

# Use of tick surveys and serosurveys to evaluate pet dogs as a sentinel species for emerging Lyme disease

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**Objective**—To evaluate dogs as a sentinel species for emergence of Lyme disease in a region undergoing invasion by *Ixodes scapularis*.

**Sample Population**—353 serum samples and 78 ticks obtained from dogs brought to 18 veterinary clinics located in the lower peninsula of Michigan from July 15, 2005, through August 15, 2005.

**Procedures**—Serum samples were evaluated for specific antibodies against *Borrelia burgdorferi* by use of 3 serologic assays. Ticks from dogs were subjected to PCR assays for detection of pathogens.

**Results**—Of 353 serum samples from dogs in 18 counties in 2005, only 2 (0.6%) contained western blot analysis–confirmed antibodies against *B burgdorferi*. Ten of 13 dogs with *I scapularis* were from clinics within or immediately adjacent to the known tick invasion zone. Six of 18 *I scapularis* and 12 of 60 noncompetent vector ticks were infected with *B burgdorferi*. No ticks were infected with *Anaplasma phagocytophilum*, and 3 were infected with *Babesia* spp.

**Conclusions and Clinical Relevance**—Serosurvey in dogs was found to be ineffective in tracking early invasion dynamics of *I scapularis* in this area. Tick chemoprophylaxis likely reduces serosurvey sensitivity in dogs. Ticks infected with *B burgdorferi* were more common and widely dispersed than seropositive dogs. In areas of low tick density, use of dogs as a source of ticks is preferable to serosurvey for surveillance of emerging Lyme disease.

**Impact for Human Medicine**—By retaining ticks from dogs for identification and pathogen testing, veterinarians can play an important role in early detection in areas with increasing risk of Lyme disease. (*Am J Vet Res* 2009;70:49–56)

*Ixodes scapularis*, the black-legged tick, is abundant in parts of the northeastern and upper midwestern United States and is expanding geographically into previously uninfested areas. Invasion of *I scapularis* results in increased risk of disease to dogs and humans from pathogens vectored by this tick, including *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia* spp, agents of Lyme disease, granulocytic anaplasmosis,

Received January 12, 2008.

Accepted April 14, 2008.

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Supported by Michigan State University's College of Veterinary Medicine Companion Animal Fund and the Centers for Disease Control and Prevention (assistantship to SAH).

The authors thank Christina C. Barber, Katherine E. Boatman, Steven R. Bolin, Nicole L. Grosjean, Alice J. Murphy, Michelle E. Rosen, and Pamela L. Roy for technical assistance.

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## ABBREVIATIONS

C6	Sixth invariant region
IFA	Indirect fluorescent antibody
VlsE	Variable major protein-like sequence-expressed
WB	Western blot

and babesiosis, respectively. Lyme disease is the most prevalent vectorborne disease of humans in the United States and Europe<sup>1</sup> and a common cause of acute arthritis and arthralgia in dogs.<sup>2</sup>

One area of recent *I scapularis* invasion is in the lower peninsula of Michigan<sup>3,a</sup> where *I scapularis* presence and incidence of *B burgdorferi* infection in humans, dogs, and wildlife have been increasing since the early 2000s. Putative sources of invasion are recently established populations of *I scapularis* immediately to the south, in northern Indiana<sup>4</sup>; initial surveys of *B burgdorferi*-infected ticks and wildlife in the lower peninsula of Michigan revealed a gradient of *I scapularis* density with the highest density in the southwestern corner of the state. The infested zone is of considerable public health importance because the Lake Michigan coast is a popular tourist site and harbors many state parks and recreation areas. Hence, it would be useful to quantify

and delineate geographically the current disease risks so as to update public health recommendations in the lower peninsula.

Assessing risk in zones of emerging Lyme disease, as opposed to in endemic zones, is challenging. Lack of public and medical awareness often hinders accurate diagnosis. Prevalence of Lyme disease in humans is initially low, so the positive predictive value of any diagnostic test is similarly low. Field surveys of ticks and mice, deer, and other vertebrates can reveal trends in risk for Lyme disease in these areas, but such surveys are laborious and costly. Consequently, numerous researchers have proposed that companion animal dogs are a useful sentinel species that can assist in assessing risk in areas endemic for Lyme disease.<sup>5-8</sup> Companion dogs typically have a close association with their human owners, but are more active in habitats where they can come into close contact with infected ticks. Eng et al<sup>9</sup> estimated that pet dogs were on average 6 times as likely to be seropositive for *B burgdorferi*, compared with their owners, and dog ownership is a significant risk factor for tickborne disease in humans.<sup>10</sup> Most serosurveys in dogs, however, have been conducted in areas endemic for *I scapularis* where human risk for Lyme disease is already appreciated. Exceptions include Rand et al,<sup>11,12</sup> who found in Maine that seropositive dogs identified areas of human risk in advance of human cases of Lyme disease, and Duncan et al,<sup>13</sup> who deemed dogs an appropriate sentinel species in North Carolina where seroprevalence in dogs was < 1% and transmission to humans in that state was rare. Nevertheless, the usefulness of serosurveys in dogs in areas where risk is low but emerging remains unclear.

