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ANTIBODIES TO *BORRELIA BURGDORFERI* IN DEER AND RACCOONS

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ABSTRACT: An enzyme-linked immunosorbent assay (ELISA) was developed to detect serum antibodies to *Borrelia burgdorferi*, the causative agent of Lyme borreliosis, in deer (*Odocoileus virginianus*) and raccoons (*Procyon lotor*). Blood samples were collected from these mammals in Connecticut, Maryland, North Carolina, Georgia and Florida. Seropositivity for deer was highest in Connecticut (56% of 353 sera) and Maryland (51% of 35 sera). Raccoons in Connecticut, Maryland, North Carolina, and Florida also had antibodies to *B. burgdorferi*, but prevalence of positive sera was highest in Maryland (79% of 14 samples). Based on adsorption tests, the immunoglobulins detected in these mammals were probably specific to *B. burgdorferi*. The ELISA was more sensitive than an indirect fluorescent antibody staining method and was more suitable for analyzing large numbers of serum samples.

Key words: *Borrelia burgdorferi*, Lyme borreliosis, antibodies, enzyme-linked immunosorbent assay, deer *Odocoileus virginianus*, raccoons *Procyon lotor*.

INTRODUCTION

Ticks in the *Ixodes ricinus* complex parasitize and transmit *Borrelia burgdorferi* to human beings, domestic animals and wildlife (Carey et al., 1980; Burgdorfer and Keirans, 1983; Burgdorfer, 1989; Anderson, 1989; Anderson et al., 1990; Magnarelli et al., 1990a). Presence of this bacterium in ticks, mammals and birds has been verified by isolation and immunochemical characterization procedures (Burgdorfer et al., 1982; Burgdorfer, 1989; Anderson et al., 1990). In addition, serum antibodies to *B. burgdorferi* have been detected by indirect fluorescent antibody (IFA) staining procedures or enzyme-linked immunosorbent assays (ELISA) (Magnarelli et al., 1984a, b, 1986, 1990a, b; Magnarelli and Anderson, 1989; Magnarelli, 1989; Godsey et al., 1987). The latter method is often preferred because of automation, ease of standardization and more objective test results that can be statistically analyzed.

White-tailed deer (*Odocoileus virginianus*) and raccoons (*Procyon lotor*) are parasitized by *Ixodes dammini* and develop antibodies to *B. burgdorferi* (Magnarelli et al., 1984a, 1986). Since sera can be collected from deer during the hunting season and from raccoons that are easily captured, these animals are particularly suitable for testing and surveillance programs. The main objective of this study was to develop and evaluate an ELISA for deer and raccoons to facilitate seroanalyses in the surveillance of Lyme borreliosis. Another goal was to further determine the geographic distribution of this disease in the eastern United States.

MATERIALS AND METHODS

Study sites and sampling

Blood samples were collected from white-tailed deer, Sika deer (*Cervus nippon*) and raccoons captured in tick-infested areas of Connecticut, Maryland, North Carolina, Georgia and Florida (USA) in 1984, 1987 to 1989 (deer) or during 1977 to 1984 (raccoons). Examination of these mammals for *I. dammini*, *I. scapularis* and other ticks and prevalence of tick parasitism will be reported separately. Blood specimens were collected from deer that had been killed during fall hunting seasons. In Connecticut, samples were obtained at state check stations located in the towns of Barkhamsted, Bethel, East Haddam, East Lyme, Haddam, Marlborough, West Stafford, and Voluntown. *Ixodes dammini* is common in many of these towns. Additional deer sera (normal specimens) were collected in northwestern Connecticut (check stations at Sharon, Litchfield, and Middlebury), where *I. dammini* and Lyme borreliosis are rare. Other deer sera were collected in Maryland and

