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Serologic Analysis of White-Tailed Deer Sera for Antibodies to *Borrelia burgdorferi* by Enzyme-Linked Immunosorbent Assay and Western Immunoblotting

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White-tailed deer serum samples were collected in the Minneapolis-St. Paul, Minn., metropolitan area during the fall and winter months from 1989 to 1992 and analyzed for antibodies to *Borrelia burgdorferi*, the etiologic agent of Lyme borreliosis. Ninety-eight percent of the serum samples were collected from regions where currently the vector tick, *Ixodes dammini*, is nonexistent. Antibodies to *B. burgdorferi* were detected in 2.2% of 508 samples by enzyme-linked immunosorbent assay, and their presence was confirmed by Western immunoblot analysis. Western immunoblotting yielded mean numbers of reactive bands of 0.1 and 6.0 for samples that were negative and positive for antibodies by enzyme-linked immunosorbent assay, respectively. The molecular weights of the antigens in many of the reactive bands from positive samples were similar to the molecular weights of antigens reactive with samples from humans with Lyme borreliosis. An antibody response to the major outer surface proteins A and B was not detected. Serologic analysis of deer sera may provide a valuable method for surveillance programs designed to monitor the spread of *B. burgdorferi* in nature.

Lyme borreliosis is the most common tick-borne disease in the United States (6). The etiologic agent, *Borrelia burgdorferi* (5, 14, 16), is transmitted through the bites of ticks of the *Ixodes ricinus* complex. In the north-central states of Minnesota and Wisconsin, the vector is *Ixodes dammini* (1). Lyme borreliosis can cause acute and chronic abnormalities in one or more major organ systems in humans. The clinical manifestations occur in major organ systems such as the skin, nervous system, heart, and joints (23).

The areas in which Lyme borreliosis is endemic are expanding. In areas with confirmed cases of the disease but no documentation of a local vector, a method for monitoring the movement of the spirochete into new areas would be of public benefit. Evaluations of human case reports can be helpful but sometimes are misleading or inaccurate because of the difficulty in establishing a precise geographic area of exposure. Surveillance studies using examination of ticks and captured animals for *B. burgdorferi* are very labor-intensive. Serologic surveillance programs involving domestic animals are useful but have problems such as the one created by the introduction of a vaccine for canine borreliosis. In certain geographic areas, serologic surveillance of white-tailed deer (*Odocoileus virginianus*) sera may be conducted by relatively easy procedures. Sera can be collected by hunters during hunting seasons or by hired sharpshooters as part of deer herd reduction programs and submitted for serologic analysis. Our objective was to collect sera of wild white-tailed deer from several sites where the disease is nonendemic and one site where it is endemic and examine these specimens for the presence of antibodies to *B. burgdorferi* by enzyme-linked immunosorbent assay (ELISA) and Western immunoblotting.

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MATERIALS AND METHODS

Study sites and sampling. White-tailed deer serum samples were collected from seven different sites in the seven-county metropolitan area of Minneapolis-St. Paul, Minn., during the fall and winter months of 1989 to 1992 (Fig. 1). Samples were obtained from male and female deer ranging from fawn to adult either by hunters during special area hunts or by sharpshooters hired to reduce overabundant local deer populations. All sample sites, except the Carlos Avery Wildlife Management Area in the northeastern section of the metropolitan area, were situated in areas where Lyme disease and the deer tick are nonendemic.

Control sera. One group of positive-control deer ($n = 4$) was immunized with killed, sonicated *B. burgdorferi* NY90-14, a virulent isolate from the white-footed mouse, *Peromyscus leucopus*, from New York state, passaged four times in BSK medium (2). Cells were washed three times in sterile phosphate-buffered saline (PBS), pH 7.4, and the protein content was determined by using a commercially available kit (Bio-Rad Laboratories, Richmond, Calif.). The first immunizing dose was given subcutaneously and intramuscularly with Freund's incomplete adjuvant. The second and third doses were subcutaneous and intramuscular injections without adjuvant. The fourth and final dose, given without adjuvant, was by intravenous injection. Each immunizing dose was 1 ml containing 1.9 mg of cell protein per ml. Doses were given at 2-week intervals. Serum samples were collected preinoculation, immediately prior to each immunizing dose, and 2 weeks after the final dose. A second group of positive-control deer ($n = 2$) was immunized with killed *B. burgdorferi* B31 grown in BSK medium. Strain B31 (ATCC 35210) is the type strain of *B. burgdorferi* originally isolated from *I. dammini* in New York state. The first dose was given subcutaneously in Freund's complete adjuvant, and the final dose was given 9 days later intramuscularly in Freund's

