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SEROLOGIC SURVEY FOR ANTIBODIES TO *BORRELIA BURGDORFERI* IN WHITE-TAILED DEER IN GEORGIA

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ABSTRACT: A serologic survey for antibodies to *Borrelia burgdorferi* utilizing an enzyme-linked immunosorbent assay (ELISA) was conducted on white-tailed deer (*Odocoileus virginianus*) serum samples collected in Georgia (USA) from 1979 to 1990. Serologic results from four regions (Barrier Islands, Coastal Plain, Piedmont, and Mountain) and three age classes (0.5, 1.5, and ≥2.5 yr) were compared. Antibody prevalence, as determined by positive results at a 1:64 dilution or higher, was 36% in the Barrier Islands, 14% in the Coastal Plain, 8% in the Piedmont, and 4% in the Mountain regions. Statewide antibody prevalence was 19%. Antibody titers generally were low, and if a more conservative cutoff titer of 1:128 were used, the statewide prevalence estimate would have been reduced to 5%. Antibody prevalence as determined at this higher cutoff value, however, still remained highest in the Barrier Islands and lowest in the Mountains. Prevalence estimates were lower in the 0.5-yr age class than in the 1.5-yr or ≥2.5-yr age class ($P < 0.05$). A more in-depth retrospective study of the Barrier Islands region from 1971 to 1985 revealed a 50% overall antibody prevalence; positive results were found in every year represented except 1990. Based on these results, we propose that *B. burgdorferi* has been present in Georgia since at least 1971.

Key words: *Borrelia burgdorferi*, Lyme borreliosis, white-tailed deer, *Odocoileus virginianus*, enzyme-linked immunosorbent assay, ELISA, antibody prevalence, Georgia.

INTRODUCTION

Antibodies to *B. burgdorferi* have been detected in white-tailed deer (*Odocoileus virginianus*) in many of the endemic areas of the northeastern and northcentral United States, suggesting a significant exposure to this spirochete. Antibodies were detected with an indirect fluorescent antibody (IFA) test in white-tailed deer in Connecticut (Magnarelli et al., 1984a), New York (Magnarelli et al., 1986), and Wisconsin (Anderson et al., 1987) (USA). Antibodies to *B. burgdorferi* in white-tailed deer also have been detected with an enzyme-linked immunosorbent assay (ELISA) in New York (O'Connell et al., 1988). Only two serological studies have involved deer in the southeastern United States: North Carolina (Magnarelli et al., 1986), and Georgia (Magnarelli et al., 1991).

There has been a recent increase in the number of reported human Lyme disease cases in the southeastern United States. In Georgia, reported human cases increased from 59 in 1988 to 715 in 1989 (Georgia Department of Human Resources, 1991). Although changes in case definition have reduced the number of annual cases of reported Lyme borreliosis in Georgia to 31 as of 1991 (Centers for Disease Control, 1992), the disease continues to be detected in the southeastern United States each year. The vectors and reservoir hosts of Lyme borreliosis in this region, however, remain unknown. *Ixodes dammini* is not present in Georgia, and both tick and small mammal species composition differs greatly from endemic areas of the northeastern United States.

Because antibodies to *B. burgdorferi* occur in white-tailed deer, this species may provide an indication of areas of human risk. Therefore, the specific objectives of this study were: 1) to determine the prevalence of antibodies to *B. burgdorferi* in white-tailed deer in Georgia; 2) to test for differences associated with age class and physiographic region; and 3) to determine, through retrospective serologic surveil-

lance, whether *B. burgdorferi* antibodies were present in white-tailed deer in Georgia prior to the recent recognition of human cases.

MATERIALS AND METHODS

ELISA procedure

Antigen for the indirect ELISA was prepared from purified sonicated whole *B. burgdorferi* (A. G. Barbour B31 strain) (Greene et al., 1988) at a protein concentration of 770 mg/dl (Bradford, 1976). The optimum antigen dilution as determined by checkerboard titration was 1:700 in 0.06 M carbonate buffer (final concentration approximated 10 μg protein/ml). Antigen at this dilution was coated on 96-well flat-bottomed polystyrene plates (Fisher Scientific, Norcross, Georgia, USA) by pipetting 50 μl of diluted antigen into each well in column 2 through 12. Column one was used for color (substrate) controls and received only the reagents necessary for the enzymatic oxidation-reduction reaction. Microtiter plates were refrigerated at 4 C for 12 to 24 hr to allow for antigen adsorption.

