Assessment of FRONTLINE® Plus Efficacy at 24-Hour Counts Against Tampa 2014 Isolate *Ctenocephalides felis* Flea Infestations on Cats and Dogs on Days 1, 7, 14, 21, and 28

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KEY WORDS: Key Words

ABSTRACT

Two studies were performed to determine the 24-hour efficacy of FRONTLINE® Plus (fipronil/(S)-methoprene) against the Tampa 2014 isolate of *Ctenocephalides felis* fleas. This isolate of fleas was collected during a field study where there was a perceived lack of efficacy of FRONTLINE Plus for Dogs against *Ctenocephalides felis* fleas. Two well-controlled laboratory studies were performed spatially and temporally separate, in two different facilities, against this isolate. In the first study, eight cats were treated with FRONTLINE Plus for Cats on Day 0, and eight cats remained as mineral oil-treated controls. Cats were infested with 100 fleas of the Tampa 2014 isolate on Days 1, 7, 14, 21, and 28, and the fleas were removed and counted 24 hours later. In the second study at a separate facility, 8 dogs were treated with the appropriate dose of FRONTLINE Plus for Dogs for their weight on Day 0, and 8 dogs remained as mineral oil-treated controls. Dogs were then infested with 100 fleas of the isolate on Days 1, 7, 14, 21, and 28, and the fleas were removed and counted 24 hours later. For Study 1, cats treated with FRONTLINE Plus had significantly ($p<0.01$) fewer live fleas than the controls 24 hours post-infestation on each assessment day. Efficacy for FRONTLINE Plus for Cats was 90.3%, 99.6%, 98.8%, 93.3%, and 85.6% on Days 2, 8, 15, 22, and 29, respectively. For Study 2, dogs treated with FRONTLINE Plus also had significantly ($p<0.01$) fewer live fleas than the controls 24 hours post-infestation on each assessment day. Efficacy for FRONTLINE Plus for Dogs was 99.6%, 100%, 100%, 100%, and 97.6% on Days 2, 8, 15, 22, and 29, respectively. FRONTLINE Plus for Cats and FRONTLINE Plus for Dogs demonstrated high efficacy against the Tampa 2014 isolate

of *Ctenocephalides felis* fleas throughout the two 29-day studies. It was later discovered that, in the original field study, the owners of the homes in which the perceived inactivity against fleas had taken place had been bathing their dogs in oil-cutting shampoos and/or dish detergent during the study. This is one of many potential confounders that can affect the performance of a topical flea control product. The results of the present studies demonstrate that the true effectiveness of FRONTLINE Plus against *Ctenocephalides felis* fleas was consistent with previous well-controlled studies over the last 20 years. These results indicate that, when there appears to be lack of effectiveness with a flea control product, one should always consider and explore all possibilities.

INTRODUCTION

A *Ctenocephalides felis* flea isolate designated "Tampa 2014" was collected during a field study in Tampa, Florida, in 2014, in which there was a concern with an observed lack of effectiveness of FRONTLINE Plus for Dogs. In a 2014 in-home flea product investigation in the Tampa FL USA area, high numbers of live fleas were observed on several dogs from different enrolled households 3 weeks post-treatment. Three dogs were then removed from the households and taken to a local veterinary clinic to reduce any re-infestation potential present in the homes. Dogs were combed with a fine-toothed flea comb, fleas were removed and counted, then returned to the dogs, and the dogs were observed overnight. Once the dogs had been maintained at the clinic free from potential re-infestation for a 24-hour period, dogs were combed again and flea counts were performed. At 24 hours, there was minimal change in the number of fleas infesting each dog. Upon entering the clinic, 127 live fleas were found on the first dog, and at the 24-hour flea check, 83 live fleas were found. Nine fleas were found on the second dog, upon entering the clinic, and at the 24-hour check, 9 fleas were again found. Eighty-eight fleas were found on the third dog upon entering the clinic, while 63 fleas were found at the 24-hour check. Additionally, flea eggs were observed in the cages in which these dogs were housed at the veterinary clinic. Live fleas from these dogs and flea eggs collected from their cages where shipped by overnight express to the Kansas State University flea research laboratory. Upon arrival eggs were placed into standard flea rearing media in an insect rearing chamber and live fleas were placed on cats. The majority of these flea eggs hatched and ultimately developed into adult fleas. Based on the observations of flea infestations on the dogs in the enrolled households, the in-home field study was terminated. Since there had previously been no validated evidence of resistance to either fipronil or (S)-methoprene, the fleas found on all three dogs in this study were used to develop a new colony, the aforementioned Tampa 2014 isolate.

