

Original Article

Efficacy profile of Cypermethrin and Chlorpyrifos based acaricides on *Rhipicephalus microplus* control on cattle in the rearing phase, naturally infested and exposed to tick fever agents in central Brazil

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ABSTRACT

The objective of this work was to evaluate the efficacy of two cypermethrin- and chlorpyrifos-based acaricides in controlling *Rhipicephalus microplus* in a naturally infested bovine herd and in *in vitro* tests, as well as to monitor the animals for tick fever. Male bovines in the rearing phase were used, with 30 Brangus and 30 Nellore animals naturally infested. The groups were composed as follows: 15 Nellore treated, 15 Nellore control, 15 Brangus treated and 15 Brangus control. Every 18 days, the animals were monitored for tick count, acaricide treatment, weight, blood pack cell volume, and clinical signs. For *in vitro* tests, the larval packet test, adult immersion test and DNA amplification for tick fever diagnosis were performed. In the first animal treatment period, product 1 (cypermethrin, 15 g + chlorpyrifos, 25 g + citronellal, 1 g) was used; in the second period, product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g) was used. In Brangus animals, the mean efficacy was 35.1% and 95.8% in the first and second periods, respectively, for the same animals. For Nellore animals, the efficacy in periods one and two was 51% and 97.1%, respectively. The *in vitro* results showed efficacy above 95% for the two challenged acaricides. The Brangus animals showed a high production of ticks associated with the presence of tick fever agents, which could generate risks for the disease's enzootic stability

1. Introduction

Rhipicephalus microplus is widespread, making it one of the great obstacles of the cattle industry; it is a one-host tick, parasitizing mainly bovines (Andreotti et al., 2016). In Brazil, it is found all over the country, and estimates indicate that it causes an annual loss of 3.24 billion dollars (Grisi et al., 2014). One of the major causes of such losses, the “Bovine Parasitic Sadness”, is a disease complex with high morbidity and mortality that is caused by *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale*, all of which utilize *R. microplus* as their main vector (Gonçalves, 2000; Gonçalves et al., 2011).

Western-central Brazil is an important region for beef cattle

production, and the high genetic value bovine market attempts to produce breeds with better performance and high productive lineage able to generate descendants with greater weight gain and better quality (Andreotti et al., 2016; Battistelli et al., 2013; Wambura et al., 1998).

In this context, the Brangus cross showed superiority with respect to Nellore and other crosses in this region (Battistelli et al., 2013); however, cross-bred animals are generally more susceptible to ticks, and the control of these ectoparasites is achieved through the use of acaricides. This increase in infection pressure places these populations of ticks at a higher risk of developing resistance, including multi-chemical resistance to the different chemical bases used (Higa et al., 2015) in

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different regions (Andreotti et al., 2011, Reck et al., 2014, Higa et al., 2016, Klafke et al., 2017) of Brazil.

Another aggravating factor in the use of acaricides is the risk of environmental contamination and residues in meat, milk and its derivatives (De Meneghi et al., 2016, Gaus and Furlong, 2002, Kunz and Kemp, 1994).

The objective of this work was to evaluate the efficacy of two cypermethrin- and chlorpyrifos-based acaricides in the control of *R. microplus* in naturally infested beef cattle and to compare the performance of these acaricides with *in vitro* tests, as well as to monitor the presence of tick fever agents.

2. Material and methods

2.1. Study area

The study was conducted between June and November 2016 on a farm owned by Agropecuária Sanyo that was located in Água Clara County, MS, Brazil at latitude 20°46'24"S longitude 52°32'24"W and an altitude of 309 m. The climate is characterized as humid tropical with a dry season of one to three months and an average temperature above 18 °C in all months of the year (IBGE, 2002; Flumignan et al., 2015).

2.2. Use of animals

All the performed procedures using animals were in accordance with the norms published by the National Council of Control of Animal Experimentation/CONCEA and were approved by the Ethics Commission of the Use of Animals/CEUA at Embrapa Gado de Corte, protocol no. 01/2016.

