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LARVICIDAL ACTIVITY OF NEMATOPHAGOUS FUNGI *Duddingtonia flagrans* AGAINST COMMON STRONGYLE ROUNDWORMS OF BUFALOES *(Bubalus bubalis)*

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ABSTRACT

Infestation of gastrointestinal nematodes is a major problem in grazing animals. Control is achieved through administration of anthelmintics; however, because of indiscriminate use, there have been increased reports of resistance to chemical anthelmintics which led to the failure of parasite control. This study determined the efficacy of the chlamydospore of *Duddingtonia flagrans* as biological control against common strongyle roundworms of buffaloes. Using corn meal agar assay, strongyle infective larvae were treated with and without *D. flagrans*. The chlamydospore/ gram (CG) assay tested a dose-dependent concentration wherein feces with 2,100 eggs/ gram (EPG) strongyles were treated with *D. flagrans* at an increasing doses of CG (100,000, 250,000 and 500,000). Results showed an 84.39% larval reduction after treatment with 500,000 CG. The chlamydspore/ egg assay evaluated increasing ratios of egg to chlamydospore dose (1:0, 1:100, 1:500, 1:1000) using the 2,100 EPG feces. The ratio 1:500 achieved the highest percent larval reduction (78.88%). *D. flagrans* was directly fed to buffaloes at varying concentrations (50,000, 150,000, 250,000 chlamydospores/kg BW). A 78.77% larval reduction was observed at 50,000 chlamydospore/kg BW oral administration for 5 days. This study showed the efficacy of *D. flagrans* as a potential alternative for anthelmintics in buffaloes.

Keywords: biological control, *Duddingtonia flagrans*, larval reduction, nematophagous fungi, strongyles

INTRODUCTION

Gastrointestinal parasitism caused by nematodes, especially in pasture-grazed animals, is of a great concern since control failure leads to reduced production efficiency and profits (Ojeda-Robertos *et al*., 2008; Sagues *et al*., 2011; Waller, 1997; Waller *et al*., 2001). The most conventional method of control used by farmers is the frequent administration of anthelmintics. However, when anthelmintics become the primary method of control, resistance is most likely to happen (Sangster, 1999).

Anthelmintic Resistance (AR) occurs when the nematode populations that have been normally susceptible to the anthelmintic treatment lose that susceptibility through selection. In the Philippines, a study has confirmed the resistance of different nematodes using chemical anthelmintics in different regions of Luzon, Visayas and Mindanao (Venturina *et al*., 2003). An alternative method of parasitic control which produces promising effects over the past years is biological control (BC). The idea of BC is to use

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one organism to gain control of another target organism (Thamsborg *et al.*, 1999). In the context of anthelmintics, it is a preventive measure which has no effect on the adult stages inside the animal but its goal is to reduce the pasture larval infectivity and simultaneously reducing the parasitism in the grazing livestock (Larsen, 1999).

Duddingtonia flagrans, a nematode-trapping fungi, has stimulated intense interest by the researchers due to its ability to survive the gastrointestinal tract because of its abundant thick walled resting spores (chlamydospores) as well as destroy the developing parasite in the dung by forming specialized 3-dimensional sticky networks that trap developing parasite soon after it has been deposited on the ground (Larsen *et al.*, 1991, 1992; Faedo *et al.,* 1997; Gronvold *et al.*, 1993, Waller *et al.*, 1994; Nansen *et al.*, 1995; Wolstrup *et al.*, 1996; Fernandez *et al.*, 1997; Githigia *et al.*,1997).

While there are various studies showing the practical use of nematophagous fungi in other countries, it remains to be investigated whether use of nematophagous fungi as a potential biological control agent against internal parasites would be feasible in the Philippines. Since gastrointestinal parasitism and anthelmintic resistance are major concern of farmers, especially in the ruminant industry, this study which aimed to determine the efficacy of *D. flagrans* against strongyle-type roundworms of buffaloes and be an alternative for chemical treatment.

