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Commercial formulation of *Metarhizium anisopliae* for the control of *Rhipicephalus microplus* in a pen study

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ABSTRACT

The present study evaluated, for the first time, the effect of the commercial formulation Metarril® SP Organic of *Metarhizium anisopliae* plus 10% mineral oil to control *Rhipicephalus microplus* in a pen study. Three groups were formed with six animals each: the first group was exposed to Metarril® plus 10% mineral oil and 1% Tween 80; the second group was exposed to sterile distilled water, mineral oil and Tween 80 (oil control group); and the third group received no treatment (control group). The fungal formulation contained 1×10^8 conidia ml^{-1}. Each animal was sprayed with 3 L of formulation. Fallen ticks were counted daily and a sample of 20 engorged females per day was incubated for assessment of biological parameters. Throughout the study period, Metarril® oil-based formulation showed an efficacy ranging from 19.20% to 67.39% in comparison with the control group; and from 8.18% to 61.38% in comparison with the oil control group. The average efficacy of Metarril® oil-based formulation was 47.74% and 40.89% in comparison with control and oil control groups, respectively. Changes in the biological parameters of engorged *R. microplus* females were observed in the first three days after treatment, with a significant reduction in hatching percentage and egg production index. We concluded that Metarril® SP Organic plus 10% mineral oil was efficient against *R. microplus* in pen studies. However, further *in vivo* studies are required to increase the efficacy and to establish a protocol for the use of this product in the field against the cattle tick.

1. Introduction

Rhipicephalus microplus Canestrini, 1888 (Murrel and Barker, 2003), known as the cattle tick, is considered one of the most important ectoparasites of livestock worldwide (Estrada-Peña et al., 2006) due to associated economic losses. In Brazil, the damage caused by this parasite costs up to 2 billion dollars per year (Grisi et al., 2002) due to decreased meat and milk production and tick control expenses. The cattle ticks are controlled by applying chemical acaricides. Improper use of chemical acaricides has resulted in the selection of populations of *R. microplus* that are resistant to almost all acaricides from existing chemicals groups (Furlong et al., 2007) and that have contaminated animal products and the environment. These issues render the search for alternatives to control this parasite important. In this context, biological control using

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acaripathogenic fungi is a potential alternative for tick control.

Among acaripathogenic fungi, *Beauveria* and *Metarhizium* have been the most studied genera for tick control (Fernandes et al., 2012). Several studies have demonstrated the importance of using these fungi in a formulation, not just suspended in water, for controlling ticks (Kaaya, 2000; Kaaya and Hassan, 2000; Maranga et al., 2005; Polar et al., 2005; Alonso-Díaz et al., 2007; Leemon and Jonsson, 2008; Leemon et al., 2008; Souza et al., 2009; Ángel-Sahagúna et al., 2010; Kaaya et al., 2011). Some of these studies evaluated the fungal formulations against ticks through *in vivo* tests; however, more studies are required to obtain formulations that maintain the viability and efficacy of acaripathogenic fungi for tick control in the field. Mineral and vegetable oils have been used as adjuvants in formulations to maintain virulence and even to increase the efficiency of acaripathogenic fungi against ticks (Maranga et al., 2005; Polar et al., 2005; Angelo et al., 2010; Camargo et al., 2012). When added to fungal suspensions, oils protect conidia from unfavorable environmental conditions and promote greater adhesion of conidia to the arthropod's surface.

According to Faria and Wraight (2007), a large number of mycoinsecticides and mycoacaricides have been developed in recent decades. Approximately 171 commercial products based on fungi have been registered and, among these, only 28 products have been used for mite control, *i.e.*, the vast majority of commercial products were developed for insect control (Faria and Wraight, 2007). Metarril® SP Organic is a commercial product based on *Metarhizium anisopliae* developed for pest control in agriculture. The purpose of this study was to investigate the effect of this formulation – Metarril® SP Organic plus 10% mineral oil – to control *R. microplus* in pen studies.