Microtiter plates were removed from the refrigerator and washed four times with a Vaccupette/96 microplate washer (DBM Scientific Corporation, Vangard Labs, Neptune, New Jersey, USA) with 200 μl per well of phosphate buffered saline (PBS) containing 0.05% Tween-80 (PBS-tween) (Fisher Scientific, 711 Forbes Avenue, Pittsburgh, Pennsylvania, USA).

Serum samples to be tested were thawed at 22 C immediately prior to use. Test and control samples were diluted to 1:64 in PBS-tween. Two positive control serum samples, four negative control serum samples, and two enzyme controls were placed in column two. Each assay of individual serum samples was performed in triplicate with 50-μl aliquots of diluted serum. Following addition of serum, microtiter plates were incubated at 37 C for 30 min. After incubation the plates were washed four times with 200 μl per well of PBS-tween.

Horseradish peroxidase-labeled rabbit affinity purified antibody to deer IgG (gamma), heavy- and light-chain specific (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland, USA), was diluted 1:800 with PBS-tween, and 50 μl were pipetted into all but the color control wells in column one. After 30 min incubation at 37 C, plates were washed four times with 200 μl per well of PBS-tween.

Fifty μl of o-phenylenediamine (OPD) diluted 1:10 with 0.05 M sodium acetate buffer and hydrogen peroxide was pipetted into all wells in columns 1 through 12. The plate then was placed in the dark at 22 C for 30 min. At the end of this time, 30 μl of 8 N sulfuric acid was added to each well to stop the reaction. The first column, which was used as the color control, was used to calibrate the automated plate reader (Titertek Multiscan spectrophotometer, Flow Labs Inc., McLean, Virginia, USA) to zero, and the optical densities were measured at 492 nm.

An individual serum sample was considered to be test positive for the presence of antibodies to *B. burgdorferi* if the mean absorbance of the triplicate samples at a 1:64 dilution was equal to or greater than a value 3 standard deviations above the mean absorbance of the four negative control sera (Grodzicki and Steere, 1988). Sera with positive test results at the screening dilution of 1:64 were retested by diluting the source serum to 1:32 with PBS-tween and performing twofold serial dilutions up to 1:4,096 to determine the endpoint titer utilizing the interpretive criterion previously described. The reported positive antibody titer was calculated by determining the dilution equal to or greater than three standard deviations above the mean absorbance of the four negative control sera at a 1:64 dilution.

Control serum samples

Positive control serum samples were collected from two adult pen-raised female white-tailed deer. Deer were hyperimmunized with a subcutaneous injection of a 0.75-ml suspension of killed *B. burgdorferi*, containing approximately 1.5 mg protein in Freund's complete adjuvant (Sigma Chemical Company, St.Louis, Missouri, USA). Nine days later, deer received a 0.75-ml intramuscular injection of antigen (approximately 1.5 mg) in Freund's incomplete adjuvant (Sigma Chemical Company). Purified *B. burgdorferi* antigen was obtained from Drs. Russell Greene and Jay Levine (North Carolina State University, Raleigh, North Carolina, USA) and was prepared from sonicated whole *B. burgdorferi* (A. G. Barbour B31 strain) (Greene et al., 1988) at a protein concentration of 240 mg/dl (Bradford, 1976). Deer were bled at 36 days postinoculation (PI).

Negative control serum samples were obtained from four additional pen-raised white-tailed deer (2 adult females and 2 male fawns) with no known exposure to *B. burgdorferi*. Serum samples from positive and negative control deer were sent to Dr. Louis A. Magnarelli, The Connecticut Agricultural Experiment Station, New Haven, Connecticut, USA, for interlaboratory comparison and standardization of the ELISA. To address the possibility that there might be cross-reactivity between antibodies for *B. burgdorferi* and some serovars of *Leptospira interrogans*, postinoculation serums from both

positive control deer were tested with a microscopic agglutination test (Cole et al., 1973) for antibodies to the following five serovars of *Leptospira interrogans*: *canicola*, *grippotyphosa*, *hardjo*, *icterohemorrhagiae*, and *pomona*.