MATERIALS AND METHODS

The fungus *D. flagrans* strain ARSEF 5701 was obtained from the Lapemi-Laboratorio De Pesquisa Micologicas, Campus Universitatio-Universidade Federal Santa Maria, Brazil.

Approximately 5 grams of sorghum grain-enriched *D. flagrans* were placed in Corn Meal Agar (CMA) plates and grown for 7 days. This was added with 5 ml of Tween® 20 (Life Technologies, USA) to stimulate release of chlamydospores from the mycelia. Subsequently, the mycelia were scraped with a spatula and gently rinsed with 5 ml of sterile water. The suspension was centrifuged in a Beckman Coulter Allegra® X-22R Centrifuge at 1500 rpm for 5 minutes. After the supernatant was removed, it was washed and 10 ml of distilled water was added. Finally, it was centrifuged at 1500 rpm for 5 minutes and a Neubauer haemocytometer (Marienfeld®, Germany) was used to approximate the number of chlamydospore per ml.

Strongyle-infested feces obtained from backyard-raised native buffaloes from Brgy. Villa Cuizon, Muñoz, Nueva Ecija was the source of infective larvae. The collected feces were evaluated using centrifugal fecal flotation technique (Dryden *et al.*, 2005). Ten grams of fresh feces was added to 20 grams of fragmented vermicast. It was thoroughly mixed to achieve homogeneity. The culture was compacted and water was added along the walls of the 250 ml culture bottle to allow moisture accumulation for a period of seven days. Following the procedure adapted by Tongson and Dimaculangan (1983): after 7 days, the setup was allowed to stand for 12 hours and then the water in the petri dish containing larvae was transferred into a 15 ml conical tube (BD Falcon®, USA). The content was reduced to 1 ml where larval count was measured in 50 µl aliquot. Finally, the larvae were killed by adding Lugol's iodine before counting.

In-vitro Assay

A CMA assay was done to test the predatory activity of *D. flagrans to* strongyle larvae grown on CMA**.** In this assay, two sets of plates with four replicates each were used to test the predatory activity of *D. flagrans*. The treatment descriptions were NC – negative control; CMA + 400 strongyle infective larvae and DF – CMA + *D. flagrans* + 400 strongyle infective larvae. The petri dishes were kept in an incubator at 27-29˚ C. After 48 hours,

all the petri dishes were washed with 5 ml distilled water and the water was collected in a 15ml conical tube (BD Falcon®, USA). An aliquot of 1 ml was taken to count the number of larvae. A drop of Lugol's iodine was added to immobilize the larvae in the harvested water. Larvae were counted in 50 µl aliquots.

A CG Assay was done to evaluate the reduction percentage of strongyle larvae by *D. flagrans* in fecal larval culture on a per gram basis of chlamydospores. Five grams of thoroughly mixed feces (2,100 EPG) counted with Modified McMaster Technique using a McMaster slide (Weber England® McMaster Slide) were added with 10 grams of vermicast and placed in sixteen 250 ml bottles. These were allocated into four treatment groups with four replicates each in a complete randomized design (CRD). Four levels of predetermined concentration of *D. flagrans* were subjected to increasing levels of chlamydospore concentration. Larvae recovery was also done by counting in 50 ml aliquots. The treatment descriptions were: NC – feces only (negative control); T1 – feces + 100,000 *D. flagrans* chlamydpspores/gram feces; T2 – feces + 250,000 *D. flagrans* chlamydospores/ gram feces; T3 – feces + 500,000 *D. flagrans* chlamydospores/ gram feces.

Then a CE assay was performed to assess the reduction percentage of strongyle larvae by *D. flagrans* in fecal larval cultures on a per egg basis of chlamydospores thus, to determine the effective dose level given a definite load of EPG from an animal's feces. Concomitant to CG Assay, the same number of fresh buffalo fecal samples and vermicast were placed in sixteen 250 ml bottles. The treatments' descriptions are NC – feces + 1 EPG/0 *D. flagrans* chlamydospores (1:0); T1 – feces + 1 EPG/ 100 *D. flagrans* chlamydospores (1:100); T2 – feces + 1 EPG/ 500 *D. flagrans* chlamydospores (1:500); T3 – feces + 1 EPG/ 1000 *D. flagrans* chlamydospores (1:1000). A total of 0; 210,000; 1,050,000 and 2,100,000 in all of the treatment assignments were added to every five grams of the strongyle-positive feces. Each treatment was replicated four times following CRD. Larval recovery was done following the procedure of Tongson and Dimaculangan (1983).

