Efficacy of an amitraz-impregnated collar in preventing transmission of *Borrelia burgdorferi* by adult *Ixodes scapularis* to dogs

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Objective—To determine whether an amitraz-impregnated collar could prevent transmission of *Borrelia burgdorferi* by *Ixodes scapularis* to dogs.

Design—Laboratory trial.

Animals—8 specific-pathogen-free Beagles.

Procedure—On days –15 and –1, all dogs had negative ELISA results for serum antibodies against *B burgdorferi*. On day 0, 4 dogs were each fitted with an amitraz-impregnated (9%) collar, and 4 dogs served as untreated controls. On day 7, all dogs were infested with 100 *I scapularis* (approx 50 females and 50 males) with a known *B burgdorferi* infectivity rate of 39.4%. On days 21, 28, 35, 42, 56, 70, and 84, each dog was tested for serum antibodies against *B burgdorferi* via ELISA and a western blot technique. Additional ELISA were also performed for serum antibodies against antigenically similar organisms.

Results—By day 70, all control dogs had developed serum ELISA responses ranging from 328 to 510 kinetics-ELISA units (equivalent to end-point titers of approx 43,500 to 60,000), whereas treated dogs remained seronegative throughout the study. Western blot assays performed on all serum samples confirmed that antibodies detected in control dogs reflected responses to specific antigens of *B burgdorferi*, whereas treated dogs had no such antibodies. Additional serologic analyses confirmed that antibody responses observed in control dogs were not attributable to antigenically similar organisms.

Conclusions and Clinical Relevance—Amitraz-impregnated collars prevented transmission of *B burgdorferi* in 4 of 4 treated dogs and may be a useful management tool for prevention of borreliosis in dogs. (*J Am Vet Med Assoc* 2001;219:185–189)

Borreliosis (Lyme disease) is a multisystemic illness caused by the spirochete, *Borrelia burgdorferi*,[1,2] and is transmitted by ticks in the *Ixodes ricinus* complex. In the northeast United States, 50% of dogs in endemic areas have been reported to be infected with the organism,[3] and substantial morbidity and mortality caused by damage to musculoskeletal, cardiac, and renal systems have also been reported.[3-9] *Ixodes scapularis* is the vector of *B burgdorferi* in the northeast United States and is responsible for 90% of the national incidence[10] of disease caused by this organism.

Numerous acaracidal products have been recommended for control of ticks, but reports indicating that use of these products causes interruption of transmission of *B burgdorferi* from the vector tick to the mammalian host have not been published. It is generally accepted that prevention of tick attachment and feeding can prevent transmission of tick-borne disease, but no controlled studies have been performed to confirm this hypothesis. The purpose of the study reported here was to determine whether an amitraz-impregnated collar could prevent transmission of *B burgdorferi* by *I scapularis* to dogs.

Materials and Methods

Dogs—Eight specific-pathogen-free Beagles[a] (5 males weighing 9.6 to 14.4 kg [21.1 to 31.7 lb] and 3 females weighing 7.9 to 13.0 kg [17.4 to 28.6 lb]) that were ≥ 6 months of age were housed singly in standard cages during a 21-day conditioning period prior to tick infestation. Animal husbandry and housing complied with National Research Council standards. On day 7, when the dogs were infested with ticks, dogs were randomly allocated into 2 groups (treated and control) so that each group contained males and females. On days 7 through 23, dogs were housed individually in 4 × 8-ft marine aluminum chambers. On day 23, 1 week after ticks were removed from dogs, dogs were returned to standard caging and maintained until the completion of the experiment on day 84. The chambers used in this study were designed with high smooth solid walls to contain ticks. Additional barrier devices were used at the entrance to each chamber to prevent contamination of the premises. Study dogs were humanely euthanatized at the conclusion of the experiment.

Study personnel were required to follow standardized biohazard precautions when handling dogs and ticks. These measures were instituted to prevent contamination of the premises by the ticks and possible infection of personnel with *B burgdorferi*. Additionally, study personnel were evaluated serologically for antibodies against *B burgdorferi* prior to and upon completion of the study.

Treatment—On day 0, an amitraz-impregnated collar[b] was placed around the neck of each dog in the treatment group, according to manufacturer's instructions. Collars remained on treated dogs for the duration of the 84-day study. Control dogs were not fitted with amitraz collars.

