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SEROPREVALENCE OF LYME DISEASE IN GRAY WOLVES FROM MINNESOTA AND WISCONSIN

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ABSTRACT: To determine the seroprevalence of Lyme disease in gray wolves (*Canis lupus*) from various counties of Minnesota and Wisconsin (USA), 589 serum samples were collected from 528 wolves from 1972 to 1989. An indirect fluorescent antibody (IFA) test was used to detect the presence of antibodies against *Borrelia burgdorferi*. Titers of ≥1:100 were considered positive. Results were confirmed by testing a few selected sera by Western blotting. Of the 589 sera tested, 15 (3%) had IFA titers of ≥1:100. Three of the positive samples were collected from Douglas County in Wisconsin and twelve were from Minnesota counties. This study indicates that wolves are exposed to *B. burgdorferi* and are susceptible to Lyme disease.

Key words: Lyme disease, gray wolves, Lyme antibodies, *Borrelia burgdorferi*, serologic survey.

INTRODUCTION

Lyme disease in humans was first reported in the United States in 1975 in Lyme, Connecticut. Prior to this, a similar illness called 'erythema chronicum migrans' was reported from Wisconsin (Scrimenti, 1970). Lyme disease was subsequently found to be caused by a spirochete (*Borrelia burgdorferi*) which is carried and transmitted by the deer tick *Ixodes dammini* (Burgdorfer et al., 1982). The primary hosts of this tick include the white-footed mouse (*Peromyscus leucopus*), the deer mouse (*P. maniculatus*) and the white-tailed deer (*Odocoileus virginianus*) (Burgess, 1986a). In addition to man, *B. burgdorferi* infects dogs (Schulze et al., 1986; Burgess, 1986a, b; Greene et al., 1988), coyotes (*Canis latrans*) (Burgess and Windberg, 1989), gray wolves (*C. lupus*) (Kazmierczak et al., 1988) and rabbits (Burgess and Windberg, 1989; Lane and Burgdorfer, 1988).

Attempts to infect wolves by oral inoculation have been unsuccessful (Kazmierczak et al., 1988) but the transmission of *B. burgdorferi* in dogs by direct contact has been shown to occur (Burgess, 1986b). Dogs with Lyme disease develop fever, lymphadenopathy, arthralagia and arthritis (Lissman et al., 1984; Kornblatt et al., 1985). Of the 423 dogs tested in New Jersey, 147 (35%) had antibody titers of ≥1:64 when tested by the indirect fluorescent antibody (IFA) test (Schulze et al., 1986). Seven of these dogs showed clinical signs of borreliosis and had titers of 1:512. In another study, 206 of 380 (54%) dogs from Wisconsin had IFA titers of 1:64 or above (Burgess, 1986a). In an experimental study, a gray wolf injected intravenously with approximately 8,000 live spirochetes developed a Lyme disease infection (Kazmierczak et al., 1988). The antibody titers in this wolf increased steadily, peaked at 1:512 on day 43 post-infection (PI), and remained at that level until day 75 PI when the wolf was euthanized. Lymphadenopathy was the only clinical sign observed (Kazmierczak et al., 1988).

Natural Lyme disease infection also has been found in wild gray wolves. Thus, two (3%) of the 78 wolves captured in Minnesota and Wisconsin from 1977 to 1984, had IFA titers of ≥1:64 (Kazmierczak et al., 1988). The present study was conducted to determine the serologic prevalence of Lyme disease antibodies in a large

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TABLE 1. Gray wolf sera examined by the indirect fluorescent antibody test for Lyme disease antibody from 1972 to 1989.

Years	Number of samples examined[a]	Number negative at 1:10	Number positive at indicated titers[b]					
			1:20	1:50	1:100	1:200	1:400	1:1,600
1972–74	20 (20)	20	0	0	0	0	0	0
1975–77	92 (78)	83	7	2	0	0	0	0
1978–80	83 (74)	63	15	4	1	0	0	0
1981–83	153 (125)	106	43	1	1	1	0	1
1984–86	113 (107)	81	21	2	2	2	1	4
1987–89	127 (123)	68	44	13	1	0	0	1
Unknown[c]	1 (1)	0	0	1	0	0	0	0
Total	589 (528)	421	130	23	5	3	1	6

[a] Number of wolves is given in parentheses.
[b] None of the samples had end point titers of 1:800.
[c] Year of sample collection was unknown for one wolf.

sample of gray wolves from Minnesota and Wisconsin and to compare these results with those of the earlier reports.

