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Research paper

Combined use of ivermectin, dimethyl sulfoxide, mineral oil and nematophagous fungi to control *Rhabditis* spp.

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ABSTRACT

Rhabditis spp., is a nematode known to cause otitis externa, an infection difficult to control, in cattle reared within tropical regions. The objective of this study was to assess the combined use of ivermectin 1%, dimethyl sulfoxide 1% and mineral oil 100% containing nematophagous fungi of both *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) species to control *in vitro Rhabditis spp.* Thus, 12 experimental groups were designed with eight replicates each: G1 (nematodes + AC001); G2 (nematodes + NF34); G3 (nematodes + ivermectin 1%/positive control); G4 (nematodes + dimethyl sulfoxide 1%/positive control); G5 (nematodes + mineral oil 100%/positive control); G6 (nematodes + AC001 + ivermectin 1%); G7 (nematodes + NF34 + ivermectin 1%); G8 (nematodes + AC001 + mineral oil 100%); G9 (nematodes + NF34 + mineral oil 100%); G10 (nematodes + AC001 + dimethyl sulfoxide 1%); G11 (nematode + NF34 + dimethyl sulfoxide 1%); G12 (nematode + distilled water/negative control). The results demonstrated that all experimentally treated groups differed statistically ($p < 0.01$) from the control group. In the present study, the use of dimethyl sulfoxide 1% and mineral oil 100% in conjunction with conidia fungi portrayed noteworthy outcomes, which represents a future premise for the combined use of nematophagous fungi within these vehicles in both controlling *Rhabditis* spp.

1. Introduction

The nematode *Rhabditis* spp., triggers otitis externa in livestock dwelling in tropical countries, leading to enormous economic losses (Round, 1962; Msolla et al., 1985; Barbosa et al., 2016). Ivermectin is an anti-helminthic drug belonging to macrocyclic lactones group, and it is worldly used to control gastrointestinal nematodes in ruminant. However, the results of treating bovine otitis with ivermectin are ineffective (Verocai et al., 2009; Barbosa et al., 2016). Thus, studies that associate the use of ivermectin with control alternatives of *Rhabditis* spp. are important.

In this sense, certain studies recommended alternative measures encompassing biological control, *e.g.* the use of nematophagous fungi, which could be associated with chemical control of this parasite (Araújo and Guimarães, 2002; Braga and Araújo, 2014; Sobral et al., 2019). However, further studies are still needed on the association of antiparasitic drugs with nematophagous fungi in the field to control *Rhabditis* spp. (Sobral et al., 2019).

Hence, before promoting the combined treatment containing nematophagous fungi, uncovering chemical and/or organic compounds that function as vehicles when associated with these organisms as well as denote synergic action is still a necessary limiting process (Mota et al., 2003). In this context, the mineral oil 100% and dimethyl sulfoxide 1% stand out as vehicles for industrial products, assisting in several topic formulations (Campos et al., 2004; Williams and Barry, 2004; Patzelt et al., 2012; Manjunath and Shivaprakash, 2013; Picoli

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et al., 2015). Thus, these products could act as efficient vehicles in topical route administration of nematophagous fungi.

Therefore, the objective of this study was to evaluate the combined use of ivermectina 1%, dimethyl sulfoxide 1% and mineral oil 100% with nematophagous fungi from *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) species to control *in vitro Rhabditis* spp.

2. Material and methods

2.1. Rhabditis spp

The nematodes were collected by rinsing the ontological area of animal's external auditory canal. At total were used 6 Gir cows between three and eight years old from a bovine herd composed of 200 animals, including cows, heifers, calves and bulls of Gir and Girolando breeds (Sobral et al., 2019).

2.2. Treatments

Ivermectin 1% (Merial, Brazil), dimethyl sulfoxide 1% (Vetnil, Brazil) and mineral oil 100% (União química, Brazil) were utilized. These compounds were obtained from local companies and have been used in routine clinical procedures in the veterinary hospital of the University of Vila Velha, Vilha Velha, Brazil (Vieira et al., 2018).

In addition to these compounds, two isolates of nematophagous fungi from the species *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) were used. Both isolates are present in Brazilian soils and were provided by the Laboratory of Parasitology of the Federal University of Viçosa, Viçosa, Brazil. Then, the isolates were placed in 9 cm diameter Petri dishes, containing 2% potato-dextrose-agar culture medium (PDA2%). Mycelial growth was observed after 7 days. In order to obtain a conidia solution from both isolates, 5 ml of distilled water were added in each Petri dish and both conidia and mycelia fragments were transferred by a spatula into 15 ml Falcon tubes (Araújo and Maia, 1993).

2.3. Experimental assay

Twelve (12) experimental groups were designed in microtubes and each group had eight replicates. The values of *Rhabditis* spp., and conidia used in those groups were standardized by aliquots, maintaining concentrations of approximately 500 nematodes/143 µl, 500 conidia/30 µl of AC001 and 500 conidia/192 µl of NF34. The experimental groups are described in Table 1.

