Prevention of Transmission of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* from Ticks to Dogs Using K9 Advantix and Frontline Plus Applied 25 Days Before Exposure to Infected Ticks

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ABSTRACT

A blinded, replicated, randomized controlled study was carried out in 24 Beagle dogs randomly allocated to one of 3 groups: K9 Advantix (imidacloprid/permethrin) (group 1; n = 8); Frontline Plus [fipronil/(S)-methoprene] (group 2; n = 8); and untreated controls (group 3; n = 8). Dogs in groups 1 and 2 were treated according to product label directions 25 days before infestation with adult *Ixodes scapularis* ticks (n = 80/dog) that were naturally infected with *Borrelia burgdorferi* and/or *Anaplasma phagocytophilum*. Efficacy of prevention was determined using a kinetic enzyme-linked immunosorbent assay (kELISA) assay for *B burgdorferi* and an indirect fluorescent antibody test (IFAT) for *A phagocytophilum*. Presence of *B burgdorferi* was confirmed in biopsies of sites of tick attachment to untreated dogs using a polymerase chain reaction (PCR) procedure. One dog was eliminated from group 2 before study initiation because of a low vaccinal titer to *B burgdorferi*. All remaining dogs were seronegative for *B burgdorferi* and *A phagocytophilum* prior to treatment and infestation. Seven of 8 untreated control dogs seroconverted to *B burgdorferi*. Seven

of 8 untreated control dogs seroconverted to *A phagocytophilum*. One of 7 dogs treated with Frontline Plus seroconverted to *B burgdorferi.* None of 8 dogs treated with K9 Advantix seroconverted to *B burgdorferi*. None of the dogs treated with K9 Advantix or Frontline Plus seroconverted to *A phagocytophilum.* Ten of 11 biopsy sites in untreated control dogs were positive for *B burgdorferi* DNA.

INTRODUCTION

Previous strategies for controlling vector-borne diseases involved either vaccination or the use of antimicrobial agents after exposure to potential vectors. Efficacy of antimicrobial therapy depends on susceptibility of the infectious agent to treatment, and the compliant use of effective antimicrobials. However, improper or indiscriminate use of these agents can aid in the development of antimicrobial resistance.[1]

Vaccination can be a safe and effective method of preventing disease, although it is not without inherent problems. The ability of organisms to evade host immune responses, as well as documented pathogen variability, can affect vaccine efficacy.[2] Also, vaccines are not yet available for several important vector-borne agents.

There is increasing interest in preventing vector-borne diseases by prohibiting arthropod vectors from attaching and feeding on animals and humans.[3–5] This strategy can employ agents that act as true repellents (agents that cause arthropods to move away from treated animals) or those that exert their antifeeding effects quickly enough to prevent successful transmission of vector-borne agents. Given the difficulties of vaccination and/or use of antimicrobial therapy, the most effective means of controlling vector-borne diseases remains the compliant use of long-acting repellents or acaricides.

It has been shown that available topical tick-control products can prevent transmission of *Borrelia burgdorferi* from *Ixodes scapularis* to dogs if they are applied prior to exposure to infected ticks.[6–10] We have previously demonstrated that a combination of imidacloprid and permethrin (K9 Advantix, Bayer Corporation, Monheim, Germany) prevents transmission of both *B burgdorferi* and *A phagocytophilum* when dogs were challenged with infected ticks 7 days after treatment.[6,7] In the present study we demonstrate that K9 Advantix prevented transmission of both *B burgdorferi* and *A phagocytophilum* when dogs were challenged with infected ticks 25 days after treatment.

MATERIALS AND METHODS

Source of Ticks

Adult male and female *I scapularis* ticks that were naturally infected with *B burgdorferi* and/or *A phagocytophilum* were collected from the wild by dragging infested habitats in Bridgeport, Connecticut (Fairfield County), USA.[6,7] The infection rate of *B burgdorferi* in the ticks was determined to be approximately 38% using an indirect fluorescent antibody test (IFAT). The presence of *A phagocytophilum* in ticks was not confirmed in this study. However, we demonstrated previously that ticks collected from the same site were infected with *A phagocytophilum*.[7]

Ticks were placed in glass vials and secured with air-permeable fabric covers. The ticks were shipped by overnight courier to the laboratory of the primary author (B.L.B.). The glass vials were placed in a secure humidified glass enclosure for several weeks prior to placement on dogs.

