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Research paper

The nematophagous fungus *Duddingtonia flagrans* reduces the gastrointestinal parasitic nematode larvae population in faeces of orally treated calves maintained under tropical conditions. Dose/Response assessment

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ABSTRACT

This research was performed to assess the *Duddingtonia flagrans* chlamydospore dose/response administered in gastrointestinal parasitic nematode (GIN) naturally infected calves and its effect in reducing the GIN infective larvae (L_3) population in faeces in a cattle farm in the Mexican tropics. This study was carried out at the experimental research station "Las Margaritas" in Hueytamalco Municipality, Puebla State, Mexico. Forty zebu calves, between 6 to 12 months of age were randomly distributed into four groups of ten calves each. One control group and three treated groups with an aqueous suspension containing three different oral doses of *D. flagrans* chlamydospores based on their body weight (BW) were established as follows: Group 1 (Control); group 2, (Lowest fungal dose) 0.250×10^6 chlamydospores per kg/BW; group 3, (Medium fungal dose) 0.5×10^6 chlamydospores per kg/BW and group 4 (highest fungal dose), 1×10^6 chlamydospores per kg/BW. The corresponding fungal chlamydospore doses were daily administered for 10 consecutive days. Every group of calves was confined into individual pens and they received a maintaining nutritional regime based on Buffel grass, nutritional concentrated supplement and water ad libitum. Every third day; since one week before treatments each animal was sampled to collect faeces taken directly from rectum to determine the number of nematode eggs eliminated per g of faeces (epg), through the McMaster technique. Four faecal cultures of 20 g each from each individual faecal sample were elaborated in plastic Petri Dishes and incubated for 14 days at room temperature (18–25 °C). Criterion to estimate the efficacy of the different treatments was based on the mean number of the GIN infective larvae recovered from faecal cultures of treated groups and compared with the control group. Data were analysed using a completely randomized design through an ANOVA analysis, followed by a Duncan multiple range test. The efficacy in reducing the larvae population in faeces attributed to the effect of the different treatments were based on the Abbot´s formula giving as a result a larval rate reduction. Results showed a great variation in the epg account in the different groups along the experiment. The reduction of the larvae population attributed to the effect of the fungal administration in calves was observed from 4th to 11th day post-treatment in the three assessed dose. Results in group 2, treated with the lowest fungal dose showed 88.5, 57.6, 55.9 and 30% (58% overall reductions) in the GIN infective larvae populations in faeces of animals after 4, 7, 9 and 11 days post-treatment; respectively. In the group 3, treated with the medium fungal dose; 95.8, 80.4, 63.4 y 52.7% larvae reduction (73% overall reduction) were recorded. At the highest chlamydospore dose used (1×10^6) the results were: 88.9, 78.0, 59.3 and 67.3% (73.5% overall reduction) ($p < .05$). The species of identified nematodes through larvae morphometric and molecular taxonomy were *Cooperia* sp. and *H. contortus.*

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From the three *D. flagrans* chlamydospore assessed doses, the medium dose (0.5×10^6 *D. flagrans* chlamydospores per kg/BW) was sufficient to provide an important reduction of the nematode larvae population in faeces of calves in production under the Mexican tropics.