Ticks—Adult *I scapularis* were collected in Fairfield County, Conn, in November 1999. Infection rate with *B burgdorferi* was 39.4%, as determined by indirect fluorescent antibody staining of midgut tissues of 160 male and female ticks with murine monoclonal antibody H5332 directed against *B burgdorferi* **outer surface protein A (OspA)**.[c]

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On day 7, each dog was infested with 100 ticks (approx 50 males and 50 females) by gently pouring the ticks along the dorsal midline at the thoracic region. Unlike previous studies[11-13] that used containment capsules for laboratory tick infestations of dogs, this method was used in an attempt to approximate heavy natural infestations and to ensure that dogs were exposed to sufficient numbers of infected ticks for transmission of the organism. On days 8 through 16, ticks that were found free in the containment cages were collected and stored at 4 C to ensure that the premises remained clear of *I scapularis*. On day 17, all remaining ticks were removed from dogs or collected from each containment chamber and stored in the same manner. After completion of the study, 5 randomly selected ticks, including females and males, recovered from each dog, cage, or both were analyzed for the presence of *B burgdorferi* via **polymerase chain reaction (PCR)** testing to confirm the presence of the organisms in the ticks.

Serologic testing—Serum was separated from blood samples collected from each dog on days –15, –1, 21, 28, 35, 42, 56, 70, and 84. An ELISA for detection of antibodies against *B burgdorferi* was performed on each serum sample.[d] Serum from blood samples collected from each dog on days –15, 56, and 84 were also submitted to another diagnostic laboratory[e] for detection of antibodies against *Babesia canis, Ehrlichia canis,* and *Rickettsia rickettsii* via ELISA. Laboratory personnel at both institutions were unaware of the sources of all serum samples.

Western immunoblot[d] analysis was performed on each serum sample to confirm that the antibody response was specific for natural *B burgdorferi* infection and not a result of vaccination antibody or antigenically similar organisms (eg, *B canis, E canis*, and *R rickettsii*). At each collection, approximately 7 ml of blood was collected by jugular venipuncture (on day 28 blood was collected from the cephalic vein of dog 1748-M), allowed to clot, and centrifuged for serum collection. Serum from each dog was collected in 2 aliquots of 1 ml each and frozen at –15 C. One of the serum aliquots collected on days –15 and –1 from each dog was evaluated by use of **kinetics ELISA (k-ELISA)** on days –8 and 0, respectively, to verify that experimental dogs had no prior exposure to *B burgdorferi*. The remaining sera from serial samplings of each dog were stored at –15 C until tested by use of k-ELISA at various intervals during the study and by western blot at the end of the study. Similarly, the serum aliquots used for detection of antibodies against *B canis, E canis*, and *R rickettsii* were stored at –15 C until tested.

Kinetics ELISA—The system used for detection of antibodies against *B burgdorferi* [d] was performed essentially as described.[11] Serum (dilution, 1:100) was added to duplicate wells in microtiter plates containing *B burgdorferi* antigen. The antigen was prepared from an early passage of *B burgdorferi,* characterized as the N-40 strain by use of PCR. This strain was cultured in Barbour-Stoenner-Kelly medium[14] supplemented with 10% heat-inactivated fetal calf serum and antimicrobials, including kanamycin (6 µg/ml), rifampicin (45 µg/ml), and nystatin (240 U/ml). The organisms were washed 3 times in **phosphate-buffered saline solution** (**PBSS**; 10 m*M*; pH 7.2), concentrated by centrifugation, and subjected to 5 cycles in a French press at 1.126 kg/cm^2. The soluble supernatant from the French press product was used as antigen. Bound antibody was detected by use of goat anti-dog antibody of heavy and light chain specificity[f] conjugated to horseradish peroxidase. Color development using the chromogen tetramethylbenzidine with H_2O_2 as a substrate was measured kinetically and expressed as the slope of the reaction rate between enzyme and substrate solution. The cutoff point between positive and negative results, based on validation of the assay for use with clinical canine samples, has been determined as 100 k-ELISA units. The relationship between k-ELISA units and endpoint titers has been described elsewhere.[15]

Western blot technique—Western blotting was performed as described[11] with slight modification. The antigen was prepared identically to that used for the k-ELISA and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide) at 200 V for 4 to 6 hours. Immunoreactive proteins were detected on nitrocellulose membranes by use of a miniblotter.[g] Antibody was detected from a 1:10 dilution of serum from dogs followed by a conjugate consisting of goat anti-dog IgG conjugated to horseradish peroxidase.[f] Results of western blots were considered positive if bound antibody was detected to at least 3 bands from among p39, p29-30, p25-26, and p19.