2.3. Experimental animals

Sixty male bovines in the rearing phase were used, with 30 Brangus and 30 Nellore animals naturally infested with *R. microplus* ticks. The animals were kept in a 48-ha picket under a continuous grazing system with *Brachiaria* spp. The rearing phase corresponded to the period of animal life post-weaning, which occurs around seven months of age, until they reached the age of 22 months. In the present study, the animals were an average of nine months of age.

2.4. Tick count, acaricide application and weighing

The experimental design was based on pre-tick counts between 4.5 and 8 mm (Wharton and Utech, 1970) throughout the body length of the two cattle sides on days –2 and –1 (pretreatment). Posteriorly, the counts on both sides of the animals were added and divided into four homogeneous groups regarding tick count and race as follows: Nellore treated, Nellore control, Brangus treated and Brangus control.

The treated Nellore and Brangus animals were treated with commercial acaricide formulations every 18 days. In the first 4 treatments (first period), product 1 (cypermethrin, 15 g + chlorpirifos, 25 g + citronellal, 1 g; Colosso® - Ouro Fino Saúde Animal Ltda, Ribeirão Preto, SP, Brazil) was applied as a *pour on* formulation; after the fourth treatment (second period), product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g; Colosso FC-30® - Ouro Fino Saúde Animal Ltda., Ribeirão Preto, SP, Brazil) was applied by topical spray.

The products were used as recommended by the manufacturer. For the first period, the counts were performed on days +18, +36, +54, and +72 (treatment 1), and for the second period, the counts were performed on days +90, +108, +126, and +144 (treatment 2).

The acaricide efficacy percentage calculation in the field was performed according to the formula proposed by Corrêa et al. (2015) and Cruz et al. (2015):

$$\text{Efficacy percentage} = \left(1 - \frac{\text{Ta} \times \text{Cb}}{\text{Tb} \times \text{Ca}}\right) \times 100$$

In this equation, **Ta** represents the mean number of partially engorged female ticks counted on the treated animals after medication; **Tb** is the mean number of partially engorged female ticks counted on the treated animals during the two days that preceded the treatment; **Ca** is the mean number of partially engorged female ticks counted on the control group after the experiment was initiated; and **Cb** is the mean number of partially engorged females counted on the untreated animals (control) during the two days that preceded the treatment.

Simultaneously, with the counts (every 18 d), the animals were individually weighed using a Coimma® digital scale.

2.5. Blood collection

Blood was collected from the animals every 36 days for the detection of tick fever agents. All material was subjected to DNA extraction and subsequent polymerase chain reaction (PCR). A hematocrit evaluation was also performed, and hematocrit concentration was obtained using a Daiki® CM-12000 microhematocrit centrifuge (Alves et al., 1986).

2.6. Detection of pathogens

2.6.1. DNA extraction

For the genomic DNA extraction, 300 µl of bovine blood plus 2 µl of proteinase K (20 mg/ml) and 500 µl of sodium dodecyl sulfate (20%) were used; the samples were incubated for 1 h in a water bath at 65 °C. After the incubation period, 800 µl of chloroform was added.

The samples were vigorously vortexed for homogenization, 350 µl of protein precipitation solution (6 ml of potassium acetate, 1.1 ml of glacial acetic acid, and 2.9 ml of water) was added, and the mixture was centrifuged at 18,000 x g (10 min). The aqueous phase was transferred to a new tube, 1 ml of 100% ice-cold ethanol was added, and the samples were kept in a freezer at –20 °C overnight for DNA precipitation.