After 24 h of exposure to treatments containing ivermectin 1%, dimethyl sulfoxide 1%, mineral oil 100%, AC001 and NF34, the content from all microtubes from G1 to G12 was read in a 10x objective light microscopy and the quantity of nematodes was totaled (Mukhtar and Pervaz, 2003; Braga et al., 2013).

Table 2
Means and reduction percentage of the recovered *Rhabditis* spp., in the experimental groups (G1 to G12) after 24 h of interaction.

Experimental groups	Mean	% Reduction
G1 - AC001	53	64.2%
G2 - NF34	30.6	79.3%
G3 - Ivermectin 1%	48.9	67%
G4 - Dimethyl sulfoxide 1%	85.9	42%
G5 - Mineral oil 100%	107.8	31.2%
G6 - AC001 + ivermectin 1%	84	43.2%
G7 - NF34 + ivermectin 1%	103.5	30.1%
G8 - AC001 + mineral oil 100%	26.8	82%
G9 - NF34 + mineral oil 100%	17	88.5%
G10 - AC001 + dimethyl sulfoxide 1%	1.9	98.7%
G11 - NF34 + dimethyl sulfoxide 1%	2.5	98.3%
G12 - Distilled water	148*	0%

Significant difference ($p < 0.01$) between the treated group and the control are denoted by an asterisk - Tukey test.

2.4. Statistical analyzes

Afterwards, the results were evaluated by analysis of variance (ANOVA) followed by Tukey post-test at the 1% probability level, using BioEstat 5.0 software (Ayres et al., 2003). The reduction percentage was calculated utilizing the following equation: % Reduction = mean of nematodes recuperated from the control group - mean of nematodes recuperated from the treated group x 100 / mean of nematodes recuperated from the control group (Mendoza-De Gives and Vazquez-Prats, 1994).

3. Results

Mean acquired and standard deviation of *Rhabditis* spp., retrieved from experimental groups (G1 to G12) are demonstrated in Table 2. The groups G1 (AC001) and G2 (NF34), treated merely with conidia, denoted a 71.8% mean reduction of nematodes. In group G3, ivermectin 1% incorporation led to a 67% decrease of nematodes retrieved after 24 h. Groups G4 and G5, which were respectively treated with dimethyl sulfoxide 1% and mineral oil 100%, exhibited 42% and 31.20% depletion of nematodes, correspondingly. Moreover, the combinations comprising ivermectin 1% + AC001 or NF34 (reduction percentages of 43.2% and 30.1%, respectively) were less affective when compared to their activity separately. After 24 h, the combinations with AC001 or NF34 + mineral oil 100% (G8 and G9, respectively) decreased the number of retrieved *Rhabditis* spp., in 82.0% and 88.5%, correspondingly. The combination of AC001 or NF34 + dimethyl sulfoxide 1% (G10 and G11, respectively) reduced the mean quantity of nematodes in 98.5%.

Table 1
Experimental groups (G1 to G12) designed to evaluate the combined use of ivermectina 1%, dimethyl sulfoxide 1% and mineral oil 100% containing nematophagous fungi of both *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) to control *Rhabditis* spp.

Groups	Experimental design
G1	500 nematodes/143 µl + 500 conidia/30 µl of *D. flagrans*
G2	500 nematodes/143 µl + 500 conidia/192 µl of *M. thaumasium*
G3 (positive control)	500 nematodes/143 µl + 8 µl of ivermectin 1%
G4 (positive control)	500 nematodes/143 µl + 8 µl of dimethyl sulfoxide 1%
G5 (positive control)	500 nematodes/143 µl + 1000 µl of mineral oil 100%
G6	500 nematodes/143 µl + 500 conidia/30 µl of *D. flagrans* + 8 µl of ivermectin 1%
G7	500 nematodes/143 µl + 500 conidia/192 µl of *M. thaumasium* + 8 µl of ivermectin 1%
G8	500 nematodes/143 µl + 500 conidia/30 µl of *D. flagrans* + 1000 µl of mineral oil 100%
G9	500 nematodes/143 µl + 500 conidia/192 µl of *M. thaumasium* + 1000 µl of mineral oil 100%
G10	500 nematodes/143 µl + 500 conidia/30 µl of *D. flagrans* + 8 µl of dimethyl sulfoxide 1%
G11	500 nematodes/143 µl + 500 conidia/192 µl of *M. thaumasium* + 8 µl of dimethyl sulfoxide 1%
G12 (negative control)	500 nematodes/143 µl + 100 µl of distilled water

4. Discussion

Controlling *Rhabditis* spp., is a challenge for tropical dairy farming due to the lack of standardized therapeutic protocols, which lead to reduced efficiency and infection recurrence (Msolla et al., 1986, 1993; Leite et al., 2013). Additionally, the treatment based on anthelmintic drugs are ineffective most of the time. In this way, the use of nematophagous fungi can represent a promising alternative for biological control. Our results shows that *D. flagrans* (AC001) e *M. thaumasium* (NF34) presented predatory activity over *Rhabditis* ssp. Nonetheless, the authors emphasize that the future use of these fungi *in vivo* can be challenge, once it is necessary to promote fungi adhesion in the ear canal.