The Tampa 2014 isolate of *Ctenocephalides felis* was used in the two studies described in this manuscript to evaluate, in a well-controlled laboratory setting, the 24-hour post-treatment and post-reinfestation efficacy of FRONTLINE Plus against flea infestations on cats and on dogs on Days 1, 7, 14, 21, and 28. The purpose of these studies was to determine if the cause of the poor efficacy of FRONTLINE Plus observed in the flea field study was due to development of resistance or other potential mitigating circumstances.

MATERIALS AND METHODS

Animal Welfare

Both of these studies were conducted by an experienced, independent contract research facility (Kansas State University for Study 1, and BerTek, Inc. for Study 2). Animals in both studies were managed similarly and with due regard for their welfare. All animals were handled in compliance with the Merial (Merial is now a part of Boehringer Ingelheim) Institutional Animal Care and Use Committee (IACUC) approvals. Both studies were approved by their respective facility local committees, the feline study (Study 1) by Kansas State University IACUC #3533 approved on February 13, 2015

Table 1. *Individual cat and dog information (as assessed prior to Day 0) and results of allocation to treatment groups (based on pre-treatment flea counts prior to Day -2)*

Study number	Animal ID	Sex	D.O.B.	Weight (lb)	Dose	Treatment group
1	19-15	M	9/6/2014	7.34	1.0 mL	Mineral oil-treated control cats
	14-15	M	9/11/2014	8.29	1.0 mL	
	28-15	F	9/15/2014	5.58	1.0 mL	
	27-15	F	9/24/2014	5.29	1.0 mL	
	12-15	M	9/15/2014	6.66	1.0 mL	
	18-15	M	9/20/2014	5.95	1.0 mL	
	30-15	F	9/12/2014	5.62	1.0 mL	
	11-15	M	9/28/2014	6.26	1.0 mL	
	23-15	F	9/12/2014	5.56	0.5 mL	FRONTLINE Plus-treated cats
	24-15	F	9/24/2014	5.40	0.5 mL	
	15-15	M	9/23/2014	6.70	0.5 mL	
	17-15	M	9/25/2014	7.30	0.5 mL	
	13-15	M	9/15/2014	6.62	0.5 mL	
	22-15	F	9/20/2014	5.71	0.5 mL	
	26-15	F	9/23/2014	5.11	0.5 mL	
	21-15	F	9/24/2014	5.62	0.5 mL	
2	F0066	M	5/5/2012	20.0	0.5 mL	Mineral oil-treated control dogs
	MC4026	F	10/29/2014	17.2	0.5 mL	
	MC4080	F	5/11/2014	16.3	0.5 mL	
	MC2889	M	9/24/2014	18.0	0.5 mL	
	MC7775	F	8/17/2012	22.5	0.5 mL	
	9223	M	11/27/2010	19.3	0.5 mL	
	MC8186	M	8/23/2012	21.0	0.5 mL	
	F0092	M	7/20/2012	14.2	0.5 mL	
	MC4078	F	7/20/2014	21.3	0.67 mL	FRONTLINE Plus-treated dogs
	MC4175	F	1/4/2013	22.3	0.67 mL	
	529	M	8/6/2011	15.7	0.67 mL	
	5038	F	7/18/2008	18.3	0.67 mL	
	F0047	F	2/22/2012	13.9	0.67 mL	
	F0144	F	10/26/2012	15.9	0.67 mL	
	1131	M	5/2010	31.0	1.34 mL	
	MC1007	F	8/20/2012	20.9	0.67 mL	

and the canine study (Study 2) by BerTek, Inc. IACUC approved on June 3, 2015. The trial facilities used for both studies meet USDA-APHIS animal welfare requirements. The Investigators ensured that all personnel were appropriately trained, and that procedures were in compliance with each protocol. Concomitant veterinary care and therapy, as well as any adverse events, were recorded.

Animals were allowed to acclimate to the test facility for at least 14 days for Study 1, and for 10 days for Study 2, prior to the initiation of each study. All animals were housed individually in accordance with the Animal Welfare Act. For Study 1, all cats received food meeting their daily nutritional requirements, and fresh water was pro-

Table 2. *Summary of geometric mean[1] flea (Tampa 2014 isolate Ctenocephalides felis) counts, percent efficacies, and p-values at 24 hours post-infestation, on each study day, for animals treated with mineral oil or FRONTLINE Plus*

Study number	Day	Control geometric means	FRONTLINE Plus geometric means	Efficacy (%)	P-value
Study 1	2	46.5	4.5[B]	90.3	0.0005[U]
	8	53.6	0.2[B]	99.6	<0.0001[E]
	15	54.1	0.6[B]	98.8	<0.0001[E]
	22	42.6	2.9 [B]	93.3	<0.0001[U]
	29	51.6	7.4 [B]	85.6	0.0011[U]
Study 2	2	90.1	0.3 [B]	99.6	<0.0001[U]
	8	90.2	0.0 [B]	100	<0.0001[U]
	15	87.4	0.0 [B]	100	<0.0001[U]
	22	87.0	0.0 [B]	100	<0.0001[U]
	29	94.7	2.3 [B]	97.6	0.0003[U]

