

Screening of nematophagous-fungi from fresh faeces of grazing animals and soils

Farah Haziqah, M.T.¹, Nur Hikmah, A.M.¹, Mat Hasan, H.¹, Hamdan, A.^{1,2} and Nik Him, N.A.I.I.^{1*}

¹School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

²Vector Control Research Unit, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

*Corresponding author e-mail: nikirwan@usm.my

Received 3 October 2018; received in revised form 6 May 2019; accepted 8 May 2019

Abstract. An investigation was undertaken for screening and isolating nematophagous-fungi from the faecal samples of various grazing animals and soils in Malaysia. Total of 111 faeces and 50 soil samples were collected and the samples were cultured on 2% water agar plates. The growth of nematophagous-fungi was stimulated by sprinkling-baiting technique. The conidia of suspected nematophagous-fungi were inoculated on 2% water agar plates. All isolated were maintained on 2% cornmeal agar plates. *Verticillium* spp., *Fusarium* spp. and *Arthrobotrys* spp. were identified from the faecal and soil samples. 62.5% of the faecal samples and 100% of the soil samples were shown to be positive with nematophagous-fungi. This study highlights the present of nematophagous-fungi population in faecal and soil samples. Much study remains to be done to better understanding some fungi especially their mode of action and their predatory behaviour against parasitic nematodes.

INTRODUCTION

In livestock production, various issues can challenge controlling parasitic nematodes. The animal health issues and the economic losses that associated with these parasitic nematodes are constant concern. Studies of sheep production from the main producer countries (Australia, Brazil, and Uruguay) indicated that parasitic nematode infections are responsible for the most economic losses of this animal species (Waller, 2006). More recently, Geurden *et al.* (2015) had reported a lower efficacy of ivermectin and moxidectin (based on the reduction in egg excretion after treatment) on European cattle farms (Germany, UK, Italy, and France), with confirmed anthelmintic resistance on 12.5% of the farms tested. Papadopoulos *et al.*, (2012) reported the widespread incidence of multidrug-resistant (benzimidazoles, imidazothiazoles and macrocyclic lactones) populations of *Haemonchus contortus*, *Teladorsagia* spp. and *Trichostrongylus* spp.

in sheep throughout Europe. This is due to intensive use of commercial anthelmintic as control measures which resulted in increasing cases of anthelmintic resistance in parasitic nematodes. In Malaysia, resistance problem of parasitic nematodes towards anthelmintic had been reported since 1990s (Dorny *et al.*, 1993, 1994). This has led to the need for alternative measures in controlling of such infection. Biological control is one of the available options in theory, which can diminish parasitic nematodes in animals as well as the infective larvae on pasture. In biological control implementation, one organism is used to gain control of another target animal (as reviewed by Larsen, 2006). Generally, there are numerous species of antagonistic organism against nematodes has been described within the group of viruses, bacteria, fungi, amoeba, dung beetles, springtails and mites (Grønvoold *et al.*, 1996). One of those interesting and promising biological control for parasitic

nematodes nowadays is the contribution of nematophagous-fungi in their life cycle.

Nematophagous-fungi is known as nematode-destroying fungi belong to the heterogeneous group of microfungi that utilizes nematodes either as a source for the nutrients or supplementary to a saprophytic existence (Hyde *et al.*, 2014). Nematophagous-fungi is soil inhabitants and can be found in most soil types throughout the world. Study on nematophagous-fungi had begun as early in the 1940' but with little success at the time (Hertzberg *et al.*, 2002) The major breakthrough was observed at the beginning of the 1990's when a group of researcher from Denmark showed that a selected fungi were able to survive *in vitro* conditions simulating the passage through the gastrointestinal tract of cattle (Larsen *et al.*, 1991). Since then many trials have been undertaken in several countries in which fecal samples of ruminant animals and samples of soil have been screened for nematophagous-fungi such as in Australia (Larsen *et al.*, 1994), Brazil (Saumell *et al.*, 1999), Fiji (Manueli *et al.*, 1999), New Zealand (Skipp *et al.*, 2002), Iran (Shams-Ghahfarokhi *et al.*, 2004) and South Africa (Durand *et al.*, 2005).

