OPTIMIZING THE USE OF *Duddingtonia flagrans* CHLAMYDOSPORES AGAINST *Haemonchus contortus* IN FECES OF SHEEP

[USO ÓPTIMO DE CLAMIDOSPORAS DE *Duddingtonia flagrans* CONTRA *Haemonchus contortus* EN HECES DE OVINOS]

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SUMMARY

The aim of this study was to identify the proportion of *Duddintonia. flagrans* chlamydospores that is needed to the achieve an optimum trapping efficacy in the presence of eggs or L_3 larvae of *Haemonchus contortus*. Firstly, five groups of 15 coprocultures each were prepared, with increasing proportions of *H. contortus* eggs and chlamydospores (E:C) starting with Group I (control group) with a ratio of 1:0 and groups II to V with ratios of 1:1, 1:10, 1:100, and 1:1000, respectively. A second batch of fifty coprocultures was then prepared, with five groups of 10 coprocultures each and *H. contortus* infective larvae with chlamydospores, each group with increasing L:C ratios. The evaluation included a control group and four different L:C ratios similar to those of the first study (Groups VI, VII, VIII, IX and X). Larval reduction percentages were calculated to estimate trapping efficacy. A lower number of L_3 larvae were harvested from cultures seeded with eggs and chlamydospores at E:C ratios of 1:10 or higher, compared to the number of larvae harvested in the control group ($P<0.05$). Similarly, when L_3 larvae were used as bait, the reduction percentage initially increased with higher chlamydospore density, but efficacy did not continue to increase at L:C proportions beyond 1:10 ($P>0.05$). This study demonstrated the importance of adjusting the dose of *D. flagrans* chlamydospores. The optimal efficacy would be achieved with a proportion of 10 chlamydospores for each nematode egg or each infective larvae present in the faeces.

Keywords: Chlamydospores; *Duddingtonia flagrans; Haemonchus contortus;* optimal proportion

RESUMEN

El objetivo del estudio fue identificar la proporción de clamidosporas de *Duddintonia flagrans* necesarias para alcanzar la eficacia de atrapamiento óptima en la presencia de huevos o larvas L_3 de *Haemonchus contortus*. Primeramente, se formaron cinco grupos de 15 coprocultivos cada uno, con proporciones crecientes de huevos de *H. contortus* y clamidosporas (H:C) empezando por el Grupo I (grupo control) con una relación de 1:0 y los grupos II al V con proporciones de 1:1, 1:10, 1:100 y 1:1000, respectivamente. Un segundo grupo de 50 coprocultivos se dividió en cinco grupos de 10 coprocultivos y se les agregaron larvas infectantes de *H. contortus* y clamidosporas, cada grupo con proporciones crecientes de L:C. Se evaluaron los grupos control y cuatro proporciones de L:C similares al primer experimento (Grupos VI, VII, VIII, IX y X). Se obtuvieron los porcentajes de eficacia de reducción larvaria en ambos experimentos. Menores cantidades de larvas L_3 fueron recuperadas de cultivos sembrados con huevos chlamydosporas en proporciones de 1:10 o mayores comparados con la cantidad de larvas cosechadas del grupo control ($P<0.05$). De manera similar, cuando se usaron L_3

como carnada, el porcentaje de reducción inicialmente incrementó a mayor densidad de clamidosporas, pero la eficacia no siguió incrementando en proporciones mayores de 1:10 (P>0.05). Este trabajo demuestra la importancia de ajustar la dosis de clamidosporas de *D. flagrans.* La eficacia óptima se alcanzaría con una proporción de 10 clamidosporas por cada huevo de nematodo o larva infectante presente en las heces.

Palabras clave: Clamidosporas; *Duddingtonia flagrans; Haemonchus contortus;* proporción óptima.

INTRODUCTION

The success of *Duddingtonia flagrans* as a biological agent to control gastrointestinal nematodes (GIN) in ruminants and other animals has been associated with the concentration of chlamydospores in the faeces of the host (Grønvold *et al.,* 1985). Grønvold *et al.* (2004) determined that increasing the concentration of chlamydospores increased nematode trapping capability. This result was confirmed by other *in vitro* studies reporting that an increase in the density of chlamydospores incubated in the faeces increased the trapping efficacy of GIN infective larvae in small ruminant faeces (Sanyal, 2004, Sanyal *et al.,* 2008) and horse faeces (Bird and Herd, 1995). Those studies confirmed that large quantities of spores must reach the faeces of the host to control the free-living larvae (Sanyal *et al.,* 2008). These inundation protocols are necessary because approximately 90% of the spores dosed could be digested in the alimentary tract at least in the case of small ruminants (Ojeda-Robertos *et al.,* 2009).