[1] *Based on transformation to natural logarithm of (count+ 1)*
[B] *Significantly different from control (p<0.01)*
[E] *Results from t-test for means with poolable variances*
[U] *Results from t-test for means with unequal variances*

vided from a single source and available ad libitum. For Study 2, all dogs received 1-2 cups of commercial dry canine ration meeting their daily nutritional requirements, and fresh water from the local city water supply was provided ad libitum.

Fleas

All of the fleas used for both of these studies originated from a closed colony, and maintained from the original fleas and flea eggs collected from the three treated field study dogs following being housed 24 hours in the clinic (described in the introduction).

Animal Management and Study Inclusion

For Study 1, 16 cats (8 males and 8 females) were selected for study inclusion based on pre-treatment flea counts. The cats were between the ages of 6 and 7 months, and weighed between 5.11 and 8.29 pounds, as weighed prior to Day 0. For Study 2, 16 dogs (7 males and 9 females) were selected for study inclusion based on pre-treatment flea counts. The dogs were between the ages of 8.5 months and 7 years, and weighed between 13.9 and 31.0 pounds, as weighed prior to Day 0.

No cats younger than 8 weeks of age or under 1.5 pounds, nor dogs younger than 12 weeks of age or under 4 pounds, were considered for use in the respective studies. No animals which may have been debilitated, suffering from disease or injury, fractious, presenting abnormalities at the application sites, or otherwise unsuitable for inclusion, were considered for use. All animals were in good health, and none had been treated with a monthly ectoparasiticide within 3 months prior to study initiation. Individual cat and dog information, as assessed before study inclusion, are listed in Table 1.

Allocation

Prior to Day -2, a total of 20 cats for Study 1, and 22 dogs for Study 2, were infested with approximately 100 *Ctenocephalides felis* fleas of the Tampa 2014 isolate of and comb-counted 24 hours later. The 16 cats for Study 1, and 16 dogs for Study 2, with the highest flea counts were selected and ranked by flea count. In each study, eight replicates of two animals each were formed, and the two animals with the highest pre-treatment

flea counts formed Replicate 1, the next two animals with the highest flea counts formed Replicate 2, and so on, until all animals were allocated. For both studies, within replicates, each animal was randomly allocated to either the control group, or the FRONTLINE Plus-treated group. All animals remained in their assigned groups throughout the duration of each study. The results of the allocation processes are recorded in Table 1.

Study Design

Conducted both spatially and temporally separate, in two different facilities, both studies were well-controlled efficacy studies using a randomized block design based on animal pre-treatment flea infestation counts, and all evaluations of efficacy were performed by personnel in blinded conditions. Each animal was an experimental unit.

Treatment

All animals in both studies were weighed prior to Day 0, and the appropriate dose of FRONTLINE Plus was applied based on the animal's species and weight. In Study 1, on Day 0, a single dose of 0.5 mL of FRONTLINE Plus (fipronil/(S)-methoprene) for Cats was applied to each cat in the treated group, and 1.0 mL of mineral oil was applied to each cat in the control group. In Study 2, on Day 0, a single dose of FRONTLINE Plus (fipronil/(S)-methoprene) for Dogs (0.67 mL for dogs up to 22 pounds, and 1.34 mL for dogs between 23 and 44 pounds) was applied to each dog in the treated group, and 0.5 mL of mineral oil was applied to each dog in the control group. All applications in both groups were made according to FRONTLINE Plus label directions: topically by parting the hair between the shoulder blades, and applying the entire formulation in one single spot, directly onto the skin. All animals in both groups were checked hourly for 4 hours post-administration to ensure there were no adverse reactions to the treatments.

Flea Counts

On Days 1, 7, 14, 21, and 28, in both studies, each animal was infested with 100 live Tampa 2014 isolate of *Ctenocephalides felis* fleas, which were placed on the lateral aspect of the body to avoid direct contact with the product application site. At 24 hours following each infestation, on Days 2, 8, 15, 22, and 29, animals were systematically combed with a fine-toothed flea comb, and all fleas were removed and counted.

STATISTICAL ANALYSIS

For both studies, the statistician was responsible for the calculation of efficacy. The statistical unit was the individual animal, and the primary assessment variable in this study was the decrease in the number of live fleas. The average percent reduction in flea counts for each group in each study was calculated using geometric means:

Efficacy (%) against fleas = 100 x (GMC–GMT)/GMC, where GMC = geometric mean number of live fleas in the control group, and GMT = geometric mean number of live fleas in dogs in the FRONTLINE Plus-treated group.