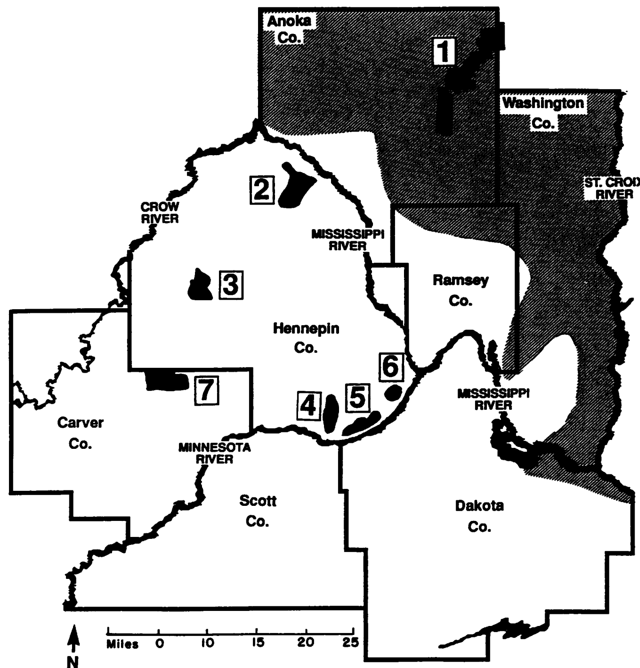

FIG. 1. Map of the seven-county metropolitan area of Minneapolis-St. Paul, Minn., showing the sites used for white-tailed deer serum sampling from 1989 to 1992 and deer tick distribution based on small-mammal trapping data from 1990 to 1991. Deer serum sampling sites: 1, Carlos Avery Wildlife Management Area; 2, Elm Creek Park Reserve; 3, Baker Park Reserve; 4, Hyland Lake Park Reserve; 5, city of Bloomington; 6, Minnesota Valley National Wildlife Refuge; 7, Carver Park Reserve. Deer ticks were found frequently throughout all test sites in the shaded portion. Deer ticks were mostly nonexistent in the unshaded portion, with a few (7 of 254) widely scattered positive sites.

incomplete adjuvant. Each dose consisted of washed cells containing 0.45 mg of cell protein in 0.75 ml of adjuvant. Serum samples were collected 36 days after the final injection. A third group of positive-control deer (n = 1) was immunized with live spirochetes by one intradermal injection of 3.4×10^7 cells in 1.2 ml of BSK medium and one subcutaneous injection of 2.8×10^7 cells in 1.0 ml of BSK. The strain used was SH-2-82, a mouse isolate from New York state, passaged six times in BSK medium and confirmed to be virulent in hamsters. All control deer, including the negative control, were tick free and pen raised in areas outside the range of the deer tick, *I. dammini*.

Rabbit immune serum was prepared by using rabbits injected intravenously with approximately 10^8 cells of *B. burgdorferi* virulent strain 297, a human spinal fluid isolate from Connecticut, every 1 or 2 months a total at least 10 times. Cells, grown in BSK medium, were washed two times and suspended in PBS at 10^8/ml before injection. The titer by indirect immunofluorescence assay (IFA) was 1:1,024.

Serologic analyses. IFA and ELISA were run on negative- and positive-control deer sera. Because there was excellent agreement between results of control sera by IFA and ELISA, subsequent serologic assays to further characterize the antibody responses of wild deer were conducted by ELISA and Western immunoblotting. The procedure for IFA (8) established the highest dilution of serum at which fluorescence of homologous whole-cell antigen that was acetone fixed to glass slides could still be detected by using fluorescein isothiocyanate-labelled goat anti-deer immunoglobulin G diluted 1:20 (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

ELISA was done by using 0.5 μg of washed, sonicated NY90-14 or B31 cell antigen per well in 96-well Immulon 4 plates (Dynatech Laboratories, Chantilly, Va.) to test control sera (8). Sera from wild deer were tested by using washed, sonicated NY90-14 cell antigen. Test plates were blocked with 3% fetal calf serum for 60 min to reduce nonspecific reactivity. The presence of deer antibodies was detected with horseradish peroxidase-labelled goat anti-deer immunoglobulin G (Kirkegaard & Perry Laboratories) diluted 1:7,000 to the optimal working concentration. Optical density (OD) values were read at 450 nm. All deer sera were diluted 1:100 in PBS before testing. Serum samples were run in duplicate wells, and each test was repeated. Samples were considered positive if the OD values were ≥3 standard deviations above the mean (OD = 0.08) of the negative-control samples (5 pen-raised deer) and samples from 40 wild deer from areas in which Lyme disease is nonendemic.