MATERIALS AND METHODS

Source of serum samples

Blood samples were collected from 528 gray wolves live-trapped in areas of northern Minnesota and Wisconsin from 1972 through 1989. Some gray wolves ($n = 42$) were bled on two to six different occasions and hence the total number of sera examined was 589. The blood was allowed to clot, centrifuged, and the serum obtained was stored at −20 C until used.

Antigen preparation

Antibodies to *B. burgdorferi* were detected by IFA (Wilkinson, 1984). *Borrelia burgdorferi* derived from a *Peromyscus* spp. isolate (Loken et al., 1985) was cultivated in BSK II medium (Barbour, 1984) at 34 C. Log phase cultures of the organism (8×10^7 to 3×10^8 cells per ml) were harvested and centrifuged at 30,000 × *g* for 30 min at 10 C. The pellet was washed three times in 0.01 M phosphate buffered saline (PBS, pH 7.2) followed by its resuspension in PBS. The resulting suspension was diluted in PBS so that 100 spirochetes per 400× microscopic field were obtained. Ten microliters (approximately 1,000 spirochetes) of the diluted antigen were applied to each well of a ten-well slide (Cell-Line, Newfield, New Jersey 08344, USA). The slides were fixed with 95% ethanol for 30 min and stored at −70 C until used.

Indirect fluorescent antibody test

Initially, sera were tested at 1:10, 1:20 and 1:50 dilutions. Samples positive at 1:50 were serially diluted to 1:1,600 and retested. The end-point was considered as the serum dilution that yielded distinct fluorescence of at least 80% of the spirochetes. Dilutions of gray wolf sera were applied to one well each of the antigen slide at 15 µl per well. The slides were incubated in a moist chamber for 30 min, washed in PBS, and dried. A 1:80 dilution of fluorescein labelled anti-dog IgG (heavy and light chain) conjugate (ICN Biomedical, Lisle, Illinois 60532, USA) was then placed on all wells of the slide at 15 µl per well. After incubation for another 30 min in the moist chamber, the slides were washed and mounted followed by examination under a fluorescent microscope. Positive control consisted of serum from a Lyme disease-infected domestic dog with a titer of 1:1,280. Known negative sera from non-infected dogs were included as controls.

Western blot analysis

The Western blot (immunoblot) was performed on 14 selected sera using a modification of the protocol initially described by Towbin et al. (1979). Purified *Borrelia burgdorferi* cells were disrupted with sodium dodecyl sulphate (SDS) and mercaptoethanol and were separated by SDS-polyacrylamide gel electrophoresis. Resolved bacterial proteins were transferred to nitrocellulose sheets which were then blocked with 1% solution of bovine serum albumin. Individual strips were cut from sheets and were incubated with 1:500 dilutions of test sera for 2 hr. Serum dilutions were made in phosphate-buffered saline containing 0.05% tween-20, 1% bovine serum albumin, and 30% calf serum. The strips were washed extensively and then reacted with horseradish peroxidase labelled anti-dog IgG conjugate for 60 min. After repeating the wash cycle, the precipitating chromogen (4-chloronaphthol) and substrate (hydrogen peroxide) were added. The reaction was stopped

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TABLE 2. The effect of sex and age of gray wolves on Lyme disease antibody titer as determined by the indirect fluorescent antibody test.

Age and sex[a]	Number of samples examined[b]	Number negative at 1:10	Number positive at indicated titers						
			1:20	1:50	1:100	1:200	1:400	1:800	1:1,600
Adults	372 (323)	254	93	14	4	1	1	0	5
Pups	200 (188)	156	34	7	1	2	0	0	0
Males	274 (251)	202	59	9	2	0	0	0	2
Females	314 (276)	221	68	14	3	3	1	0	4

[a] The ages and sex of 17 and one wolves, respectively were not known.
[b] The number of wolves is given in parentheses.

by washing with deionized water and the results were interpreted immediately. Commercially available molecular mass standards were used to assign apparent molecular masses for spirochetal antigens. Reactive bands were identified by using a set of 37 sera from dogs showing symptoms of Lyme disease and with positive IFA and/or ELISA (enzyme-linked immunosorbent assay) titers. Significant antibody reactivity was most commonly observed against proteins with apparent molecular masses of 14, 21, 30–31, 34, 41, 55, 60–61, 66, and 83 kilo daltons. Significant reaction to four or more bands was considered to be positive.