In vivo Assay

In order to fully confirm the effectivity *of D. flagrans* as a biological anthelmintic, an *in vivo* study was conducted to test the chlamydospore suspension previously prepared and orally administered at different concentrations to the experimental buffaloes. Four Brazillian Murrah buffaloes of the Philippine Carabao Center (PCC) Dairy Farm, Central Luzon State University, Muñoz, Nueva Ecija with zero strongyle eggs per gram (EPG) were used for treatment. Each animal was designated to four treatment groups: NC – negative control (fed the standard diet [SD] only); T1 – 50,000 *D. flagrans* chlamydospores/ kg BW; T2 – 150,000 *D. flagrans* chlamydospores/ kg BW; T3 – 250,000 *D.flagrans* chlamydospores/ kg BW. The prepared chlamydospore suspension was administered to buffaloes under T1 to T3 orally once daily for 5 consecutive days, before their feeding schedule in the afternoon. A withdrawal period of chlamydospore suspension feeding was done for two days, while the feces were collected continuously.

Rectal fecal samples were collected for 7 consecutive days. The thoroughly mixed feces were collected daily from the donor animal with a defined number of EPG. Individual animal fecal cultures were performed in triplicate, where 10 g of *D. flagrans* chlamydospores treated or untreated feces (control) were mixed with 10 g of donor feces in a 250 ml bottle. Total number of larvae recovered per treatment was counted.

Fecal Egg Count was computed as number of eggs counted x 50, expressed as EPG.

The Percent Larval Reduction was computed as:

$$\frac{\text{Control Mean Larvae Recovered - Treatment Mean Larvae Recovered}}{\text{Control Mean Larvae Recovered}} \times 100$$

When results were negative, the percent reduction was considered zero. Data were submitted to analysis of variance (ANOVA) and Tukey's Test. Subsequently, the means were compared using least significant difference test.

RESULTS

CMA Assay. Table 1 shows the effect of addition of larvae on CMA plates as compared to fully developed *D. flagrans* plates in terms of larval survivability when exposed to CMA and *D. flagrans* petri dishes, respectively. The CMA assay showed that treatment DF fungal plate is 95.85% efficient in reducing the larvae compared to NC demonstrating the predatory activity of *D. flagrans* by inducing entrapment of the L3 of strongyle roundworms.

CG Assay. In the CG assay, results showed the effect of addition of increasing numbers of *D. flagrans* chlamydospore at four different dose rates in the feces (Table 2). Results showed that the mean larvae recovered from T2 and T3 were significantly lower (P <0.05) than the control. The highest percent larval reduction (84.39%) was seen in T3 while mean larvae of T1 has still achieved 42.06%.

Table 1. Mean larvae recovered and percent efficacy for CMA and *D. flagrans* petri dishes after 48 hours incubation.

Treatment	Total Larvae Recovered/ml	Mean Larvae Recovered/ Treatment	Percent Efficacy
NC[1]	1326	331.5 [a]	17.12
T1[2]	66	16.5 [b]	95.85

Means with no common superscript(s) were not significantly different P < 0.05
[1]NC – CMA + 400 infective larvae of strongyles
[2]DF – CMA + *D. flagrans* + 400 infective larvae of strongyles

CE Assay. In the CE Assay, results showed the effect of adding chlamydospore of *D. flagrans* per egg of strongyle positive feces in four different dose ratios. The same feces, along with culture procedures used in CG Assay, were used in this study to show the reduction in the mean number of the larvae recovered among the chlamydospore per egg ratios.

Table 2. Mean larvae recovered and percent reduction in increasing levels of spore per gram administration.