1. Introduction

The conventional system of control of gastrointestinal parasitic nematodes in cattle is based on the regular administration of anthelmintic chemical drugs in cattle herds; although farmers excessively use these drugs due to their growing inefficacy (Ramos et al., 2016). This strategy has various inconveniences in their use; for example, after being pressured for repeated occasions with chemical treatments, parasites develop mutations in their genome and some genes have been identified as responsible of overcoming the lethal effect of anti-parasitic drugs (Encalada-Mena et al., 2014; EMA, 2016; Laing et al., 2016). On the other hand, the use of anthelmintic drugs brings other problems *i.e.,* the possible presence of drug residues in meat, milk or even in sub- products for human being consumption, which is considered as an important potential risk of public health (NMPF, 2016; Beyene, 2016). Likewise, the anthelmintic compounds administered in the animals are eventually spelled together with the animal faeces to the soil contaminating the environmentand affecting beneficial microorganisms (Lumar et al., 2013; Adler et al., 2016). These are some disadvantages in the use of anti-parasitic drugs against parasitic nematodes; however, other alternative methods of control of these parasites have been proposed and they include the use of grazing rotation, supplying a source of protein and energy incorporated into nutritional supplement food, vaccination, the use of plants and their products with a high content of proteins and additionally some of theses plants contain metabolites with anthelmintic activity (Stear et al., 2007). On the other hand, the use of nematophagous fungi has being considered as an important biological toll of GIN control. Nematophagous fungi are a group of natural-nematode enemies living in soil and they act as regulators of the nematode populations in nature. This group of micro- fungi are soil saprophyte organisms; however, in presence of nematodes they form trapping devices, especially designed to catch nematodes, destroy them and feeding on their internal tissues. Cattle health should not depend under a chemotherapy system; since clean production of meat and milk developed under a non-chemical usage is a growing demand worldwide. The Mexican tropics offer an enormous potential source of forage to feed cattle adapted to extreme climate conditions. Unfortunately, humidity and warm conditions with abundant grass and the presence of calves are exactly the necessary elements to favour high populations of GIN infective larvae contaminating grass and promoting endless re-infections of the animals. The use of nematophagous fungi has displayed their benefits in the control of ruminant parasitic nematodes; although, establishing the adequate dose of chlamydospore to achieve a satisfactory result in the control of GIN larvae is crucial to achieve the best practical and realistic commercial conditions. This study was designed to assess three different *D. flagrans* chlamydospore doses in reducing the GIN infective larvae population in calves naturally infected in a cattle farm in the Mexican tropics.

2. Materials and methods

2.1. Allocation

This study was carried out at the experimental research station “Las Margaritas” in Hueytamalco Municipality, Puebla State, Mexico. This farm is located in the Northeast

Mountain Range at - an -O; at an altitude range between 450 and 500 metres above the sea level. Prevalent weather is classified as Af (m) (warm and humid with rain during the whole year) with 22 ºC mean temperature; 90% relative humidity and 2400–3000 mm rainfall (Espino-Barros et al., 2005).

2.2. Biological material

2.2.1. Duddingtonia flagrans chlamydospore production

The *D. flagrans* FTHO-8 strain belonging to the CENID-Parasitología Veterinaria, INIFAP fungal collection, was used. This strain was originally isolated from sheep faeces from a sheep farm in Fierro del Toro community, Huitzilac municipality, Morelos State, Mexico (Llerandi-Juárez and Mendoza de Gives, 1998). Fungal production was achieved transferring some fungal mycelia to sterile wheat agar plates and incubating at room temperature (18–25 °C) for 30 days. After this period an abundant production of chlamydospores were produced and they were recovered by adding some millilitres of sterile distilled water and scrapping the agar surface with a scalpel to remove most of produced spores from agar. The estimation of the mean number of fungal chlamydospores in an aqueous suspension; as well as the elaboration of fungal Inocula was achieved using the NeuBauer´s chamber technique.

2.3. Experimental animals

From a zebu calves herd grazing on GIN contaminated pasturages were randomly selected forty animals with an age range between 6 to 12 months. All the animals resulted positive to the presence of GIN eggs in their faeces through the McMaster´s chamber technique. It is important to remark that the animal welfare and the unnecessary animal suffering are Good Management Practices policies well established in our institution. For these reasons, to ensure animal welfare when this experiment was carried out the Norma Oficial Mexicana (Official Rule number) NOM- 052-ZOO-1995 (https://www.gob.mx/senasicaas) well as the Ley Federal de Sanidad Animal (Federal Law for Animal Health) DOF 07-06-2012 (http://www.diputados.gob.mx/LeyesBiblio/ref/lfsa.htm), were strictly abided and all the procedures performed in this study were carried out in accordance with the ethical standards at INIFAP.

2.4. Experimental design

Animals were randomly distributed into four groups of ten animals each. Group conformation was balanced based on their mean of elimination of GIN faecal eggs. One control group and three groups treated with an aqueous suspension containing three different oral doses of *D. flagrans* chlamydospores (BW) were established, based on their body weight as follows: Group 1 (Control); group 2, 0.250×10^6 chlamydospores per kg/BW; group 3, 0.5×10^6 chlamydospores per kg/BW and group 4, 1×10^6 chlamydospores per kg/BW. The corresponding fungal chlamydospore doses were daily administered for 10 consecutive days. Every group of calves was confined into individual pens and they received a maintaining nutritional regime based on Buffel grass, nutritional concentrated supplement and water ad libitum. Every third day; since one week before treatments, each animal was sampled directly from rectum to collect faeces to determine the number of nematode eggs eliminated per g of faeces (epg), through the McMaster technique. Four faecal cultures of 20 g each from each individual faecal sample were elaborated in plastic Petri Dishes (100 × 15 cm) and incubated for 14 days at room temperature (18–25 °C). Criterion to estimate the efficacy of the different treatments was based on the mean number of the GIN infective larvae recovered from faecal cultures of treated groups and compared with the control group.