Afterwards, the samples were centrifuged at 13,000 rpm (5 min), the supernatant was discarded for the addition of 1 ml of 70% ethanol, and the mixture was centrifuged at 13,000 rpm (2 min). The pellet was oven-dried at 37 °C, and the DNA was resuspended in 50 µl of ultrapure water and eluted in a water bath for 30 min at 65 °C. The samples were quantified by spectrophotometry (NanoDrop ND-1000, Uniscience) and diluted to 100 ng for PCR performance.

2.6.2. Polymerase chain reaction (PCR)

For pathogen detection, specific pairs of primers for *Anaplasma marginale* (Echaide et al., 1998), *Babesia bigemina* and *Babesia bovis* (Guerrero et al., 2007) were used to amplify fragments of 458, 262 and 217 base pairs (bp), respectively.

To perform PCR, the following reagent concentrations were used: 2.5 µl of 10× buffer (1×); 0.75 µl of MgCl₂ (50 mM); 0.5 µl of dNTPs (2.5 mM, Invitrogen by Life Technologies™); 0.5 µl of forward and reverse primers (10 picomoles); 0.3 µl of Taq (Ludwig Biotec); 1 µl of DNA (100 ng) and ultrapure water. The final volume was 25 µl.

The reactions were performed in a Bio Rad T100™ thermal cycler. For *A. marginale*, the following program was used: 95 °C/3 min; followed by 40 cycles of 95 °C/30 s; 65 °C/1 min; 72 °C/45 s; with a final extension of 72 °C/10 min. For *B. bigemina*, the performed program was as follows: 95 °C/2 min; followed by 40 cycles of 95/1 min; 60 °C/30 s; 72 °C/1.5 min; with a final extension of 72 °C/7 min. For *B. bovis*, the program that was used was as follows: 95 °C/2 min; followed by 40 cycles of 94/1 min; 60 °C/30 s; 72 °C/1 min; with a final extension of 72 °C/7 min. After amplification, the products were visualized on a 1.5% agarose gel stained with ethidium bromide (EtBr).

2.7. Field bioassays

To verify acaricide efficiency in the different field situations, the adult immersion test (AIT) was performed. For each AIT, three groups of ten engorged female ticks (triplicates), totaling 30 engorged female ticks per treatment, were used in an adapted test from Drummond et al. (1973).

The product tested in the field bioassay was product 2, and the engorged female ticks used in the test were manually collected just before the animals were topically sprayed. Three treatments were performed: dirty solution, clean solution and control.

For controls, water was used for engorged female tick immersion. For the clean solution treatment, the solution was collected directly from the spray pump at the time of spraying. For the dirty solution, the solution that had been drained from the body of the animal treated at the time of the spraying was collected.

After being submitted to the AIT, the engorged female ticks were conditioned in Petri dishes, sent to the laboratory and maintained in ideal conditions of temperature and humidity (28 °C and 80% relative humidity) to evaluate the reproductive parameters and posterior acaricide efficiency.

The evaluation of the *in vitro* efficacy of product 1 on engorged females was carried out according to Garcia et al. (2011). To evaluate the hatchability rate of larvae, a technique described by Szabó et al. (1995) was used.

2.8. Laboratory bioassays

To verify acaricide efficacy throughout the experiment (product 1 and product 2), engorged female ticks were collected and taken to the laboratory for *in vitro* bioassays. To obtain efficacy, the adult immersion test (AIT) (Drummond et al., 1973) was performed, and a sample of engorged female ticks were kept in BOD to obtain larvae and later for use in the larval packet test (LPT) (FAO, 2004).

2.9. Statistical analysis

For the statistical analysis, the program Bioestat 5.0 was used. The Kruskal-Wallis and Mann-Whitney tests were performed.

3. Results

3.1. Tick count

The general tick production in average numbers is shown in Table 1, and the number of ticks during the whole experiment is shown in Fig. 1. In Table 2, the acaricide efficacy throughout the experiment is shown.