RESULTS

Five hundred eighty nine gray wolf sera were examined, of which 421 (72%) were seronegative at 1:10 (Table 1). Of the 168 reactive samples, 153 fluoresced at titers of ≤1:50. Only 15 (2.5%) of the 589 samples tested were positive at titers of 1:100 or above. Of these, 6 samples had titers of 1:1,600. None of the 15 samples was collected prior to 1980, and 9 of them were collected between 1984 and 1986. Fourteen sera were examined by Western blotting; four had IFA titers of >1:100, three were at 1:100, five were between 1:10 and 1:50, and two sera were negative at 1:10. All sera with IFA titers of ≥1:200 were positive by Western blot while those at ≤1:50 were negative. Of the three sera that had a titer of 1:100, two were strongly positive by Western blot and one was weakly positive.

Three hundred twenty-three adult gray wolves (≥1-yr-old), 188 pups, and 17 wolves of unknown age were tested. The numbers of sera from known adults and pups were 372 and 200, respectively. Of these, eleven (3%) adult sera and three (2%) pup sera were positive at ≥1:100 (Table 2). Of the adult wolves which had antibodies to *B. burgdorferi*, five had titers of 1:1,600. The age of one of the wolves with a titer of 1:1,600 was not known. The sex was known for 527 animals; 251 (48%) being males and 276 (52%) being females. Four sera (2%) from males and 11 sera (4%) from females were positive at IFA titers of ≥1:100.

Multiple serum samples were collected from 42 wolves; six and five samples from one wolf each, four samples each from two wolves, three samples each from eight wolves, and two samples each from 30 wolves. All but four of these multiple serum samples had titers of ≤1:10 each time they were examined. Three of these showed different titers on multiple sampling. As examples, wolf 17 which was negative on 7 August 1987 showed a titer of 1:50 on 19 August 1987 and wolf 141 had titers of 1:20 and 1:50 on 1 July 1989 and 26 July 1989, respectively; and wolf 6117 had a titer of 1:10 in both 1982 and 1983 but had a titer of 1:50 in 1987. Three consecutive samples from wolf 139 showed that the titers of this wolf remained constant at 1:20 between 29 June 1989 and 22 October 1989.

Samples were collected from wolves in 18 counties (Table 3). Thirteen of these counties are in northern Minnesota, two in northwestern Wisconsin and three in central Wisconsin. Thirteen of the 18 counties did not yield any sample with titers of ≥1:

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TABLE 3. The distribution of Lyme disease antibodies in gray wolves in counties of northern Minnesota and western Wisconsin.

County name and number[a]	Number of samples examined[b]	Number negative at 1:10	Number positive at indicated titers[c]						Percent positive at ≥1:100
			1:20	1:50	1:100	1:200	1:400	1:1,600	
1 Aitkin	1 (1)	1	0	0	0	0	0	0	0
2 Bayfield, Wisconsin	1 (1)	1	0	0	0	0	0	0	100
3 Becker	6 (6)	2	3	1	0	0	0	0	0
4 Beltrami	30 (30)	20	9	0	1	0	0	0	3.3
5 Carlton	2 (2)	1	0	0	0	0	0	1	50.0
6 Douglas, Wisconsin	13 (13)	8	3	0	0	1	0	1	15.4
7 Itasca	39 (39)	28	9	1	1	0	0	0	2.6
8 Kittson	3 (3)	2	1	0	0	0	0	0	0
9 Koochiching	47 (47)	36	10	1	0	0	0	0	0
10 Lake	226 (186)	172	43	8	1	1	0	1	1.3
11 Lake of Woods	17 (17)	15	2	0	0	0	0	0	0
12 Lin, Wisconsin	1 (1)	0	0	0	0	0	1	0	0
13 Marshall	3 (3)	2	1	0	0	0	0	0	0
14 Oneida, Wisconsin	1 (1)	1	0	0	0	0	0	0	100
15 Pine	4 (4)	0	0	0	1	0	0	3	100
16 Price, Wisconsin	1 (1)	0	1	0	0	0	0	0	0
17 Roseau	28 (28)	24	4	0	0	0	0	0	0
18 St. Louis	161 (140)	106	42	11	1	1	0	0	1.3
19 Unknown	5 (5)	2	2	1	0	0	0	0	40.0
Total	589 (528)	421	130	23	5	3	1	6	

[a] See Figure 1 for location of counties in Minnesota. County numbers in this table correspond to those in Figure 1. Counties were unknown for five wolves.

[b] Number of wolves is given in parentheses.

[c] None of the samples had end point titers of 1:800.

100. The highest number of samples examined (38%) were from Lake County, Minnesota. However, Lake County had the least number of samples with ≥1:100 titer; only three of 226 examined (1%). From Carlton County, Minnesota, only two (<1%) samples were collected of which one was positive at 1:1600. All four (100%) samples from Pine County, Minnesota, had titers of ≥1:100 whereas three (18%) of the 17 samples from Wisconsin had titers of ≥1:100.