Experimentally inoculated deer

In order to validate observed antibody titers in naturally exposed or infected white-tailed deer, a 10-mo-old pen-raised male white-tailed deer was experimentally inoculated with live *B. burgdorferi* strain SH2-82 which had been passed in culture six times (Rocky Mountain Laboratories, Hamilton, Montana, USA). The deer was inoculated intradermally with 1.2 ml and subcutaneously with 1.0 ml of Barbour-Stoenner-Kelly (BSK II) medium (Barbour, 1984) containing approximately 10^7 spirochetes per ml. Subsequent hamster inoculations with this isolate verified infectivity of the SH2-82 strain. Serum samples collected from this animal 1 wk before and immediately prior to inoculation were negative for antibodies to *B. burgdorferi* on the ELISA. Postinoculation serum samples were collected at weekly intervals for 8 wk and tested with the ELISA.

Serum samples collected from this deer before inoculation and at 8 wk PI were screened for antibodies to the *Leptospira interrogans* serovar *bratislava*, as well as for the same serovars as described previously for the control deer.

To verify infection with *B. burgdorferi*, whole blood was collected in EDTA at 1, 2, 3, 4, 6, and 8 wk PI, and ear tissue samples (5 mm punch biopsy) were taken at 1, 3, and 8 wk PI for culture in modified BSK II medium containing the following: 0.15% soft agarose (SeaPrep; FMC Bioproducts, Rockland, Maine, USA), 0.023% L-cysteine hydrochloride, 0.015% DL-dithiothreitol, 1 μg of L-glutamine per ml, 50 μg of rifampin per ml, 20 μg of phosphomycin per ml, and 2.5 μg of amphotericin B (Fungizone) per ml (Sigma Chemical Company, St. Louis, Missouri, USA) (Sinsky and Piesman, 1989). Ear punches were cleaned with hydrogen peroxide and 95% alcohol, then placed in 4.5 ml BSK II in snap cap tubes. Blood (0.1 ml) was inoculated in 7.0 ml of BSK II. All cultures were incubated at 33.5 C and examined for spirochetes by darkfield microscopy twice each week for 2 wk, then once each week for 4 additional wk.

Serologic survey

White-tailed deer serum samples were collected throughout Georgia from 1971 through 1990 and included hunter-killed deer and deer collected during deer herd health evaluations conducted by the Southeastern Cooperative Wildlife Disease Study (SCWDS). Samples from hunter-killed deer were collected from October through December. Samples from herd health evaluations which represented <20% of the total sample were collected from July through September. Test serums were stored at −32 C until ELISA testing was performed.

For data analysis, collection sites were divided into physiographic regions as delineated by Fenneman (1946). However, the Georgia barrier islands which are a part of the Coastal Plain physiographic region were treated as an individual region. Data from the Blue Ridge and Ridge and Valley regions of Georgia also were pooled and were collectively referred to as the Mountain region.

For initial evaluation, serum samples from 787 deer were tested: 277 samples from the Barrier Islands, 203 from the Coastal Plain, 200 from the Piedmont, and 107 from the Mountains. A more in-depth serologic survey was performed on 109 additional samples from the Barrier Islands because it was the region exhibiting the greatest prevalence of high antibody titers.

Age classes of deer were determined by tooth wear (Severinghaus, 1949). Ages for 182 deer were not available and these were not included in the age class comparisons.

Differences in antibody prevalence among physiographic regions and age classes were tested for independence using the *G*-test with Williams' correction (Sokal and Rohlf, 1981). For statistical analyses, white-tailed deer were categorized into age classes of 0.5, 1.5, and ≥2.5 yr. Deer with ages falling between these categories were placed in the closest age class above their determined age.