A recent *in vivo* dose titration study performed on hair sheep infected with *Haemonchus contortus* showed that, while higher dose rates resulted in larger quantities of chlamydospores per gram of faeces (CPG), the trapping efficacy of *D. flagrans* was not always improved with higher chlamydospore doses (Ojeda-Robertos *et al.,* 2008). The authors found that the percentage reduction in larval yields was associated with the CPG expressed as a proportion of the nematode eggs per gram of the faeces (EPG). The highest trapping efficacy was obtained with a proportion of 1 egg per 10 chlamydospores in the faeces (1:10 E:C). Higher proportions of chlamydospore per egg did not achieve higher trapping efficacies. Those results suggested that there is a level of saturation, above that more chlamydospore in the faeces fail to achieve higher trapping efficacies. The use of excess numbers of chlamydospores would be costly in terms of the large quantities of biological material that could be wasted in animals with low nematode egg excretion and may in part explain the variability in trapping efficacy found in dose titration trials. The relationship between trapping efficacy and the ratio of eggs to chlamydospores in the faeces needs to be confirmed under *in vitro* conditions, in which the proportions of spores to eggs can be controlled. The *in vitro* validation must also consider if the optimal proportion of chlamydospores is the same for nematode eggs and infective L_3 larvae present in the faeces. The objective of this study was thus to identify the proportion of *D. flagrans* chlamydospores that is needed to achieve an optimum trapping efficacy in the presence of eggs or L_3 larvae of *H. contortus* in sheep faeces.

MATERIALS AND METHODS

Duddingtonia flagrans chlamydospores

This study used the Mexican *D. flagrans* strain (FTHO-8). Chlamydospores were produced at the Helminthology Laboratory at CENID-PAVET, Jiutepec, Morelos, México. Fungal spores were produced in wheat agar harvested, and suspended in distilled water (Llerandi-Juárez and Mendoza de Gives, 1998). The number of chlamydospores was determined with a Neubauer chamber. After quantification, spores were maintained in a beaker to allow fungal sedimentation, and the supernatant was subsequently discarded in order to diminish the amount of water. The experimental doses were stored in 2.5 ml vials at 4 ºC until used (Llerandi-Juárez and Mendoza de Gives, 1998).

Haemonchus contortus eggs

The *H. contortus* eggs were isolated from fresh faeces of a male donor sheep with a mono-specific infection. Extraction of nematode eggs was performed following the technique described by Hubert and Kerboeuf (1984), Jackson and Hoste (2010) with some modifications. Briefly, 20 g of faeces were obtained from the rectum of sheep. Faeces were slightly crushed without breaking the pellets and then soaked in tap water for 10 minutes. The faecal suspension was passed through a 0.297 mm mesh to exclude large particles of organic material and debris. Material that passed through the sieve was collected in a beaker and passed through a 0.038 mm mesh. The material caught in the mesh was then washed thoroughly until a clear liquid passed through the mesh. The material was then transferred to a petri dish and the presence of eggs was verified with a stereoscopic microscope at 10x. Eggs were then washed again with distilled water, and thick particles were removed by different density gradients with a

sucrose solution (density 1200 g/l). Eggs were removed immediately in order to avoid further damage. Clean eggs were suspended in distilled water and maintained at 4 ºC. The number of *H. contortus* eggs per ml was estimated by counting 10 times 5-ml aliquots. After determining the quantity of eggs, they were left to settle in the tubes for 1 hour in order to allow sedimentation. After that period, the supernatant was extracted carefully in order to concentrate 7000 eggs per 1000 µl, and eggs were used within 30 minutes of collection.

***Haemonchus contortus* infective larvae (L_3).**

The *H. contortus* L_3 were obtained from the same artificially infected male sheep. Faeces were deposited in a plastic container and covered to avoid contamination. Faeces were incubated at room temperature for 8 days and mixed every second day to promote aeration of nematode eggs. After incubation, L_3 larvae were recovered using the Baermann technique. The number of L_3 larvae was determined by counting ten 5-ml aliquots. The mean number of larvae was estimated according to the method described by Fontenot *et al.* (2003). After counting, the volume of liquid was reduced to yield a total of 7000 L_3 per 1000 µl. Larvae were then stored in centrifuge tubes at 4 ºC until further use.

