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Full Length Article

Documentation of Naturally Occurring Nematophagous Fungi in Faeces/Soil in Hilly Areas of Pakistan and Investigation of the Effect of Season on their Frequency Distribution

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Abstract

The gastrointestinal nematodes (GIN) affect the growth rate and milk production of the small ruminants, resulting in considerable economic loss. For their control, the use of broad-spectrum anthelmintic is common, but due to drug resistance, their use is very restricted now. To overcome this dilemma, the utilization of nematophagous fungi (NF) is a promising alternative. The current research was the first study planned to check the natural occurrence of NF in Mansehra, KPK (Hilly area), Pakistan. Samples were collected in two seasons (Summer and Winter) from small ruminant livestock farms. Some soil along faecal deposit area was also collected as naturally occurring NF from the soil surrounding the faecal culture enters the faecal material to predate the larvae. Samples were cultivated on individual Petri dishes comprising 2% water agar with nematode larvae as bait. Petri dishes were examined underneath the stereomicroscope weekly to check any fungal growth. The NF cultured on the Petri plates were then isolated and refined in pure cultures for identification procedure. Twenty-one species of NF were identified with the help of various identification keys. The highest overall frequency of occurrence was observed in the Summer season as compared to Winter. The most abundantly occurring NF were, *Arthrobotrys robusta*, *A. oligospora*, *A. cladodes*, *A. conoides* and *Harpsporium anguillulae* while, *Dactylella heterospora* and *Cystopage lateralisa* were the least identified predatory fungi in faecal/soil samples collectively in both seasons for all three years of sampling. The single most abundant NF species observed was *A. oligospora* and *Arthrobotrys* was the overall predominant genus which was observed in Summer and Winter of all three years. It is concluded from the results of this study that NFs are naturally prevalent in faecal/soil samples of the study area, but their occurrence is more frequent in an environment with moderate temperature and relatively higher humidity compared to cold dry weather. © 2020 Friends Science Publishers

Keywords: Nematode trapping fungus; Nematophagous fungi; Sheep; Biological control; Gastrointestinal parasites

Introduction

Profitability in livestock farming is adversely affected due to different types of parasitic and vector borne infections (Imran *et al*. 2018; Rashid *et al*. 2019; Zafar *et al*. 2019) and gastrointestinal nematodes (GINs) is one of the major impediments. The most common method for control of GINs is the use of synthetic anthelmintics. The development of resistance in parasites to several families of anthelmintics (Leathwick *et al*. 2001; Ijaz *et al*. 2018) has posed a threat in affective chemotherapeutic parasite control. In the last two decades, resistance has developed rapidly throughout the world and increasing incidents of anthelmintic resistance (AR) in Brazil, Argentina, New Zealand, USA, and the UK have been reported (Waghorn *et al*. 2006; Stafford *et al*. 2007). This increased prevalence of AR and the issue of drug residues in animal products (Manzoor *et al*. 2019) have provided a spur for research into 'alternative/novel' approaches for control of GINs. The search for novel approaches is not only driven by AR but also by the need for techniques that are feasible for application by resource-poor farmers in non-commercial subsistence farming systems. The utilization of nematophagous fungi (NF), which parasitise nematodes, is one of such alternatives to control the nematode population (Khattak *et al*. 2018).

Various species of NF have been identified as potential biological control agents to decrease the infection level of GINs in ruminants (Chandrawathani *et al*. 2004; Waller *et al*. 2004). These NF belong to all major taxonomic groups but the majority belongs to phylum Ascomycetes (Gams *et al*. 1998). All NF can be divided into two major groups *i.e*., predacious and endoparasitic, which are further categorized based on killing or trapping behavior. The predacious fungi construct extensive hyphal structures in their surrounding

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environment that produce adhesive traps or nets at intervals that trap and kill the nematode. In contrast, the endoparasitic NF produces encysting spores that adhere to and penetrate the egg-shell or conidia that are ingested and grow within the nematode (Basualdo *et al.* 2000).