Evaluation of serologic results

Since initial serologic results were based on cutoff values derived from only four negative control serum samples, serologic results were recalculated using 175 field collected serum samples as negative controls. All of these samples were collected from the upper Piedmont and Mountain physiographic regions from which there are no reports of *Ixodes scapularis* from white-tailed deer (V. Nettles, Southeastern Cooperative Wildlife Disease Study, unpubl. files).

The normal curve for this 175-deer sample was derived by dividing mean optical density (OD) of each sample at the 1:64 dilution by the OD for one of the original control negative serum samples at the 1:64 dilution (S/N ratio). This was done to eliminate individual plate variation and to standardize results. Samples with S/N ratios exceeding the mean for the 175 field control negatives plus 3 standard deviations were considered positive.

TABLE 1. Prevalence of serum antibodies to *Borrelia burgdorferi* in white-tailed deer from four physiographic regions in Georgia.

Physiographic region	Number tested	Years collected	Number positive (% positive)			
			≥1:64	≥1:64*	≥1:128	≥1:256
Mountain	107	1979–90	4 (4%)[b]	2 (2%)[b]	1 (1%)[b]	0 (0%)[b]
Piedmont	200	1986–90	16 (8%)[b,c]	11 (6%)[b,c]	5 (2%)[b]	1 (<1%)[b]
Coastal Plain	203	1980–89	28 (14%)[c,d]	19 (9%)[c,d]	6 (3%)[b]	1 (<1%)[b]
Barrier Island	277	1986–89	100 (36%)[e]	61 (22%)[e]	30 (11%)[c]	10 (4%)[c]
Total	787		148 (19%)	93 (12%)	42 (5%)	12 (<2%)

* Results based on the distribution of sample optical density (OD)/control negative OD (S/N ratio) for 175 white-tailed deer serum samples from north Georgia. Cutoff S/N ratio equals mean ratio (0.60) plus three standard deviations (1.76).

[b,c,d,e] Prevalence estimates with the same superscripts within each column were not significantly different ($P < 0.05$).

RESULTS

Control and experimentally inoculated deer serum samples

The two positive control deer given killed B-31 strain *B. burgdorferi* had antibody titers of 1:2,048 and 1:4,096 at 36 days PI. Antibody titers for these two serum samples as tested by Dr. Louis A. Magnarelli were 1:2,560 and 1:5,120. Serum samples from both deer were negative for antibodies to all five previously stated *Leptospira interrogans* serovars.

Titers of serum samples collected at weekly intervals from the deer inoculated with live *B. burgdorferi* strain SH2-82 increased slowly and reached a peak at 1:128 at 4 wk PI. Test results for serum samples collected from the experimentally inoculated deer at preinoculation and at 8 wk PI were negative for antibodies to all six previously stated *Leptospira interrogans* serovars. Spirochetes were not isolated from blood or ear tissue cultures throughout the 8-wk period.

Serologic results

Serum IgG antibodies to *B. burgdorferi* were detected at the 1:64 dilution or greater in 19% of (n = 787) white-tailed deer serum samples that were tested throughout Georgia from 1979 to 1990 (Table 1). Antibody prevalence was significantly higher ($P < 0.05$) in deer from the Barrier Islands region (36%, n = 277) than in deer from the other three physiographic regions. A significant difference ($P < 0.05$) also was detected between antibody prevalence estimates for the Coastal Plain and Mountain regions. Antibody prevalence in deer was 4% (n = 107) in the Mountain region, 8% (n = 200) in the Piedmont region and 14% (n = 203) in the Coastal Plain region. Physiographic regions of Georgia, counties tested, and counties where serum samples with antibodies to *B. burgdorferi* were detected are shown in Figure 1.