Western immunoblots were conducted as previously described (8). *B. burgdorferi* 297 was used as the test antigen. Antigen was run under reducing conditions with sodium dodecyl sulfate on 5 to 20% linear gradient polyacrylamide gels (14 by 16 cm) (17) and electrophoretically transferred (24) to polyvinylidene difluoride paper (Millipore Corp., Bedford, Mass.). Approximately 150 μg of antigen was loaded into one large trough well for electrophoresis, and after transfer, the polyvinylidene difluoride paper was cut into strips about 2 mm wide. Ten microliters of serum was diluted 1:1,000 for testing of antigen strips. Reactive antigen bands were detected by the method of Blake et al. (4) using alkaline phosphatase-labelled goat anti-deer immunoglobulin G diluted 1:1,000 (Kirkegaard & Perry Laboratories) and the nitroblue tetrazolium–bromo-chloro-indolyl phosphate system (Sigma Chemical Co., St. Louis, Mo.). Nonspecific binding was blocked with 0.5% nonfat dry milk.

RESULTS

A total of 508 white-tailed deer serum samples were collected from the Minneapolis-St. Paul metropolitan area and analyzed by ELISA and Western immunoblotting (Table 1). More than 90% of the samples were negative by ELISA, while only 2.2 and 6.5% were positive and borderline positive, respectively. Samples were considered borderline positive if their OD readings were positive on one test and just below the cutoff of 3 standard deviations from the mean of the negative-baseline value upon retest or if the OD readings from both tests were just below the cutoff for positives. The percentage of samples that were negative by ELISA from individual sampling sites ranged from 97 to 82%.

All serum samples that were positive and borderline positive by ELISA analysis and a random selection of negative ELISA samples (80 of 464 negative samples) were examined by Western immunoblotting (Tables 2 to 4). Only 10% of the negative ELISA samples had bands indicating the presence of deer antibodies (Table 2). Of the eight samples reactive on Western immunoblots, seven reacted with only one band and the remaining sample reacted with two bands (Table 3). Of the borderline positive and positive ELISA samples, 61 and 82%, respectively, were reactive on Western immunoblots (Table 2). The average number of reactive bands increased from 0.1 per blot for the negative ELISA samples to 6.0 per blot for the positive ELISA samples (Table 3). The molecular masses of the antigens and the

TABLE 1. ELISA results for white-tailed deer sera collected during the fall and winter months of 1989 to 1992 from the Minneapolis-St. Paul metropolitan area

Site	Yr	No. of samples (%)			
		Tested	Positive	Borderline positive	Negative
Minnesota Valley National Wildlife Refuge	89–90	36	0 (0)	1 (2.8)	35 (97)
Baker Park Reserve	89–90	33	0 (0)	1 (3.3)	32 (97)
Carlos Avery Wildlife Management Area	89–90	11	1 (9.1)	1 (9.1)	9 (82)
Elm Creek Park Preserve	90–91	110	2 (1.8)	3 (2.7)	105 (95)
Carver Park Reserve	91–92	86	1 (1.2)	10 (12)	75 (87)
City of Bloomington	91–92	82	0 (0)	11 (13)	71 (87)
Hyland Lake Park Reserve	91–92	150	7 (4.7)	6 (4.0)	137 (92)
Total		508	11 (2.2)	33 (6.5)	464 (91)

