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Research Article

Isolation of Nematophagous Fungus of Different Eco-Climatic Zones of Nepal and its Use as a Biological Control of Gastrointestinal Nematodes of Goat

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Abstract

This study aimed to isolate the nematophagous fungi from different eco-climatic zones of Nepal and to visualize its effect against gastrointestinal nematode of goat. The descriptive study was conducted. Fecal sample collected from the different eco-climatic zone (Sindhupalchok, Rupandehi, Arghakhanchi, Kaski, Syangja and Kavrepalanchok district) were inoculated in Potato dextrose agar and incubated at 27°C for 1 month. The isolated fungal spores were tested invitro with nematode eggs under 3 different experimental designs. The result of this study indicates that the eggs and larvae present in 5gms feces of infected goat were snared when the spores rate of nematophagous fungi increases from 1500 - 6000. Whereas the ovicidal and larvicidal activities of nematophagous fungi were tumbled when the fungal spores were kept constant about 10,000 and the rate of the nematode eggs were increased from 0 to 4000. Meanwhile when the concentrations of nematode egg were kept constant about 1,000 and the level of spores was gradually elevated, their ovicidal and larvicidal activities were also found to increase gradually. Hence, the isolated nematophagous fungal spores were found to be effective in reducing the number of nematode larvae to an acceptable level but further study should be done to observe the efficiency of these fungi for environmental control over nematodes in naturally infected goat and pasture.

Keywords: *Goat; Gastrointestinal Nematode; Biological Control; Nematophagous Fungi*

Abbreviations

PDA: Potato Dextrose Agar; GDP: Gross Domestic Product; GI: Gastrointestinal Nematode; EPG: Egg Per Gram; CRD: Completely Randomized Design; L3: Third Stage Larva; BW: Body Weight; KG: Kilogram

Introduction

Agriculture is the largest economic sector in the world and the Nepalese economy is also agricultural dependent where about 65% of the population is engaged in agriculture; which is a typical combination of crops, livestock, and forests under the integrated mixed farming system. Among these components, livestock contributes about 31% to the total GDP of the country [1] and it acts as a cornerstone of rural economy and is the source of soil fertility, energy (biogas) as well as source of income and food [11]. Cattle, buffalo, goat, horse, and sheep are the major components of livestock farming systems in Nepal, having said that the resource-poor farmers who cannot invest large sum of money on cattle, buffalo and horse prefer goat and sheep which play an important role in improving the welfare of poor families in this country.

However, one of the impediments to their production worldwide is gastrointestinal helminthiasis. This gastrointestinal parasitic infection is most often responsible for vandalism to livestock since almost all animals produced in the field possess one or more species of helminth and once these animals are exposed to zenith parasitic load they may capitulate, mostly the younger ones which are more susceptible [2].

The ordinary method for suppressing such gastrointestinal parasites is to use synthetic anthelmintic drugs [7]. But the excess use of these chemical anthelmintic drugs has provoked the problem of anthelmintic resistance among the small ruminant livestock producers [5]. Moreover, the excessive use of chemical drugs leads to the water bodies, groundwater and soil contamination which affect humans and other animal's health due to the release of chemical residues by the treated animals in their active form [3]. Thus, the development of non-chemical approaches or biological control appears to be a suitable and efficacious alternative toward anthelmintic drugs [16].

One of the main natural enemies of nematodes is the nematophagous fungi whose demands as a nematode bio-control agent have been increased abruptly in recent years [17]. Nematophagous fungi are cosmopolitan microorganisms found widely in the soil where the animals grazing in pastures digest it and excrete it in their feces and further they are recycled in the environment [8]. These fungi are capable of altering their saprophytic behavior to carnivorous which allows them to feed on nematodes even under unfavorable nutritional conditions. Nematophagous fungi are traditionally classified into four groups: (i) Nematode-trapping/predatorial, (ii) Opportunistic or ovicidal, (iii) endoparasitic and (iv) Toxin producing fungi [15].

The genera *Arthrobotrys* and *Duddingtonia* are intensively studied and are considered to be the most important nematophagous fungi, especially in nematode management programs. These nematophagous fungi can reduce the number of infective larvae of gastrointestinal parasitic nematodes in grazing lands which in turn lowers the risk of re-infections due to which it has become an essential tool for controlling parasitic diseases of livestock [5].