Antibodies were detected at the 1:64 dilution or greater in 50% of 109 additional

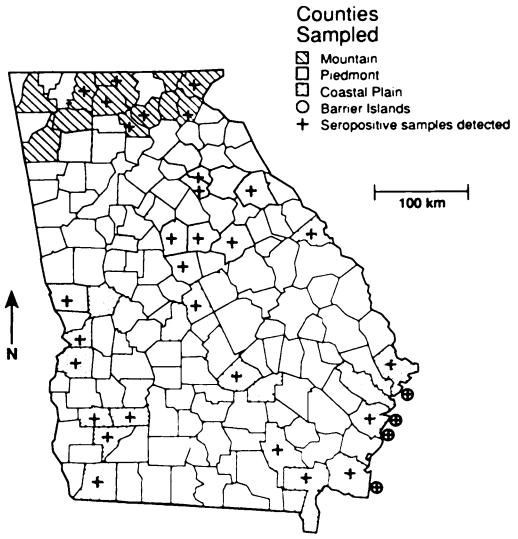

FIGURE 1. Physiographic regions and areas where white-tailed deer serum samples with antibodies to *Borrelia burgdorferi* were detected.

TABLE 2. Prevalence of serum antibodies to *Borrelia burgdorferi* in white-tailed deer from the barrier islands 1971 to 1985.

Year	Number tested	Number positive (% positive)			
		≥1:64	≥1:64[a]	≥1:128	≥1:256
1971	5	2 (40%)	2 (40%)	1 (20%)	0 (0%)
1972	5	5 (100%)	5 (100%)	3 (60%)	0 (0%)
1974	5	4 (90%)	2 (30%)	3 (60%)	1 (20%)
1975	5	3 (60%)	1 (20%)	2 (40%)	0 (0%)
1981	18	14 (78%)	18 (100%)	8 (44%)	1 (20%)
1982	4	2 (50%)	2 (30%)	0 (0%)	0 (0%)
1983	14	4 (29%)	2 (14%)	3 (21%)	0 (0%)
1984	28	12 (43%)	13 (46%)	8 (29%)	3 (11%)
1985	25	9 (36%)	11 (44%)	5 (20%)	0 (0%)
Total	109	55 (50%)	56 (51%)	33 (30%)	5 (5%)

[a] Results based on the distribution of sample optical density (OD)/control negative OD (S/N ratio) for 175 white-tailed deer serum samples from north Georgia. Cutoff S/N ratio equals mean (0.60) plus three standard deviations (1.76).

serum samples collected from the Barrier Islands from 1971 to 1985 (Table 2). Prevalence of antibodies to *B. burgdorferi* for all of the Barrier Islands serum samples collected from 1971 to 1989 was 40%. Positive results were found in every year tested from 1971 to 1990 except 1990 when only 10 samples were tested.

Titers ranged from 1:64 to 1:2,048. Most seropositive animals, however, tested positive only at the minimum 1:64 dilution (Table 1). Higher titers (≥1:128) were detected most frequently in deer from the Barrier Islands; this also was the region where the one 1:2,048 titer was detected.

Although antibody prevalence increased with age for results at both the 1:64 and 1:128 dilutions, significant differences ($P < 0.05$) in antibody prevalence were detected only between the 0.5-yr age class and the older age classes at the 1:64 dilution (Table 3).

TABLE 3. Prevalence of serum antibodies to *Borrelia burgdorferi* among three age classes of white-tailed deer from Georgia (1979 to 1990).

Age class (yr)	Number of samples tested	Number (%) seropositive	
		≥1:64	≥1:128
0.5	104	10 (10%)[a]	3 (3%)[a]
1.5	200	49 (24%)[b]	11 (5%)[a]
≥2.5	480	127 (31%)[b]	25 (6%)[a]
Total	712	186 (25%)	39 (5%)

[a,b] Prevalence estimates with the same superscripts within each column were not significantly different ($P < 0.05$).

Evaluation of serologic results

The mean S/N ratio for the 175 field control negatives was 0.60. With 3 standard deviations added to the mean, the final S/N cutoff value was 1.76. Prevalence estimates using this criteria were similar to previous results (Tables 1, 2). Significant differences in prevalence by physiographic region were the same as reported at the 1:64 dilution as calculated using the four control negative serum samples.