Surveys for documentation of NF in the faecal environment have been conducted in various countries of the globe like; Australia (Larsen *et al*. 1994), Brazil (Saumell *et al*. 2000), Iran (Ghahfarokhi *et al*. 2004), Malaysia (Chandrawathani *et al*. 2002), New Zealand (Skipp *et al*. 2002) and South Africa (Durand *et al*. 2005). As far as literature could be ascertained, In Pakistan, no survey to check the prevalence of naturally occurring NF has been conducted. Keeping in view the significance of NF in control of GINs and no availability of data from Pakistan, this study has been planned with objectives to document the species of naturally occurring NF present on pastures in hilly areas. To check the effect of temperature and rainfall on the prevalence of NF, the seasonal variation was also estimated in the current study. This study will provide baseline data on prevalent species of NF and will help in designing a biological control program.

Materials and Methods

The samples were collected in both the Summer and Winter seasons for three years to record the maximum number of naturally occurring NF in hilly areas. Faecal and soil samples were collected from sheep farms to isolate all naturally occurring NF in the study area. Information obtained was recorded in the form of the frequency of occurrence.

Study area

Pakistan is a country with varying climatic conditions having four seasons annually *i.e*., Winter, Spring, Summer, and Autumn. It also has a variety of terrain conditions, mountains, hills, arid lands, and desserts. This study was carried out in the hilly area (District Manshera) to check the difference in the natural occurrence of NF in Summer and Winter Winterseasons for three consecutive years. District Mansehra is mainly a hilly area located in Khyber Pakhtunkhwa, Pakistan. It is located 34.6744° N and 73.3709° E at an altitude of 1,088 meters above sea level. The annual rainfall (1445 mm) is concentrated in the Summer. The climate of the region is rainy in the Summer and dry in Winter. The estimates of temperatures and rain falls during these seasons have been mentioned in Table 1. The average annual temperature in the region falls around 22.5°C.

Documentation of nematophagous fungi

Collection of faecal samples

During each season, a total of 150 random samples were collected (5 g each) from small ruminant livestock farms. Faecal material was acquired directly from the rectum of animals and the ground after wearing gloves. Some soil along faecal deposit area was also collected as naturally occurring NF from the soil surrounding the faecal culture enters the faecal material to predate the larvae. After collection, all the samples were pooled into one big container and mixed to maximize the chances of isolation of NF. All containers were clearly and completely labeled (location, animal, date of collection) and samples were brought to the laboratory at the Department of Parasitology, the University of Agriculture Faisalabad within 12 h after collection.

Sample processing

In the laboratory, the collected samples were homogenized thoroughly. Out of the homogenized faecal material, 10 samples (2 g each) were crumpled and cultivated on individual Petri dishes comprising 2% water agar with nematode larvae as bait (1000 larvae/Petri dish). Petri dishes were then enclosed and retained at 21–26°C for 3 weeks. Petri dishes were examined underneath the stereomicroscope weekly to check any fungal growth.

Isolation and identification of fungi

The NF cultured on the Petri plates were then isolated and refined in pure cultures for identification procedure. First, they were shifted to clean water agar plates and L_3 from mixed infection of GINs were added to the Petri plates as a source of bait ensuring eminent proliferation. After growth on water agar Petri plates, conidia were then shifted to cornmeal (2%) agar plates and net development was encouraged by the addition of L_3 of GINs to pure cultures for identification. The identification was carried out based on morphological features (as the morphology of conidiophores and conidial size in the micro-culture) and trapping structure produced by the fungus on nematode infected culture. This was achieved primarily by following the explanations of Subramanian (1963), Cooke and Godfrey (1964), Cooke and Dickinson (1965), Schenck *et al.* (1977), van Oorschot (1985), Gams (1988), Liu and Zhang (1994) and Rubner (1996).

Results

A total of 21 NF belonging to seven different genera were observed. The maximum number of species from genus *Arthrobotrys* was recorded (n=7) followed by genus Dactylaria (n=5) and Nematoctonus (n=4). Name of individual NF species and their total frequency of occurrence along with different infection structures observed in Summer and Winter seasons have been presented in Table 2 and 3 respectively.