2. Materials and methods

2.1. Location of the experiment

The pen study was conducted between May and July 2012, at Embrapa Gado de Leite, Campo Experimental Santa Mônica, Valença, Rio de Janeiro, Brazil. This region presents a highland tropical climate, with an average annual temperature of 21.3 °C, an average annual rainfall of 1411 mm and a height of 446 m. The monitoring and analysis of biological parameters of *R. microplus* females was conducted between June and September 2012, in the Laboratory of Microbial Control at the Wilhemn Otto Neitz Parasitological Research Station, which is part of the Department of Animal Parasitology, Veterinary Institute, Rio de Janeiro Federal Rural University (UFRRJ), located in Seropédica, Rio de Janeiro, Brazil.

2.2. Animals

Eighteen female Holstein and Gir crossbred cattle between 11 and 20 months old (mean age 16 months) and weighing between 149 and 230 kg (mean weight 188.2 kg) were used. These animals were kept in individual roofed stalls before (during the infestation period) and after the treatment. There was a wooden pallet on the floor of each stall, which prevented the animals from stepping on the ticks. They were fed twice a day with grass and commercial feed and were offered water *ad libitum*. The research project in which this pen study is included was submitted to the ethics committee of UFRRJ and has the following protocol number 23083.011620/2011-68.

2.3. Fungal formulation

The product tested, Metarril® SP Organic, is composed of *M. anisopliae* isolates ESALQ 1037 and ESALQ E9 and is produced by Koppert Biological Systems. A formulation containing only water, 10% mineral oil (Vetec Química Fina Ltda., Rio de Janeiro, RJ, Brazil) and 1% Tween 80 was prepared for treating the oil control group. The fungal formulation was prepared using Metarril® SP Organic, water, 10% mineral oil and 1% Tween 80. The formulation had 1×10^8 conidia ml^{-1}. The viability of conidia was checked by inoculating an aliquot of 20 μL of formulation onto Potato Dextrose Agar culture medium and incubating it at 25 °C for 24 h. Viability was determined through direct observation with an optical microscope, and the calculation of their germination was performed according to Alves (1998).

2.4. Experimental design

All of the methodology used in the pen test – animal infestation with tick larvae, the number of animals and the division between groups, the treatment of cattle with the formulations and the evaluation of its efficacy – was carried out according to the methodology recommended by the Ministry of Agriculture, Livestock and Supply (MAPA) of the country, which is based on the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of acaricides against ticks (Ixodidae) on ruminants (Holdsworth et al., 2006).

Each calf was infested with approximately 3000 *R. microplus* larvae, aged between 7 and 21 days, on days −24, −21, −19, −17, −15, −11, −9, −7, −5, −3 and −1 with treatments applied to animals on day zero. The larvae were applied along the back of the animals, which remained with their heads and tails tied with ropes for at least an hour in order to prevent the removal of the larvae. This methodology of infestation allows all developmental stages of ticks to be present in cattle at the day of treatment. The 18 animals were sorted in three groups according to the tick burden of each animal, assessed by counting the total fallen ticks in the three days prior to treatment (−3, −2 and −1). Then, the three animals with the lowest mean score were placed randomly, one in each group, and so on until the formation of three groups with six animals. Thus, the three groups began the test with a very similar average number ticks.

The pen test consisted of three groups: a control group that did not receive any treatment (control), a group treated with oil-based formulation of Metarril® SP Organic and a group treated only with water, mineral oil and Tween 80 (oil control group). The formulations were sprayed all over the body of animals, in the opposite direction of the fur, giving greatest attention to the areas most affected by

Table 1
Daily and average efficacy (%) with its standard deviation of the formulation of Metarril® SP Organic plus 10% mineral oil in the control of *Rhipicephalus microplus* in the pen study.