Aim of the Study

This study aimed to isolate and identify the nematophagous fungal spectra in the feces of cow, sheep, and goat found in Nepal and to test their effect on the larva of gastrointestinal nematodes of goats.

Materials and Methods

Culture, isolation, and identification of the fungal spectra found in Nepal

Sample collection for the culture of fungi

Fecal (fresh and old) and soil/manure samples of cow, sheep, and goat were collected from different eco-climatic zones (Sindhupalchok, Rupandehi, Arghakhanchi, Kaski, Syangja, and Kavrepalanchok) in clear zip-lock bags and was carried to the lab in thermo-coated containers. The fecal and soil samples were refrigerated at a temperature below 5°C until the sub-samples were taken and inoculated in the culture medium.

Fungal culture, isolation and identification

The collected fecal and soil/manure samples were well pulverized and three subsamples of 2 gram each were taken. The sub sample was uniformly spread over PDA plates and then it was incubated at 27°C for one month. The culture plates were observed every week under 40x magnifications to detect the presence of nematophagous fungus.

The obtained fungus was sub-cultured to fresh PDA plates and was allowed to grow for 2 weeks in pure culture before the fungal specimen were mounted on Lacto-phenol cotton blue for morphological identification. Different types of fungus thus grown were observed and identification was done under 40x magnification power.

Cultivation of fungi and harvesting of fungal spores

For the preparation of the subculture of the isolated fungi, the conidia were transferred from pure culture to PDA plates. These plates were incubated at 27°C for 3 weeks. After full maturation, the culture spores were collected by washing the culture with a fine stream of distilled water and then stored in sterilized containers. Spore count was done in the Mc Master counting chamber to find out the initial concentration of spore in the collected solution. A stock solution of known concentration of fungal spores was prepared after concentrating the spores in limited volume and subsequently diluting to a required volume of required concentration and was stored for further use. A germination test of the spore was done by inoculating the collected spore in PDA agar on 7 and 21 days of storage and incubating at 27°C for 7 days.

Fecal sample collection

A fecal sample of the goat which was naturally infected with gastrointestinal nematodes was collected from Rupandehi, Arghakhanchi, Kaski, Syangja and Kavrepalanchok area in clear zip-lock bags and was carried to the lab. The fecal examination was done to calculate egg per gram (EPG) of the pooled feces and stored as stock in refrigeration until further experiments were conducted.

Experiments and experimental designs

Experiment to study the trapping Mechanism of fungi

A drop of water suspension containing a known number of third stage larvas of (*Strongyloides* and *Haemonchus*) of GI nematodes of the goat was introduced in one week old pure cultures of fungi. Each plate was observed after every 6 hours and then daily for a week to detect any change in the morphological structure of the fungi and its interaction with the larva.

Experiment on the effect of an increasing rate of spore per gram of feces of goat

Fresh fecal samples from the goats naturally infected with GI nematodes were collected, minced well and pooled. Microscopic examination was done to calculate the EPG of the feces. Five gram of such sample was placed in 20 different 100-ml beakers. These are allocated in five treatment groups of four bottles (replicates) each in a completely randomized design (CRD). Five different levels of the spores (0, 1500, 3000, 4500 and 6000 spores per gram) were mixed with five grams feces each. The required concentration of the spores was obtained by dilution of the original stock solution of the spores. Such beakers were cultured for a week at room temperature and L3 was recovered following the procedure of [18].

Experiment on the effect of constant number of spores on increasing rate of nematode egg

GI nematodes eggs were recovered from the fresh goat feces using a different gradient of sugar and salt solution. The eggs recovered by this method were mixed in sterile dry goat feces at the rate of 0, 1000, 2000, 3000 and 4000 per gram of feces. 20 experimental units (five treatments with four replication each) containing 3gm feces each were prepared in a sterilized Petri plates and a constant number of spores (10,000 per replication) were added to each experimental unit. The experiment was allocated in a completely randomized design. The feces were incubated at 27°C for 7 days and then larvae were recovered.

Effect of increased level of spores per egg of GI nematode of goat

Fresh feces were collected from a goat naturally infected with GI nematodes. Microscopic examination was done to calculate the EPG of the feces. 20 experiment units (5 treatments with 4 replication each) containing 3gm of feces in each were prepared. Spores were added to make an egg to spore ratio of 1:0, 1:10, 1:20, 1:30 and 1:40. The required concentrations of the spore were made by serial dilution of the original stock solution of the spores. The feces were incubated at 27°C for 7 days and then the larvae were recovered

Recovery and counting of larvae

The larva was recovered following the procedures adopted by [28].