Georgia, states with records of *I. dammini* or *I. scapularis*, respectively. Positive and negative serum controls were available from previous work (Magnarelli et al., 1986). Raccoons were captured alive in Tomahawk traps during summers from sites in southern Connecticut and in Maryland, North Carolina, and Florida. Animals were anesthetized as previously described (Magnarelli et al., 1984a) before blood samples were collected by cardiac puncture. Following a recovery period, animals were released unharmed into their natural habitats. Sera collected during 1980 in Newtown, Connecticut, a town where *I. dammini* and Lyme borreliosis was uncommon, served as negative controls. For positive serum controls, blood samples were obtained from two raccoons 6 to 8 wk after they were each inoculated four times over 19 days intraperitoneally or in the region of the left axillary lymph node with living *B. burgdorferi* (CT strain 2591). Numbers of spirochetes in inocula (0.5 ml of BSK II medium) ranged from 2.0 to 7.0 $\times$ 10^7. Additional positive serum samples used previously (Magnarelli et al., 1984a) also were included. All serum samples were stored at −60 C until analyses could be performed.

Serologic tests

Indirect fluorescent antibody staining methods and ELISA were used to detect antibodies to *B. burgdorferi* (Magnarelli et al., 1984a, b, 1986). Procedures for ELISA were similar to those described for analyses of human and dog sera (Magnarelli et al., 1984b). Washed whole cells of *B. burgdorferi* (5 to 15 μg protein/ml of CT. 2591) were coated to the solid phase at 37 C for 18 to 20 hr. Plates were filled with blocking solutions and washed. Test sera were diluted to 1:160, 1:320, and 1:640 in phosphate buffer saline (PBS) solution containing 0.05% Tween 20, 5.0% horse serum, and 50 μg of dextran sulfate/ml. Plates with diluted sera were incubated at 37 C for 1 hr and washed to remove unbound antibodies. Horseradish peroxidase-labeled rabbit anti-deer and goat anti-raccoon immunoglobulin G antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland 20879, USA) were diluted in PBS solution to 1:4,000 and 1:800, respectively. Plates containing these polyvalent conjugates were incubated and washed as in previous steps. Following the addition of 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonate) substrate (Kirkegaard and Perry Laboratories) to each well and a 45-min incubation period, absorbance values (optical densities) were recorded at 414 nm by using a microtiter spectrophotometer. Net absorbance values were calculated by subtracting readings for PBS controls (i.e., wells without antigen) and compared to values determined for negative serum controls. Critical regions for positivity were determined by statistical analyses (three standard deviations + mean) of net absorbance values for 30 normal deer sera or 30 normal raccoon sera. Readings for deer sera were considered positive if net optical densities exceeded 0.10, 0.08, and 0.07 for the respective serum dilutions of 1:160, 1:320, and $\geq$1:640. Although antiserum to white-tailed deer was used to prepare the conjugated reagent, the polyvalent, peroxidase-labeled goat anti-deer antibodies reacted similarly in assays for both genera of deer. Therefore, the same cut-off optical density values were used to determine positive results. A net optical density of >0.08 was considered positive for diluted raccoon sera. All analyses were standardized by using the same negative and positive control sera. Newly purchased enzyme-labeled antibodies were tested to determine optimal working dilutions, and tests on reproducibility of results were conducted to verify that standardization had been achieved.

Specificity

To determine if deer and raccoon sera contained antibodies to *Leptospira* sp. or *Treponema* sp. spirochetes, unadsorbed and adsorbed samples were screened by an IFA staining method or ELISA. In tests for *Leptospira* sp. antibodies, *Leptospira interrogans* serovars *canicola* (strain Moulton) or *pomona* (strain MLS) were used as antigens in an IFA assay. Details on the sources and preparation of these and other reagents and on adsorption procedures have been described (Magnarelli et al., 1986, 1990b). The following were used in adsorption experiments: a 1:2 or 1:5 dilution of commercially prepared sorbent (*Treponema phagedenis* biotype Reiter) or washed cells of *B. burgdorferi* or *L. interrogans* serovars *canicola* or *pomona*. Sorbents were added to test sera in wells of microtiter plates prior to analyses to remove heterologous or homologous antibodies. Protein contents of washed *L. interrogans* or of *Borrelia* sp. cells were determined by performing a commercially available assay (Bio-Rad, Richmond, California 98404, USA) and were standardized to 30 μg protein/ml after preliminary testing of a range of concentrations (20 to 60 μg protein/ml). Each sorbent was mixed in equal volumes (1:1) with test sera. Adsorbed and untreated (control) sera were held at room temperature (23 ± 3 C) for 90 min and subsequently tested against *B. burgdorferi* in antibody assays.

