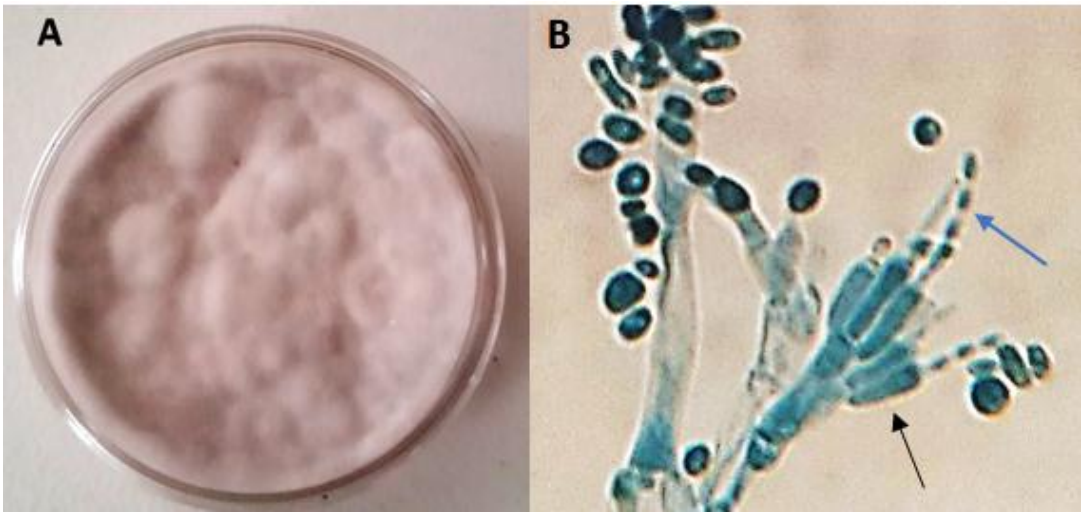


Fig. 2. *P. lilacinus* isolated from a soil sample. (A) Colonies of the fungus on PDA culture medium, (B) morphological features of the fungus; conidiophores bearing phialides with ellipsoid conidia are indicated with black and blue arrows, respectively.

Table 1. Invasion of helminths eggs by *P. lilacinus* at different time intervals

Exposure time/day	Helminths eggs affected by *P. lilacinus*		
	E. granulosus	*S. obvelata*	*H. diminuta*
7	20	41	56
14	27	46.6	62
21	34.3	52.3	68

DISCUSSION

In this study, we demonstrated the destructive effects of *P. lilacinus* on three different helminths eggs, *S. obvelata, H. diminuta, and E. granulosus*. This fungus has shown to utilize the helminths eggs as its nutritional source [18]. Previously, *P. lilacinus* showed a significant *in vitro* ovicidal activity on *Toxocara canis* eggs. The ovicidal acitivity of this fungus was higher than the closely related species, *Paecilomyces marquandii*, and the number of affected eggs increased with the exposure time [5]. Also, this fungus had an adverse effect on the viability of oncospheres of *Taenia hydatigena* [19]. Other studies have reported colonizing the eggs of pathogenic nematode *Meloidogyne incognita* under *in vitro* condition [20], and destructive effects on *Taenia saginata* eggs by this fungus species [21]. The destructive effects of *P. lilacinus* on *Fasciola hepatica* eggs have been demonstrated by a scanning electron microscope in detail [13]. Infection of helminths egg begins with adherence of the hyphae of parasitic fungi to the eggshells followed by penetration through the small pores existing in the egg. This process leads to changes in permeability of the shell and increases its volume which results in the disintegration of egg layers [22]. *Paecilomyces lilacinus* penetrates into helminths egg through the particular mycelial structures, known as appressoria, causing mechanical damage via exerting pressure on eggshell [5]. Further damage by the fungus hyphae is mediated through release of hydrolytic enzymes resulting in digestion of the eggshell [23]. This fungus also produces antibiotics, *e.g.*, leucinostatin and lilacin as well as chitinolytic enzymes, acetic acid [18], and the unsaturated fatty acids including oleic, linoleic and linolenic [24]. The mode of action of leucinostatin is based on the inhibition of mitochondrial ATP synthesis and

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phosphorylation pathways [24]. Leucinostatin and all three fatty acids have shown to have nematocidal activities [24]. Chitinases and proteases produced by *P. lilacinus* play a major role during eggshell penetration and lead to the disintegration of eggshell layers [18]. In the present study, invasion of *H. diminuta* eggs by *P. lilacinus* was higher than the eggs of the two other helminth species. The penetration rate of hyphae into eggshells seems to be associated with the structure of eggshell [25]. *Syphacia obvelata* eggs have a thick shell consisting of five imentexternal uterine, internal uterine, vitelline, chitinous, and lipid layers [26] making penetration of hyphae difficult. Our results showed that the number of *E. granulosus* eggs affected by the fungus were significantly lower than those of *S. obvelata* and *H. diminuta*. The resistance of *E. granulosus* eggs to environment factors and biological agents is attributed to its protective layer, embryophore, membrane or wall around the hexacanth embryo of tapeworms, forming the inner portion of the eggshell making it able to survive in the soil up to one year [27]. In the current study, the ovicidal effect of the *P. lilacinus* on all three species increased with the exposure time which is in agreement with previous works [28]. Nematophagous fungi have shown to be ingested and excreted from the animals' gastrointestinal tract along with helminth eggs. Nematophagous fungi including *P. lilacinus* can grow on fresh feces and destroy the eggs of the helminthic parasites [22]. Therefore, the combination of its hyphae with feces or administration of the spores in the animals' food may reduce the helminths eggs in the environment and intervene with the transmission of these parasites in the animal houses.