Dogs

Twenty-four adult Beagle dogs [12 male; 12 female; mean age, 20 months (range, 18–22 months)] were acquired from a commercial supplier (Harlan Sprague Dawley, Madison, WI). All dogs were housed individually in stainless steel cages and provided with a standard laboratory ration once daily and water ad libitum. During the period in which live ticks were present, dogs were housed in a biosafety level-2 facility. After

Table 1. Treatment Groups and Weights of Dogs

Sex	Group 1 K9 Advantix (imidacloprid/ permethrin)	Weight (lbs)	Group 2 Frontline Plus (fipronil/(S)- methoprene)	Weight (lbs)	Group 3 (Untreated)	Weight (lbs)
Male dogs	BSH2	22.4	ALF2*	21.8	SXJ2	20.3
	VSJ2	23.0	0LH2	22.9	XZH2	23.9
	UFH2	25.1	BVH2	24.9	0PH2	24.1
	URJ2	29.2	WUJ2	26.9	UKJ2	26.3
Female dogs	G002	16.8	P0Q2	16.3	0T02	18.6
	WB02	19.9	FXG2	19.2	TPK2	18.9
	OH02	20.6	IKG2	20.3	ETK2	20.6
	CEO2	23.0	ES02	24.7	0G02	23.2
Mean group weight		22.5		22.2 (n = 7)		22.0

*ALF2 was eliminated from the study due to a low vaccinal antibody titer. Pretreatment immunoblot confirmed vaccinal antigens.

all ticks had been removed from the dogs, the dogs were placed in conventional indoor/outdoor kennels for the remainder of the study. This study was reviewed and approved by the Auburn University Institutional Animal Care and Use Committee (AUIACUC No. 2003–0614).

Experimental Design

The 24 dogs were allocated to 3 treatment groups using a randomized block design. All dogs were separated by sex and ordered by increasing body weights. Eight replicates of 3 dogs were created by moving down the list from least weight to greatest weight. Within each replicate, dogs were assigned to 3 treatment groups by use of a random number table. Treatment groups were determined by random drawing. Body weights were obtained and allocations to groups were performed on days –4 and –3. Treatment groups were as follows (Table 1):

Group 1: Dogs were treated on day 0 with a combination of 8.8% weight/weight imidacloprid and 44.0% weight/weight permethrin (K9 Advantix).

Group 2: Dogs were treated on day 0 with a combination of 9.8% weight/weight fipronil and 8.8% weight/weight (S)-methoprene (Frontline Plus, Merial, Duluth, GA, USA).

Group 3: Dogs were untreated controls.

Treatment of Dogs

K9 Advantix was applied topically to the dogs based on weight bands indicated in the package insert (treatment group 1). Treatment consisted of either a single spot application at the mid-scapular skin surface or as multiple spot-on applications at the skin surface along the dorsal midline as instructed in the package insert.

Frontline Plus was also applied topically based on weight bands indicated in the package insert (treatment group 2). Treatment consisted of a single spot application at the mid-scapular skin surface as instructed in the package insert.

Untreated control dogs in treatment group 3 remained untreated.