As reviewed by de Soares *et al.* (2018), currently nematophagous-fungi was classified into five different groups based on their predation characteristics on nematode. The first (nematode-trapping/ predatorial group) they produce modified hyphae that bind and digest only nematode larvae. The second group (opportunistic or ovicidal group), also produce the modified hyphae that bind, digest the eggs, cysts and nematode females. The third group (endoparasitic group) they use spores as infection structures, which may adhere to nematode cuticle or be ingested. The fourth group (toxin producing group) they secrete toxin that immobilize the nematodes. The fifth group (producers of special attack devices) produce special attacking devices that cause mechanical damage to the nematode cuticle.

In Malaysia, the only survey of nematophagous-fungi was carried out by Chandrawathani *et al.* (2002) which several species of nematophagous-fungi had been

identified and fulfilled the classification criteria of *Arthrobotrys* spp. and *Duddingtonia flagrans*. However, few other studies have isolated and utilized native strains (Manueli *et al.*, 1999; Sanyal 2000), which is crucial because fungi adapt easily to regional climatic conditions. Therefore, isolating and characterizing fungal strains with predatory activity is essential for further research. In this study, we aimed to identify the nematophagous-fungi present in the faeces of grazing animals and in soils in order to select a native nematophagous-fungal strain with the potential for use in the biological control program in livestock animals.

MATERIALS AND METHODS

Sample collection

Faecal samples were collected from two farms of grazing animals in Penang (Sg. Pinang and Permatang Pauh) and Perlis (Padang Besar). All faeces samples for goats were freshly obtained directly from the rectum of animals by inserting fingers to stimulate defecation. Meanwhile, faecal samples of cattle, horses and deer were collected immediately after observing defecation. All samples were kept in a sealed plastic bag and were kept in an ice box with cold packs and brought to the laboratory. All samples were then refrigerated at 4°C prior to use. Soil samples were collected from the grazing areas of cattle, horse and deer in Permatang Pauh, Penang and Padang Besar, Perlis. The soil samples were randomly collected from the open area of the grazing paddock and treated in a similar manner with faecal samples. There were 36 faecal samples of goats, 15 samples faecal samples of cattle, 30 faecal samples of horses and 30 faecal samples of deer. There were 50 soil samples has been collected from various grazing areas.

Acquisition of nematode larvae for baiting technique

Nematode larvae culture for baiting was conducted according to standard protocols by Soulsby (1987) for identification and

collection of infective larvae of trichostrongylid in faeces of goat. The weighed faeces (10 g) gently were crushed using mortar and pestle which addition a small amount of water. The mixture was then transferred into a clean wide-mouthed glass jar and compressed gently by a flat-bottomed tube. Then, a glass jar was covered with a Petri dish and left at 27°C for 7 days. Then culture was monitored daily and distilled water was sprayed as needed to maintain the moisture condition in the glass jar. On the 7th day, the glass jar was filled to the top with distilled water. One Petri dish was put over the mouth of the jar and carefully was overturned. The distilled water of 10 ml was added to the periphery of the Petri dish. The jar was left for 1 hour to allow the larvae to migrate from the culture to the Petri dish. Then the water in the Petri dish that containing the migrated larvae were collected and transfer into conical tubes, centrifuged for 2 minutes at 1000 rpm. The supernatant was removed and the sediment that contains the larvae was collected in small glass vials. The volume was adjusted to a final concentration of 200 larvae per one ml of distilled water containing 5 µg/ml of amphotericin B (Sigma-Aldrich, USA) to prevent the growth of fungi. The larvae suspension was kept at 4°C prior to use.

Fungi culture and sprinkling-baiting technique

All samples of fresh faeces and soils were subjected for screening of the presence of nematophagous-fungi. The procedures were in accordance with those previously reported by Waller and Faedo (1996) and Chandrawathani *et al.* (2002). The fresh weighed 5 g of faeces were mashed and 0.5 ml aliquots of sample materials were sprinkled onto 2% water agar in Petri dish. Then mix species of infective larvae (L3) (*Haemonchus contortus*, *Trichostrongylus* spp. and *Cooperia* spp.) (approximately 200 larvae per ml) were added to the Petri dish as bait to stimulate the growth of fungi. The samples were incubated at 30°C. The examinations were first made after three days of incubation. The plates were examined daily to determine

the presence of trapped larvae. Approximately 2 weeks after initial baiting, plates were re-baited if the production of conidiophores or conidia was low to promote fungal growth and subsequently examined. The same procedures were carried out for the soil samples.