RESULTS

Deer sera from Connecticut, Maryland and Georgia were tested for antibodies

TABLE 1. Number of serum samples from *Cervus nippon* (Sika deer) or *Odocoileus virginianus* (white-tailed deer) in eastern United States with antibodies to *Borrelia burgdorferi* by IFA staining methods or ELISA.

Sites	Years	IFA		ELISA	
		Total sera tested	Number (%) positive[a]	Total sera tested	Number (%) positive[a]
Connecticut[b]	1984, 1989	387	83 (22)	353	197 (56)
Cumberland Is., Georgia[b]	1987	124	2 (2)	42	3 (7)
Assateague Is., Maryland[c]	1987, 1988	38	13 (34)	35	18 (51)
Totals		549	98 (18)	430	218 (51)

[a] IFA positive = ≥1:64; ELISA positive = ≥1:160.

[b] White-tailed deer; IFA results for some sera obtained in Connecticut have been reported earlier (Magnarelli et al., 1986) and are included here for comparison.

[c] Sika deer.

to *B. burgdorferi*. There were positive specimens from each state (Table 1). Prevalence of seropositive specimens was highest in Connecticut and Maryland, regardless of the assay method used; the ELISA appeared to be more sensitive than the IFA method. For example, more than 350 samples from Connecticut were tested by each method. Seropositivity in ELISA (56%) was more than 2-fold greater than results obtained by IFA staining (22%). Titration endpoints for high-titered samples were comparable; maximal antibody concentrations in ELISA and IFA analyses were 1:10,240 and 1:4,096, respectively. To determine reproducibility, 25 positive and 15 negative serum samples were reanalyzed in ELISA. In the second test, 24 of 25 specimens remained positive, while results for all 15 negative samples were unchanged. A single serum sample was positive at a dilution of 1:160 in the first trial and was negative in the second test. Differences in the remaining antibody titers were unchanged (n = 9) or varied by 2 fold (n = 14) or 4 fold (n = 1).

Serum samples of raccoons captured in Connecticut, Maryland, North Carolina, and Florida contained antibodies to *B. burgdorferi*. Seropositivity in ELISA (Table 2) varied from 15% (Connecticut) to 79% (Maryland). Although antibody titers ranged between 1:160 and 1:40,968, the majority were relatively low (1:160–1:1,280). In Connecticut, antibody concentrations were highest (≥1:5,120) for sera collected during June through August. Serum samples from all four states (n = 206) were included in comparative analyses of testing procedures. Of these, results for 155 samples were positive (n = 11) or negative (n = 144) by IFA staining and ELISA. For the remaining 51 serum samples, ELISA was more sensitive. Forty seven specimens were positive by this method and negative by IFA staining. There were four sera positive by immunofluorescence staining and non-reactive in ELISA. Maximal antibody titers were higher in ELISA (1:40,960) than by the IFA method (1:2,048). To verify reproducibility, 40 serum samples were reanalyzed by an ELISA on different days. Of these, 24 of 25 positive samples remained reactive in the second trial. Titration end points were identical (n = 8) or differed by 2 fold (n = 13) or 4 fold (n = 3). One sample was positive initially (1:160) and non-reactive subsequently. In the retesting of 15 negative serum samples, 14 remained negative in the second trial. One sample was negative in the first test and positive (1:160) after reanalysis. Antibody titers for inoculated raccoons were 1:128 and 1:256 by an IFA staining method and 1:5,120 by ELISA.