Results

Known concentration of *Haemonchus* larvae were introduced upon one-week pure culture of nematophagous fungi. After 6 hours of introduction of larvae on fungal culture, no progressive changes were seen with fungus. In figure 2 tunnels were seen made by the moving larvae. On the first day of culture, fungal hyphae were seen developing. On the second day, hyphae were seen entrapping the moving larvae. From third-day fungus, hyphae started growing on larvae, killing the infected larvae.

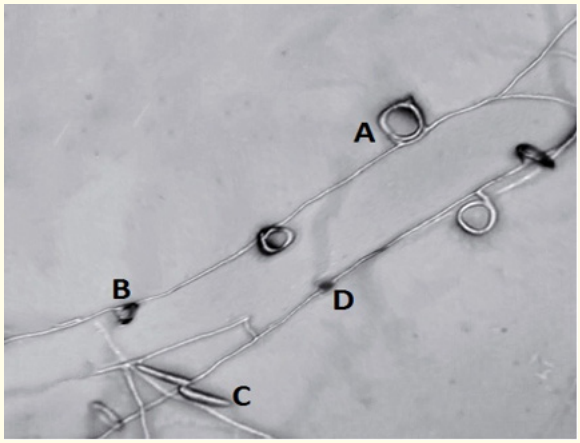

Figure 1: *Predaceous or trap forming nematophagous fungi: (A) Constricting rings (B) Adhesive conidia (C) Adhesive column (D) Adhesive knob. Optical Microscopy (object lens 40x).*

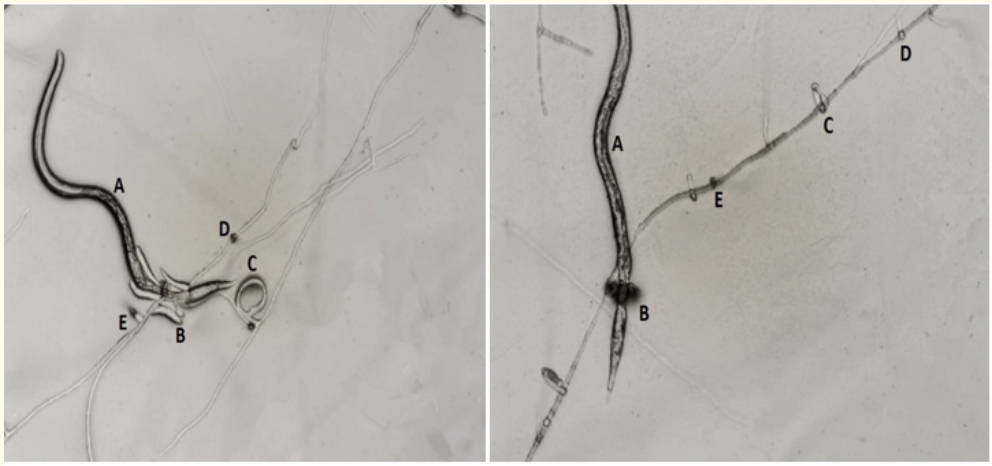

Figure 2: *Optical Microscopy of the trap formation process and interaction between GI nematode and nematophagous fungi: (A) GI nematode larvae (B) Adhesive column (C) Constricting rings (D) Adhesive knob (E) Adhesive conidia (object lens 40x).*

Effect of an increasing rate of spore on per gram of feces of goat

Eggs and larvae present in the feces of infected goat were not snared when no spores of nematophagous fungi were introduced, but as the spores rate increases to 1500 - 6000, its ovicidal and larvicidal activity were escalated.