2.5. Metereological parameters

2.5.1. Temperature and rainfall

The environmental temperature values (maximum, medium and minimum) and rainfall values were recorded every two days, since the beginning of the experiment. Meteorological data were obtained from the closest meteorological station into the farm.

2.6. Statistical analysis

Data were analysed using a completely randomized design through an ANOVA analysis, followed by a Duncan multiple test. Data were square root transformed to get a normal distribution. The efficacy in reducing the larvae population in faeces attributed to the effect of the different fungal treatments was based on the Abbot's formula and the result was expressed as larvae reduction percentage (Aguilar-Marcelino et al., 2016).

$$\text{Larvae Reduction \%} = \frac{\text{X recovered larvae from control group-X recovered larvae from treated group}}{\text{X recovered larvae from treated group}} \times 100$$

3. Results

Results showing the mean elimination of nematode eggs per g of faeces of one control group and three groups of calves receiving three different *Duddingtonia flagrans* chlamydospore doses along the experiments are summarized on Table 1.

The results of the larvae counts recovered from faecal cultures from the different groups are summarized on Table 2. The effect in reduction of the faecal larvae population attributed to the administration of the three different fungal chlamydospore regimes was observed from the fourth until the 11th day post-treatments in the three different fungal assessed doses. The mean reductions in the faecal larvae population recovered from the different sampling days were: 88.5, 57.6, 55.9 and 30% at 4th, 7th, 9th and 11th day post-treatments at the lowest assessed dose (0.250×10^6 *D. flagrans* chlamydospore/kg BW). The overall mean faecal larval reduction by effect of the fungal administration during these days was 58% with respect to the control group. Meanwhile, 95.8, 80.4, 63.4 and 52.7% reductions were recorded during the same sampling days using the medium fungal dose (0.5×10^6 per kg BW). These percentages gave 73% overall larval reduction, during this time. On the other hand, the highest fungal dose (1×10^6 per kg BW) resulted in 88.9, 78.0, 59.3 and 67.3% larval reductions ($p < .05$); which resulted in 73.3% overall larvae reduction ($p < .05$) (Table 2). The morphometric analysis of recovered larvae in faecal cultures showed the presence of two gastrointestinal parasitic nematodes *Haemonchus contortus* and *Cooperia* sp. The environmental temperature values (maximum, medium and minimum) and pluvial precipitation values along the 8 sampling days in the area of this study are shown in Figs. 1 and 2; respectively.

4. Discussion

The present research, shown clear evidence about the efficacy of the three assessed *D. flagrans* chlamydospore doses in reducing two important gastrointestinal parasitic nematode infective larvae (*H. contortus* and *Cooperia* sp.) in faeces of treated zebu calves naturally infected under grazing tropical conditions. It is interesting the fact that the effect in reducing the faecal larvae population was recorded 24 after the first inoculation of chlamydospores in calves and it was maintained until one day after stop the fungal administration. From the three assessed fungal doses, the highest reduction percentages were recorded with the medium and high chlamydospore administered doses. The highest faecal larval reduction percentage in the three assessed doses

Table 1
Mean of GIN eggs per g of faeces recorded from zebu calves orally treated for 10 days with three different *Duddingtonia flagrans* chlamydospore doses.