When evaluating the tick counts with their averages, a daily average in the first period of 121.5 ticks/animal in the Brangus control was observed, while for the treated Brangus animals, in the same period, an

Table 1

Mean of ticks, *Rhipicephalus microplus*, in cattle in the rearing phase after treatment with product 1 (cypermethrin, 15 g + chlorpyrifos, 25 g + citronellal, 1 g) and product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g) in the municipality of Água Clara, MS, Brazil.

	Average tick count								
	Pre ^b	Product 1				Product 2			
		+18 ^a	+36 ^a	+54 ^a	+72 ^a	+90 ^a	+108 ^a	+126 ^a	+144 ^a
Brangus control	143	62	70	202	152	100	106	65	124
Brangus treated	147	38	49	125	111	6	5	3	2
Nellore control	7	12	8	19	20	31	20	9	23
Nellore treated	7	7	4	9	8	2	1	0	0

^a Day of tick counting and treatment of animals.
^b Pretreatment (days -1 e-2).

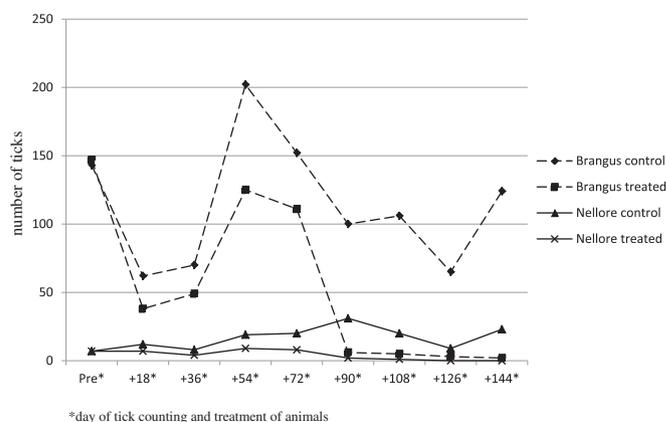


Fig. 1. Profile of the average number of *Rhipicephalus microplus* ticks on cattle in the rearing phase after treatment with product 1 (cypermethrin, 15 g + chlorpyrifos, 25 g + citronellal, 1 g) and product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g) in the city of Água Clara, MS, Brazil.

Table 2

Treatment efficacy with product 1 (cypermethrin, 15 g + chlorpyrifos, 25 g + citronellal, 1 g) and product 2 (cypermethrin, 15 g + chlorpyrifos, 30 g + fenthion, 15 g) on *Rhipicephalus microplus* in Brangus and Nellore animals in the rearing phase, Água Clara county, MS, Brazil.

	Efficacy in percentage (%)							
	Product 1				Product 2			
	+18 ^a	+36 ^a	+54 ^a	+72 ^a	+90 ^a	+108 ^a	+126 ^a	+144 ^a
Brangus	40,0	31,9	39,8	28,9	94,1	95,4	95,5	98,4
Nellore	41,6	50,0	52,6	60,0	93,5	95,0	100	100

^a Day of tick counting and treatment of animals.

average production of 80.7 ticks/animal was observed, demonstrating an average efficacy of 35.1% for product 1 when used in Brangus. For Nellore animals, in the same period, the counting averages were 14.7 and 7 ticks/animal for control and treated animals, respectively. Thus, an average efficacy of 51% was obtained.

In the second period, the counting averages were 98.7 and 4 ticks/animal for the control and treated Brangus animals, respectively, and the average efficacy was 95.8%. For the Nellore animals of the control group during the same period, a mean of 20.7 ticks/animal was obtained, while the treated ones showed an average of 0.7 tick/animal, demonstrating an average efficacy of 97.1% for product 2.

The Nellore group of control animals produced an average of 17.7 ticks/animal throughout the experimental period, while in that same period, the average of the control Brangus was 110.1 ticks/animal. The results showed that Nellore animals presented 16.1% of the total production of Brangus ticks, meaning that Brangus produced 6.2 times

more ticks than Nellore when both were untreated and exposed to the same conditions.