Treatment	Total Larvae Recovered/ml	Mean Larvae Recovered/ Treatment	Percent Reduction
NC[1]	1890	472.5 [a]	-
T1[2]	1095	273.75[ab]	42.06%
T2[3]	595	148.75[b]	68.51%
T3[4]	295	73.75 [b]	84.39%

Means with no common superscript(s) are not significantly different P < 0.05
[1]NC – 0 chlamydospores/g feces
[2]T1 – 100,000 chlamydospores/g feces
[3]T2 – 250,000 chlamydospores/g feces
[4]T3 – 500,000 chlamydospores/g feces

Results showed that all the chlamydospore treatments were statistically significant (P< 0.05) as shown by evident reduction in larvae recovery. All the chlamydospore treated feces had a reduced larvae recovered by more than 50% as compared to the control. T3 had the highest larval reduction at 76.34%.

In vivo assay. Tables 4 and 5 show the effect of the addition of increasing levels of chlamydospores of *D. flagrans* when fed to buffaloes for a period of 5 days and two days of withdrawal.

Table 3. Mean larvae recovered and Percent reduction in increasing levels of Chlamydospore per Egg administration.

Treatment	Total Larvae Recovered/ml	Mean Larvae Recovered/ Treatment	Percent Reduction
NC[1]	1965	491.25[a]	-
T1[2]	980	245[b]	50.13%
T2[3]	415	103.75[b]	78.88%
T3[4]	465	116.25 [b]	76.34%

Means with no common superscript(s) are not significantly different P < 0.05
[1]NC – 1:0
[2]T1 – 1:100
[3]T2 – 1:500
[4]T3 – 1:1000

On day 2, larval reduction was observed to increase between 18-26% across all treatments. The following day, the larvae continued to decrease from 38%-55%. Until the last day of chlamydospore administration, the highest larval reduction (78.77%) was achieved by T1. T3, despite the highest chlamydospore count, only had 65.55%. After discontinuing chlamydospore administration, on the sixth day, it was interesting to note that all of the chlamydospore treated animals had a 7.42% (T2) and 29.38% (T3) decrease in larval reduction than the previous day. The lowest chlamydospore treatment (T1) only had negative larval reduction on the same day which means the control had lower larval count than the chlamydospore treatment after withdrawal at day 6. The trend continued to decrease on day 7 as larval reduction reached 25.07% and 37.6 % compared to the last day of chlamydospore administration. However, T1 had a decreased larval reduction compared to the previous day. Finally, reduction rate demonstrated by T1 to T3 were all significant when compared to the control (P<0.05), which is an indication that the lowest dose of *D. flagrans* was more than enough to affect larval reduction under *in vivo* conditions in individual animals tested in this study.

Table 4. Mean larvae recovered on administration of *D. flagrans* in the 5-day feeding and 2-day withdrawal period of buffaloes.

Treatment	Mean larvae recovered per ml at different day treatment						
	d1	d2	d3	d4	d5	d6	d7
NC[1]	471.67	863.00	503.30	408.30	378.30	415.00	626.67[b]
T1[2]	611.67	395.00	321.70	225.00	118.30	606.67	428.30[a]
T2[3]	618.30	446.67	258.30	206.67	161.70	221.67	316.67[a]
T3[4]	550.00	381.67	288.30	203.30	185.00	331.67	415.00[a]

Means with no common superscript(s) are not significantly different P < 0.05
[1]NC – 0 chlamydospores/g feces
[2]T1 – 100,000 chlamydospores/g feces
[3]T2 – 250,000 chlamydospores/g feces
[4]T3 – 500,000 chlamydospores/g feces

DISCUSSION

The significant difference (P<0.05) as shown by the low survivability of the larvae (16.05) from the control plates compared to the *D. flagrans* plates (331.50) in the CMA assay is a manifestation of the predatory activity of the fungi.