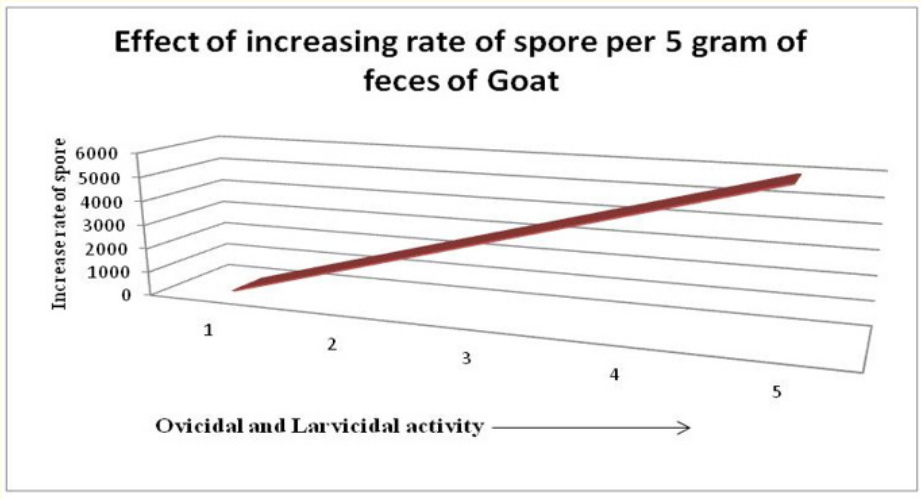

Figure 3: *Effect of an increasing rate of spore per 5 gram of feces of a goat.*

Effect of constant number of spores on increasing rate of nematode egg

The ovicidal and larvicidal activity of nematophagous fungi were tumbled when the fungal spores were kept constant about 10,000 and the rate of the nematode egg were increased from 0 to 4000.

S.N	Spores (about 10,000)	Increasing rate of nematode egg				
		0	1000	2000	3000	4000
1	10,000	-	++++	+++	+++	++
2	10,000	-	++++	++	++	+++
3	10,000	-	++++	+++	+++	+++
4	10,000	-	++++	++++	++++	++

Table 1*: Effect of Constant Number of Spores on the increasing rate of nematode egg.*

Negative (-): No ovicidal and larvicidal activity.

Positive (+ to ++++): Escalate level of ovicidal and larvicidal activity of nematophagous fungi.

Effect of increased level of spores per egg of GI nematode of goat

Meanwhile when the concentration of nematode egg was kept constant and the level of spores was gradually elevated to make the ratio of 1:0, 1:10, 1:20, 1:30 and 1:40, its ovicidal and larvicidal activity were also found to increase gradually.

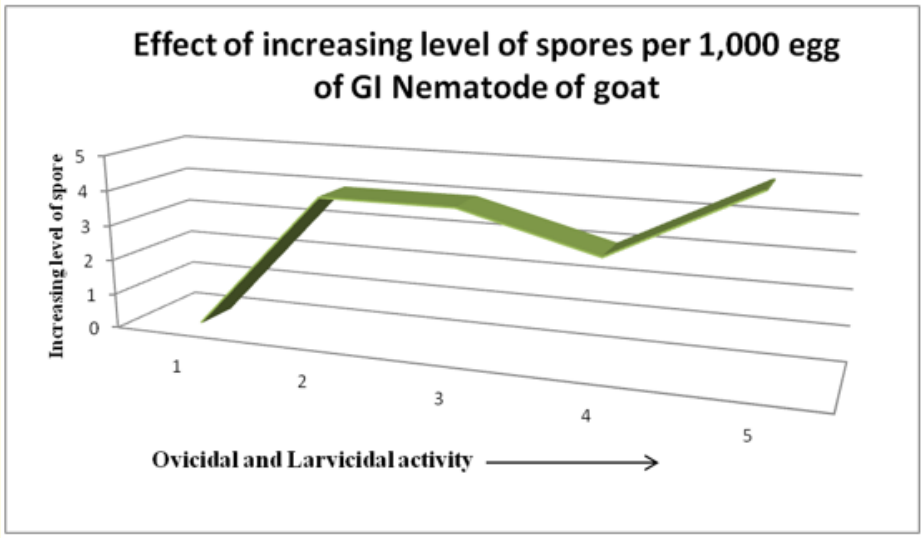

Figure 4: *Effect of increasing level of spore per 1,000 egg of GI Nematode of Goat.*

Discussion

Studies evaluating the activity of nematophagous fungi as a biological control are scarce in a developing country like Nepal, and this work is one of the first to evaluate the efficiency and use of the nematophagous fungi as a biological agent for the control of gastrointestinal nematodes of goats. In this study, the presence of nematophagous fungi with ovicidal and larvicidal activity was detected with the introduction of its spores with the eggs of nematode and then visualizing the change in the morphological structure of the fungi and its interaction with the larva. The effect of nematophagous fungi was observed through 3 different experimental designs (i) By increasing the rate of spores per gram of feces of goat (ii) by increasing the rate of nematode egg and keeping the spore number constant and (iii) By increasing the level of spore per egg of GI nematode of the goat.