	Daily efficacy					Average efficacy	
	Control group[a]	Oil control group[b]		Control group[a]	Oil control group[b]	Control group[a]	Oil control group[b]
Day 1	25.75 ± 9.66	8.23 ± 12.17	Day 13	50.28 ± 6.06	45.17 ± 6.73		
Day 2	30.81 ± 8.57	28.13 ± 8.95	Day 14	47.41 ± 6.36	49.46 ± 6.12	47.74 ± 13.20	40.89 ± 11.88
Day 3	38.33 ± 7.55	32.14 ± 8.37	Day 15	33.73 ± 7.94	32.51 ± 8.11		
Day 4	19.25 ± 9.81	22.19 ± 9.46	Day 16	48.86 ± 6.39	43.63 ± 7.10		
Day 5	49.02 ± 6.14	27.15 ± 8.93	Day 17	67.41 ± 4.19	49.96 ± 6.59		
Day 6	27.35 ± 9.64	42.59 ± 7.51	Day 18	65.42 ± 4.39	47.17 ± 6.86		
Day 7	50.22 ± 6.94	37.87 ± 8.83	Day 19	65.41 ± 4.47	50.00 ± 6.60		
Day 8	53.23 ± 6.50	38.96 ± 8.68	Day 20	58.44 ± 5.46	61.40 ± 5.06		
Day 9	52.57 ± 6.47	52.02 ± 6.56	Day 21	62.08 ± 4.72	53.02 ± 5.91		
Day 10	53.53 ± 5.60	36.43 ± 7.78	Day 22	37.57 ± 7.90	34.04 ± 8.40		
Day 11	44.96 ± 7.07	49.58 ± 6.46	Day 23	49.52 ± 7.84	45.40 ± 8.56		
Day 12	42.76 ± 6.96	45.35 ± 6.65					

[a] Efficacy of the Metarril® SP Organic oil-based formulation calculated with respect to the control group.
[b] Efficacy of the Metarril® SP Organic oil-based formulation calculated with respect to the oil control group.

ticks, such as inner thighs, dewlap and ears. Each animal was sprayed with 3 L of the formulation, which is enough to distribute over the surface area of an animal with the aforementioned average characteristics (a mean age of 16 months and 188 kg). The animals were examined before and during the length of the experiment to assess their physical condition and to monitor them for any possible adverse reactions.

All of ticks that fell from the body of cattle from day −3 until day 23 after treatment were collected from the stall floors. The number of ticks per group was recorded daily, and a sample of 20 engorged females per group per day was randomly selected for the assessment of biological parameters. They were washed with water, dried with paper towels, individually weighed and attached to large Petri dishes, one for each group containing 20 females. Special care was taken while attaching the females to the Petri dishes so that their postures did not mingle. Afterwards, the female ticks were kept at 27 ± 1° C and relative humidity ≥80% for the evaluation of biological parameters. The egg mass of females was weighed and incubated under the same conditions of temperature and humidity mentioned above for further evaluation of hatching larvae.

The biological parameters hatching percentage and egg production index (EPI = weight of egg mass/initial weight of engorged female × 100) (Bennett, 1974) were evaluated. The efficacy of Metarril® formulation was assessed according to the following formula (Henderson and Tilton, 1955):

$$\text{Efficacy} = \left(1 - \frac{T_a \times C_b}{T_b \times C_a}\right) \times 100$$

where T_a, average number of fallen ticks from treated animals, after the day of treatment; T_b, average number of fallen ticks from treated animals for 3 days prior to treatment; C_a, average number of fallen ticks from control animals after the day of treatment; C_b, average number of fallen ticks from control animals for 3 days prior to treatment.

2.5. Statistical analysis

To assess the parametric data, analysis of variance (ANOVA) was performed, followed by the Student–Newman–Keuls test (SNK), and to assess the nonparametric data, the Kruskal–Wallis test followed by the SNK test was used. In all analyses, the significance level was set at 5% ($p \leq 0.05$) (Sampaio, 2002).