Several factors influence the success of trap formation and larval entrapment of nematophagous fungi such as motility and size of larvae (Bishnu, 2004). The predatory activity of another nematophagous fungi, *Arthobotrys oligospora*, has been shown to be influenced by locomotive behavior and size. Nansen *et al.* (1988) confirmed this correlation in their study wherein the most motile L3 (i.e. ruminant *trichostronylid* nematodes) were able to induce the greatest number of traps compared to the least motile ones, such as cattle lungworm, which was trapped poorly by *A. oligospora.* Meanwhile, in this study, since the L3 placed in the agar plates were from the fecal culture of strongyle positive buffalo, it supports the studies that motile ruminant trichostronylids is capable of inducing traps as there was a significant difference in the mean larvae recovered after 48 hours post rinsing.

Table 5. Percent larval reduction on administration of *D. flagrans* in the 5-day feeding and 2-day withdrawal period of buffaloes.

Treatment	Percent larval reduction at different day treatment					
	d2	d3	d4	d5	d6	d7
NC	0	0	4.63	3.90	0	0[b]
T1	23.87	38.43	51.32	78.77	0	28.63[a]
T2	18.15	55.27	62.31	69.42	62.00	42.73[a]
T3	26.06	49.55	60.72	65.55	36.17	27.95[a]

Means with no common superscript(s) were not significantly different (P > 0.05)
[1]NC- 0 chlamydospores/kg BW
[2]T1- 50,000 chlamydospores/kg BW
[3]T2- 150,000 chlamydospores/kg BW
[4]T3- 250, 000 chlamydospores/kg BW

In the CG Assay, the highest percent reduction rate of 84.39% seen in the highest chlamydospore treatment (500,000 chlamydospores/g feces) indicated that the chlamydospore suspension can be applied directly to the feces of a nematode infected animal. This is in agreement with a study in Spain, where increasing amounts of chlamydospores were directly spread on fecal pats of naturally infected horses and was found to have an overall 94% reduction as well as decrease in EPG count (Paz-Silva *et al.*, 2011).

All the treatment ratios (1:100, 1:500, 1:1000) in the present study were able to demonstrate reduction in fecal larvae compared to the control (P <0.05). This supports the findings in goats (Bishnu, 2004) which showed 89.8% larval reduction at a very low concentration of 1:1 – 1:10 egg to chlamydospore ratio compared to the 78.89% (1:1000) of the present study.

Ojeda-Robertos *et al.* (2008) explored the relationship between chlamydospore per gram (CPG) and egg per gram of feces (EPG) in reducing the infective larvae of *Haemonchus sp.* as well as quantify the effective number of chlamydospores per gram of feces administered orally. A higher chlamydospore dose was able to produce higher recovery of chlamydospore per gram but a clear-dose dependent effect did not manifest in the larvae reduction or in CPG:EPG ratio. It is interesting to note however that a

threshold was reached in the present study where the two fold increase in the highest spore concentration (1:500 vs. 1:1000) did not produce any increase in reduction rate. Therefore, 1:500 is sufficient to reduce larvae present in the feces of the animal which would later on infect the pasture area of the grazing animals.

In the *in vivo* assay, wherein increasing doses of chlamydospores were administered to one buffalo in each treatment group, it was observed to have a decrease in larval reduction on day 6 (no chlamydospore administration), after a continuous increasing trend since day 2 which signifies development of mycelial traps in the fecal larval cultures that can reduce larval counts in buffaloes treated for five consecutive days. The significant difference (P <0.05) shown between the control and chlamydospore treated animals indicates that *D. flagrans* may be a beneficial part of the standard diet of the animal due to the substantial increase of larval reduction after larval culture during the 5-day treatment period.

The results of this *in vivo* study confirmed that chlamydospores were able to bypass the gastrointestinal tract as there was a continuous larval reduction after fecal larval culture was observed throughout the 5-day feeding period and a sudden decrease in the same parameter after the withdrawal of the daily chlamydospore suspension. However, although the chlamydospore suspension prepared was able to reduce the larvae in the feces, the decrease was not as substantial as the one reported by Peña *et al.* (2002) where as early as day 2, there was 80.9 – 99% larval reduction in treatment groups of goats which were administered with 50,000 – 1,000,000 chlamydospores/ kg BW. It also continued to remain within this range until day 7 of spore administration. Two days after the spore feeding was stopped, reduction had dropped substantially and remained low until the last day of the trial which was also observed in the present study.