The samples collected (n=150/sampling) in Summer 2013, 2014 and 2015, showed total frequencies of n = 166, n=142 and n= 145, respectively. In Summer 2013 the

Table 1: Avergae temperature and rainfall of district Mansehra, Pakistan

Seasons	Summer				Winter		
Months	June	July	August	September	December	January	February
Avg. Temperature (°C)	28	26.7	25.3	23.9	10	8	9.8
Min. Temperature (°C)	21.3	21.3	20.4	18	4.2	2.6	4.3
Max. Temperature (°C)	34.8	32.2	30.3	29.9	15.9	13.4	15.3
Precipitation /Rainfall (mm)	91	302	266	113	55	90	127

Adopted from: https://en.climate-data.org (Anonymous, n. d)

Table 2: Frequency of occurrence of nematophagous fungi out of collected samples from Jabba farm, Mansehra, KPK, Pakistan in Summer 2013-2015

Nematphagus Fungi	Predatory mechanism	Frequency in Summer 2013	Frequency in Summer 2014	Frequency in Summer 2015	Total Frequency
Arthrobortrys robusta	Adhesive nets	21	17	18	56
A. conoides	Adhesive nets	12	9	9	30
A. dactyloides	Constricting rings	4	5	6	15
A. brochopaga	Adhesive spores	5	6	4	15
A. cladodes	Adhesive nets	18	9	22	49
A. oligospora	Adhesive nets	27	19	16	62
A. musiformis	Adhesive nets	8	4	3	15
Cystopage lateralisa	Adhesive mycelia	1	0	1	2
Dactylaria candida	Non-constricting rings	2	1	1	4
D. haptospora	Conidia with adhesive branches	3	0	1	4
D. leptospora	Conidia with adhesive branches	4	6	1	11
D. pyriformis	Conidia with adhesive branches	3	1	2	6
D. sclerohypha	Conidia with adhesive branches	1	2	0	3
D. heterospora	Conidia with adhesive branches	1	0	0	1
D. leptospora	Non-constricting rings	1	1	1	3
Harpsporium anguillulae	Ingested conidia	17	15	12	44
Monacrosporium spp.	Adhesive nets	1	3	1	5
Nematoctonus campylosporus	Conidia with adhesive knobs	3	2	0	5
N. robustus	Adhesive conidia	7	3	6	16
N. haptocladus	Conidia with adhesive knobs	2	0	1	3
N. tylosporus	Conidia with adhesive knobs	1	0	2	3
Total frequency		142	103	107	352

Table 3: Frequency of occurrence of nematophagous fungi out of collected samples from Jabba farm, Mansehra, KPK, Pakistan in Winter 2013-2016

Nematphagus Fungi	Predatory mechanism	Frequency in Winter 2013-2014	Frequency in Winter 2014-2015	Frequency in Winter 2015-2016	Total Frequency
A. robusta	Adhesive nets	8	7	4	19
A. conoides	Adhesive nets	1	3	6	10
A. dactyloides	Constricting rings	0	1	2	3
A. brochopaga	Adhesive spores	2	0	1	3
A. cladodes	Adhesive nets	1	2	1	4
A. oligospora	Adhesive nets	6	4	8	18
A. musiformis	Adhesive nets	0	1	3	4
Cystopage lateralisa	Adhesive mycelia	0	0	0	0
D. candida	Non-constricting rings	3	1	0	4
D. haptospora	Conidia with adhesive branches	0	0	0	0
D. leptospora	Conidia with adhesive branches	1	0	1	2
D. pyriformis	Conidia with adhesive branches	1	3	4	8
D. sclerohypha	Conidia with adhesive branches	2	1	2	5
D. heterospora	Conidia with adhesive branches	0	0	1	1
D. leptospora	Non-constricting rings	0	1	0	1
H. anguillulae	Ingested conidia	5	2	7	14
Monacrosporium spp	Adhesive nets	1	2	2	5
N. campylosporus	Conidia with adhesive knobs	1	0	0	1
N. robustus	Adhesive conidia	0	0	5	5
N. haptocladus	Conidia with adhesive knobs	0	0	3	3
N. tylosporus	Conidia with adhesive knobs	0	0	4	4
Total frequency		32	28	54	114

occurrence of NF with different infection structures were; Adhesive nets (n = 87), Constricting rings (n = 4), Adhesive spores (n = 5), Adhesive mycelia (n = 1), Non-constricting rings (n = 3), Conidia with adhesive branches (n = 12), Ingested conidia (n = 17), Conidia with adhesive knobs (n = 6) and Adhesive conidia (n = 7). *A. oligospora* (n = 27), *A.*