3. Results

The conidia of formulated Metarril® SP Organic containing 10% mineral oil gave 100% germination within 24 h of incubation on PDA at 25 ± 1 °C and RH ≥ 80%.

The animals remained healthy throughout the experiment. No physical, behavioral or physiological change that could be interpreted as an adverse reaction to treatment was observed.

The formulation of Metarril® SP Organic containing 10% mineral oil, when compared to the control group, showed an efficacy of up to 50.19, 53.50 and 67.39% in the first, second and third week after treatment, respectively (Table 1). When compared to the oil control group, the oil formulation of Metarril® SP Organic showed an efficacy of up to 42.56, 52.00 and 61.38% in the first, second and third week after treatment, respectively (Table 1).

The average efficacy of the fungal formulation during the study period compared to the control group and to the oil control group was 47.74 and 40.89%, respectively (Table 1).

The efficacy of the tested product increased over the days of collection of ticks, with a peak during the last week after treatment.

Animals bathed with the oil-based formulation of Metarril® SP Organic showed a reduced number of ticks when compared to animals in the control groups (Fig. 1). The group treated with the oil-based formulation of Metarril® SP Organic presented, on all evaluation days, an inferior tick count and followed the same trend as the control groups. The total number of ticks collected from the floors of the stalls on each day after treatment is shown in Fig. 1.

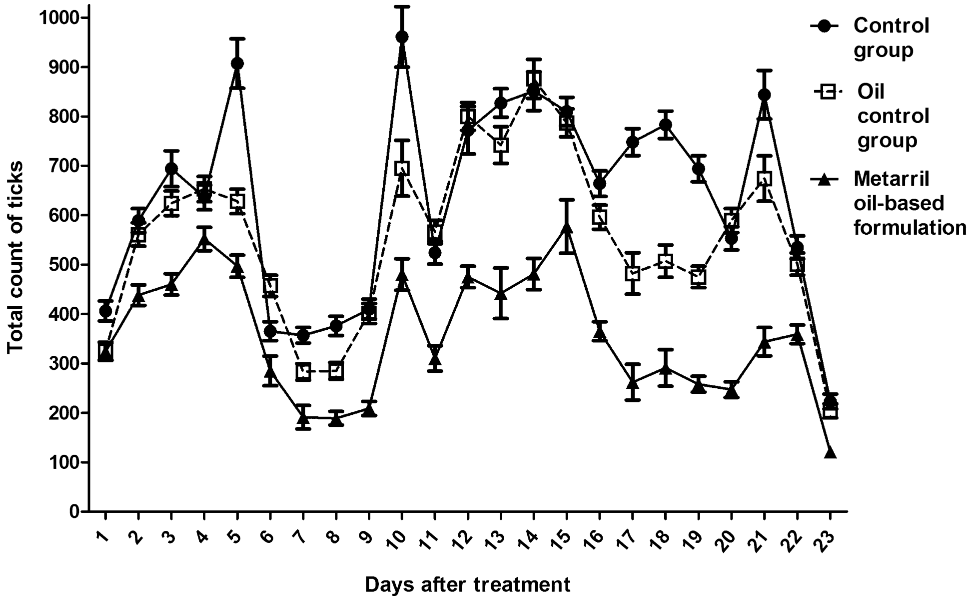

Fig. 1. Total daily count and standard deviation of *Rhipicephalus microplus* ticks collected after the treatment of the animals with the formulation of Metarril® SP Organic plus 10% mineral oil in the pen study.

Changes in biological parameters of engorged *R. microplus* females were observed mainly on days +1, +2 and +3 (the first three days after treatment), with a significant reduction in hatching percentage and egg production index (Table 2).