Sterile faeces for coproculture

Two nematode-free sheep were, maintained in metabolism crates and the total fecal volume produced in 24 hours was collected, weighed, and packed for sterilization. The sterilization process was performed at 15 PSI for 30 minutes. After sterilization, faeces remained overnight at room temperature (26 ºC) until their use. Sterile faeces were used in all the incubation procedures used in the study.

Experimental design

In the first trial, seventy-five coprocultures were established by mixing 7 g of faeces with a fixed quantity of *H. contortus* eggs (1000 eggs g^{-1} faeces) and increasing quantities of chlamydospores. Five experimental groups with 15 coprocultures per group were formed. Proportions of eggs:chlamydospores (E:C) for Groups I through V were 1:0 (Control group), 1:1, 1:10, 1:100, and 1:1000, respectively.

The second part of the study used fifty coprocultures formed by adding 7000 *H. contortus* L_3 as bait to 7 g of faeces and increasing proportions of *D. flagrans* chlamydospores. Five experimental groups were formed with 10 coprocultures per group. Proportions of larvae:chlamydospores (L:C) in Groups VI to X were 1:0 (Control), 1:1, 1:10, 1: 100, and 1 to 1000, respectively.

Coprocultures

Sterile sheep faeces were crushed and deposited in Petri dishes (13 cm diameter). Then, 1000 µl of an aqueous suspension containing either *H. contortus* eggs or L_3 larvae were added with an automatic pipette. Chlamydospore suspensions with differing dose levels were then added to the cultures, and coprocultures were incubated for eight days at room temperature (25 ºC) in the laboratory. Faeces were moistened and aerated daily. Larvae were recovered using the Baermann technique and stored in 50 ml centrifuge tubes at 4 ºC until counted. The number of L_3 harvested was estimated in 10 aliquots of 5 µl each.

***Haemonchus contortus* larvae reduction efficacy**

Larvae reduction percentage was determined differently for the coprocultures seeded with eggs and those seeded with L_3 larvae. In coprocultures seeded with eggs, the reduction percentage was estimated based on the formula described by Terril *et al*. (2004):

Reduction % = 100 - (yield of treated group / yield of control group).

The larvae yield was obtained by expressing the number of larvae recovered as a percentage of the total number of eggs g^{-1} in the faeces (Paraud *et al.*, 2004).

For coprocultures seeded with L_3 larvae, the percentage larval reduction was estimated as:

Larval Reduction % = 100 - (mean number of larvae recovered in the respective treatment group / mean number of larvae recovered in the control group) x 100.

Statistical analysis

Means and medians of recovered larvae per group were estimated. Non-parametric procedures were used to test treatment differences because distributions of larvae number were not normal and could not be normalized by use of data transformation procedures. Thus, medians for larvae numbers for each treatment were compared to their respective control groups using the non-parametric Kruskall-Wallis test and Minitab software (Minitab Inc. release 15 1998). Comparisons between groups were made with the Dunn test for multiple contrasts (Hollander and Wolfe 1973).

RESULTS

Arithmetical means, medians, larvae yields and reduction percentages of *H. contortus* L_3 larvae

recovered from coprocultures with different E:C or L:C proportions are shown in Table 1. Coprocultures seeded with eggs at an E:C proportion of 1:1 did not differ from the control group (P >0.05). However, groups III, IV and V (E:C proportions of 1:10, 1:100 and 1:1000) all differed from the control group (P<0,05). Increased chlamydospore densities beyond 1:10 did not result in a further reductions in larvae numbers because medians were zero for E:C ratios of 1:10, 1:100 and 1:1000.

For coprocultures seeded with L_3 larvae, the median number of L_3 larvae recovered from the control coprocultures was higher than that obtained when spores were added to the cultures (P<0.05). Furthermore, an L:C ratio of 1:1 yielded more larvae than an L:C ratio of 1:10. However, higher L:C densities (Groups IX and X with L:C ratios of 1:100 and 1:1000, respectively) were not associated with further reductions in number of L_3 larvae compared to numbers observed at an L:C ratio of 1:10.

Table 1. Means and medians for *Haemonchus contortus* L_3 larvae numbers, larvae yield (%) and reduction percentages obtained from faecal cultures seeded either with eggs or L3 larvae and exposed to increasing ratios of *Duddingtonia flagrans* chlamydospores.