DISCUSSION

Animals with titers of ≥1:100 were considered to be exposed to Lyme disease spirochete in this study. This is a conservative estimate of infection in view of the titers reported in naturally infected animals such as dogs, deer and wild rodents (Magnarelli et al., 1984a, b, 1985). Magnarelli et al. (1984a) considered IFA titers of ≥1:64 to be positive in a study in which they tested sera from 251 deer, 220 dogs and 42 wild rodents in Connecticut. Of these, 68 deer, 53 dogs and 42 wild rodents were found to be serologically positive at titers of ≥1:64. It was proposed that at titers of <1:64, the antigen can fluoresce non-specifically causing false positive results (Magnarelli et al., 1984a).

In one study, Magnarelli et al. (1984b) found that 34 of 76 humans, five of nine Swiss mice, and five of 20 dogs were positive for Lyme disease antibody at titers of ≥1:64. Based on IFA results from 153 dog sera from areas where Lyme disease had not yet been found, titers of ≥1:64 were considered positive (Magnarelli et al., 1985; Kazmierczak et al., 1988; Schultze et al., 1986).

Magnarelli et al. (1985) conducted an epizootiological study in which 60 of 210 (29%) dogs from areas of high Lyme dis-

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ease prevalence were found to be seropositive at titers of ≥1:64, whereas all 97 dogs from low prevalence areas had titers of <1:64. Considering ≥1:100 positive, 2.5% of the gray wolf sera from Minnesota and Wisconsin were positive for antibody to *B. burgdorferi* in the present study. These results are in agreement with those of Kazmierczak et al. (1988) who tested 78 live-captured gray wolves from Minnesota and Wisconsin between 1977 and 1984 and found 3% to be serologically positive at ≥1:64. Although experimental infection of a single gray wolf with *B. burgdorferi* produced clinical signs of Lyme disease (Kazmierczak et al., 1988), the effects of Lyme disease in the wild cannot be determined without further examination of naturally exposed gray wolves with positive titers. Also, more experimental studies using larger numbers of animals are needed to determine the degree of susceptibility of wolves to Lyme disease. The results of multiple sample examination in this study were inconclusive. Although three of the wolves from which multiple samples were collected showed a rise in titers between samplings, these wolves can not be claimed to have seroconverted because titers of all these samples were always <1:100. Similarly, age and sex of the wolves were found to have no effect on the serological prevalence of Lyme disease.

The county with the highest prevalence of antibody activity was Pine County, Minnesota, with a prevalence rate of 100%, although the number of samples tested was small (n = 4). Carlton County, Minnesota, had the second highest number of positive gray wolf sera (50%) followed by Douglas County, Wisconsin (18% positive). It is interesting to note that these three counties adjoin one another (Fig. 1) and that this general area coincides with the area known to be endemic for Lyme disease in Minnesota (Osterholm et al., 1984) and with locations from where high numbers of *I. dammini* have been found (Drew et al., 1988). Further studies on the prevalence of *B. burgdorferi* in the wild and domestic animal populations of Minnesota will help define the pattern of spread of Lyme disease including information on the range where *I. dammini* is most commonly found and the animals on which they feed and breed.

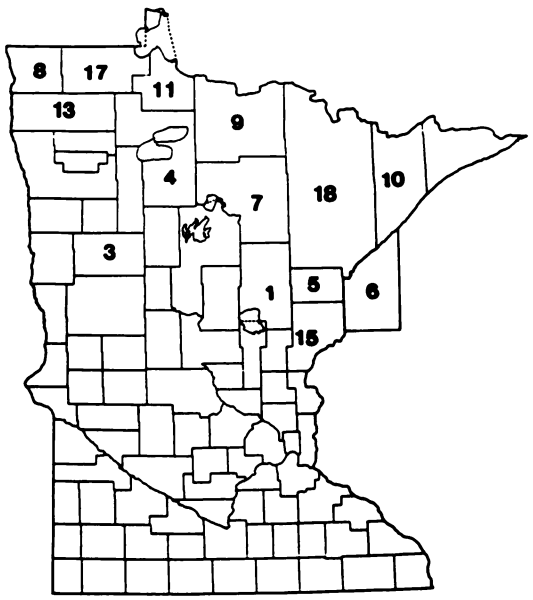

FIGURE 1. Minnesota counties from which samples were taken are shown. Number 6 is Douglas County, Wisconsin. See Table 3 for county names.

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