Deer and raccoon sera were tested for antibodies to *L. interrogans* spirochetes by an IFA staining method. Of the 47 samples tested from deer killed in Connecticut, Maryland and Georgia, none had serologic

TABLE 2. Number of serum samples from raccoons in eastern United States with antibodies to *Borrelia burgdorferi* in ELISA.

Sites	Total sera tested	Number (%) positive	Number samples at reciprocal titers			
			160–320	640–1,280	2,560–5,120	≥10,240
Connecticut	304	45 (15)	17	19	7	2
Assateague Island, Maryland	14	11 (79)	5	5	1	0
Cape Hatteras, North Carolina	40	15 (38)	6	7	0	2*
Merritt Island, Florida	12	4 (33)	1	3	0	0
Totals	370	75 (20)	29	34	8	4

* Maximum titer recorded (1:40,960).

evidence of previous or current *L. interrogans* infections. Similar results were recorded for 55 of 57 raccoon serum samples collected in Connecticut and North Carolina. The remaining two specimens reacted to *L. interrogans* serovars *canicola* and *pomona* at dilution of 1:64, but antibody titers to *B. burgdorferi* were considerably higher (1:20,480 and 1:40,960).

Adsorption procedures were used to determine whether antibodies that reacted with *B. burgdorferi* were specific or cross-reactive. Following treatment of 23 positive deer sera with a 1:5 dilution of *T. phagedenis* biotype Reiter sorbent, there was a 2-fold decline in antibody titers to *B. burgdorferi* in 21 specimens. A 4-fold drop in antibody concentration was recorded for two samples. In separate experiments with washed *L. interrogans* cells as sorbent, IFA titers to *B. burgdorferi* were identical for adsorbed and unadsorbed sera. However, application of washed whole cells of *B. burgdorferi* to positive sera in duplicate trials resulted in 8-fold or greater declines in antibody titers for 13 specimens, regardless of the assay method used. This change in results was beyond normal, two-fold variability. The remaining titers varied by 4 fold or less. Five raccoon sera, having IFA titers of 1:512 to 1:2,048 to *B. burgdorferi*, were chosen for similar adsorption tests. Following treatment with either *T. phagedenis* sorbent (1:5 dilution) or washed cells of *L. interrogans* serovars, changes in titration end points were 4 fold or less by IFA staining. There were no changes in antibody titers when sera from inoculated raccoons were tested. However, the addition of *B. burgdorferi* cells to sera, including the positive controls, in duplicate trials resulted in an 8-fold decline in antibody concentrations. Negative serum samples (controls) for deer and raccoons remained non-reactive in tests for *B. burgdorferi* with or without the use of sorbents. In reanalyses of deer and raccoon sera, increased concentrations of *T. phagedenis* sorbent (1:2 dilution) did not reduce antibody concentrations.

DISCUSSION

Indirect fluorescent antibody and ELISA methods are suitable for detecting serum antibodies to *B. burgdorferi*. Both assays have aided clinical diagnoses of Lyme borreliosis infections in humans (Steere, 1989; Magnarelli et al., 1987; Magnarelli, 1989) and domestic animals (Magnarelli and Anderson, 1989; Magnarelli et al., 1984a, b, 1990a) and have provided information on the geographic distribution of *B. burgdorferi* in wildlife populations (Magnarelli et al., 1984a, 1986). In the present study, ELISA seemed to be more sensitive.

The geographic distribution of Lyme borreliosis is strongly correlated with the presence of ticks in the *I. ricinus* complex (Steere, 1989; Burgdorfer, 1989; Anderson, 1989). Attack rates for human infections are relatively high in parts of the northeastern United States (Petersen et al., 1989; Steere, 1989) where *I. dammini* abounds. Numerous studies have verified the presence of *B. burgdorferi* in ticks and mammals (including raccoons) collected in Connecticut, New York State and Rhode

Island (Anderson, 1989). Based on our serologic results for deer and raccoons and on reports of human infections in tick-infested areas of Maryland, North Carolina, Georgia and Florida (Tsai et al., 1989), it appears that Lyme borreliosis occurs in many sites along the eastern coast of the United States. The relatively lower prevalence of seropositive raccoons, as compared to deer, in Connecticut might be due to differences in the degree of exposure to infected ticks, host immune responses to *B. burgdorferi* and/or in persistence of antibodies.