Fungal isolation and identification

Fungi were isolated according to the method described by Duddington (1955) and modified by Santos *et al.* (1991). Nematophagous-fungi were detected by the characteristic appearance of conidia and trapping structures from and around trapped nematodes (Cooke and Godfrey, 1964; Juan Li *et al.*, 2014). Isolates culture were established by transferring the fragments of the culture contained the nematodes that were trapped within the fungal structures on a new Petri dish. The plates were re-baited again with infective larvae. Sub-cultures were then carried out until pure cultures were obtained, which were there after incubated on cornmeal agar (2%) and stored at 4°C. In this preliminary study, the identification of the fungi was carried out based on the trapping mechanism, the involved structure and the morphology of conidiophores, conidia and the presence or absence of chlamydospores in mature fungal culture. Confirmation of the genus and species was validated by the expert.

RESULTS

Faecal samples were derived from goat, cattle, horses and deer from two different locations in Penang (Sg. Penang and Permatang Pauh) and Perlis (Padang Besar). Table 1 shows the distribution of the faecal samples that were screened for the presence of nematophagous-fungi according to animal species and location. The overall prevalence of the goat samples was 62.5% were shown to be positive with the nematophagous-fungi from the farm in Sungai Pinang, Penang. While 54.3% of the goat and cattle samples from Permatang Pauh, Penang were positive with nematophagous-fungi and 76.6% of horse and deer samples from Padang Besar, Perlis

Table 1. Distribution of faecal samples from various animal species that were screened for the presence of nematophagous-fungi and percentage that were found to be positive

Animal species	Number of samples			TOTAL	Nematophagous-fungi (%) Percentage (No) ^a
	Sg. Pinang, Penang	Permatang Pauh, Penang	Padang Besar, Perlis		
Goat	16	20	0	36	50.0 (18)
Cattle	0	15	0	15	73.3 (11)
Horse	0	0	30	30	53.3 (16)
Deer	0	0	30	30	100 (30)
Total	16	35	60	111	
Nematophagous-fungi Percentage (No) ^a	62.5(10)	54.3(19)	76.7(46)		67.6 (75)

a = Number of sample positive for nematophagous-fungi.

Table 2. Fungi identification from animal faecal samples

Animals	No of samples collected	No of samples positive	Percentage (%)	Fungi
Goat	36	18	50	<i>Verticillium</i> spp. ^a
		18	50	<i>Botrytis</i> spp. ^b
Cattle	15	11	73.3	<i>Verticillium</i> spp. ^a
		4	26.7	NA
Horse	30	14	46.7	<i>Botrytis</i> spp. ^b
		16	53.3	<i>Verticillium</i> spp. ^a
Deer	30	30	100	<i>Fusarium</i> spp. ^a

NA – Not available / unknown species.

^a = Nematophagous-fungi group.

^b = Non nematophagous-fungi group.

were also shown positive results with nematophagous-fungi.

Three species of fungi have been identified in the faecal samples of animal however only two were belong to nematophagous-fungi group (*Verticillium* and *Fusarium* genus) (Table 2). There are 50% of the goat faecal samples were shown to be positive for *Verticillium* spp. and 100% of the deer faecal samples were positive for *Fusarium* spp. *Verticillium* spp. was found to be the most abundant in most samples of goat, cattle and horse. On the other hand, *Fusarium* spp. only present in faecal samples of deer. One non nematophagous-fungi of *Botrytis* spp. was present in the faecal samples of the animals.

A total of 50 soil samples were screened for the presence of nematophagous-fungi (Table 3). The same group of nematophagous-

fungi were identified from soil samples of the cattle grazing area in Permatang Pauh, Penang. *Fusarium solani* were observed to be present in all soil samples of deer grazing area in Padang Besar, Perlis. While for the soil samples of horse grazing area, *Verticillium* spp. and *Fusarium* spp. were observed.