In the present study, the predatory activity of both *D. flagrans* (AC001) and *M. thaumasium* (NF34) was efficient in controlling the nematodes. This outcome harmonizes with the recent study carried out by Sobral et al. (2019), whom observed the efficacy of AC001 and NF34 isolates on limiting *Rhabditis* spp., under laboratory conditions. In that occasion, the authors drew attention to these fungi as a future alternative to control *Rhabditis* spp., but they did not proposed any methodology for fungi administration in animals.

According to Soares and Monteiro (2011), the combined use of chemical and biological controls could represent a feasible strategy for livestock, decreasing costs, resistance, toxicity and management, as well as reducing the residues in both animal products and the environment. Nevertheless, it is noteworthy that there is a discrepancy in the literature regarding this topic and certain authors pointed out that the environmental reproduction of biological controllers could be compromised by this process (Sanyal et al., 2004; Anhalt et al., 2010). Thus, the results show that all chemical compounds tested did not interfered negatively with the fungi activity, what makes possible to use an association of those in future.

The ivermectin have been used for decades for nematodes control in ruminant, but it present already an established drug resistance (Coles et al., 1992; Chaparro et al., 2017). The scientific community are still struggling to control *Rhabditis* spp., in Brazil (Verocai et al., 2009; Barbosa et al., 2016). In Africa, Msolla et al. (1985) registered significant results after using ivermectin to limit *Rhabditis bovis* proliferation. In the present study, the utilization of pure ivermectin 1% without the fungi AC001 or NF34 was more efficient than when the application was combined with these fungi.

Similarly, the predatory activity of AC001 and NF34 was more effective when not associated with ivermectin. This finding is interesting and may be a determining factor for future strategies aiming at using the topical combination of nematophagous fungi with ivermectin. However, further studies are still needed to overcome certain hurdles in the general use of nematophagous fungi and their route of administration as previously discussed. Vieira et al. (2017) brought to light that antiparasitic compounds have an *in vitro* inhibitory effect on nematophagous fungi, including *D. flagrans*, compromising their activities as biological control agents.

In contrast, Vilela et al. (2018) reported efficacy in the controlling gastrointestinal nematodes of sheep when these animals were treated with *D. flagrans* (AC001) encapsulated with sodium alginate associated to levamisole hydrochloride 5%. Nevertheless, it is noteworthy that encapsulating nematophagous fungi is a viable and safe strategy to transport the fungus through cattle's gastrointestinal tract, protecting the fungi against intrinsic and extrinsic factors (Braga and Araújo, 2014; Luns et al., 2018). Thus, further compatibility tests between both nematophagous and anthelmintic fungi are necessary to evaluate the developmental changes of these organisms resulted from their combined use with anthelmintic drugs.

Mineral oil 100% was tested as a potential vehicle that could be used in the future for topical administration of the aforementioned fungi to control *Rhabditis* spp. Since the use of diluted fungi (conidia) in aqueous medium has been widely explored (Silva et al., 2011; Mendoza-de Gives et al., 2018), mineral oil utilization could assist in a superior adhesion of the conidia to the nematode cuticle, fomenting fungal predatory activity (Araújo and Maia, 1993; Paz Silva et al., 2011).

Mineral and vegetal oils have been extensively used as adjuvants in formulations aimed at maintaining virulence because when added to fungal suspensions, oils protect the conidia from unfavorable conditions and even increase entomopathogenic fungi effectiveness (Camargo et al., 2012). Nevertheless, this is the first work carried out with nematophagous fungi combinedly used with mineral oil 100%. It was observed that mineral oil presented a high affinity for conidia fungi, what makes it to have a potential use as adjuvant for the biological control of *Rhabditis* spp.

In the present study, dimethyl sulfoxide 1% was used in order to be tested as a permeabilizer in animals' auditory canal, which could eventually receive the combined use containing nematophagous fungi. The dimethyl sulfoxide 1% also presented high affinity for conidia fungi. Willian and Barry (2004) affirmed that human skin, for instance, functions as a barrier that causes difficulties for the transdermal release of therapeutic agents. According to them, penetration enhancers, which are chemicals that interact with cutaneous constituents to promote the medicine flow, are needed to overcome this barrier. Therefore, the dimethyl sulfoxide 1% exhibits a biological property of increasing the penetration of hydrophilic and lipophilic molecules in the skin (Williams and Barry, 2004; Simon et al., 2009). Nucleotides are examples of this mechanism since their passage through cell membranes is enhanced when these compounds are solubilized in organic solvents (Mangia et al., 2014).

Araújo and Guimarães (2002) brought to light during a pioneering *in vitro* study that the NF34 isolate can destroy *Rhabditis* spp. Recently, Sobral et al. (2019) reported that *D. flagrans* (AC001) and *M. thaumasium* (NF34) could be used *in vivo* to control *Rhabditis* spp., in infected animals. Notwithstanding, these authors recognized the importance of a correctly standardized methodology for topical routes of administration. In the current study, the use of dimethyl sulfoxide 1% and mineral oil 100% in conjunction with fungal conidia portrayed an interesting outcome, which scaffolds a premise for the combined use of nematophagous fungi with these vehicles to control *Rhabditis* spp., enhancing the parasitic otitis treatment in the field.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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