4. Discussion

The development and use of commercial products based on fungi for the control of insects and mites has increased substantially worldwide over recent decades. However, most of these products are developed for the control of pests in agriculture, with a small number produced for controlling ticks (Faria and Wraight, 2007). Among these is Metarril® SP Organic, a product based on concentrated spores of the fungus *M. anisopliae* and developed for the control of insects that attack agricultural crops. This is the first study to evaluate the effect of this commercial product with added mineral oil against *R. microplus* ticks in pen studies. This study aimed to evaluate the effect of this product on cattle ticks because it is already in a formulation and therefore is easy to transport, store, handle and apply in addition to already being produced on a large scale. The supplemental 10% mineral oil was added to Metarril® SP Organic to enhance its fungal effect against ticks as well as to protect it from temperatures and moisture in the environment may have been possibly inadequate for fungal growth.

The present study showed that the formulation of Metarril® SP Organic containing 10% mineral oil is effective in reducing the number of *R. microplus* ticks after treatment of cattle in the pen study. The average efficacy of the evaluated formulation was 47.74%, meaning a reduction of almost 50% of the ticks in the treated group during a period of three weeks with a single application of the product. Leemon et al. (2008) evaluated the effect of an oil formulation of *M. anisopliae* to control *R. microplus* in pen studies and observed high mortality in unengorged ticks incubated *in vitro* and a significant reduction of egg production by engorged ticks collected in the first 3 days after treatment. However, they did not observe a statistically significant effect on tick mortality *in vivo*. The authors believe that the high temperature interfered negatively in the fungus' pathogenicity to ticks. Besides that, there was a wide variability in the tick count, resulting in a lack of statistical power. The difference between the results obtained by Leemon et al. (2008) and the results presented in this study may be due to several factors, such as differences in the methodology used for the treatment of animals, the evaluation of the treatment's efficacy, the different *M. anisopliae* isolates used, the distinct populations of ticks, or the breeds of cattle in addition to varying environmental conditions at the sites where the pen studies were performed.

The strategic control of ticks proposes to focus on cattle bathing treatments during seasons that are unfavorable for the development of ticks in the environment, usually performing a bath with a contact acaricide every 21 days or more frequently (Furlong and Prata, 2005). In the present study, the tested fungal formulation had an average efficacy of 47.74% and caused deleterious effects on the biological parameters of females collected during the first three days after treatment, with only one bath per animal. It is believed that this efficacy can be enhanced with the development of a treatment protocol, *i.e.*, by establishing a number of animal baths with fungal formulations for a period of time. This theory was strengthened by Kaaya et al. (2011), who evaluated the effect of an oil formulation of *M. anisopliae* by spraying it once every three weeks for one year in cattle infested with ticks *Rhipicephalus evertsi evertsi* and *Rhipicephalus* (*Boophilus*) *decoloratus* under field conditions. These researchers observed a reduction of up to 83% of tick populations. Alonso-Díaz et al. (2007) observed efficacy between 0 and 91.2% of a suspension of *M. anisopliae* when cattle that were naturally infested with *R. microplus* were bathed every 15 days for two months under field conditions. These results corroborate the need to develop a treatment protocol for cattle aimed at enhancing the action of fungal formulations for the control of ticks *in vivo*.

The efficacy of the tested fungal formulation increased during the weeks after the treatment of cattle, with up to 50.19% during the first week, increasing during the second week (up 53.50%) and peaking at 67.39% during the last week of evaluation. These results indicate the effect of the fungal formulation on adult, nymphal and larval stage ticks, respectively, that were present in cattle on the day of treatment, showing that the fungal formulation caused a greater deleterious effect on larvae than nymphs and adults present in cattle on the day of treatment. Therefore, a smaller number of ticks were collected during the last week of the experiment (third week after treatment), during which the larvae present on the cattle on the day of treatment would have reached the adult stage. Previous studies have shown a difference in susceptibility to acaripathogenic fungi between different developmental stages of ticks (Castro et al., 1997; Kaaya and Hassan, 2000; Bahiense et al., 2007; Camargo et al., 2012). Kaaya and Hassan (2000), while assessing the effect of oil formulations of *Beauveria bassiana* and *M. anisopliae* on *Rhipicephalus appendiculatus* and *Amblyomma variegatum* in pastures, observed mortality of 100%, 80–100% and 80–90% in larvae, nymphs and adults, respectively, demonstrating that the immature developmental stages of ticks are more susceptible to acaripathogenic fungi.

