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Additive Efficiency of Fipronil + Methoprene Compared to Fipronil Alone against *Rhipicephalus sanguineus* Ticks in Naturally Infested Dogs

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Abstract

The acaricidal efficiency of fipronil alone and fipronil + methoprene compared to commercial fipronil and commercial fipronil + methoprene, applied by the epicutaneous route (spot-on) in dogs naturally infested with ticks, was assessed. Thirty dogs infested with high loads of ticks were used. On day 0, the dogs were divided into 5 groups of 6 animals each. Each animal was duly identified for individual and group monitoring. Treatments were made based on body weight according to manufacturer's instructions. Group 1 (G1) received 10.0% fipronil at a single dose of a pipette applied by epicutaneous route in the base of the neck. G2 received 10% fipronil + 10% methoprene in single application similarly to G1. G3 was treated with 9.8% commercial fipronil as before mentioned. G4 received commercial 9.8% fipronil + 9.8% methoprene applied as in previous groups. G5 served as an infected untreated control. Animals were examined by thumb tick counts on days 0 (Treatment day), 3, 7, 14, 21 and 28. Efficacy was measured as a percentage of tick reduction in the treated groups relative to the untreated control. Results indicated an overall efficacy of 88.2%, 93%, 90.4% and 99.3%, respectively. There was no significant statistical difference between the treated groups ($P < 0.05$), neither in sex nor in weight. Engorged ticks were the most frequent, followed by the nymph group. However, by the end of the study these data were reversed due to the fact that only small larvae were found, which we interpreted came from engorged ticks that released their progeny before dying and a month later their offspring began to be observed presently and in full development. It is concluded that the combined formulations of fipronil + metho-

prene compared to fipronil applied alone, showed an additive effect against *Rhipicephalus sanguineus* ticks in naturally infested dogs kept in captivity.

Keywords

Ticks, Dogs, Efficacy, Fipronil, Fipronil + Methoprene

1. Introduction

The *Riphicephalus sanguineus* or brown dog tick, is one of the most widely distributed tick species, occurring world-wide between latitudes of 35˚C and 50˚C [1].

They are recognized not only as large blood suckers, but as important vectors of tick-borne diseases such as Lyme disease, ehrlichiosis, babesiosis, Rocky Mountain spotted fever, Colorado tick fever, Tularaemia, Q fever, tick paralysis, spotted fever and tick encephalitis. [2] [3] [4] [5]. For many years different methods of tick-control have been used [6] [7] [8] and the most popular method to control them is the application of chemical acaricides such as organophosphates, organochlorines, pyrethroids and amitraz, among others [9].

Considering its relevance to animal and human health, the need for specific tick products is increasingly pressing.

Today, there is a wide variety of ixodicidal products on the market prepared as collar formulations [10] [11], shampoo, powders, topical spot-on [12] [13], oral formulations [14], etc.

On the other hand, compounds based on fipronil alone or in combination with another drug are widely used by veterinarians due to the high efficacy and safety [15]-[20]. Therefore, the pharmaceutical industry produces new formulations each time looking for synergy, or some additive effect with the idea of increasing the effectiveness of the combined formulation.

The aim of the present study was to compare the effectiveness of fipronil + methoprene compared to fipronil alone against *Rhipicephalus sanguineus* ticks in naturally infested dogs.

2. Materials and Methods

2.1. Study Location

The study was carried out in October 2018, in a dog's shelter in the state of Morelos (central part of Mexico). This shelter has enough space to accommodate more than 70 dogs and incorporated feeders and troughs with ad libitum water.

During the development of the test, there was support from two people to provide cleaning and feeding to the canines, as well as two clinical veterinarians who were experts in handling dogs for the general health check-up and review of the animals for the tick count.

2.2. Animals

Thirty male or female dogs naturally infested with ticks (regardless of weight or age) were used. This selection was made based on the highest tick load and general health status using dogs with more than 15 ticks/animal.

Ten days prior to the start of the test, the animals were dewormed with Panacur® Plus (Intervet) containing fenbendazole + praziquantel to remove gastrointestinal worms and tapeworms, respectively. The dose used was 1 tablet/each/5 kg of body weight.