Diagnosis of Lyme disease in dogs is typically based on serologic test results alone and, as in humans, has been problematic since the emergence of the disease because of cross-reactivity to spirochetes including *Treponema denticola* and *Leptospira interrogans* and lack of standardization of laboratory protocols. Recommended practice has therefore been a 2-step diagnostic algorithm for determining exposure of dogs to *B burgdorferi*, whereby IFA tests or ELISAs are used to screen serum samples and WB analysis<sup>14,15</sup> is used to confirm results in serum samples with suspect-positive results on the basis of antibody titer. Recently, a *B burgdorferi* peptide antigen, the C6 of the VlsE, has been used effectively in a commercially available ELISA kit that provides sensitivity and specificity equaling that of WB analysis.<sup>16</sup>

In 1992 to 1993, prior to the current *I scapularis* invasion of southwestern Michigan, a serosurvey in dogs was undertaken in 6 Michigan counties including Menominee County in the upper peninsula, the only Michigan county endemic for Lyme disease at that time.<sup>17</sup> On the basis of ELISA and IFA test results, 25 of 299 (8.4%) dogs from Menominee County were seropositive for *B burgdorferi* by 1 or both screening methods, whereas only 1 of 919 (0.1%) dogs from the lower peninsula had positive IFA test results (all had negative ELISA results). These serosurvey findings thus establish the almost complete absence of exposure of dogs to *B burgdorferi* in the lower peninsula of Michigan in the early 1990s.

The purposes of the study reported here were the following: to determine the extent of exposure of dogs

to *B burgdorferi* in an area undergoing active invasion by *I scapularis*; to determine the extent of infestation of dogs by *I scapularis* and other ticks; to determine the prevalence of *B burgdorferi*, *A phagocytophilum*, and *Babesia* spp in ticks collected from dogs; and to assess patterns of seroprevalence, vaccination, *I scapularis* presence, and tick infection in relation to the zone of *I scapularis* invasion in southwestern Michigan. It was hypothesized that dogs that were seropositive for *B burgdorferi* would be found throughout the known geographic distribution of *I scapularis*, and similarly, that *I scapularis*-borne pathogens would be restricted to that same geographic range. Specifically, all these measures were predicted to be highest at clinics in the southwestern corner of the state (the putative site of earliest invading ticks) and would decline with increasing distance from this focus.

## Materials and Methods

**Sample acquisition**—In spring 2005, 18 veterinary clinic owners consented to participate in a tick survey and serosurvey of dogs; these clinics were selected along 3 transects that originated in the recently invaded southwestern corner of Michigan and extended radially to the north, northeast, and east. An initial 133 clinics operating within the area of interest were identified from online directories and geocoded.<sup>b</sup> Six clinics distributed along each transect were then randomly selected and contacted by phone to solicit participation in the study. For analysis, the clinics were grouped into 3 zones on the basis of their proximity to the recently established *I scapularis* populations. Zone 1 included all clinics in counties where *I scapularis* was known to be established (n = 6; establishment defined as documented presence of all 3 life stages of *I scapularis* on drag cloth, wildlife, or humans). Zone 2 included all clinics in counties that bordered zone 1 (4). Zone 3 included all clinics in counties outside of zones 1 and 2 (8; Figure 1).

Veterinarians were asked to retain 30 serum samples acquired from dogs in the course of routine blood sample collection from July 15, 2005, through August 15, 2005. This number of serum samples was requested on the basis of the number needed to have a > 95% chance of detecting  $\geq 1$  dog seropositive for *B burgdorferi*, assuming a response of 50% by veterinarians and an invasion area prevalence of 1%. In recently invaded southwestern Michigan, *I scapularis* adult and nymphal host-seeking behavior peaks in April and June, respectively,<sup>a</sup> so the collection period chosen was the time of year when dogs would have likely seroconverted (ie, > 4 to 6 weeks after exposure<sup>2</sup>) from infection caused by exposure to nymphs or adults. Veterinarians were instructed to remove serum from blood samples and to store serum in cryogenic vials at  $-20^{\circ}\text{C}$  until pickup time.

Veterinarians were also asked to collect all ticks found on dogs during the same July through August period. Dogs that had ticks were not necessarily the same dogs from which blood was collected. All ticks from each dog were stored in vials of 70% ethanol. For all dogs from which blood or ticks were collected, the veterinarian completed a short questionnaire to indicate