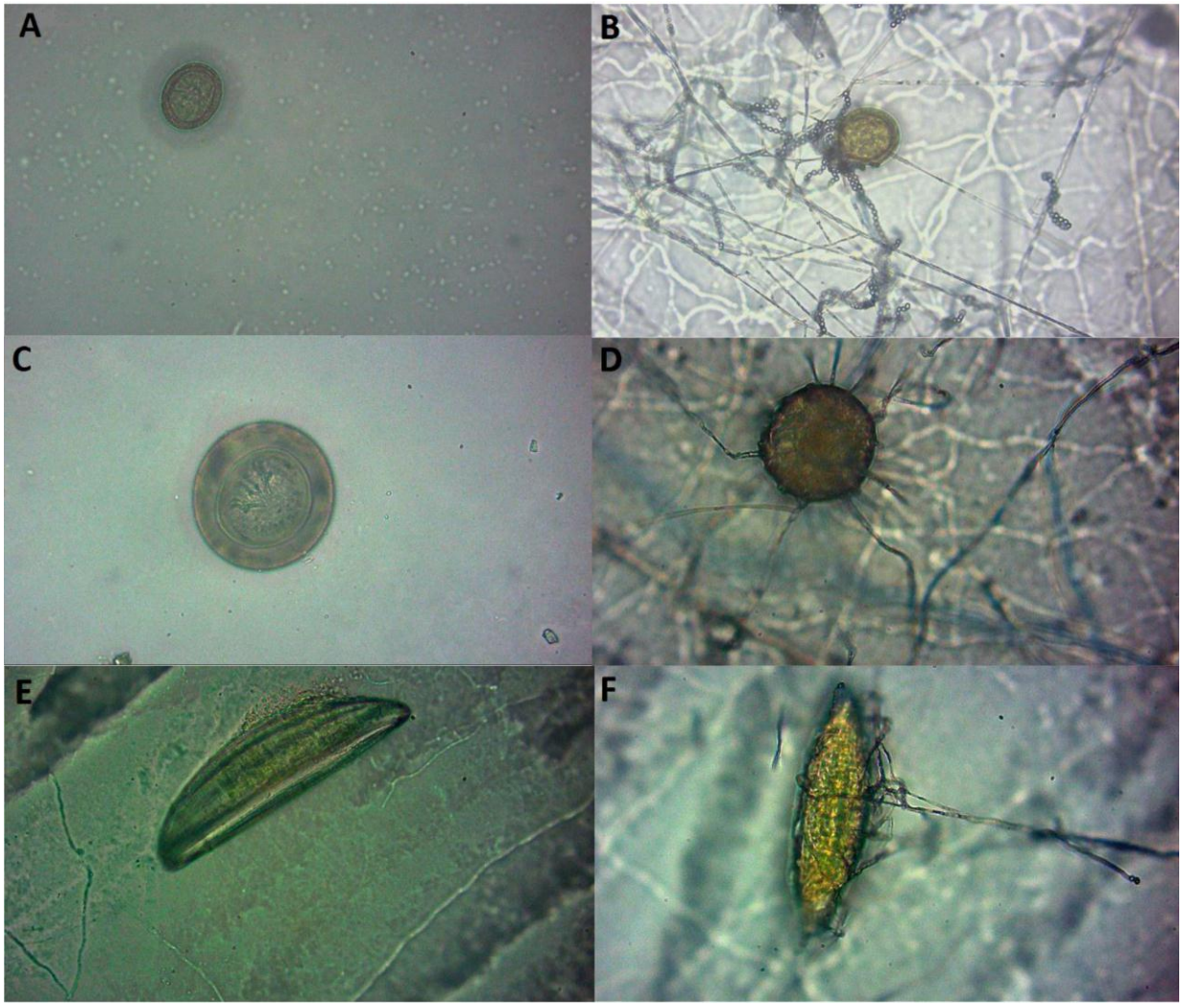


Fig. 3. The effect of *P. lilacinus* on helminths eggs. (A) Intact *E. granulosus* egg in control group, (B) *P. lilacinus* hyphae and the spores inside *E. granulosus* egg, (C and D) intact and parasitised *H. diminuta* eggs, (E) intact *S. obvelata* egg in control group, (F) penetration of hyphae in the eggshell of *S. obvelata*.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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