In contrast to the field trials conducted by Braga *et al.* (2009) where a commercially available *D. flagrans* with precounted dose of 10^6 spores per gram, the present study had a manual preparation of chlamydospore suspension. Several factors have influenced release of chlamydospores from mycelia. To the authors' knowledge, no published studies were done yet using the detergent Tween® 20 as an agent to release the chlamydospore from mycelia although Santurio *et al.* (2011) mentioned Triton X, a similar detergent, can also serve to release chlamydospore from mycelia. In addition, several factors can affect chlamydospore germination such as temperature and pH (Okazaki, 1975). This may explain the slower rate of percent larval reduction throughout the whole feeding period as compared to the substantial larval reduction, as early as day 2, in the study of Peña *et al.* (2002).

Several investigations have already been conducted to define the optimal number of chlamydospores dosage orally in order to achieve maximum larval reduction from sheep (Peña *et al.*, 2002; Terril *et al.*, 2004) or goats (Paraud *et al.*, 2005) however; there are no published data yet in buffaloes. Faedo *et al.* (2002) revealed in his study that higher chlamydospore doses ($1x10^5$-$1x10^6$/kg BW) had an efficacy approaching to 100% in L3 of *T. colubriformis* in cattle. This is due to the expected larval reduction as higher number of chlamydospores are expected to pass into the feces. Terrill *et al.* (2004) used the same chlamydospore levels (50,000 chlamydospores/kg BW & 250,000 chlamydospores/kg BW) of the present study. Their study disclosed a 60.8% and 80.2% respectively in the larval reduction of the mixed nematode infection in goats (*H. contortus*, *T. colubriformis*, *Cooperia sp*). The present study was able to show that even the lowest chlamydospore treatment (50,000 spores/kg BW) had the highest percent larval reduction (78%) while the highest chlamydospore treatment (250,000 chlamydospores/kg BW) had the lowest percent larval reduction (65%) among the fungal treated animals.

To date, presence of *D. flagrans* has not yet been reported in the environment of the Philippines. Routine detection and identification of nematophagous fungi typically involves nematode baiting, repeated incubations and re-isolation in order to obtain a pure

culture, followed by microscopic examination of hyphae and spores. Nowadays, molecular approaches are increasingly being used to develop PCR-based species specific diagnostic assays for broad range of organisms (Atkins and Clark, 2004).

Modern approaches to parasitic control need to move away from complete reliance on chemical treatments to a more integrated form of parasite management. Biological control, using nematophagous fungi *D. flagrans*, offers an alternative method as a prophylactic worm control which serves to reduce the living parasite population thereby reducing pasture infectivity and contamination (Waller, 1997). *D. flagrans* has the ability to disrupt the cycle of parasite development by simultaneous reduction of the number of infected animals and number of infective larvae on pasture (Lalosevic *et al.*, 2008).

Biological control is not a total substitute for anthelmintics. It should be used in combination with other anthelmintics (in reduced amount) as *D. flagrans* has no chemotherapeutic effect on the worm population inside the animal and therefore will not be affected (Waller, 1997). Moreover, with the increasing awareness of the consumers to animal products without drug residues (Charon, 2004; Diez-Tascon *et al.*, 2005), this alternative approach of parasitic control provides an opportunity for livestock producers to capitalize on chemical- free products.

Results herein provide preliminary data on the effect of *D. flagrans* on buffaloes (*Bubalus bubalis)*. There were few attempts to study other species of nematophagous fungi in the country yet no *in vivo* studies have been done to date. Isolation and confirmation of local strains of *D. flagrans* in feces and soil environment is recommended as this is found to be more potent than foreign isolates. Larval reduction experimentation on grazing pastures for longer periods is also highly suggested since it will provide information on the efficacy in different seasons. The economic impact of the use of *D. flagrans* also needs to be studied in the future since it will provide a background on financial losses and gains that a farmer will obtain from a herd with non- parasitized and parasitized infection while using *D. flagrans* as an alternative control method.

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