The transformed data of both studies were analyzed using t-tests for means with poolable variances or for means with unequal variances, as appropriate. Variances were compared using the maximum-F test, and Sattertwaite's Approximation was used to determine the degrees of freedom for the unequal variance tests. When one group had zero variance, variances were declared unequal by definition. The t-tests are equivalent to one-way ANOVA when variances are poolable and are more appropriate when variances are found to be unequal. For both studies, the FRONTLINE Plus-treated group was compared with the control group at each assessment.

All analyses and calculations for both studies were performed using SAS Version 9.3, and statistical significance was declared at a two-sided p-value of 0.05.

RESULTS

Adverse Reactions

For both studies, all animals remained in apparent good health throughout the studies, no product-related adverse events were noted, and no animals were removed from

either study. One of the control dogs in Study 2 (F0092) received a puncture wound to its tongue in an attempted fight with a neighboring dog. Actions were taken to prevent recurrence, and the dog was treated as per facility veterinarian recommendation.

Flea Efficacy

For Study 1, cats treated with FRONTLINE Plus had significantly ($p<0.01$) fewer live fleas than the controls 24 hours post-infestation on each assessment day (Table 2). Efficacy for FRONTLINE Plus for Cats was 90.3%, 99.6%, 98.8%, 93.3%, and 85.6% on Days 2, 8, 15, 22, and 29, respectively (Table 2).

For Study 2, dogs treated with FRONTLINE Plus also had significantly ($p<0.01$) fewer live fleas than the controls 24 hours post-infestation on each assessment day (Table 2). Efficacy for FRONTLINE Plus for Dogs was 99.6%, 100%, 100%, 100%, and 97.6% on Days 2, 8, 15, 22, and 29, respectively (Table 2).

DISCUSSION AND CONCLUSIONS

FRONTLINE Plus for Cats and FRONTLINE Plus for Dogs demonstrated efficacy against the Tampa 2014 isolate of *Ctenocephalides felis* fleas throughout the two 29-day studies. In the cat study, the only feature of note was the low numbers of fleas retained for 24 hours on the control cats, which reduced the apparent efficacy. When cats are acclimated and tolerant of fleas, 24-hour counts typically range from 85-100 fleas. In this study, juvenile purpose-bred cats were used, and prior to qualification for this study, they had not been exposed to fleas to determine their tolerance. Obviously these cats weren't very tolerant, and the average flea counts on the controls ranged from 42.6 to 54.1 fleas per assessment. It is well documented that cats can be very adept at grooming fleas,[1-3] and in this case, affected the measured efficacy.

These results were distinctly different than the observations in the original field study in Tampa, Florida.

While it is difficult to ascertain exactly why these performance differences occurred, it was later discovered that the owners in at least two of the homes in which the perceived inactivity against fleas took place had been repeatedly bathing their dogs during the study, and in one case, was using a dish washing detergent. Use of flea shampoos and in some cases dish washing detergents will remove oils from the skin surface. In fact, the Animal Poison Control Center recommends the use of detergent shampoos or dish washing detergent to remove topical ectoparasiticide products from pets accidentally exposed or over-exposed. Given the lipophilic nature of many topical flea products, including fipronil based FRONTLINE Plus, repeated bathing during the month after application would be expected to remove some quantity of active ingredient and thereby reduce efficacy.

The results of the two studies reported here demonstrate that perception of effectiveness of flea control products in real home conditions can be confounded by factors other than the intrinsic effectiveness of the product. In a previous study in Tampa, Florida, many of these confounding factors were identified, and included the presence of stray cats, untreated visitor pets, wildlife that can harbor *Ctenocephalides felis* fleas, and fluctuations in environmental conditions that can affect the flea development cycle.[4-7] Our ability to collect fleas with suspected resistance to FRONTLINE Plus for Dogs and evaluate the effectiveness of both FRONTLINE Plus for Dogs and FRONTLINE Plus for Cats under well controlled laboratory conditions allowed us to conclude that there was no evidence for resistance to these fleas in these households.

Conflict of Interest

These clinical studies were funded by Boehringer Ingelheim, of which Doug Carithers is an employee, and Jordan Crawford is a contractor. Michael Dryden is employed by Kansas State University, where the first present study was performed. BerTek, Inc.,

of which William Russell Everett is an employee, is an independent contract research facility contracted to conduct the second present study. Sheila Gross is an independent statistician.

All authors voluntarily publish this article and have no personal interest in these studies, other than publishing the scientific findings in which they have been involved via planning, initiating, monitoring, and conducting the investigations, as well as analyzing the results.

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