frequencies of reactivity of antigens on Western immunoblots are given in Table 4. For the negative ELISA samples, the molecular masses of the antigens ranged from 9 to 72 kDa, with a response to the 46-kDa band being most common. The responses of the borderline positive and positive ELISA samples showed reactivities to antigens ranging in size from 9 to 85 kDa. There was a substantial number of responses to antigens of 85, 60, 46, 41, 39.5, 35, and 19.5 kDa. Interestingly, no response to the major outer surface proteins OspA and OspB was seen. Representative Western immunoblots are shown in Fig. 2. Control deer immunized with killed spirochetes and hyperimmunized control rabbits, however, did show strong antibody responses to OspA and OspB in addition to many other antigens (Fig. 2, lanes 1 and 3; Table 4). Deer immunized with live *B. burgdorferi* showed barely detectable responses to OspA, OspB, flagellin (41 kDa), and the 39.5-kDa antigen while showing significantly strong responses to other antigens (Fig. 2, lane 4; Table 4). There were 2 positive ELISA samples of 11 total that were apparently false positive and did not react on Western immunoblots (Table 3). A total of 14 borderline positive and positive ELISA samples reacted with one or two bands on Western immunoblots (Table 3). More than half of these

TABLE 3. Reactive bands on Western immunoblots probed by white-tailed deer sera collected during the fall and winter months of 1989 to 1992 from the Minneapolis-St. Paul metropolitan area

ELISA result	No. of samples					Mean no. of bands ± SD
	Tested	With a band(s)	With 1 band	With 2 bands	With ≥3 bands	
Negative	80	8	7	1	0	0.1 ± 0.4
Borderline positive	33	20	6	4	10	2.0 ± 3.0
Positive	11	9	3	1	5	6.0 ± 6.1

samples reacted with one or more of the 85-, 41-, 37.25-, 19.5- and 9-kDa antigens.

DISCUSSION

The area from which the deer serum samples were collected was the seven-county metropolitan area of Minneapolis-St. Paul, covering approximately 3,000 square miles (ca. 7,767 km^2) with a human population greater than 2,000,000. Many undeveloped sections of nature preserves, parks, woodland, and lowlands that support relatively large deer herds are dispersed throughout this large metropolitan area. The closeness of large numbers of deer to humans poses for people and domestic animals a potentially high risk of contracting Lyme borreliosis through the bite of the deer tick, *I. dammini*, the primary vector of *B. burgdorferi*. The Metropolitan Mosquito Control District has, at the request of the legislature of the state of Minnesota, undertaken a major multiyear study to determine the presence and distribution of *I. dammini* within the seven-county metropolitan area (22). Preliminary results (21) from trapping 9,217 small mammals, of which 7,229 were *P. leucopus*, over 2 years have shown that *I. dammini* is found regularly only in the northeastern section of the metropolitan area (Fig. 1). Because the deer population is well dispersed throughout the metropolitan area, we believe that during the next few years *I. dammini* ticks, some of which will be infected with *B. burgdorferi*, will probably spread over the entire region. By establishing a surveillance program in which white-tailed deer sera are screened for antibodies against *B. burgdorferi*, we feel that we can monitor the spread of the spirochete and, indirectly, of the deer tick into areas in which they are

TABLE 2. Western immunoblot analysis of white-tailed deer sera collected during the fall and winter months of 1989 to 1992 from the Minneapolis-St. Paul metropolitan area

Site	No. of samples (%)					
	Positive by ELISA		Borderline positive by ELISA		Negative by ELISA	
	Tested by WB[a]	With bands by WB	Tested by WB	With bands by WB	Tested by WB	With bands by WB
Minnesota Valley National Wildlife Refuge	0	0	1	1 (100)	4	0 (0)
Baker Park Reserve	0	0	1	0 (0)	4	1 (25)
Carlos Avery Wildlife Management Area	1	1 (100)	1	1 (100	2	0 (0)
Elm Creek Park Reserve	2	1 (50)	3	1	11	0 (0)
Carver Park Reserve	1	1 (100)	10	5 (50)	9	4 (44)
City of Bloomington	0	0	11	7 (64)	34	2 (5.9)
Hyland Lake Park Reserve	7	6 (86)	6	5 (83)	16	1 (6.3)
Total	11	9 (82)	33	20 (61)	80	8 (10)

[a] WB, Western immunoblot.