DISCUSSION

Since antibodies to *B. burgdorferi* in white-tailed deer were detected in every year examined from 1971 to 1989, there is serologic evidence that Lyme borreliosis has been present in Georgia for at least 20 years. The statewide prevalence of antibodies to *B. burgdorferi* of 19% found in Georgia is similar to prevalence estimates reported for Wisconsin (16.5%, $n = 97$) (Anderson et al., 1987), North Carolina (11.5%, $n = 209$) (Magnarelli et al., 1986), east Texas (USA) (12.3%, $n = 73$) central Texas (15%, $n = 60$) (Rawlings, 1986), and Connecticut (17.8%, $n = 631$) (Magnarelli et al., 1986). Our prevalence estimates ex-

ceed those recently reported for Cumberland Island, Georgia (Magnarelli et al., 1991). This most likely relates to the difference in serum dilutions used between laboratories, (1:160 vs. 1:64). If a 1:128 minimum dilution is used as a cutoff value, our prevalence estimate for Cumberland Island (n = 92) would be 11% which is very close to the 7% antibody prevalence estimate reported by Magnarelli et al. (1991) at a 1:160 dilution. These differences emphasize the need for standardization of serological tests for antibodies to *B. burgdorferi* in white-tailed deer. Without such standardization comparisons of antibody prevalence estimates from different studies are inappropriate.

Reported titers of seropositive deer as determined by IFA test usually were between 1:64 and 1:512 (Magnarelli et al., 1986). Serum samples tested have comparable results regarding titration endpoints with both an ELISA and IFA test (O'Connell et al., 1988). Although infection could not be confirmed by culture of *B. burgdorferi*, a 1:128 titer in the experimentally inoculated deer in this study also was well within the most commonly determined titers detected in field samples. If a more conservative interpretation of this data set were used, setting the positive threshold titer to ≥1:128, the number of deer reacting positively would have been greatly reduced, with the statewide antibody prevalence dropping from 19% to 5% (Table 1). Using this more conservative criterion, however, did not alter the pattern of antibody prevalence among the four physiographic regions, with antibody prevalence remaining highest in the Barrier Island region and lowest in the Mountain region. Variation in antibody prevalence by physiographic region cannot be explained at this time, but may be related to tick abundance and species composition. In Georgia, the most common tick species recovered from white-tailed deer include *I. scapularis* and *Amblyomma americanum*. These species are abundant on the Barrier Islands and throughout the Coastal Plain and lower Piedmont. They are rare or absent in the upper Piedmont and Mountains. *Ixodes affinis* is not as common and is recovered from deer only on the Barrier Islands and extreme coastal areas. *Amblyomma maculatum* is restricted to the Coastal Plain and Barrier Islands. Similar patterns of coastal *B. burgdorferi* activity have been reported in endemic areas of New Jersey (Schulze et al., 1984) and Connecticut (Magnarelli et al., 1984b).

Statewide prevalence estimates also were influenced by disproportionate sample size with regard to deer age class and physiographic region. For example, older age-class deer predominated in the Barrier Islands region, and samples from this region were more available than in the other regions. Because antibody prevalence and sample size were the highest in the Barrier Islands region which represents a very small portion of the state, the actual statewide prevalence in Georgia, even at the ≥1:64 dilution cutoff, probably is lower than the 19% estimate reported in this study.

Problems have been reported with regard to specificity of tests used to detect antibodies to *B. burgdorferi* and other *Borrelia* species and bacterial organisms, including *B. hermsii*, *B. recurrentis*, *Treponema* spp., and *Leptospira* spp. (Magnarelli et al., 1987). A higher positive (≥1:128) threshold titer probably would reduce this potential cross-reactivity. Although *B. hermsii* and *B. recurrentis* are not known to be present in Georgia, the possibility of a previously undescribed and cross-reacting spirochete in white-tailed deer cannot be totally ignored. Western blot techniques have been used in past studies of other species to help confirm antibody specificity to *B. burgdorferi*, but this technique has not been applied to or tested in white-tailed deer.

The ELISA serology is a feasible method of detecting antibodies to *B. burgdorferi* in white-tailed deer, and given that white-tailed deer are widespread throughout the United States, serologic surveillance of this species could be used to identify foci of *B.*

burgdorferi for further epidemiological studies. Additional work, however, is required to both standardize and validate existing serological tests applied to this species.

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