Likewise, in order to reduce stress, the selected animals were housed in their specific premises for adaptation to food and mutually group coexistence.

2.3. Experimental Design

On day 0 (start of the experiment), the 30 dogs were divided into 5 groups of 6 animals each proportionally distributed taking care that there were high loads of ticks.

In order to carry out individual and group monitoring, each animal was identified with a numbered necklace with indelible ink, in addition to being recognized by name.

To perform the treatments, a human scale was used to dose the dogs based on body weight in addition to performing weight measurement at the beginning and end of the study. Treatments were made according to the following scheme:

Group 1 (G1) received fipronil 10.0% (KiroGard-Laboratorios Kiron Mexico), at a single dose of 6.7 mg per kg by epicutaneous route applied in the base of the neck, according to manufacturer's instructions (0.67 mL/animal pipette weighing between 1 and 10 kg or 1.34 mL/animal pipette weighing between 10 and 20 kg).

G2 received 10.0% fipronil + 10% methoprene (KiroGard-M-Laboratorios Kiron Mexico), applied as described in the previous group.

G3 was treated with 9.8% commercial fipronil (Frontline®-Merial) in a single dose at the rate of 1 pipette for dogs weighing 10 to 20 kg, also applied in the neck region (Frontline® top-spot: small dog (2 - 10 kg) or Frontline® top spot: medium dog (10 - 20 kg), following the instructions recommended by the manufacturer.

G4 received 9.8% commercial fipronil + 9.8% methoprene (Frontline Plus®-Merial) applied similarly to the previous ones.

Group 5 served as an infected untreated control.

The experimental animals were examined by thumb counts on days 0 (Treatment day), 3, 7, 14, 21 and 28 (ticks were categorized as live free, live attached, dead free or dead attached) [21].

2.4. Assessment of Efficacy

Efficacy was evaluated in accordance to the WAAVP guideline [22].

$$\text{Efficiency Percentage} = \frac{\text{Average number of ticks in the control group} - \text{Average number of ticks in the treated group}}{\text{Average number of ticks in the control group}} \times 100$$

2.5. Taxonomic Identification

At the end of the test and in order to know the genus and specie of ticks involved in the study, more than 100 living or dead specimens were morphologically examined. This examination was carried by Dr. M.T. Quintero which is an expert acarologist of our parasitology department. Actually, this procedure was merely confirmatory, since to the best of our knowledge the only tick specie that parasites Mexican dogs is the brown tick or *Rhipicephalus sanguineus* [23] [24] [25] [26].

2.6. Statistical Design

In order to compare the efficacy of each treatment, an Analysis of Variance (ANOVA) was applied that determines whether there are significant differences between the means of the efficacy of the treatments. As there was a difference between them, a mean contrast was applied to identify between which treatments these differences occurred. Subsequently, the data was grouped into segment bar graphs indicating the percentage of efficacy in each experimental group. In order to carry out the statistical analyzes, the efficacy values (%) were transformed to arcsine values.

3. Results and Discussion

G1, the efficacy exerted by KiroGard (fipronil alone) was 100%, 100%, 100%, 88.1% and 53%, for days 3, 7, 14, 21 and 28, respectively, obtaining an overall efficacy of 88.2%.

G2, the efficacy conferred by KiroGard-M (fipronil + methoprene) was 98.9%, 100%, 100%, 84.6% and 85.9% respectively, generating 93.8% of Global Efficacy.

G3, the efficiency generated by Frontline (fipronil alone) was 97.8%, 98.8%, 100%, 96.5% and 59.1%, respectively, showing a Global Efficiency of 90.4%.

G4, the efficacy obtained for Frontline Plus (fipronil + methoprene) was 100%, 100%, 100%, 98.1% and 98.7%, respectively (99.3% of Global Efficacy).