Groups	Days Post-treatment							
	Day 0	Day 2	Day 4	Day 6	Day 9	Day 11	Day 13	Day 16
	Epg values (± standard Error)							
1 (Control)	775^{a} (± 9.81)	1630^{a} (± 16.55)	1105^{a} (± 10.95)	1005^{b} (± 11.55)	2440^{a} (± 15.93)	1365^{a} (± 20.38)	1125^{b} (± 10.02)	1185^{b} (± 22.11)
2 (0.250×10^6) *D. flagrans* chlamydospores /Kg BW	1005^{a} (± 9.81)	1655^{a} (± 16.55)	1280^{a} (± 10.95)	1265^{ab} (± 11.55)	1044^{b} (± 15.93)	1611^{a} (± 20.38)	1372^{b} (± 10.02)	1517^{b} (± 22.11)
3 (0.5×10^6) *D. flagrans* chlamydospores/Kg BW	820^{a} (± 9.81)	1625^{a} (± 16.55)	1365^{a} (± 10.95)	1345^{ab} (± 11.55)	2040^{a} (± 15.93)	3690^{a} (± 20.38)	2140^{a} (± 10.02)	1838^{b} (± 22.11)
4 (1×10^6) *D. flagrans* chlamydospores /Kg BW	1510^{a} (± 9.81)	1645^{a} (± 16.55)	1535^{a} (± 10.95)	1650^{a} (± 11.55)	2220^{a} (± 15.93)	2850^{a} (± 20.38)	1840^{ab} (± 10.02)	4544^{a} (± 22.11)

Note: Means in the same column with different literal are statistically different ($P > .05$).

Table 2
Means of gastrointestinal parasitic nematode larvae (L_3) recovered from faecal cultures from zebu calves orally treated with three different *Duddingtonia flagrans* chlamydospore doses and faecal larvae population reduction attributed to the fungal effect.

Group	Sampling days	Days post-treatment	Means of recovered larvae (L3)	Reduction percentage	Mean of overall faecal larvae reduction per group
	0	0	3900	———	
	1	2	2820	———	
	2	4	10680	———	
	3	7	7500	———	
1) Control	4	9	4800	———	
	5	11	1500	———	
	6	14	1680	———	
	7	16	2100	———	
	8	18	3540	———	
	0	0	3900	0	
	1	2	5580	0	
	2*	4	1200	88.6*	
	3*	7	3180	57.5*	58%
2) 0.250 × 10^6	4*	9	2100	55.9*	
chlamydospores/	5*	11	1020	30.1*	
kg BW	6	14	7920	0	
	7	16	2700	0	
	8	18	12060	0	
	0	0	8400	0	
	1	2	2700	4.3	
	2*	4	420	95.8*	
	3*	7	1440	80.4*	
3) 0.5 × 10^6	4*	9	1740	63.4*	73%
chlamydospores/	5*	11	720	52.7*	
kg BW	6	14	1200	27.2	
	7	16	6060	0	
	8	18	3240	8.9	
	0	0	5760	0	
	1	2	3900	0	
	2*	4	1140	88.9*	
4) 1 × 10^6	3*	7	1620	78.0*	
chlamydospores/	4*	9	1920	59.3*	73.3%
kg BW	5*	11	480	67.3*	
	6	14	2520	0	
	7	16	1380	32.5	
	8	18	3960	0	

* Data with a significant statistic differences ($p < .05$).

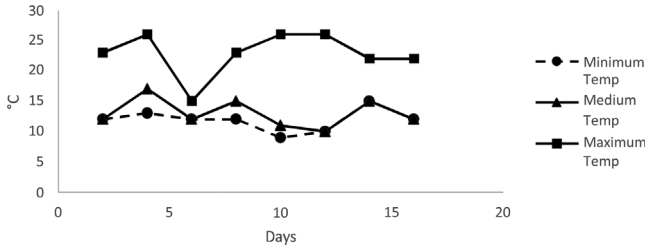

Fig. 1. Environmental temperature values recorded at the Hueytamalco meteorological station, in Puebla, Mexico during the period of 8 faecal sampling days of creole calves orally treated with three different *Duddingtonia flagrans* chlamydospore doses.

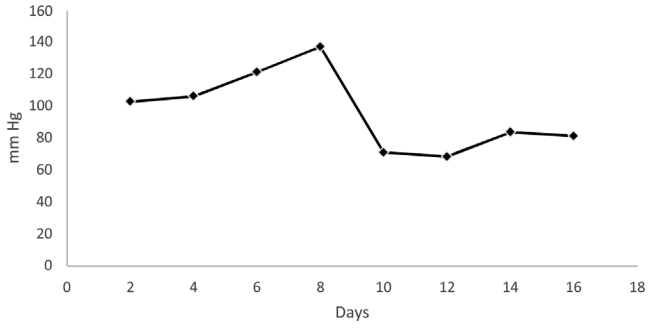

Fig. 2. Pluvial precipitation values recorded at the Hueytamalco meteorological station, in Puebla, Mexico during the period of 8 faecal sampling days of creole calves orally treated with three different *Duddingtonia flagrans* chlamydospore doses.