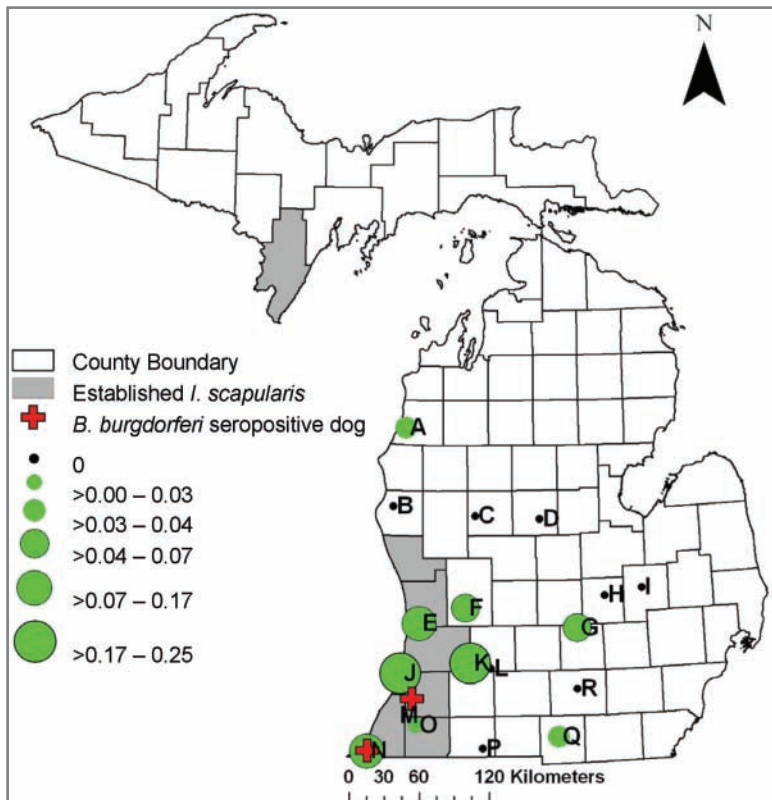


Figure 1—Locations of the 18 veterinary clinics (A through R) that participated in the 2005 tick survey and serosurvey in dogs of the lower peninsula of Michigan. Shading indicates counties in which *I. scapularis* has recently invaded (lower peninsula) or is endemic (upper peninsula); shaded counties are those within which all 3 life stages of *I. scapularis* have been documented.<sup>a</sup> Circles indicate the estimated proportion of dogs vaccinated against *B. burgdorferi* infection at each clinic. Locations of the 2 dogs seropositive for *B. burgdorferi* resulting from natural exposure are shown.

dog breed, sex, age, zip code, travel history, and Lyme disease vaccination and diagnosis status. After pickup from clinics, serum samples were stored at  $-80^{\circ}\text{C}$  before processing at the Michigan State University Medical Entomology Laboratory.

As a follow-up, in 2008, we conducted a short phone survey of veterinarians and licensed veterinary technicians at 9 of the 18 participating veterinary clinics (3, 2, and 4 clinics in zones 1, 2, and 3, respectively) to assess the prevalence of use of anti-tick and anti-tickborne pathogen measures at the clinics. Veterinarians specifically were asked to recall the amount of use in summer 2005, when our sample collection was underway.

**IFA testing**—Serum samples were thawed and centrifuged for 10 minutes at  $15,700 \times g$ , then serially diluted with sterile PBS solution to 1:320 and 1:640. Both dilutions were screened for antibody presence by use of 12-well IFA testing slides fixed with whole cell *B. burgdorferi* organisms (low passage mixture of isolates B31 and 297).<sup>c</sup> Slides were warmed to room temperature (approx  $21^{\circ}\text{C}$ ), and each well was loaded with  $20 \mu\text{L}$  of test serum or control serum. One positive and 1 negative control sample<sup>d</sup> were included on each slide. Slides were incubated in a humidifying chamber for 30 minutes at  $37^{\circ}\text{C}$ . Samples were washed by rinsing with PBS solution 3 times after which excess PBS solution was tapped off onto a paper towel. While wells were

still moist,  $20 \mu\text{L}$  of stained fluorescein isothiocyanate-conjugated anti-dog IgG was added to the wells. This conjugate was prepared by use of sterile 0.1% Evans blue solution in PBS solution to make a 1:100 dilution fluorescein isothiocyanate-conjugated anti-dog IgG.<sup>e</sup> The conjugated secondary antibody was incubated on the slide for 30 minutes at  $37^{\circ}\text{C}$  in the humidifying chamber and washed as described.

A coverslip was placed onto the moist slide, which was then viewed under UV illumination at  $400\times$  magnification (ie,  $10\times$  ocular piece and  $40\times$  objective) by use of a fluorescent microscope.<sup>f</sup> All slides were read by 1 observer (SAH), who was blinded to the identity of the slides. Approximately 15 seconds were spent scanning each of 10 fields within each well, with both the intensity and abundance of fluorescing spirochetes recorded and compared with that of the negative control sample. Wells with well resolved, and consistently bright yellow-green spirochetes were considered positive for antibodies against *B. burgdorferi* at the tested dilution. Samples with no fluorescing spirochetes at a dilution of 1:640 were considered negative for antibodies against *B. burgdorferi*. All serum samples positive for antibodies against *B. burgdorferi* at a dilution of 1:640 were tested in a series of 2-fold dilutions until an endpoint antibody titer was found. Serum samples positive for antibodies against *B. burgdorferi* at  $\geq 1:640$  dilutions were considered suspect and were subjected to confirmatory testing by use of WB analysis.

**WB analysis**—Western blot analysis was used as a confirmatory test for all serum samples with suspect-positive results on IFA testing, all serum samples from dogs that were reported as having been vaccinated against *B. burgdorferi* infection regardless of endpoint antibody titer, and randomly selected serum samples with negative results on IFA testing. Western blots were performed by use of a commercially available kit<sup>g</sup>; blots were prepared by the manufacturer and contained 18 separated antigens of *B. burgdorferi* strain B31. Interpretive criteria were as follows: 2 or more bands in the p30 to p14 region without both p31 (outer surface protein A) and p34 (outer surface protein B) indicated a positive result for *B. burgdorferi* caused by natural exposure; 2 or more bands in the p30 to p14 region plus both p31 and p34 were indicative of a positive result caused by vaccination; and  $< 2$  bands in the p30 to p14 region were considered a negative result for specific antibodies against *B. burgdorferi*.

Overall seroprevalence was computed as follows:

$$Sp = \frac{\text{Number of natural exposure positives on WBA}}{(\text{total number of IFA tested samples} - \text{number of vaccine positives on WBA})}$$

where Sp stands for seroprevalence and WBA stands for WB analysis.