With regard to the two acaricide treatments, a significant difference ($p < .05$) was observed between the treated animals of the first and second periods, and in the second period, treatment with product 2 showed satisfactory efficacy (averaging over 95%).

The presence of myiasis was observed during the experiment in 5 of 15 animals of the Brangus breed belonging to the control group, which had a high tick infestation.

3.2. Results of the field bioassays

The results of the field bioassays with product 2 were 100% effective in AIT, both for the clean and dirty solutions. The engorged female ticks from the control groups performed posture, and the larval hatching rate was 98%. Both of the groups from the clean and dirty solutions showed 100% mortality.

3.3. Results of the laboratory bioassays

All AIT bioassays for both products, performed in the laboratory, presented 100% efficacy. The engorged female ticks used for the control performed posture, and the hatching rate was of 98%. With respect to the LPT bioassays, there was an efficacy above 95% for both products.

3.4. Results for tick fever

Regarding the detection of pathogenic agents by PCR, the circulating agents in the experimental animals were, mainly, *A. marginale* and *B. bigemina*, and both were detected in all animals. *B. bovis* was detected only once in a Brangus animal of the treated group. Despite the molecular detection of tick fever agents, no animal showed clinical signs of disease during the experiment.

3.5. Hematocrit

In the present study, no correlation was observed between the parasitic load and the presence of below-normal values in the hematocrit. The animals, regardless of the parasitic load, had normal hematocrit values.

3.6. Weight gain of the animals in the period

When comparing the weight gain of the animals in the rearing phase in which the experiment was performed, there was no significant difference between the different groups (treated and controls) and breeds ($p > 0.05$).

4. Discussion

During the whole experimental period, an average of 110 ticks/day were found for Brangus animals, which is well above the amount reported by Gomes et al. (1989), who, using the same animal and breed categories, observed 59.7 ticks/day in infested animals in the field in the same region of the present study.

Higher values were also obtained for Nellore animals, with this study yielding 17.7 ticks/day, while Gomes et al. (1989) reported 3.3 ticks/day. The differences in the number of ticks found between the studies may be due to changes in the biological and ecological behavior of the ectoparasite (Rodrigues et al., 2017), as well as changes in the host, given the constant genetic improvements only with respect to the animals' weight gain.

The treatment of the Brangus and Nellore animals with product 2 provided an average efficiency above 95%, which was similar to the *in vitro* tests and in compliance with the requirements and recommendations of the competent authorities (Ministry of Agriculture, Livestock

and Food Supply, Brazil, 1997 and FAO, 2004). On the other hand, the use of product 1 showed an efficacy above 95% *in vitro*, but when used on animals in the field, it presented much lower results, with only 35.1% and 51% effectiveness for Brangus and Nellore, respectively.

The significant difference of the *in vitro* and *in vivo* (in the field) results suggests that this difference may be associated with the application method, since in the field, product 1 was used *via* the *pour on* system, and product 2 was applied by topical spray. These data corroborate results presented by Corrêa et al. (2015), who evaluated the performance of acaricides *in vivo* using animals in the field and using the stall test method, as well as *in vitro* examination, and concluded that depending on the formulations used, the *in vitro* tests can overestimate the effectiveness of a certain acaricide or formulation, thus generating unreliable results.

According to the methodology used and the degree of susceptibility of a *R. microplus* tick population to the acaricide, differences in the effectiveness of the products in the different tests can classify a tick population as sensitive or resistant (Corrêa et al., 2015). According to Corrêa et al. (2015), AIT provides a greater penetration of acaricides in ticks, and the five-minute immersion time may actually be more than sufficient for the tests using acaricides in association.

The acaricides efficacy evaluation studies in Brazil are not systematic, and the presence of resistance to different chemical bases is a reality reported in several regions of the country (Andreotti et al., 2011, Reck et al., 2014, Higa et al., 2015, 2016, Klafke et al., 2017). In the region in which this study was conducted, there are populations of this tick that are highly resistant to cypermethrin-based acaricides, a fact that was previously identified by Andreotti et al. (2011) and Mendes et al. (2013).