Table 1 shows the average percentage of efficacy according to the days of treatment for each group under study. Even though the efficacy conferred by KiroGard and KiroGard-M was slightly lower, the statistical analysis indicated that there is no statistically significant difference when compared to their Frontline and Frontline Plus counterparts. To determine if the number of ticks was decreased due to the application of the treatments, an ANOVA was applied. The analysis indicated significant differences between treatments ($P < 0.05$). The untreated control group registered the highest number of ticks and differed from all treatments. The treatment with the lowest number of ticks was the Frontline Plus treatment (Figure 1). To determine if the efficiencies of the treatments were

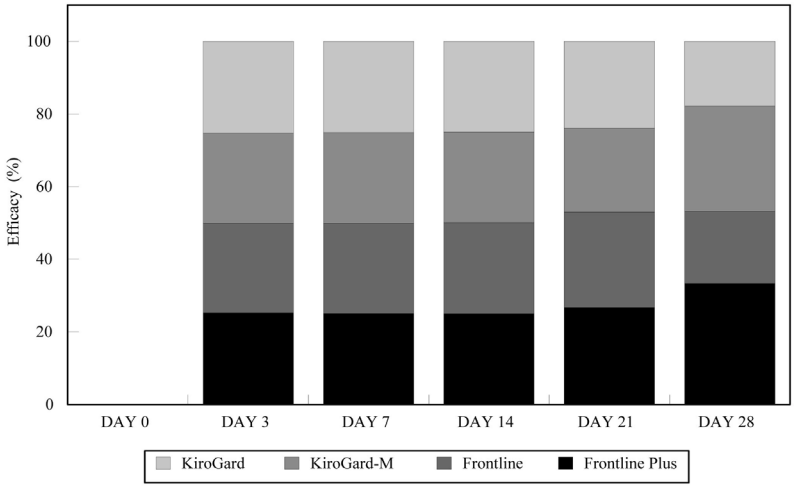

Figure 1. Efficacy of compounds per treatment per day.

Table 1. Efficacy percentage of four compounds against *Rhipicephalus sanguineus* ticks in dogs naturally infested.

Group No./compound	Efficacy (% in days)						
	0	**3**	**7**	**14**	**21**	**28**	**Global %**
1							
fipronil (KiroGard)	0	100	100	100	88.1	53	**88.2**
2							
fipronil + methoprene (KiroGard-M)	0	98.9	100	100	84.6	85.9	**93.8**
3							
fipronil (Frontline)	0	97.8	98.8	100	96.5	59.1	**90.4**
4							
fipronil + methoprene (Frontline Plus)	0	100	100	100	98.1	98.7	**99.3**
5							
untreated control	-	-	-	-	-	-	-

the same, an ANOVA was performed, where it was determined that between treatments there were no significant differences regarding efficacy ($P > 0.05$) since the treatments registered similar efficiencies. Regarding the evaluation days there were significant differences ($P < 0.05$).

In Figure 1 it can be seen that the efficacies of the treatments are the same, but regarding the day it can be seen that the days 3, 7 and 14 are the ones that report the best efficiencies, and as of the 21st they decrease in all the treatments except for the Frontline Plus that did not decrease its effectiveness, on the contrary registered an increase.

Table 2 shows the number of ticks counted before and after treatment with the drugs. In general, it is appreciated that from day 3 of evaluation, the number

Table 2. Number of ticks found before and after treatment with different ixodicides in naturally infested dogs.