In the present study, significant changes were observed in the biological parameters of females collected during the first three days after treatment. This fact must be due to the greater contact of engorged females with the fungal formulation during this period because, over time, the fungal concentration in contact with the ticks on the animal's body tends to decrease due to the animal licking, scratching and lying down on the ground, thus eliminating the product. Bahiense et al. (2008), while evaluating the effect of *M. anisopliae* suspension associated with deltamethrin in the *R. microplus* control in pen studies, also observed significant changes in female biological parameters collected during the first two days after treatment. Leemon et al. (2008) and Kaaya et al. (2011) observed a 100% mortality of female ticks after treatments *in vivo* with oil formulations of *M. anisopliae* as well as a significant reduction in egg mass. However, these researchers collected females directly off the body of cattle for the assessment of mortality and biological parameters prior to complete engorgement and detachment. In the present study, on the other hand, females were collected from the stall floors after full engorgement and detachment from the bovine's body, were washed under running water to remove dirt and feces of cattle and were dried on a paper towel to remove excess water. This washing and drying procedure may have removed part of the conidia still present on the surface of ticks and interfered in the assessment of the effect of the fungal formulation on the biological parameters of the ticks.

Table 2
Means and standard deviations of hatching percentage and egg production index (EPI = weight of egg mass/initial weight of engorged female × 100) of engorged females collected from the stall floors in the first three days after the treatment of the animals with the formulation of Metarril® SP Organic plus 10% mineral oil in the pen study. Females were cleaned, dried, individually weighed, fixed in Petri dishes and incubated at 27 ± 1 °C and relative humidity ≥ 80% for the evaluation of biological parameters. The egg mass of females was weighed and incubated under the same conditions for further evaluation of hatching of larvae.

	Day +1			Day +2			Day +3		
	Control group	Oil control group	Metarril oil-based formulation	Control group	Oil control group	Metarril oil-based formulation	Control group	Oil control group	Metarril oil-based formulation
Hatching percentage (%)	97.21a ± 6.26	92.19a ± 17.28	90.38a ± 24.86	98.94a ± 3.61	99.32a ± 1.57	90.16b ± 24.40	98.29a ± 3.35	97.21a ± 7.09	93.95a ± 22.56
EPI (%)	54.12a ± 19.41	58.60a ± 5.46	34.70b ± 25.95	59.66a ± 11.63	61.74a ± 4.92	52.41b ± 19.00	51.28a ± 12.74	52.09a ± 16.47	38.54b ± 16.15

The means followed by the same letter in the same line and the same day did not differ significantly ($p \geq 0.05$) by the Student–Newman–Keuls test.

In the present study, no adverse reactions in cattle could be attributed to the treatment with a formulation of *M. anisopliae*, suggesting that the commercial formulation Metarril® SP Organic is safe for cattle. These results are in agreement with the findings of Zimmermann (2007), who concluded that *M. anisopliae* is safe for cattle and presents minimal risk to mammals and humans. The researchers Kaaya et al. (2011) and Alonso-Díaz et al. (2007) also reported no adverse reactions in cattle plated with fungal formulations of *M. anisopliae*.

5. Conclusion

The results of this study suggest that the commercial formulation Metarril® SP Organic containing 10% mineral oil is efficient against *R. microplus* in pen studies and can be an important tool in the control of this tick. However, studies with more than one application of this formulation in cattle for the development of a treatment protocol are important to enhance the effect of this biopesticide and will contribute to the strategic control of the tick *R. microplus* in the field.

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