was recorded in the second sampling (48 h post-treatment). The results in the epg count shown great variability as we expected along the experimental sampling days. No direct correlation between temperature and pluvial precipitation with the larval reduction in faeces of treated calves was found. During the last decades the study of nematophagous fungi has gained great attention as a possible biotechnological and environmentally friendly way to reduce the gastrointestinal parasitic nematodiasis in ruminants (Ortiz Pérez et al., 2017; Aguilar-Marcelino et al., 2016). The nematode trapping fungi *Duddingtonia flagrans* has gained a good reputation as an alternative of control, different to the chemical anthelmintic drugs; mainly because of the continuous and frequent use of such compounds have triggered an imminent presentation of anthelmintic resistance in parasites against most of commercially available anthelmintic drugs (García et al., 2016; Andrioli-Salgado and de Paula-Santos, 2016). There are some records about the use of *D. flagrans* chlamydospores with different results. For instance, the efficacy a fungal formulation of *D. flagrans* chlamydospore in alginate pellets was evaluated in a Nelore herd in Brazil at a dose of 1 g of pellets per 10 kg BW, twice a week for 12 months; authors reported 56.7% faecal epg reduction of gastrointestinal nematodes and 60.5% faecal larval reduction (Assis et al., 2012). Later on same authors

carried out another experiment and elaborated two kind of alginate pellets; one with *D. flagrans* and other with *Monacrosporium thaumasium* at 0.2 g of each fungus per 10 kg BW; they reported 56.6 and 47.8% epg reductions; respectively after a year of grazing in cattle (Assis et al., 2013). In a recent study, conducted in a livestock organic milk farm in Malpaso, Chiapas (Southern Mexico), zebu/Swiss crossbreed calves receiving a *D. flagrans* dose of 2×10^6 chlamydospores, twice a week for 30 days resulted in 53.8% reduction in the gastrointestinal parasitic nematode infective larvae in faeces, with 75.3% as a maximum faecal larval reduction; although no statistical difference was observed. Authors also reported 25.2% larval reduction in grass with respect with a control group ($p > 0.05$) (Ortiz Pérez et al., 2017). It is important to mention that in our studies *D. flagrans* chlamydospore production is performed by transferring fungal material to potato dextrose agar plates only in a low scale; only to satisfy the experimental requirements of fungal material. This production of chlamydospores islaborious, consuming time and expensive. The commercialization of a product containing *D. flagrans* chlamydospores to be used in the control of ruminant parasitic nematodes requires developing a large scale chlamydospore production system in order to optimizing the materials to make a profitable and realistic production system with marketing competing prices. It is important to remark that the benefits in using nematophagous fungi as a biological tool of control of gastrointestinal parasitic nematodes are not only reflexed in the reduction of parasite populations, improving either the animal health and production; but in the environmental care, reducing the use of chemical anthelmintic drugs that contaminate soil and affecting beneficial microorganisms and can contaminate the phreatic aquifer (Wagil et al., 2014; Bártíková et al., 2016). On the other hand, it is interesting that the lowest chlamydospore rate used in the present study achieved almost 60% larvae reduction in faeces of the experimental herd. Meanwhile, the medium dose (0.5×10^6 chlamydospores per Kg BW) and the highest dose (1×106 chlamydospores per kg BW) achieved larval reduction percentages higher than 70%. These results motivate us to keep on study with this promising line of research and we planned in the near future assessing the cost-benefit values of these chlamydospore doses to establish a potential product containing *D. flagrans* chlamydospores for controlling ruminant parasitic nematodes to be used together with other sustainable control measures in an integrated control system.

5. Conclusions

The results of the present study shown evidence that the three selected doses of *D. flagrans* chlamydospore orally administered in calves, are effective in reducing the population of gastrointestinal parasitic nematodes in faeces of treated animals. The medium dose (0.5×10^6 chlamydospores/Kg BW) is sufficient to achieve 70% reduction of the faecal larval population and this result can have an important implication in future works focused to establish a biological control system against nematodiasis in calves maintained under tropical conditions

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