Eggs and larvae present in the feces of infected goat were not snared when no spores of nematophagous fungi were introduced, but as the spores rate increases to 1500 - 6000, its ovicidal and larvicidal activity were escalated. This ovicidal activity may be due to the penetration organ that physically damages the egg shell hence the damaged layer will no longer be able to perform its role of the osmotic barrier. The embryo, inside it, can be killed by the injurious substances from the outer environment, which can freely diffuse into the egg [13]. Similarly, Hern., *et al.* (2017) isolated seven fungal genera from the feces of captive animals with ovicidal activity type 3.

C Chartier and I Pors (2003) conducted a study where half of the goats infected with GI nematode were periodically given *D. flagrans* chlamydospores at a daily dose of 2.5×10^5 spores/kg BW (bodyweight) while the remaining animals were kept as controls found that the fecal material derived from the animals fed with *D. flagrans* chlamydospores at a dose rate of 2.5×10^5 spores/kg BW, generally resulted in lower numbers of nematode larvae on the pastures, compared with the control plots.

Similarly, when the fungal spores were kept constant about 10,000 and when the rate of the nematode egg was increased from 0 to 4000, the ovicidal and larvicidal activity of nematophagous fungi were found to be tumbled (Table 1). This may be due to the reason that the constant number of fungal spores was not enough to reduce the increase rate of nematode eggs.

In a study conducted by Larsen., *et al*. (1998) has found that the levels of nematophagous spores between approximately 1.5×10^4 and 3×10^5 gave good reduction (> 80%) in numbers of nematode larvae developing in culture.

According to Zarrin., *et al.* (2017), the *in vitro* nematophagous activity of *D. flagrans* was found to be more effective in reducing the nematode larva after 14 days of incubation as compared to *V. chlamydosporium, F. solani* and *T. harzianum.* The significant effect was seen after 7 days of incubation, therefore the live larva was decreased to 9, 11, 19 and 25. Similarly in a study conducted by Peña., *et al.* (2002) where eighteen ewes were given oral dose spore concentrations of 5×10^5, 2.5×10^5, 1.25×10^5, 5×10^4, 2.5×10^4 of nematophagous fungus per kg of body weight mixed in their feed for 7 days. Across dosages and during the 7 days of fungus feeding, the percent reduction of infective larvae ranged from 76.6 to 100.0%.

Meanwhile when the concentration of nematode egg was kept constant and the level of spores was gradually elevated to make the ratio of 1:0, 1:10, 1:20, 1:30 and 1:40, its ovicidal and larvicidal activity were also found to increase gradually. This entrapment mechanism of nematophagous fungi is all due to the recognition mediated by a lectin carbohydrate interaction. The lectins that are located on the fungal hyphae can specifically bind to the carbohydrate present in the nematode cuticle. And after the recognition event, the fungus immobilizes the nematode and secretes the extra cellular enzymes and further causes the posterior parasitism [12].

The results of this study have shown that the use of nematophagous fungal spore as a biological control reduces the parasitic nematode populations in the absence of anthelmintic treatments. However, it should be envisaged that such reduction would take a long period time before the infection is truncated to levels below which anthelmintic treatments would not be necessary and such control will only become possible after commercial organizations start to be involved in developing fungal formulations. On the other hand, the present results justify the need for studies in the field, over longer intervals, to observe the efficiency of these fungi for environmental control over nematodes in naturally infected goat and further to identify the species of nematophagous fungi.

Conclusion

The present study revealed that the gastrointestinal nematode is the major cause of helminthiasis in the small ruminant in Nepal which affects the goat productivity and further shows the detrimental effect on the goat industry. In this study, an endeavor was made to evaluate the effect of nematophagous fungal spectra as biological control of GI nematode and from this conducted research, the isolated nematophagous fungal spores were found to be effective in reducing the number of nematode larvae to an acceptable level. However, this research was conducted in the lab under the artificial controlled environmental condition, due to which there might impose some difference in the activity and efficacy of the nematophagous fungus on the nematode larva than the real natural field condition. Hence, more studies are needed to be done over a longer interval to visualize the effect of these fungi against the nematodes in naturally infected goat.

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