Aside from being chief hosts for adult *I. dammini*, the role of deer in the epizootiology of Lyme borreliosis is unclear. Spirochetes, presumably *B. burgdorferi*, have been observed by dark-field microscopy in urine and blood samples taken from white-tailed deer (Bosler et al., 1983, 1984). However, these organisms were not subcultured in Barbour-Stoenner-Kelly medium or tested by IFA staining with murine monoclonal antibody (H5332). The specific identity of these organisms is therefore unknown. In other studies (Telford et al., 1988), authors have concluded that white-tailed deer are reservoir incompetent. In southeastern states, *I. scapularis* is a suspected vector of *B. burgdorferi*, but this agent has yet to be isolated from these ticks or from lone-star ticks (*Amblyomma americanum*) there. Accordingly, further studies of arthropod vectors and vertebrate reservoirs are needed to support serologic test findings and clinical reports that indicate the occurrence of Lyme borreliosis in southern United States.

There is potential for false positive results in serologic tests for Lyme borreliosis when whole cell *B. burgdorferi* is used. For example, the flagellar components of *Borrelia* sp. and *Treponema* sp. spirochetes share common epitopes (Baker-Zander and Lukehart, 1984; Luft et al., 1989). Consequently, immunoglobulins directed to these polypeptides cross-react in heterologous antibody tests against *Borrelia* sp. and *Treponema* sp. antigens. Treponemal antibody reactivity to *B. burgdorferi* in serologic assays for human sera is reported (Magnarelli et al., 1987, 1990b), whereas false-positive reactivity due to *Leptospira* sp. antibody is minimal. In addition to flagellin, there are surface proteins of *B. burgdorferi*, such as those having a molecular mass of about 60,000 daltons, that are common to *Escherichia coli* and other bacteria (Hansen et al., 1988). Homologous antibodies to these bacteria may also contribute to non-specific reactivity in tests for Lyme borreliosis. However, adsorption with *T. phagedenis* sorbent or washed cells of *L. interrogans* had little or no effect on serologic reactivity of deer or raccoon sera to *B. burgdorferi*. Conversely, the use of *B. burgdorferi* cells as sorbent effectively removed antibodies from sera. Since cut-off levels for positive results were appropriately set to minimize or eliminate cross-reactivity of low concentrations of nonspecific antibodies, the immunoglobulins detected in the present study were probably specific to *B. burgdorferi*. The high-titered samples collected in Connecticut, where *I. dammini* and *B. burgdorferi* are widely distributed, are particularly convincing. Now that peroxidase-labeled antisera to deer and raccoons are commercially available, Western blot analyses can be conducted to verify antibody production to specific outer surface protein A or other key immunodominant polypeptides of *B. burgdorferi*. Nonetheless, isolation of *B. burgdorferi* is ultimately required to confirm that antibodies present in deer and raccoons in more southern states are indeed specific to this bacterium.

Serologic analyses can provide important information in surveillance programs. Detection of antibodies to *B. burgdorferi* in dogs has been especially useful in identifying foci of Lyme borreliosis (Magnarelli et al., 1985; Greene et al., 1988). These analyses have supplemented tick surveillance studies and evaluations of human case reports and can be particularly help-

ful in areas where tick vectors and *B. burgdorferi* are less prevalent. However, the recent introduction of an experimental vaccine for canine borreliosis in Connecticut and other states may confuse interpretations of serologic test results for dogs in the future. If this vaccine receives widespread acceptance and use, serologic assays for antibodies in deer, raccoons, white-footed mice (*Peromyscus leucopus*) and other wildlife species may become more important in national surveillance programs.

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