DISCUSSION

Nowadays, the needs for bio friendly agents for nematode management has been dramatically increased due to the emergence of multidrug resistance and problem that associated with the wide use of chemicals in animal industry and environment. Since nematophagous-fungi can survive the gastrointestinal tract of animal, it could be

Table 3. Distribution of soil samples that were screened for the presence of nematophagous-fungi and percentage that were found to be positive

Sampling site	Grazing animal	Number of soil samples	Fungi species	Nematophagous-fungi Percentage (No) ^a
Permatang Pauh, Penang	Cattle	10	<i>Arthrobotrys</i> spp.	70.0 (7)
			<i>Botrytis</i> spp. ^b	30.0 (3)
Padang Besar, Perlis	Horse	20	<i>Verticillium</i> spp.	65.0 (13)
			<i>Botrytis</i> spp. ^b	25.0 (5)
			<i>Fusarium</i> spp.	10.0 (2)
	Deer	20	<i>Fusarium solani</i>	100.0 (20)
Total		50		

^a = Number of sample positive for nematophagous-fungi.

^b = Non nematophagous-fungi group.

provided a potential option as biological control agent. In this screening, two groups of nematophagous-fungi were present in fresh faeces of grazing animals and one additional group of nematophagous-fungi in soil samples. These fungi belong to *Verticillium* spp., *Fusarium* spp. and *Arthrobotrys* spp.

Out of 111 faecal samples, 67.6% were positive for nematophagous-fungi and this percentage can be considered high compared to the previous study by Chandrawathani *et al.* (2002) where only 28% of the faecal samples from 779 were found out to be positive. In their study no data on the diversity of nematophagous-fungi were reported because they only focused on *Arthrobotrys* and *Duddingtonia* that can be used for *in vivo* study. While in Indonesia, seven genera of nematophagous-fungi have been identified from sheep faeces, namely *Arthrobotrys*, *Cladosporium*, *Fusarium*, *Gliocladium*, *Paecilomyces*, *Trichoderma* and *Cephalosporium* (Briajaya and Ahmad, 1999).

In this study, *Verticillium* spp. was observed in faecal sample of cattle and deer. *Verticillium* spp. can be distinguished based on the size of the conidia where their conidia are avoid or ellipsoid and usually single-celled (Pegg and Brady, 2002). The used of *Verticillium* sp. was proven to be affective to reduce the infection caused by plant parasitic nematodes (de Leij *et al.*, 1992). *Fusarium solani* was found in soil samples of deer grazing area. Generally, *Fusarium solani* is a natural enemy of parasitic

nematode of plants and can be found everywhere worldwide. In addition, *Fusarium* sp. also can be found in the faeces of small ruminants (Satrija and Briajaya, 1998). According to Perpinan *et al.* (2010), *Fusarium* has been isolated from the faeces of animals without any signs of disease. A study by Zareen *et al.* (2001) showed that *Fusarium solani* caused substantial mortality of *Meloidogyne javanica* juveniles, a parasitic nematode of plant root. Study on T2-toxin, moniliformin, verrucarin A and cytochalasin B produced by species of *Fusarium* and other soil fungi also reduced viability of *Meloidogyne javanica* juveniles (Ciancio, 1995). Thus, it has been suggested that toxins released by *Fusarium* spp. may play an important role in the population dynamics of plant parasitic nematodes in the field. In this study, we are unable to identify the presence of *Arthrobotrys* in faecal samples. According to Samuel *et al.* (1999) and Manuelli *et al.* (1999) *Arthrobotrys* are susceptible to rumen fluid in animals *in vitro* and *in vivo*. Thus, could explained the negative results for *Arthrobotrys* in faecal samples.

CONCLUSION

The results of the present study showed that the present of nematophagous-fungi in the faecal samples of grazing animals (goat, cattle, horse and deer) and soil samples of the grazing areas. Further trials should be

considered for the evaluation of these potential fungi against parasitic nematodes. Study on their behaviour and ecology should be considered for better understanding of this unique predatory fungi.

Acknowledgements. Special thanks go to the local farmers in Permatang Pauh and Sungai Pinang, Penang for their cooperation to use their farm for this study. Special thanks also go to staffs of Department of Veterinary Services, Perlis for their technical supports for this study and to Professor Baharuddin Salleh for his help for the identification of the fungi. This study was funded by Short Term Grant provided by Universiti Sains Malaysia (304 / PBIOLOGI / 6311112).

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