Preparation of tick samples for PCR assay—Randomly selected adult ticks, processed individually,[d] were homogenized in sterile microcentrifuge tubes by use of micropestle. Two hundred microliters of DNA extraction fluid[h] was added to each tube, and the mixture was incubated at 20 C for 1 hour. The tubes were maintained at 15 C overnight. Ticks were then further homogenized by repetitive pipetting with a micropipette. The homogenate was centrifuged for 10 minutes at 10,000 × g, and the supernatant was transferred to a new tube. The DNA was precipitated with 400 µl of 100% ethanol and harvested by use of centrifugation for 15 minutes at 13,000 × g. After washing in 70% ethanol, the DNA was dried under vacuum and resuspended in 100 µl of sterilized water.

Detection of *B burgdorferi* DNA by use of PCR assay—All tick samples were tested by duplex PCR assay. The PCR assay was performed in a reaction volume of 25 µl containing 0.625 units of *Taq* DNA polymerase, 1× PCR buffer, 1.5 m*M* $MgCl_2$, 200 µ*M* dATP, dCTP, dGTP, dTTP, 20 pmol each of primers, and 5 µl of DNA sample. Two DNA sequences were targeted in this study: the OspA gene[16] and the 23S rRNA gene.[17] The PCR assay protocol consisted of an initial denaturation at 94 C for 4 minutes, and 35 cycles with denaturation at 94 C for 30 seconds, annealing at 55 C for 30 seconds, and extension at 72 C for 1 minute. A final extension step was performed at 72 C for 15 minutes. The PCR mixture was analyzed on ethidium bromide-stained 2% agarose gel in Tris-borate-EDTA (pH 8.2).

Statistical analyses—The number of dogs that developed serum antibodies against *B burgdorferi* was compared between groups by use of the Fisher exact test. Differences were considered significant at $P < 0.05$.

Results

Ticks—Prestudy infection rate with *B burgdorferi* was 39.4% of male and female ticks. A PCR analysis of 40 ticks (5 randomly selected ticks from each dog, representing 5% of the total tick population used in the study) was also conducted after the study to confirm the presence of *B burgdorferi* in ticks recovered from the dogs, their cages, or both. Of the randomly selected 5-tick cohorts from each dog or cage, at least 1 *Borrelia*-positive tick was found in 3 of 4 dogs from each group. Although no infected ticks were discovered in the 5-tick cohort recovered from 1 control dog, this dog developed an anti-*Borrelia* antibody response by day 42 of the study and remained seropositive throughout the remainder of the study. Negative results of PCR analysis were also obtained with the tick cohort recovered from 1 treated dog. Analysis of 5 ticks from 2 treated dogs each revealed 2 infected ticks, whereas analysis of 5

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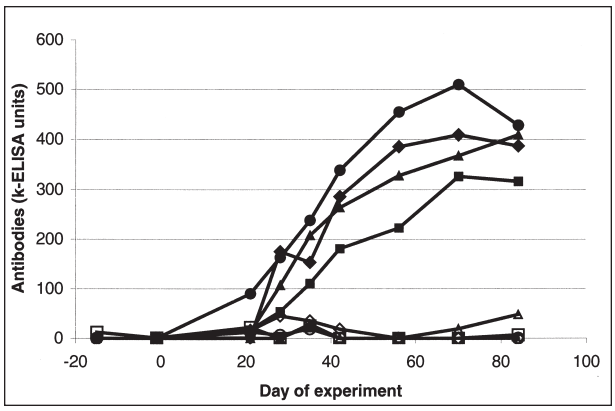

Figure 1—Serum antibodies against *Borrelia burgdorferi* in 4 dogs treated with an amitraz-impregnated collar (open symbols) and 4 untreated control dogs (solid symbols). Dogs were infested with *B burgdorferi*-infected ticks on day 7.