**C6-peptide ELISA**—In addition to the 2-step diagnostic procedure already outlined, selected serum samples were subjected to a newly available commercial ELISA-based kit<sup>h</sup> to evaluate its usefulness in an area of invading *I scapularis* and its use with the more laborious 2-step process. The kit uses a synthetic peptide (C6) with sequence homology to 1 of 6 invariable regions within the variable domain of VlsE, a surface lipoprotein of *B burgdorferi*. Antibodies to VlsE are found in field-infected dogs but not in dogs vaccinated against *B burgdorferi*.<sup>16</sup> In addition to assaying for the presence of antibodies against *B burgdorferi*, this test simultaneously assays for antibodies against *Ehrlichia canis* and an antigen of *Dirofilaria immitis* adult worms. Manufacturer's instructions were followed to assay all serum samples with IFA endpoint titers  $\geq 1:2,560$  (n = 22) plus 7 randomly selected serum samples with lower endpoint antibody titers.

**Tick processing and PCR assay**—All ticks removed from dogs were identified to life stage and species by use of standard taxonomic keys.<sup>18,19</sup> Total DNA was extracted from each tick by use of an animal tissue protocol kit.<sup>1</sup> Known infected nymphs from a CDC laboratory colony served as positive control samples, and no-template wells served as negative control samples for the extraction. Ticks were prepared for extraction by slicing through the tick exoskeleton and midgut with a scalpel in a dry microcentrifuge tube. Lysis buffers were added and the solution incubated overnight. In the case that an engorged tick clogged the spin column, a sterile pipette tip was used to dislodge the clog. The DNA was eluted in a single 100- $\mu$ L elution. Five microliters of DNA was used in each of 3 separate PCR assays of 50- $\mu$ L volumes to assay for *B burgdorferi*, *A phagocytophilum*, and *Babesia* spp. Gel electrophoresis was performed by use of 10  $\mu$ L of PCR product in a precast 4% agarose gel.<sup>l</sup> The *rrs-rrlA* gene (16S to 23S rRNA) intergenic spacer of *B burgdorferi* was amplified in a nested assay producing a 978-bp fragment.<sup>20</sup> The DNA extracted from cultured spirochetes served as a positive control sample (spirochetes were cultured from field-collected adult ticks from a different study in a complete media<sup>k</sup> and incubated at 37°C). The *p44* gene of *A phagocytophilum*<sup>21</sup> was amplified by use of a PCR program, as described by Steiner et al,<sup>22</sup> to produce a 334-bp fragment. Positive control DNA was provided.<sup>1</sup> A *Babesia* genus-specific PCR assay was performed by use of primers for the 18S rRNA gene to produce a fragment of variable size, including a 408-bp fragment for *Babesia microti* or a 437-bp fragment for *Babesia odocoilei*.<sup>23</sup> A commercially available *B microti* organism<sup>m</sup> was extracted as described and used as a positive control sample. Ticks were removed from dogs at different stages of engorgement, so their extracted DNA varied in concentration. As results of PCR assay optimization trials on serially diluted templates revealed that overly concentrated template (from engorged ticks) inhibited detection of pathogen (data not shown), all DNA samples were tested at full strength and at a 1:10 dilution in the non-nested PCR assays. In all PCR assays, negative control samples consisted of wells in which all reagents except a DNA template were added.

**Sequence analysis**—The DNA sequence analysis of PCR amplicons of *B burgdorferi* (to assess strain variation) and *Babesia* spp (to identify to the species) was undertaken. The 40  $\mu$ L of amplicon that remained after electrophoresis was purified<sup>n</sup> and used as a template for DNA sequencing at the Research Technology Support Facility at Michigan State University on a genetic analyzer.<sup>o</sup> Sequences were compared with those published in the National Center for Biotechnology Information sequence database by use of the nucleotide-nucleotide basic local alignment search tool to identify the pathogen strains with the greatest sequence homology.

**Spatial analysis**—Generalized linear models were used to assess the relationship between tick-pathogen measures (ie, seroprevalence, vaccination rate, background reactivity on IFA testing, presence of *I scapularis* on dogs, and *B burgdorferi* infection of ticks removed from dogs) and the geographic zone of each clinic. All analyses were performed by use of a standard statistical programming language.<sup>24</sup>

**Early invasion survey**—Data from an initial serosurvey that was undertaken in 2001 to 2002 are included in the study reported here. The initial serosurvey was conducted with similar methods to collect serum samples from dogs of 17 clinics dispersed throughout zone 1. This survey was initiated in response to the first direct field evidence, from drag and wildlife sample collection, of invading *I scapularis* in southwestern Michigan.<sup>a</sup> Briefly, veterinarians were asked to store surplus blood samples and place ticks from dogs in 1 common vial from June 2001 through December 2002. Serum samples were processed as described, except that serum samples with suspect-positive results were defined as having an IFA cutoff titer of  $\geq 1:320$  in 2001 and  $\geq 1:640$  in 2002 on slides read by a single observer (ESF). A subset of serum samples with suspect-positive results was subjected to WB analysis. Serologic testing and tick collection results are presented as a means for comparison with the 2005 survey data reported here.