Treatment Group[1]		Median* (Interquartilic range)	Larvae Number Mean (±S.D.)	Larvae Yield (%)	Reduction (%)
Fecal cultures seeded with eggs					
I	1 to 0	80[a] (40 – 160)	104± 66	1.5	-
II	1 to 1	40[ab] (20-120)	69 ± 63	1.0	33.3
III	1 to10	0[b] (0 – 40)	48 ± 90	0.7	53.8
IV	1 to 100	0[b] (0 – 60)	40 ± 58	0.6	61.5
V	1 to 1000	0[b] (0 – 40)	19 ± 30	0.3	82.1
Fecal cultures seeded with infective larvae					
VI	1 to 0	1510[a] (1.290 – 2910)	2020 ± 1003	-	-
VII	1 to 1	1350[b] (945 – 1575)	1272 ± 458	-	37.0
VIII	1 to 10	120[c] (60 – 330)	306 ± 488	-	84.9
IX	1 to 100	90[c] (60 – 240)	156 ± 155	-	92.3
X	1 to 1000	120[c] (0 – 270)	156 ± 172	-	92.3

[1]Treatments are defined by the proportions of eggs or larvae relative to chlamydospores.
* Different letters in the same column indicate statistical differences (P<0.05).

DISCUSSION

The control of GIN through *D. flagrans* chlamydospores is based on their capacity to reduce the number of infective L_3 larvae found in faecal material and relies on oral administration of large enough chlamydospore doses. The fungal concentration in the faeces has been considered the key factor affecting the success of the trapping activity (Grønvold *et al.*, 1985). Although previous dose-titration studies demonstrated that higher *D. flagrans* chlamydospore concentrations resulted in higher nematode trapping capability (Grønvold *et al*., 2004, Paraud *et al.*, 2005), this effect has not been consistently observed (Ojeda-Robertos *et al.*, 2008). Several factors affect the trapping efficacy of *D. flagrans* chlamydospores, including the temperature and moisture in the faeces (Grønvold *et al*., 1999), nematode species, and quantity of clamydospores in the faeces (Bird and Herd, 1995, Paraud *et al*., 2006). This evidence provides the rationale that supports the need for inundation protocols ensuring that enough chlamydospores reach the faeces to produce the traps for the nematodes (Sanyal *et al.* 2008). Consequently, more faecal chlamydospores increase the probability of GIN larvae to be vulnerable to the trapping process (Jaffe, 1993).

Effective use of *D. flagrans* must also consider the number of GIN eggs and/or larvae present in the faeces. Morgan *et al.* (1997) suggested that trapping efficacy increase when nematodes are abundant in the faeces, at least within certain threshold densities. The living nematodes are the most important biotic factor in a trapping system, acting not only to induce trap formation, but also as the main source of food for nematophagous fungi (Barron, 1977, Nordbring *et al.*, 2002). Therefore, effective use of nematophagous fungi for biological control of GIN must consider the density of spores, expressed as the number of chlamydospores in the faeces, and also the proportion of fungi in relation to the nematodes units (i.e., eggs

or larvae) (Jaffe, 1992). The present trial emphasizes the importance of both, the quantity of spores and the quantity of eggs or larvae in the faeces. The Mexican *D. flagrans* strain evaluated in the present study did not improve larvae reduction efficacy beyond the 1:10 proportion of eggs or infective larvae to chlamydospores, suggesting that the optimal proportion is 1:10. In the case of *H. contortus* eggs, a higher E:C proportion improved reduction efficacy from 53.8 % (E:C 1:10) to 61.5% (E:C 1:100) and 82.1% (E:C 1:1000) but results varied greatly among coprocultures, thus, efficacies were not significantly different.

It was evident that larvae reduction efficacies found in coprocultures seeded with *H. contortus* eggs and spores were lower than those obtained by Sanyal *et al*. (2008), even when comparable quantities of eggs and similar E:C ratios were used in both studies. The latter could have resulted from the high egg hatching rates (40%) reported by Sanyal *et al*. (2008) in their control groups, compared to the 2% egg hatching rate for the control group of the present trial. Low egg hatching may have failed to produce enough larvae to effectively induce traps in the cultures seeded with eggs, reducing the larvae reduction efficacy (Bird and Herd, 1995). However, the low egg hatchability found in the present study was similar across all the groups seeded with eggs, and therefore would have affected all groups similarly.