Group No./compound	Dog number	Dog's name	Sex	Number of ticks on day:					
				0	**3**	**7**	**14**	**21**	**28**
1 KiroGard	1	Bambi	M*	28	0	0	0	0	7
	2	Colada	F**	36	0	0	0	1	10
	3	Tabata	F	42	0	0	0	0	5
	4	Negro	M	36	0	0	0	15	16
	5	Oso	M	72	0	0	0	0	31
	6	Carpintera	F	18	0	0	0	1	8
	Total No. of ticks/group			**232**	**0**	**0**	**0**	**17**	**77**
2 KiroGard-M	7	Chaparro	M	38	0	0	0	0	0
	8	Caronte	M	60	1	0	0	0	0
	9	Guera	F	36	1	0	0	8	5
	10	Dobbi	M	98	0	0	0	14	11
	11	Rorro	M	150	0	0	0	0	4
	12	Rayita	F	32	0	0	0	0	3
	Total No. of ticks/group			**414**	**2**	**0**	**0**	**22**	**23**
3 Frontline	13	Moro	M	102	0	0	0	0	20
	14	Pili	F	32	1	0	0	0	5
	15	Mili	M	40	0	0	0	0	2
	16	Rocío	F	84	0	0	0	2	20
	17	Willy	M	68	3	2	0	3	15
	18	Lady	F	138	0	0	0	0	5
	Total No. of ticks/group			**464**	**4**	**2**	**0**	**5**	**67**
4 Frontline Plus	19	Betsy	F	30	0	0	0	2	0
	20	Rambo	M	16	0	0	0	0	0
	21	Whisky	M	60	0	0	0	0	2
	22	Güero	M	36	0	0	0	0	0
	23	Tigrillo	M	60	0	0	0	0	0
	24	Terry	M	36	0	0	0	5	0
	Total No. of ticks/group			**238**	**0**	**0**	**0**	**7**	**2**
5 Untreated control	25	Chino	M	42	45	38	27	16	23
	26	Tepe	F	24	29	32	25	15	24
	27	Naty	F	22	20	24	28	20	25
	28	Flays	F	22	25	21	31	17	37
	29	Perla	F	36	39	32	17	20	31
	30	Capulin	M	24	28	25	19	15	24
	Total No. of ticks/group			**170**	**186**	**172**	**147**	**143**	**164**

*Male, **Female.

of ticks present drastically decreased, maintaining this low percentage until day 21 after treatment. By day 28, a gradual increase in ticks is observed in all groups, indicating that possibly the residual power of each drug is very low, which allows the establishment of new larvae.

At the start of the treatment (Day 0) no differences were observed between the treatments ($P > 0.05$), however, in the first evaluation (Day 3) the number of ticks significantly decreased ($P < 0.05$) in all the treatments compared to the untreated control, this pattern was observed until day 14. In the last two evaluations a slight increase in the activity of the treatments to eliminate the ticks was noted, but the number of these always remained below the values recorded by the untreated control group.

However, the efficacy conferred by the products during the 28 days of the study was good, observing a decline by the end of the study (**Figure 2**).

Identification of the evolutionary stages: When identifying the evolutionary stage of the ticks/group, it was determined that the adult ticks were the most frequent, followed by the group of nymphs. However, by the end of the study these data were reversed due to the fact that only small larvae were collected, which we interpreted came from ticks that released their progeny before dying and a month later their offspring began to be observed present and in full development due to that by this time the residual power of the drugs under study was surely minimal.

Relationship of the sex of the dogs with the load of ticks: It was observed that the female were more efficient for elimination of ticks, as can be seen in **Figure 3**.

Weight difference in the experimental dogs:

The average initial and final weight of groups can be seen in **Table 3**.

In general, the differences in the weight of the dogs indicated a slight increase in the groups treated with reference to the untreated control.

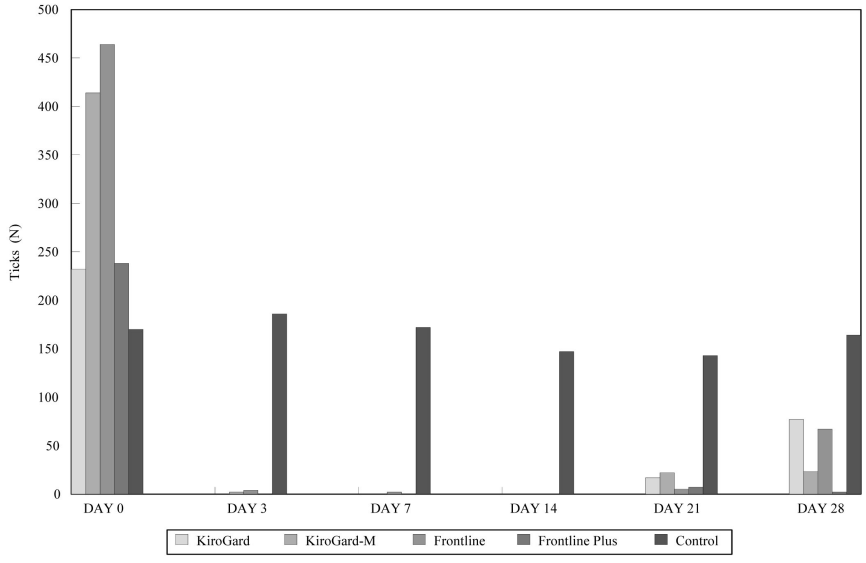

Figure 2. Number of ticks per treatment per day.