## Results

A total of 353 serum samples were obtained from dogs of veterinary clinics enrolled in this study (mean, 20 serum samples/clinic; range, 3 to 30 serum samples/clinic), of which 174 (49.3%) were from female dogs and 179 (50.7%) were from male dogs. All American Kennel Club breed groups were represented as follows: 38% sporting, 14% herding (ie, German Shepherd Dog-mixed breeds), 12% terrier, 10% working, 8% mixed (ie, mixed breed with no breed specification), 7% toy, 6% hound, 4% nonsporting, and 1% unknown. Of 353 dogs, 21 (5.9%) were reported as vaccinated against *B burgdorferi* infection, 113 (32.1%) were reported as not vaccinated, and 219 (62%) had unknown vaccination status. None were reported to have previously been diagnosed with Lyme disease or to have traveled to any other Lyme disease endemic area, although 232 (66%) had an unknown travel history or the owner did not answer this question. In our 2008 phone survey of 9 of the participating clinics, veterinary health providers estimated that 20% to 65% (mean, 50%) of the pet dogs

receiving service at their clinic were being actively protected against ticks during the summer months, with topical agents being the most common form of prophylaxis. Additionally, clinicians estimated that 5% to 25% (mean, 12%) of dogs were vaccinated against the Lyme disease pathogen.

Results of IFA testing of the 353 serum samples revealed that 79 (22.4%) had endpoint antibody titers  $\geq 1:640$  and thus were considered suspect-positive for *B burgdorferi* exposure (Figure 2). The maximum endpoint antibody titer detected was 1:81,920.

Western blot analysis was performed on 105 serum samples (79 serum samples with suspect-positive results on IFA testing [ie, antibody titer  $> 1:640$ ], 3 serum samples from dogs reported as vaccinated against *B burgdorferi* infection but having IFA endpoint titers  $< 1:640$ , and 23 randomly selected serum samples with IFA endpoint titers  $< 1:640$ ). Of the 79 serum samples with suspect-positive results on IFA testing, 2 (2.5%) had WB analysis–confirmed natural exposure–induced antibodies and 19 (24.0%) had WB analysis–confirmed vaccine-induced antibodies (including 3 serum samples from dogs reported as not vaccinated against *B burgdorferi* infection and 4 serum samples from dogs with an unknown vaccine history). Fifty-eight of the 79 (73.4%) serum samples had negative results for specific antibodies against *B burgdorferi* on WB analysis.

The highest IFA endpoint titer of serum samples that had negative results for antibodies against *B burgdorferi* on WB analysis was 1:5,120. Two of the 3 serum samples from dogs reported as vaccinated against *B burgdorferi* infection but as having IFA endpoint titers  $< 1:640$  had WB analysis–confirmed vaccine-induced antibodies. One of these 3 serum samples had negative results for specific antibodies against *B burgdorferi* on WB analysis. Serum samples with WB analysis–confirmed vaccine-induced antibodies had IFA endpoint titers ranging from 1:320 to 1:20,480 (median, 1:2,560; Figure 2). All of the 23 randomly selected serum samples with IFA endpoint titers  $< 1:640$  had negative re-

sults for specific antibodies against *B burgdorferi* on WB analysis.

The overall natural seroprevalence detected in the study was 2 of 332 (0.6%) serum samples (denominator discounted by number of serum samples with WB analysis–confirmed vaccine-induced antibodies). The 2 serum samples with WB analysis–confirmed natural exposure–induced antibodies had IFA endpoint titers of 1:81,920 and 1:10,240 and were submitted from clinics within the *I scapularis* invasion zone (Figure 1). Overall, 21 of 353 (5.9%) serum samples had WB analysis–confirmed vaccine-induced antibodies.

Twenty-nine serum samples were assayed for antibodies against *B burgdorferi* and *E canis* and the *D immitis* antigen by use of the C6-peptide ELISA. Of the 22 serum samples with IFA endpoint titers  $\geq 1:2,560$ , 2 had positive C6-peptide ELISA results for antibodies against *B burgdorferi* (these serum samples had WB analysis–confirmed natural exposure–induced antibodies). Of 7 randomly selected serum samples with low IFA titers, none had positive results by use of the C6-peptide ELISA for antibodies against *B burgdorferi*. The C6-peptide ELISA was thus 100% sensitive and 100% specific, compared with WB analysis, for detection of antibodies against *B burgdorferi*. No serum samples had positive results for antibodies against *E canis* or for the *D immitis* antigen.

A total of 78 ticks from 55 dogs were submitted (mean, 4.9 ticks/clinic; range, 0 to 14 ticks/clinic). Three dogs had reportedly traveled to *I scapularis*–endemic areas (2 to the upper peninsula of Michigan and 1 to the northeastern United States), and for 28 dogs, this information was not available. Ticks comprised nymphs and adults of 3 species: *I scapularis*, *Ixodes cookei*, and *Dermacentor variabilis*. A total of 18 *I scapularis* were removed from 13 dogs (including 1 dog that was reported to have traveled to an endemic area; all other dogs with *I scapularis* had no known travel to endemic areas). Of the dogs with *I scapularis*, 1 was from zone 1 (1 clinic), 9 were from zone 2 (1 clinic), and 3 were from zone 3 (2 clinics; Figure 3). Fifty-two and 8 *D variabilis* and *I cookei* were removed from 35 and 6 dogs, respectively.

Of the 78 ticks submitted, 18 ticks removed from 15 dogs were infected with *B burgdorferi*, which included ticks of all 3 species, as determined by use of a PCR assay. Six of 18 *I scapularis* (nymphs and adults combined) were infected with *B burgdorferi*. The prevalence of *B burgdorferi* infection in *D variabilis* was 17% (9/52; nymphs and adults combined). Three of 8 *I cookei* (nymphs and adults combined) were infected with *B burgdorferi*. Multiple infected ticks were removed from the same dogs (3 infected *D variabilis* from 1 dog at a clinic in zone 1, and 2 infected *I scapularis* from 1 dog at another clinic in zone 1). Ticks infected with *B burgdorferi* were identified at 9 of the 18 clinics (Figure 3).