robusta (n = 21), *A. cladodes* (n = 18), *Harpsporium anguillulae* (n = 17) were the top four most frequent species. Moreover, second replication of the trial was carried out in Summer of 2014. Out of the total frequency, the occurrence of NF with different infection structures were; adhesive nets (n = 61), constricting rings (n = 5), adhesive spores (n = 6), adhesive mycelia (n = 0), non-constricting rings (n = 2), conidia with adhesive branches (n = 9), ingested conidia (n = 15), conidia with adhesive knobs (n = 2) and adhesive conidia (n = 3), which also shows that NF with adhesive nets were most prevalent and adhesive mycelia were least prevalent as they were absent in this area. Although less frequent than 2013 *Arthrobotrys* (n = 69) was still the most predominant genus whereas, *A. oligospora* (n = 19), *A. robusta* (n = 17), *H. anguillulae* (n = 15) and *Arthrobortrys conoides* (n = 9), were the top four most frequent species at Jaba farm, Mansehra in Summer of 2014. In Summer of 2015, the occurrence of NF with different infection structures at this farm were; adhesive nets (n = 69), constricting rings (n = 6), adhesive spores (n = 4), adhesive mycelia (n = 1), non-constricting rings (n = 2), conidia with adhesive branches (n = 4), ingested conidia (n = 12), conidia with adhesive knobs (n = 3) and adhesive conidia (n = 6), which shows that NF with adhesive nets were predominant during all three Summers and adhesive mycelia were least. *A. oligospora* (n = 16), *A. robusta* (n = 18), *A. cladodes* (n = 22) and *H. anguillulae* (n = 12), were the top four most frequent species in Summer of 2015. Detailed results have been presented in Table 2.

The samples collected from Jabba farm (n=150/sampling) during Winter 2013–14, 2014–15 and 2015–16 showed total frequencies of 50, 40 and 95 (Table 3). Out of the total frequency, the occurrence of NF with different infection structures at this farm were; adhesive nets (n = 17), adhesive spores (n = 2), non-constricting rings (n = 3), conidia with adhesive branches (n = 4), ingested conidia (n = 5), conidia with adhesive knobs (n = 1). The NFs with adhesive nets were most prevalent and constricting rings, adhesive mycelia and adhesive conidia were least prevalent as they were absent at this sampling instance. *Arthrobotrys (*n = 18) was the most predominant genus whereas, *A. robusta* (n = 8), *A. oligospora* (n = 6), *H. anguillulae* (n = 5) and *Dactylaria candida* (n = 3), were the top four most frequent species at Jaba farm, Mansehra in Winter of 2013–2014. In the next trial replication of Winter 2014–2015, NF with different infection structures were; adhesive nets (n = 19), constricting rings (n = 1), non-constricting rings (n = 2), conidia with adhesive branches (n = 4), and ingested conidia (n = 2). The NFs with adhesive nets were most prevalent and adhesive spores, adhesive mycelia, conidia with adhesive knobs and adhesive conidia were absent in this area sampling. *Arthrobotrys (*n = 18) was the most predominant genus whereas, *A. oligospora* (n = 4), *A. robusta* (n = 7), *Dactylaria pyriformis* (n = 3) and *Arthrobortrys conoides* (n = 3), were the top four most frequent species out of all. The frequency of occurrence of NF with different infection structures in Winter 2015–16 were; adhesive nets (n = 24), constricting rings (n = 2), adhesive spores (n = 1), conidia with adhesive branches (n = 8), ingested conidia (n = 7), conidia with adhesive knobs (n = 7) and adhesive conidia (n = 5). NF with adhesive nets was most prevalent and adhesive mycelia, non-constricting rings were not observed in this area. *Arthrobotrys (*n = 25) was the most predominant genus out of all, whereas, *A. oligospora* (n = 8), *H. anguillulae* (n = 7), *Arthrobortrys conoides* (n = 6) and *Nematoctonus robustus* (n = 5), were the most frequently observed species.