If we were to consider only the efficacy results of product 1 in the field, excluding the *in vitro* test, product 1 could be considered ineffective in tick control because of the low efficacy values presented. According to studies by Gomes et al. (2011) and Higa et al. (2016) in the same region of the study were found populations resistant to this acaricide formulation.

For the same formulation in question, Brito et al. (2011) observed a minimum and maximum efficacy of 72.4% and 86.3%, respectively, in a study carried out in the Northern region of the country, confirming the need for resistance monitoring using bioassays for each locality and indistinct tick population.

The results obtained in this study regarding acaricide efficacy in the field and *in vitro* demonstrate that this tick population is sensitive to the formulation of cypermethrin associated with chlorpyrifos and fenthion, corroborating the data of Heidmann et al. (2016).

It is also worth emphasizing that the enzootic stability for tick fever was demonstrated in the present study, since the presence of the causative agents was verified through the DNA amplification of samples collected from the animals; however, no bovine from the present study presented clinical signs of the disease or the presence of hematocrit values below normal.

Animals with high infestation (> 100 adult ticks in the total count) received preventive treatment for tick fever to reduce their risk of acquiring the disease during the experiment; however, this preventive treatment consisted of the subcutaneous administration of imidocarb dipropionate at a dosage of 1.5 mg/kg of the animal's live body weight, and animals of the Brangus breed that presented a high rate of tick infestation associated with the presence of tick fever agents were potentially at risk in terms of the enzootic stability of the disease. Madruga et al. (1987) noted that Nellore is a breed with a high resistance to ticks and a mean infestation rate that is sufficient to maintain tick fever at a stable enzootic level.

Also in relation to the high infestations, the presence of myiasis can be observed in the control group animals that presented intense infestations. This result corroborates the data presented by Reck et al. (2014) who report a positive relationship between the high rate of tick infestation and the presence of myiasis. These authors also found that

animals with high parasitic loads are about four times more likely to have myiasis than animals with low parasitism.

Regarding cattle weight, treated and control animals in the short experimental period did not have significant differences in weight gain following either treatment (product 1 and product 2); in contrast, Jonsson (2006) estimated that tick parasitism led to a loss of 1.18 g/tick/day.

Although the *in vitro* tests are low cost and have a certain practicality in the development of the techniques, the results obtained need to be carefully analyzed because they do not always reliably reflect the results obtained in field trials, thus making it necessary to make careful assessments and observations, both in bioassays and in the acaricide application methods (e.g., *pour on* or topical spray).

The formulation of a commercial product containing an acaricide, or mixtures thereof, can influence its performance. How the applicator handles the product to treat the animal can also influence product performance. However, performance variability and other factors can influence the acaricidal formulation and treatment method of choice for cattle (De Meneghi et al., 2016).

We demonstrate here the importance of tick monitoring in a cattle production system in Central Brazil, especially for animals of more sensitive breeds such as Brangus. In these animals, high infestations and, in some cases, association with myiasis can be observed. Therefore, the correct use of acaricides at treatment intervals not exceeding 21 days (as demonstrated in the study) proved to be efficient in significantly reducing infestation of the animals.

Preventive treatment against cattle tick fever in animals with high infestations of ticks proved to be effective during the experimental period and may be an alternative for tick fever prevention, however additional studies should be performed to demonstrate the efficacy of this treatment in the bovine production system.

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Conflict of interests

The authors declare to have no conflict of interest.

Ethical statement

All the performed procedures using animals were in accordance with the norms published by the National Council of Control of Animal Experimentation/CONCEA and were approved by the Ethics Commission of the Use of Animals/CEUA at Embrapa Gado de Corte, protocol no. 01/2016.

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