Infestation with Ticks

Twenty-five days after treatment of dogs in groups 1 and 2 (on study day 25), each dog in all treatment groups was infested with 80 *I scapularis* ticks (40 female; 40 male) by placing ticks on the dogs' dorsal hair coat. Ticks were encouraged to enter the dogs' hair coats by gentle nudging. Five days after infestation (study day 30) all dogs were treated with K9 Advantix as described to eliminate any remaining ticks. This treatment limited poten-

tial exposure of personnel to ticks to a period of 5 days (120 hours). Based on previous research, 5 days allows ample time for infected ticks to transmit *B burgdorferi* to susceptible dogs.[11] Sites of tick attachment to untreated dogs were marked at that time for later biopsy. After all ticks had been removed, the dogs were relocated to conventional indoor-outdoor kennels for the remainder of the study. All dogs were observed twice daily by the investigator's staff, the animal care unit staff, or the project veterinarian. All personnel examining the dogs or performing laboratory procedures (see below) were blinded to treatment group allocations.

Blood Collection and Serology

A blood specimen (approximately 10 mL) was collected from each dog by cephalic venipuncture prior to treatment (study days –11 to –7) and on study days 42, 56, 70, 90, and 110. Serum was obtained from each specimen and frozen at –80˚C. Sera were shipped to the laboratories of one of the authors (M.B.P.) to conduct the serologic assay for *B burgdorferi*. The author was blinded to treatment group allocations during evaluation of the serum samples. Serum was assayed for *B burgdorferi*-specific antibodies by computerized kinetic enzyme linked immunosorbent assay (kELISA) as described previously.[10,12] kELISA titers were interpreted as follows: 0–99 = negative; 100–199 = equivocal; 200–299 = low-positive; 300–399 = mid-positive; 400–499 = high-positive; >499 = very high positive. Serum samples collected from all dogs before treatment and on study day 110 were tested in the laboratory of the primary author for antibodies to *A phagocytophilum* as previously described, except that sera were tested at a dilution of 1:50.[7] The individual performing the procedure (T.M.L.) was blinded as to what groups the samples were obtained from.

Western immunoblotting was employed to determine the cause of a positive pretreatment *B burgdorferi* antibody response in dog ALF2 (Table 1) and to confirm that the mid-positive *B burgdorferi* kELISA titer observed for dog P0Q2 after tick exposure was the result of *B burgdorferi* infection. Immunoblotting was performed as previously described and was considered positive if serum antibodies bound to at least 3 of the following bands: p39, p29–30, p28, p25–26, p22, p19.[10]

Polymerase Chain Reaction

Skin biopsies of tick attachment sites (n = 11 biopsy sites) were obtained from 3 untreated dogs (0T02 [n = 3 biopsy sites], TPK2 [n = 4 biopsy sites], 0G02 [n = 4 biopsy sites]) on day 110 to confirm the presence of *Borrelia*-specific DNA at previously identified sites of tick attachment. Biopsies were taken at sites located on the dorsal or ventrolateral neck areas. Biopsy specimens were immediately frozen (–70ºC) and shipped by overnight courier to author M.B.P. for conduct of the polymerase chain reaction (PCR) procedure. A duplex PCR procedure was performed using primers GI (Osp A) and JS1-JS2 (23S rRNA).[13,14]

An overview of the study design and procedures is presented in Figure 1.

RESULTS

Twenty-three of the 24 dogs tested negative for *B burgdorferi* antibodies prior to treatment and tick challenge. One dog (ALF2) had an equivocal result using the kELISA prior to tick challenge (titer = 174). Western blot subsequently confirmed that detected antibodies were specific for vaccinal antigens in this dog. Consequently, this dog was eliminated from the study. All 24 of the dogs tested negative for *A phagocytophilum* prior to treatment and tick challenge.