Three of 78 (3.9%) ticks were infected with *Babesia* spp; these comprised 1 adult *D variabilis* from 1 clinic and 2 nymphal

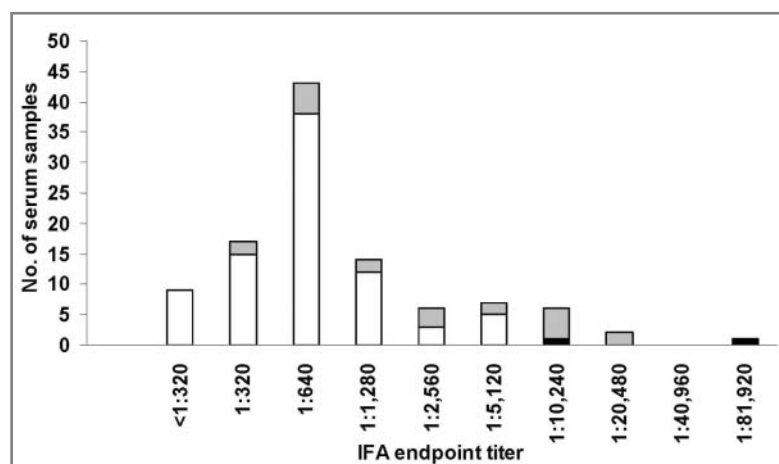


Figure 2—Frequency distribution of IFA endpoint titer against *B burgdorferi* in serum samples of 353 dogs. Serum samples with antibody titers  $\leq 1:320$  were classified as negative for *B burgdorferi* exposure (white portion of bars). Remaining serum samples with high antibody titers, plus serum samples from dogs reported as having been vaccinated against *B burgdorferi* infection, were classified on the basis of WB analysis results as positive for antibodies against *B burgdorferi* as a result of natural exposure (black portion of bars) or positive for antibodies against *B burgdorferi* as a result of vaccination (gray portion of bars).

*I cookei* from 2 other clinics (Figure 3). One nymphal *I cookei* infected with *Babesia* spp was coinfecting with *B burgdorferi*. No ticks were infected with *A phagocytophilum*. Results of positive and negative extraction and PCR controls for all pathogens were as expected.

All PCR amplicons for *B burgdorferi*- and *Babesia* spp-infected ticks were purified and sequenced. Sequences were obtained for 13 *B burgdorferi* amplicons with a mean trimmed sequence length of 790 bases. These sequences were found to have  $\geq 98\%$  sequence identity with previously reported *B burgdorferi* 16S to 23S ribosomal DNA gene sequences of the following strains: B515 (n = 4; GenBank accession No. AF467860.1), CS2 (2; GenBank accession No. DQ437492.1), B356 (2; GenBank accession No. AF467861.1), Lenz (1; GenBank accession No. EF537391.1), RSP2 (1; GenBank accession No. EF649782.1), MI415 (1; GenBank accession No. EF537369.1), HB1 (1; GenBank accession No. EF537294.1), and IP3 (2; GenBank accession No. EF537392.1). Sequences were obtained for 2 *Babesia* amplicons (both from *I cookei* nymphs) with a mean trimmed sequence length of 316 bases. The sequence from 1 *Babesia* spp-infected nymph had 93% sequence identity with *B odocoilei* Wisconsin 1 (GenBank accession No. AY237638.1), whereas the sequence from another *Babesia* spp-infected nymph had 91% sequence identity with *Babesia* sp BiCM002 (GenBank accession No. AB053216.2).

On the basis of spatial analyses, the proportion of dogs vaccinated against *B burgdorferi* infection was significantly ( $P = 0.008$ ) higher in zone 1 (an *I scapularis* established area) than in the other 2 zones (mean proportions of vaccinated dogs for zones 1, 2, and 3 were 14.0%, 1.7%, and 1.9%, respectively). Seroprevalence, background IFA activity, *I scapularis* presence, and *B burgdorferi* presence were all unrelated to clinic zone ( $P = 0.169, 0.728, 0.738, \text{ and } 0.616$ , respectively). The only clinics where multiple *B burgdorferi*-infected ticks were found were those closest to the endemic southwestern corner of the state and just north of the invasion zone. The only coinfecting tick (*I cookei* nymph infected with *B burgdorferi* and *B odocoilei*) was from the southwestern-most clinic.

In the early invasion survey of 2001 to 2002, a total of 2,030 serum samples from 17 clinics in zone 1 was screened by IFA testing, with 235 serum samples identified as having suspect-positive results. Western blot analysis was performed on 20 randomly chosen serum samples with suspect-positive results on IFA testing, resulting in 2, 12, and 6 serum samples classified as positive for antibodies against *B burgdorferi* as a result of natural exposure, positive for antibodies as a result of vaccination, and negative for antibodies, respectively. Extrapolating from this albeit small sample size of sera tested by WB analysis, these data suggest overall seropositivity, vaccination prevalence, and background IFA activity of 1.2%, 6.9%, and 30%, respectively. A total of