TABLE 4. Comparison of the frequencies at which specific bands were reactive in Western immunoblots probed by white-tailed deer sera

Sample(s) (*n*)	No. reactive to antigens with a molecular mass (kDa) of:											
	85	72	60	46	41	39.5	37.25	35	34	31	19.5	9
Negative by ELISA (80)	0	1	0	4	1	1	1	0	0	0	0	1
Borderline positive or positive by ELISA (44)	7	0	8	12	7	9	5	9	0	0	12	4
Positive control immunized with killed spirochetes (6)	2	1	4	3	6	4	2	2	6	6	4	2
Positive control immunized with live spirochetes (1)	0	0	0	1	1	1	0	1	1	1	1	0

nonendemic. This method should also be valuable in screening large, nonurban areas subject to deer hunting.

The only sample site in an area where *I. dammini* is endemic was Carlos Avery Wildlife Management Area, located in the northeastern metropolitan area. Unfortunately, we obtained only 11 samples from this site and were not able to reach conclusions about seropositivity in an area where the tick is endemic. Our goals for the future include, in addition to monitoring the spread of the spirochete in the metropolitan area, determining seropositivity for deer in areas of Minnesota where it is endemic, such as Carlos Avery Wildlife Management Area and St. Croix State Park (9).

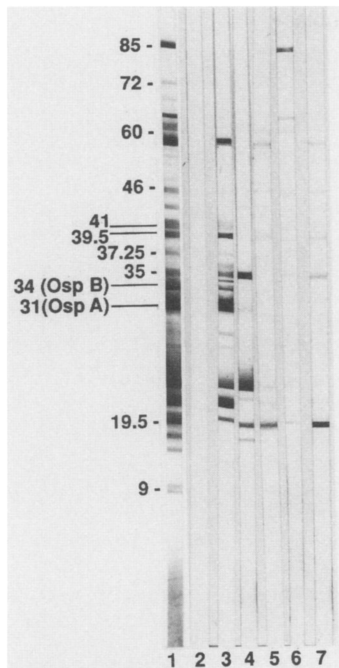

FIG. 2. Western immunoblots of *B. burgdorferi* probed with different sera. Lanes: 1, rabbit anti-*B. burgdorferi* control sample; 2, negative-control deer sample; 3, positive-control sample from a deer immunized with killed *B. burgdorferi* NY90-14; 4, positive-control sample from a deer immunized with live *B. burgdorferi* SH-2-82; 5, deer sample from Carlos Avery Wildlife Management Area that was positive by ELISA; 6, deer sample from Carver Park Reserve that was positive by ELISA; 7, deer sample from Hyland Lake Park Reserve that was positive by ELISA. Molecular sizes in kilodaltons are shown on the left.

The results of this study show that only 2.0% of the sera from areas where the spirochete is endemic were seropositive by ELISA (Table 1). There was a positive correlation of ELISA results with those of the Western immunoblots (Tables 2 and 3). In general, samples with higher OD readings by ELISA yielded greater numbers of reactive bands on Western immunoblots. Many of the reactive bands seen with borderline positive and positive ELISA samples are consistent with the bands seen on Western immunoblots of samples from humans with Lyme borreliosis (3, 7, 8, 10, 12, 15). Some of the reactive bands that may indicate specific anti-*B. burgdorferi* antibody production have antigens of 85, 41, 39.5, 35, and 19.5 kDa (Fig. 2 and Table 4). The 60-kDa antigen may represent a common bacterial antigen (13). The 46-kDa antigen may also represent a nonspecific antigen, because nearly one-half of the bands seen in response to probing with negative ELISA samples were 46 kDa. A response to the two major outer surface proteins, OspA and OspB, was not observed with sera from naturally infected deer (Fig. 2 and Table 4). This lack of response to OspA and OspB in natural infections is not uncommon. It has been shown that humans with Lyme borreliosis have no or little response to these proteins early in the disease (7, 12, 15) and that the antigenic response of naturally infected dogs is similar to that seen with human-sample immunoblot profiles (11). Rabbits and deer immunized with killed spirochetes, however, did show strong antibody responses to OspA and OspB (Fig. 2). Two of 11 positive ELISA samples may have been false positive because they failed to react on Western immunoblots (Table 3).

Serologic analysis of white-tailed deer sera has been done previously by using IFA and ELISA (18–20) but to our knowledge not by using Western immunoblotting. In addition to analyzing wild-deer sera with ELISA, we have also looked at the antibody responses of pen-raised and wild deer to *B. burgdorferi* by using Western immunoblot analysis. From our data, it appears that both immunized deer and naturally exposed wild deer generate a specific antibody response to *B. burgdorferi*.

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