Seven of 8 untreated dogs developed mid- to very high kELISA antibody titers to *B burgdorferi* by study day 110, indicating successful attachment and feeding of ticks on these dogs (Figure 2). The titers ranged from mid-positive to very high positive (range, 312–526, mean kELISA titer = 407; Figure 2).
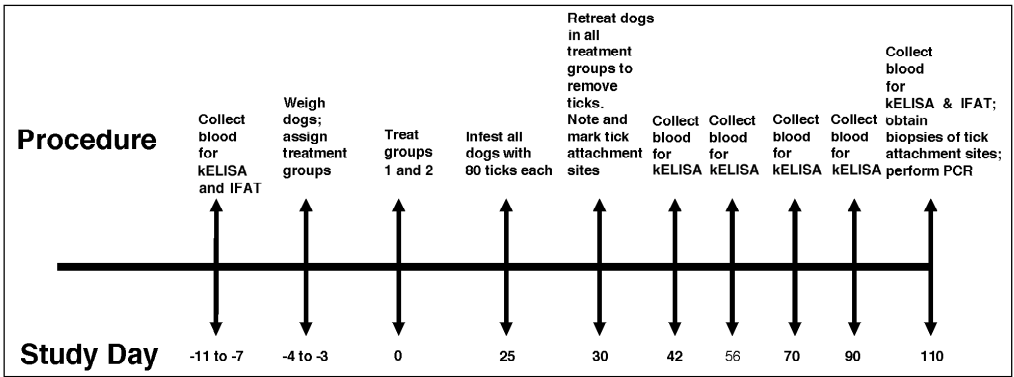
Figure 1. Overview of study design. Group 1 was treated with imidaclopromid/permethrin (K9 Advantix); group 2 was treated with fipronil/(S)-methoprene (Frontline Plus); group 3 was untreated controls. Each dog was infested with 40 female and 40 male ticks. Retreatment of all dogs limited the exposure of personnel to ticks to 5 days.
*kELISA indicates kinetic enzyme linked immunosorbent assay; IFAT, immunofluorescent antibody test; PCR, polymerase chain reaction.

None of the dogs treated with K9 Advantix on day 0 and infested with ticks on day 25 developed antibodies to *B burgdorferi* (Figure 3). Six of 7 dogs treated with Frontline Plus on day 0 and infested with ticks on day 25 remained negative for antibodies to *B burgdorferi* (Figure 4). Serum samples from dog P0Q2 demonstrated increasing kELISA titers from day 42 to day 110. Titer ranged from 5 to 343 (Figure 4). A Western blot performed on study day 110 indicated that antibodies were directed against *B burgdorferi* antigens that are expressed during active infection.

Seven of 8 control dogs were positive for antibodies to *A phagocytophilum* on study day 110. None of the dogs treated with either K9 Advantix or Frontline Plus developed antibodies to *A phagocytophilum*.

PCR performed on biopsy sites from the 3 controls resulted in successful amplification of *B burgdorferi*-specific amplicons from 10 of 11 biopsy sites, indicating that *B burgdorferi* DNA was present in most of the sites of tick attachment to control dogs.[13,14] It is interesting that 10 of 11 tick attachment sites were positive by PCR even though the infection rate in ticks was estimated to be about 38%.

Ticks were observed attached to untreated dogs between days 25 and 30, but not to dogs treated with either K9 Advantix or Frontline Plus. However, because a kELISA indicated infection in one dog treated with Frontline Plus, it is likely that one or more ticks attached to that dog but remained unnoticed. Ticks that were observed attached to control dogs were not counted.

DISCUSSION

These results indicate that *I scapularis* harbored and transmitted *B burgdorferi* and *A phagocytophilum* to untreated Beagle dogs. Results also demonstrated that administration of K9 Advantix 25 days before infestation prevented attachment and subsequent feeding of infected ticks. Similar results were obtained in our previous studies in which dogs were challenged with *B burgdorferi*- and *A phagocytophilum*-infected ticks 7 days after treatment with K9 Advantix.[6,7]