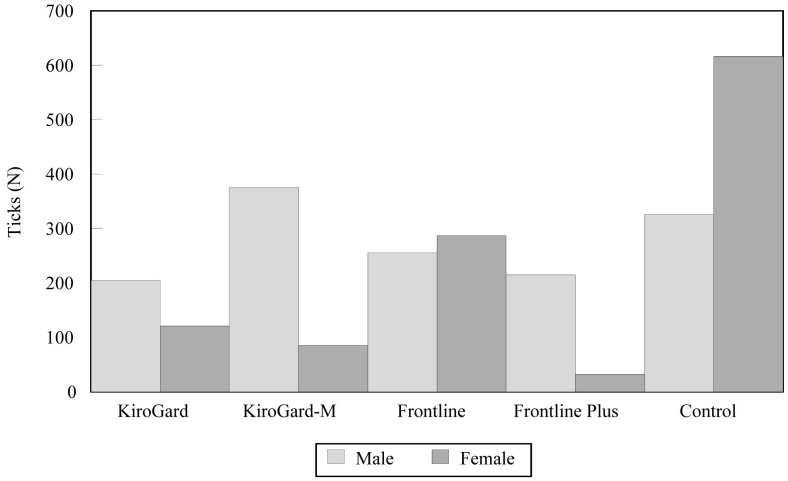

Figure 3. Number of ticks per treatment related to sex.

Table 3. Weight difference of the experimental dogs treated with different ixodicides.

Group	Compound	Day 0	Day 28	Difference of weight (kg)
		Average weight/group	Average weight/group	
1	KiroGard	73.5 (12.2)	76 (12.6)	**2.5**
2	Kirogard-M	63.5 (10.5)	69 (11.5)	**5.5**
3	Frontline	38 (6.3)	39 (6.5)	**1**
4	Frontline Plus	106 (17.6)	106.5 (17.7)	**7.5**
5	Untreated control	40.5 (6.7)	36 (6.0)	**4.5**

To determine the effect of the treatments on the weight of the dogs before and after the application of these, a paired T-test was performed. The analysis indicated that the treatment does not have an effect on the weight of the dogs ($P > 0.05$).

Regarding the number of ticks collected dead on the host and found on the floor, it was surprising, especially on day 3 post-treatment, since all the drugs under study removed a high percentage of them. It should be clarified that possibly some ticks were not detected, so due to their size, perhaps an "x" number of them was not reported, but the effect is evident and continued since very few specimens were observed in the subsequent sampling.

With reference to the identification of evolutionary stages, the presence of engorged adults was more evident, indicating that when falling to the ground a considerable number of eggs had to be deposited, thus ensuring a number of larvae as a new generation to give continuity to its evolutionary cycle. However, since the core part of this study was to determine the efficacy of the compounds, there was not the continuity required to know the long-term residual power. It is important to clarify that all the specimens collected were identified as *Rhipicephalus sanguineus*.

Regarding the differences between the tick parasitization in female and male dogs, it was observed that the female had a higher parasite load in each of the groups. However, since they were natural infections, this finding is possibly merely circumstantial, so it is suggested to analyze this parameter with a larger number of dogs and with controlled tick loads to elucidate this point.

Finally, the weight of the dogs varied at the end of a month that the study lasted, observing a slight increase in the treated individuals in all groups, compared to the untreated control. Perhaps it was necessary to keep the dogs for a longer time, with a good diet and without ticks, and possibly the difference in weight would have been really significant.

It was also observed that the treated dogs improved their coat and physical condition unlike the untreated group that always maintained an attitude of sadness. At the end of the study, untreated control dogs were also treated with these drugs to improve their physical condition.