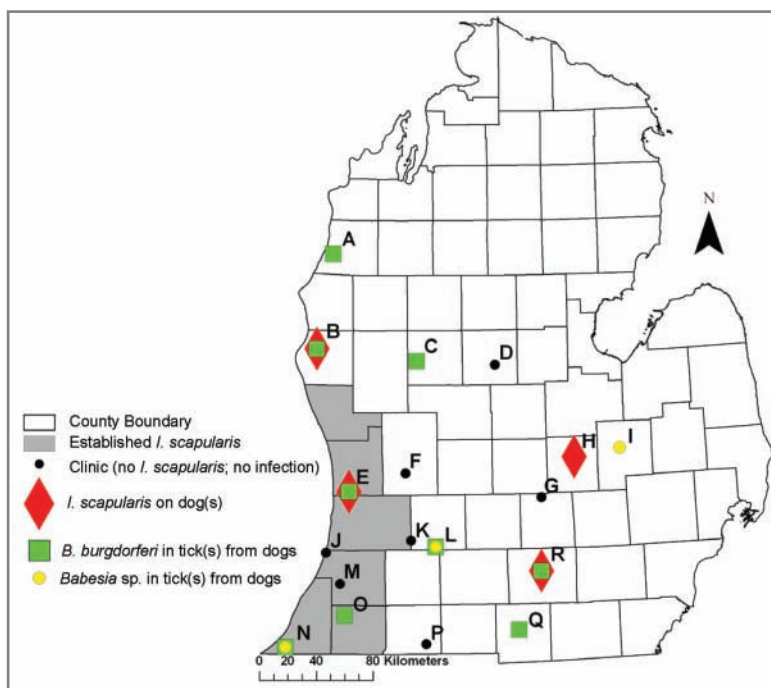


Figure 3—Distribution of 18 veterinary clinics (A through R) with dogs harboring *I scapularis*, *B burgdorferi*-infected ticks of any species, and *Babesia*-infected ticks of any species in the lower peninsula of Michigan. Shading indicates counties in which *I scapularis* has recently invaded and is now established with documented presence of all 3 life stages.<sup>a</sup>

345 ticks were removed from dogs of which 26 (7.5%) were *I scapularis*; this tick species was recovered from 8 clinics, and only adults ticks were found. Ticks of other species included *D variabilis* (n = 298; 86.4%), *Rhipicephalus sanguineus* (7; 2.0%), *I cookei* (11; 3.2%), and *Amblyomma americanum* (3; 0.9%). No pathogen testing of these ticks was undertaken.

## Discussion

Numerous researchers have proposed that companion animal dogs are a useful sentinel that can assist in assessing risk in areas endemic for Lyme disease.<sup>5-8</sup> Most such serosurveys, however, have been conducted in areas already endemic for *I scapularis*. In the study reported here, the efficacy of such surveys in areas where there is concern about low-prevalence emerging disease is considered.

Despite evidence from tick and other wildlife surveys that *B burgdorferi* is emerging in the lower peninsula of Michigan,<sup>a</sup> seroprevalence (0.6%) for *B burgdorferi* was extremely low in dogs from the lower peninsula of Michigan in this study. The 2 dogs determined to have natural exposure to *B burgdorferi* were from clinics within the *I scapularis* invasion zone, where local exposure is expected. Seroprevalence in dogs in this zone during the early invasion study of 2001 to 2002 was 1.2%, and the degree of seropositivity in neither this study of 2005 nor in the investigation of 2001 to 2002 was substantially greater than the seropositivity (0.1%) found during a 1992 to 1993 serosurvey conducted in the lower peninsula of Michigan.<sup>17</sup> This leads to the conclusion that the use of a serosurvey in dogs provides an in-

sensitive approach for detection of *B burgdorferi*-infected *I scapularis* invasion.

In previous studies,<sup>8,25-28</sup> seroprevalence in dogs for *B burgdorferi* has ranged from 53% to 67% in *B burgdorferi*-endemic areas (Wisconsin and Connecticut) to 2% to 6% in nonendemic areas (Alabama and Texas) where Lyme disease is considered largely absent. The low seroprevalence estimate found in the current study is explained, in part, by many of the clinics operating in the outer zones where *I scapularis* is apparently not yet established (Figure 1). Second, seroconversion may not yet have occurred for all exposed dogs. In laboratory studies, dogs exposed to infected adult ticks develop detectable antibodies 4 to 6 weeks after exposure; antibody titers increase for an additional 6 to 8 weeks and remain high for  $\geq 1$  year. In contrast, 50% of dogs exposed to infected nymphs fail to seroconvert or convert only after repeated exposure<sup>2</sup>; therefore, some dogs infected in the summer of 2005 may not have seroconverted by the July to August blood sample collection period. Third, flea and tick chemoprophylaxis, which is increasingly being recommended by veterinarians in southwestern Michigan, may be reducing the number of *B burgdorferi* infections and subsequent seroconversion.

In serum samples tested in this study, background IFA activity was high, with 73.4% of serum samples with suspect-positive results on IFA testing (ie, titer  $\geq 1:640$ ) not confirmed as having natural exposure-induced or vaccine-induced antibodies against *B burgdorferi* on WB analysis or by use of the C6-peptide ELISA. The geographic distribution of serum samples with high IFA titers was unrelated to the location of the Lyme disease hotspot, so false-positive results obtained by IFA testing may represent cross-reactivity with heterologous antibodies or the subjectivity involved in evaluation of IFA slides. The high background reactivity detected by IFA testing emphasizes the importance of subsequent confirmatory testing such as WB analysis or the use of a rapid ELISA, as was done in this study. The C6-peptide rapid ELISA kit was found to be 100% sensitive and 100% specific, compared with WB analysis, confirming the value of this test for studies on dogs as a sentinel species for Lyme disease.<sup>13,16,29</sup>

Most of the 13 dogs that harbored *I scapularis* during this study period were from clinics within the zone of established *I scapularis* (zone 1; 1 dog) and just north of this zone (zone 2; 9 dogs), demonstrating that dogs, in harboring *I scapularis*, serve as a useful sentinel species for presence of this tick in areas undergoing invasion. Contrary to other studies,<sup>30</sup> however, the geographic distribution of *I scapularis* was not predictive of seropositivity in dogs. Six of 18 *I scapularis* (from 6 dogs) plus 12 of 60 ticks of other species (from 9 dogs) were found to be infected with *B burgdorferi*. Thus, detection of *B burgdorferi* in ticks collected from dogs was more common than detection of antibodies against *B burgdorferi* in the serum of dogs.