Discussion

Nematode trapping fungi are considered to be a useful biological tool against parasitic nematodes in cattle and sheep (Larsen 2000; Flores-Crespo *et al*. 2001). The *Arthrobotrys* genus is distributed worldwide and is known for controlling nematodes by its predatory activity (Dalla Pria *et al*. 1991; Naves and Campos 1991; Nordbring-Hertz *et al*. 2011). The NFs belonging to this genus utilizes two different carbon sources, one in organic matter as a saprophyte and the other from the predatory activity against nematodes, which makes it adaptable to different habitats (Wachira *et al*. 2009). This ability of fungus produces a very little impact on the environment when used as a biological control agent on livestock farms.). *In vitro* testing of *H. anguillulae* on faecal cultures and agar media has shown that these fungi are capable of reducing the number of free-living larval stages significantly (Nansen *et al*. 1988; Waller and Faedo 1993; Mendoza-de Gives and Vazquez-Prats 1994; Mendoza-de Gives *et al*. 1992, 1994). Charles *et al*. (1996) also evaluated the efficacy of *H. anguillulae* in Brazil. He conducted an invitro assay to estimate its efficacy in which he targeted *H. contortus* and percetage reduction of 99.5% was observed at a dose rate of 300,000 conidia per gram of faeces. Abovementioned and various other studies throughout the world showed significant effect of nematophagous fungi against gastro intestinal nematodes making it a considerable candidate for developmental strategies for control of nematodes.

In this present study, various species of NFs were locally isolated in Mansehra and it was found that they were isolated more abundantly in the soil/faeces collected in Summer as compared to Winter, which was influenced by climatic conditions. In a tropical climate, the development of pre-parasitic forms of trichostrongylid nematodes of sheep on pasture occurs in a few days. Thus, the rapid colonization of faeces by NF would be important in the natural control of these parasites.

Overall, the highest frequency of these NF was observed in the Summer season, and a low frequency was observed in the Winter season. This may be because NF propagates more efficiently in hot humid conditions as compared to low temperatures and dry conditions. The optimum temperature recorded for the growth of NF is 25°C

(Anamika 2015). It has been reported that the consequent development of adhesive networks around nematodes, was significantly slower at the lower temperatures (5–10°C) than the higher temperatures (15–30°C). The colonization of the nematode by trophic hyphae is affected by temperature. At temperatures below 15°C, it has been observed that the development of tropic hyphae is reduced (Belder and Jensen 1994). The high prevalence of *A. oligospora* isolated from livestock farms has also been reported (Mahoney and strongman 1994). In accordance with Saumell *et al*. (2000), who reported the high prevalence of *Arthrobotrys* species in Brazil, the mean annual temperature of the location chosen for sampling was 18.5°C, which is similar to the annual temperature (20°C) in the present study area. High prevalence of *A. oligospora*, *A. conoides*, and *A. robusta* have also been reported in Iraq and Oman (Muhsin and Kasim 1998).

The ideal temperature range for the growth and development of the majority of the NF has been found between 15–30°C (Cooke 1963; Pandey 1973; Grønvold *et al*. 1985). The temperature of the study falls close to the ideal temperature of fungal growth this could be the reason for a high frequency of NF recorded during the present study. The largest number of isolates was retrieved from faeces at the beginning of the rainy season (during Summer). During this period of the year, the average minimum as well as maximum temperature is increased.Under these conditions, NF became abundant. In the rainy months, the invasion of the faeces by an abundance of free-living nematodes would facilitate the colonization of predatory fungi. When nematodes are infected by NF, they maintain their motility for some time thus facilitating dispersal. The presence of a larger number of isolates of NF in rainy months was also reported in the organic matter of the soil, where sheep pastured. Fungi, which form adhesive nets, were predominant during the dry season. However, *A. oligospora*, among the predatory fungi, was isolated in every sampling, demonstrating their abundance in the environment and their capacity to colonize faeces and develop in both the wet and dry months. Adhesive net-forming fungi *A. oligospora* , which were the most abundant predatory species in the dry months, may resist drying out, as was observed by (Gray and Bailey 1985).

Conclusion

For the treatment of nematode infections in livestock and to prevent the spread of larvae in the environment, these NF may be directly administered to animals. This may provide a strategy for the control of further environmental contamination by the nematode larvae. *In vitro* studies are pivotal to select effective NF before their application in the field. However, *in vitro* studies may give an overestimate about the efficacy of NF; as in these trials, the nematodes cannot escape and reproduce as they have in the natural environment. Also, they have limited physical space and less evaluation time. So, *in vivo* studies may also be conducted for the proper estimation of the predatory activity.

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