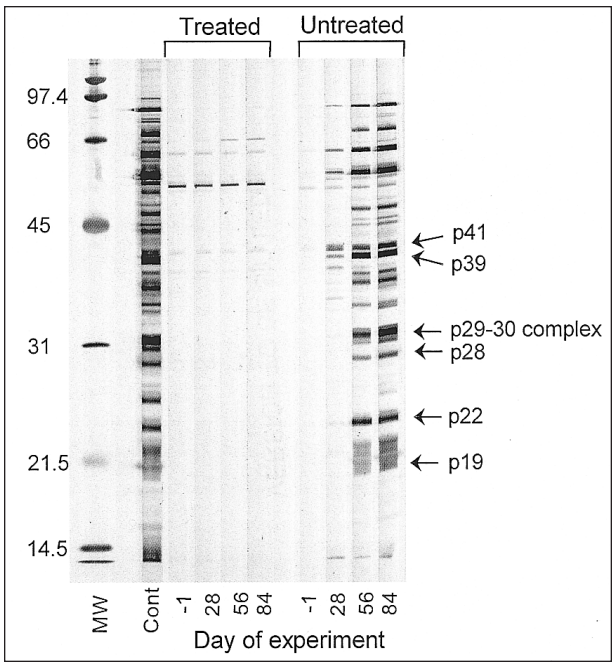

Figure 2—Results of western immunoblot analysis of serum from an untreated control dog and a dog treated by use of an amitraz-impregnated collar; both dogs were experimentally infested on day 7 with ticks that were infected with *B burgdorferi*. Molecular weight markers are indicated on the left side of the figure. Arrows and values along the right side of the figure indicate bands corresponding to *B burgdorferi* proteins. MW = Molecular weight marker lane. Cont = Control serum from a dog infected with *B burgdorferi*.

ticks from another treated dog revealed 1 infected tick. In the control group, analysis of 5 ticks from 2 dogs each revealed infection in 1 tick, whereas analysis of 5 ticks from another dog revealed 3 infected ticks.

Serologic results—Throughout the study, no treated dogs developed antibodies against *B burgdorferi* (**Fig 1**). By day 28, 3 of the 4 control dogs had seroconverted. All control dogs developed strong antibody responses during the study; difference between groups was significant ($P = 0.029$). All dogs (control and treated) had negative results of each of 3 serial analyses for antibodies against *B canis*, *E canis*, and *R rickettsii*.

Western blots—Treated dogs remained seronegative throughout the study. For each control dog, bands typical of tick-borne *B burgdorferi* infection were detected in serum samples obtained from blood taken on day 28 and subsequently. By day 56, the immune response was well-developed with distinct bands at molecular weights consistent with *B burgdorferi* antigens p19, p22, p28, p30 complex, p39, and others (**Fig 2**).

Discussion

The finding that 39.4% of the ticks collected for use in this study were infected with *B burgdorferi* was not atypical for *I scapularis* in the area of New England where the ticks were collected in which tick infestation rates may range as high as 50 to 80%.[11,12] *Ixodes scapularis* become infected with *B burgdorferi* while feeding as larvae or nymphs, and infection persists across developmental stages. Additionally, only adults express sexual dimorphism. Therefore, it is reasonable to expect that larvae and nymphs destined to become either male or female adults would be infected at the same rate. Placing 50 female ticks on each dog provided potential exposure to 20 infected female ticks per dog. As few as 1 to 3 infected female ticks may transmit the infection to dogs experimentally and lead to seroconversion.[18] In our study, each dog was exposed to a sufficient number of infected ticks to permit exposure and transmission of the organism. The fact that PCR analysis of tick cohorts recovered from each dog or cage failed to reveal infected ticks in 2 of the 8 study dogs does not rule out the likelihood that all dogs were exposed to infected ticks. Analysis of 5 ticks from 1 control dog did not reveal evidence of infection with *B burgdorferi*, although this dog developed a substantial antibody response by day 42 of the study and remained seropositive for the duration of the study. Analysis of 5 ticks from 1 treated dog also did not reveal evidence of infection with *B burgdorferi*, but the likelihood that this dog was not exposed to at least 1 infected tick was extremely remote, considering that the dog was theoretically exposed to approximately 20 infected female ticks and that only 5 of 100 ticks that were placed on the dog were tested. It should be noted that these ticks were in poor condition when tested by use of PCR, because they had been stored for several months at refrigeration temperatures; bacterial and fungal growth were not preempted under these conditions. We cannot rule out the possibility that nuclease activity within these ticks could have accounted for lack of detection of *B burgdorferi* DNA in the PCR assay.