Treatment with Frontline Plus prevented transmission of *B burgdorferi* to 6 of 7 treated dogs. One dog that was treated with Frontline Plus developed antibody levels above the kELISA cutoff, indicating that infected ticks attached to and fed on that dog. A review of treatment data for that dog indicated that it did receive the correct dosage of Frontline Plus. These results are consistent with those of a previous study in which 2 of 8 dogs treated with Frontline
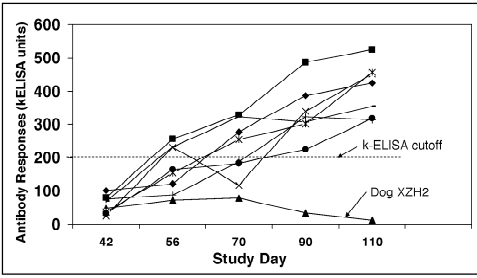
Figure 2. Serum antibody responses to *Borrelia burgdorferi* in untreated control dogs. Dogs were treated on day 0 and infested with *B burgdorferi*-infected ticks on day 25. Note that titers for 7 of 8 control dogs surpassed the cutoff (200 kinetic enzyme linked immunosorbent assay (kELISA) units), indicating that they were infected with *B burgdorferi*. One dog (XZH2) did not seroconvert.
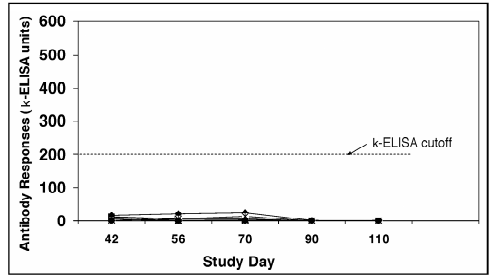

Figure 3. Serum antibody responses to *Borrelia burgdorferi* in imidacloprid/permethrin (K9 Advantix)-treated dogs. Dogs were treated on day 0 and infested with *B burgdorferi*-infected ticks on day 25. Note that titers for all dogs remained below the cutoff (200 kinetic enzyme linked immunosorbent assay (kELISA) units), showing no indication that they were infected with *B burgdorferi*.
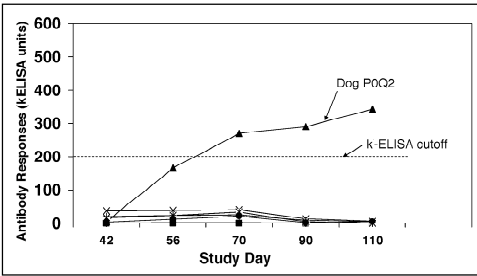

Figure 4. Serum antibody responses to *Borrelia burgdorferi* in fipronil/(S)-methoprene (Frontline Plus)-treated dogs. Dogs were treated on day 0 and infested with *B burgdorferi*-infected ticks on day 25. Note that titers for 6 of 7 dogs remained below the cutoff (200 kinetic enzyme linked immunosorbent assay (kELISA) units), showing no indication that they were infected with *B burgdorferi*. One dog (P0Q2) developed antibodies to *B burgdorferi*.

Plus and challenged with ticks 28 days after treatment seroconverted, indicating that ticks attached to the dogs and fed for a sufficient amount of time to transmit *B burgdorferi*.[10]

The present study demonstrated that K9 Advantix and Frontline Plus prevented transmission of *A phagocytophilum* when dogs are challenged 25 days after treatment with *I scapularis* ticks that are infected with this agent. Our previous study demonstrated that K9 Advantix can prevent transmission of *A phagocytophilum* from naturally infected ticks to dogs when dogs were challenged with *I scapularis* 7 days after treatment.[7] This study again confirmed, as we and others have, that ticks are often infected with multiple pathogens.[7,15] Other published studies have also demonstrated that fipronil (Frontline Spray) and amitraz (Preventic Tick Collar, Virbac Animal Health, Ft. Worth, TX, USA) can prevent transmission of *B burgdorferi* if they are applied to dogs prior to exposure to naturally infected ticks.[8,9]

The results of this and previous studies confirm that the use of available tick control products can aid greatly in preventing infection with certain tick-borne agents. This strategy would be preferable to preventive treatment of exposed dogs with antimicrobial agents and could help prevent infections in dogs for which there is no available vaccine.

We believe that a comprehensive vector-borne diseases control strategy should be employed, including compliant use of effective tick-control products, client education, use of appropriate tick avoidance behavior, and use of vaccines when available. Implementation of a comprehensive strategy can minimize the likelihood of exposure to and infection with tick-borne agents.

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