It is important to note that none of the treated dogs showed any adverse symptoms after treatment.

Leschnick *et al.*, [2] communicated that dogs have a high risk to become infected with at least one pathogen during their life. Consequently it is mandatory to apply suitable and effective measures against tick infestations in dogs, starting early in life.

On the other hand the sale and use of ectoparasiticides for the control of arthropod parasites of domestic animals constitute a major sector of the global animal health market and their control still relies heavily on the use of chemicals of whatever origin [27].

One of the goals of veterinarians are to test the effectiveness of combined formulations aimed to determine if there exists a synergistic or additive effect aimed to improve the drug's efficacy.

Prullage *et al.*, [1] reported a synergistic effect produced by the combination of fipronil, amitraz and (S) methoprene applied as a spot-on in dogs by preventing attachment of ticks in 90% during 28 days.

A similar synergistic effect was obtained by [16] using a combination of 6.76% fipronil and 50% permethrin.

Here is important to note that in our study the combined spot-on administration of fipronil + methoprene showed a higher efficacy when compared to the treatment with fipronil alone, indicating that perhaps there is a synergistic or additive effect which enhances the activity against the ticks. Then the efficacy conferred in our study, is classified as good in that all the compounds showed high percentages of tick efficiency, keeping very high protection suggesting by 24 h for 28 days monthly application can be expected to significantly reduce the chance of transmitting tick-borne pathogens.

Here it is important to know that the spot-on formulations provide convenience and ease of use and a monthly dosing interval. The currently available combination of fipronil + methoprene, have a great activity against ticks. This combination of drugs may prevent transmission of disease to dogs.

In 2008, [28] pointed out that *R. sanguineus* can cause direct harm through irritation produced during attachment. Also they may be a direct cause of tick paralysis [29] and anemia when found in sufficient numbers [30] [31]. All these, added to the transmission of tick-borne diseases makes ticks a very important target to control.

At present there are various long-lasting compounds showing a high sustained acaricidal efficacy [10] [32] [33] [34]. However, it is unknown if the long drug persistence in the dog may lead to tick resistance problems in future. Therefore, it is essential to count with different molecules aimed to avoid resistance and reduce the prevalence of ticks in infected dogs to minimize the risk of spread and transmission to other animals and humans.

Limitation of the study: The authors agree that this study must have been prolonged for a longer time because there was no opportunity to follow up on the new generation of ticks which, when exposed to frequent treatments with fipronil + methoprene, could show a certain degree of resistance. Unfortunately, the aim of the study was only focused on comparing the efficacy of the tested compounds for 28 days. Further studies aimed at elucidating the possible emergence of resistance to these and other ixodicidal compounds should be encouraged.

The results here obtained underlines the findings of other trials demonstrating the ability of fipronil combined with methoprene to enhance the ixodicidal efficiency against dog ticks. This particular combination may provide an effective means for controlling ticks infesting dogs and limiting the spread of tick transmitted diseases.

4. Conclusions

The combined formulations of fipronil + methoprene compared to fipronil applied alone, showed an additive effect against *Rhipicephalus sanguineus* ticks in naturally infested dogs.

Most ticks appeared dead on the floor from the 3rd day after-treatment.

During the study, a greater number of engorged adult ticks were observed.

A higher degree of infestation was observed in female dogs.

A slight weight gain was determined in the treated dogs with reference to the untreated control.

All ticks were morphologically identified as *Rhipicephalus sanguineus*.

No toxic effects or adverse symptoms were observed in any of the dogs under study.

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Availability of Data and Material

All datasets are included in this manuscript.

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Contributors

FIV contributed to the study design, carried out the field study, wrote the paper, manuscript revision and discussion. YVM collaborated with the field study and results interpretation. ICM and YAC performed laboratory analysis, and statistical analyses, respectively. All authors supervised the experimental procedures, read and approved the final version of the manuscript.

Animal Research

The work was carried out adhering to the guidelines of the Institutional Committee for Use and Care of Experimental Animals of the institution, according to the Mexican Official Regulation NOM-062-ZOO-1999 and Animal Research: Reporting of *in Vivo* Experiments guidelines was followed.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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