Serologic evidence of infection with *B burgdorferi* was detected in all control dogs and absent in the treated dogs. The pattern of antibody response observed in control dogs was similar to that reported in dogs experimentally exposed to infected adult ticks[11] and dogs naturally exposed to infected ticks under field conditions.[9] Western blot analysis of each sample confirmed that antibodies were directed against naturally transmitted *B burgdorferi*. Negative results of serologic analysis for antibodies against *E canis*, *B canis*, and *R rickettsii* corroborated the specificity of the antibody response against *B burgdorferi* without the potential of

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spurious results arising from cross-reactivity of these antigenically similar organisms.

Borreliosis in dogs is an important clinical entity,[3-9] especially in the northeast region of the United States where *I scapularis* is the vector for the causative organism, *B burgdorferi*. Many pesticidal and acaracidal products are effective for killing the ticks that transmit borreliosis, but prior to this study, to the authors' knowledge, an agent that prevents transmission of the organism from vector to dog had not been reported. Because transmission of *B burgdorferi* may occur in as few as 24 hours after tick attachment and feeding,[19] rapid prevention of tick attachment and feeding is necessary to prevent transmission of the disease. An acaracidal agent that prevents ticks from biting may, therefore, be expected to prevent transmission of disease-causing organisms from these ticks. Acaracidal products that permit some period of attachment and feeding prior to killing ticks may not act quickly enough to prevent transmission of *B burgdorferi* to dogs.

Amitraz prevents tick bites by affecting the CNS of the tick and paralyzing its mouth parts.[20-23] Amitraz also has tick-repellent effects.[23] Amitraz-containing collars have rapid and effective acaracidal efficacy and anti-attachment and anti-feeding efficacy against several tick species.[24-25,i-m] The collars also have an important effect in reducing numbers of live feeding ticks, hatchability of eggs, and tick larval viability.[24] In this experiment, however, the acaracidal activity and anti-attachment and anti-feeding effects of the collars (as determined by counting the numbers of viable feeding ticks observed daily on each dog) were not recorded. This study was designed to determine whether the well-established acaracidal and feeding prevention and interruption efficacy of amitraz-impregnated collars would result in the prevention of disease transmission. Therefore, the primary focus of this study was the development, if any, of a specific *B burgdorferi* antibody response in dogs wearing amitraz-impregnated collars and those without collars after exposure to infected *I scapularis* ticks.

In addition to borreliosis, other tick-borne diseases of dogs are responsible for substantial morbidity and mortality. The efficacy of amitraz-containing collars in prevention of attachment and feeding by *Rhipicephalus sanguineous* and *Dermacentor variabilis*, the vectors of *B canis, E canis,* and *R rickettsii*, has been reported.[24-25,i-m] On the basis of this ability, we hypothesize that the transmission of the organisms carried by these tick species may also be prevented.

[a]Study performed at Stillmeadow Inc, Sugar Land, Tex.
[b]Preventic, Virbac AH Inc, Fort Worth, Tex.
[c]Indirect fluorescent antibody analysis performed at The Connecticut Agricultural Experiment Station, New Haven, Conn.
[d]kELISA, western blot analysis, and polymerase chain reaction analysis performed at Cornell Diagnostic Laboratory, College of Veterinary Medicine, Cornell University, Ithaca, NY.
[e]Texas Veterinary Medical Diagnostic Laboratory, College Station, Tex.
[f]ICN Biomedical Research Products, Costa Mesa, Calif.
[g]Immunetics, Cambridge, Mass.
[h]DNAzol, Life Technologies, Md.
[i]Elfassy O, Goodman FW, Leeper-Carter L. Elimination speed, efficacy and anti-fixing, anti-feeding efficacy of 9% amitraz collars and topical selamectin in experimental *Dermacentor variabilis* infestations in dogs (abstr), in *Proceedings*. 45th Annu Meet Vet Parasitol 2000;74.
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[k]Estrada-Pena A, Ascher F. Efficacy of two ixodicide collars against natural infestation with the brown dog tick (abstr), in *Proceedings*. 42nd Annu Meet Vet Parasitol 1997;73.
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[m]Estrada-Pena A, Ascher F. Killing effect of two ixodicide collars against the brown dog tick (abstr), in *Proceedings*. 42nd Annu Meet Vet Parasitol 1997;74.

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