Of the 3 species of ticks that veterinarians submitted from dogs, only *I scapularis* is regarded as a competent vector for *B burgdorferi* and *Babesia* spp. Yet, in addition to the *I scapularis* found infected with *B burgdorferi*, in the study reported here, 12 *B burgdorferi*-infected and 3 *Babesia* spp.-infected nonvector ticks were found, including 1 coinfecting *I cookei* removed from a dog in the south-

western-most site in the study. A blood fed nonvector tick could become infected via 3 means (not mutually exclusive). First, the nonvector tick may have been collected during or soon after feeding on a spirochetemic dog, so that the PCR assay detects the spirochetemic bloodmeal within the engorged tick. In this study, 39 dogs were parasitized by *D variabilis* or *I cookei*, and *B burgdorferi*-infected ticks of these species (n = 12) were removed from 9 dogs. That 2 dogs had > 1 infected tick lends support to the spirochetemic dog scenario; however, no dogs in this study were reported to have Lyme disease (74.5% veterinarian response rate), and seroprevalence in dogs was negligible. Second, transstadial passage of pathogen acquired in a previous infectious bloodmeal (most likely from a noncanine host) may result in an infected non-engorged tick, though the infected nonvector is unable to transmit the pathogen. Walker et al<sup>31</sup> cultured *B burgdorferi* from 5 questing *D variabilis* from the endemic area in Michigan and found that 1.3% of these ticks were infected. Reports of low to moderate infection prevalence, with low spirochete loads, are consistent with results of transmission in a study<sup>32</sup> indicating that *D variabilis* is an incompetent vector for *B burgdorferi*. Third, nonvector ticks potentially could acquire spirochetes by cofeeding with vector ticks, even in the absence of systemic infection of the canine host. This mechanism, proposed for the maintenance of tickborne encephalitis virus in Europe,<sup>33</sup> has not been tested for nonvector ticks and *B burgdorferi* and has only been demonstrated for *I scapularis* under laboratory conditions with artificially high tick burdens.<sup>34</sup> Nevertheless, although the mechanism remains unclear, findings from the study reported here indicate that assays of the vector and nonvector ticks found attached to dogs can contribute to detection of pathogen presence in a given area, irrespective of the tick species' inability to transmit the pathogen of interest.

Relative to the low apparent exposure of dogs to *B burgdorferi* based on serologic findings of the study reported here, ticks testing positive for this pathogen were far more common than expected. It was hypothesized a posteriori that the tick and serum samples in this study came from different subpopulations of pet dogs. Serum samples may have come from a range of dogs that included those prophylactically vaccinated against *B burgdorferi* infection or chemically protected against tick bites (or both), whereas ticks may have been collected from dogs not chemically protected from tick exposure. The collaborating veterinarians estimated that 50% of dogs in this study were receiving tick chemoprophylaxis and 12% were being vaccinated against *B burgdorferi*, which indicates that acquisition of serum from pet dogs randomly selected at veterinary clinics (a common method of sampling in canine serosurveys<sup>8,13,27</sup>) will undersample the unprotected dogs that are likely the most effective sentinels for tickborne pathogens.

It is anticipated that *I scapularis* and associated pathogens will spread north and east from the present southwestern Michigan hotspot in coming years, so that both veterinary and human clinicians will see an increase in Lyme disease among their patients. On the basis of serologic testing, dogs are unlikely to be a sensitive sentinel species for changes in tick and pathogen activity in these areas of low tick density. The find-

ings of the study reported here indicate that *I scapularis* emergence, and consequent increase in canine and human disease risk, can be predicted more effectively by surveys of *I scapularis* and other nonvector ticks removed from dogs than by serosurvey of dogs.

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- b. ArcGIS 8.0, Environmental Systems Research Institute, Redlands, Calif.
- c. *Borrelia burgdorferi* IFA Substrate Slides, Fuller Laboratories, Fullerton, Calif.
- d. Lyme Disease Positive Control for IFA, VMRD, Pullman, Wash.
- e. Anti-Dog IgG (H+L) Antibody, FITC labeled, Kirkegaard and Perry Laboratories, Gaithersburg, Md.
- f. Eclipse E800, Nikon, Melville, NY.
- g. QuallCode Canine Lyme Disease Kit, Immunetics, Cambridge, Mass.
- h. SNAP 3Dx test, IDEXX Laboratories, Westbrook, Me.
- i. DNeasy Tissue Prep extraction kit, Qiagen, Valencia, Calif.
- j. E-gel system, Invitrogen, Carlsbad, Calif.
- k. BSK-H complete media, Sigma-Aldrich, St Louis, Mo.
- l. Provided by Dr. Durland Fish, Yale School of Public Health, New Haven, Conn.
- m. *Babesia microti* organism No. 30221, American Type Culture Collection, Manassas, Va.
- n. PCR Purification Kit, Qiagen, Valencia, Calif.
- o. ABIPrism 3100 Genetic Analyzer, Applied Biosystems, Foster City, Calif.

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