When L_3 larvae were seeded directly to the faecal cultures, the clamydospores reduced the number of larvae in all the experimental groups compared to the control ($p<0.05$). As expected, the reduction efficacy of *D. flagrans* was more evident in cultures seeded with L_3 larvae, compared to cultures seeded with similar quantities of *H. contortus* eggs. The latter illustrates the importance of the egg hatching process as a factor influencing reduction percentages obtained with *D. flagrans*, as explained above, and is consistent with the observation that living nematodes stimuli is the most important biotic factor in *D. flagrans* trapping feature (Barron, 1977, Nordbring *et al.,* 2002). Morgan *et al.* (1997) suggested that trapping efficacy increases when nematodes are abundant in the faeces, but only within certain threshold densities. The present study showed that, in spite of the large quantity of L_3 in the cultures, the reduction efficacy of *D. flagrans* was not further increased beyond L:C ratios of 1:10 ($P>0.05$). The latter was consistent with results observed for cultures seeded with eggs, and both results are consistent with results from *in vivo* work with sheep infected with *H. contortus*. These three pieces of evidence suggests that there is a maximum saturation level for the chlamydospores, and that E:C or L:C rations of 1:10 were optimal under the conditions of these experiments. Such proportion may, however, not be ideal for all types of ruminants under all types of diets and should be validated for the different *D. flagrans* isolates. However, the concept of a minimum necessary saturation level is important because it can reduce waste of chlamydospores at the farm level.

Based on the results of the present study, a protocol for oral doses adjustment of spores is proposed:

(a) Identify the number of GIN eggs per gram of faeces in the ruminant host. According to the present results, the dose should provide 10 chlamydospores for every egg per gram of faeces. Thus, if an animal excretes 2000 egg per gram of faeces (EPG), the chlamydospore dose should aim to achieve excretion of at least 20000 chlamydospores per gram of faeces (CPG).

(b) Identify the total quantity of faeces produced by the host per day. This information can help to determine the quantity of chlamydospores offered to the animals. If a sheep produces 1500 g of faeces per day, with 2000 EPG, then the total egg output would be 3 million eggs / day. Thus, the total number of spores that should be excreted would be 30 million spores / day. Procedure to determine this parameter would require collection bags attached to the tail or the perianal region of the animal (Corbett, 1978; Retama-Flores *et al*., 2012), or any other practical approach to estimate the total faecal production of a ruminant of a given weight eating in a given type of vegetation. An average estimation for a group of animals would be more practical and feasible than an individual estimation of this variable.

(c) Digestibility of *D. flagrans* spores with the respective diet. Chlamydospores offered *per os* are largely destroyed in the gastrointestinal tract of small ruminants (90% digestibility; Ojeda-Robertos *et al.,* 2009). Therefore, an oral dose of 300 million spores is required to achieve a faecal excretion of 30 million spores per day, considering a 90% digestibility of the spores. It is important to say that such dose level would represent only 8.6×10^6 spores/kg of body weight for an adult sheep of 35 kg. The latter is a common dose level reported for adult sheep (Ojeda-Robertos et al., 2008).

This A, B, C must be considerate to adjust the oral dose and to have a guide chart to use in practical way in the field. In addition, spores should be provided selectively only to hosts with significant excretions of GIN eggs. Otherwise the spores will be largely wasted.

CONCLUSION

This study demonstrates the importance of adjusting the dose of *D. flagrans* to achieve 10 chlamydospores

for every egg or larvae present in the faeces. The use of higher chlamydospores ratios fails to achieve further significant improvement in trapping efficacy against *H. contortus*. The key element in maximizing the efficacy of *D. flagrans* is the synchronization between numbers of nematode eggs and clamydospores in faeces. The challenge would be to adjust the oral dose of spores to be consistent with rates of egg elimination. The latter involves understanding both the nutritional quality (digestibility) of the diet and the level of nematode infection.

Acknowledgments

This research received financial support from CONACYT-SAGARPA-COFUPRO (project No. 12441). The experiment was part of Nadia Florencia Ojeda-Robertos PhD thesis at the Autonomous University of Yucatan. Special thanks is expressed to Ivonne Carolina Alfaro, Liliana Aguilar-Marcelino, Gema Lisete Arteaga, Gabriel Ramírez and Ulises López for their technical assistance and to Roberto Barrientos-Medina for valuable statistical advice. To Dr David Notter for his help in the English language revision of this manuscript.

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Submitted April 27, 2015 – Accepted October 09, 2015