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Serological Evidence for *Borrelia* sp. in *Peromyscus leucopus* from Western Kentucky

Zixing Wang, Leon Duobinis-Gray, Stephen White,
Ed Snoddy, and James Stuart*

[1]Department of Biological Sciences, Murray State University,
Murray, Kentucky 42071
[2]OSA 1S 104B, Tennessee Valley Authority,
Water Resources, Muscle Shoals, Alabama

Abstract

During this study, 312 *Peromyscus leucopus* were surveyed for the presence of *Borrelia* sp. Mouse serum was screened for antibodies against *Borrelia* sp. by an immunoblot technique using whole spirochete antigen. By this technique, 21 of 312 (6.7%) of the samples appeared to be serologically positive. Each positive sample was further tested by western blotting against *B. burgdorferi* whole-cell proteins. Eighteen (5.7%) of the 21 samples were confirmed to possess antibodies which bound to the 39/41 kDa protein band on western blots. Equal numbers of positive samples (9 and 9) were obtained from the eastern and western shores of Kentucky Lake. A total of 40 ticks were removed from 24 of 312 mice and examined by the indirect fluorescent antibody (IFA) technique for the presence of spirochetes. All samples were negative by this method. Mouse ear biopsies were collected and incubated in BSK medium, however *B. burgdorferi* was not recovered by this culturing technique.

Introduction

Borrelia burgdorferi is a bacterial spirochete which is the etiological agent of Lyme disease. This agent has become the most common vector-borne pathogen in the United States (7) and is transmitted by ticks.

The primary tick vector of *B. burgdorferi* in the northeastern portion of the United States is *Ixodes scapularis* (6, 7, 11), whereas *I. pacificus* is most common in the western U.S. While neither of these vectors have been reported from Kentucky, it does appear that Lyme disease transmission is occurring in the state. Pelletier et al. (7) reported that 51 Kentucky cases of Lyme disease, based upon CDC criteria, met the case definition between 1985 and 1990.

This information suggests that either *I. scapularis* is present, but not yet identified, or another vector is transmitting the disease. Recent evidence by Teltow et al. (12) in Texas and Luckhart et al. (3) in Alabama indicated that other species of ticks harbor *B. burgdorferi*. Among the species noted was the lone star tick, *Amblyomma americanum*, a common tick in western Kentucky.

Levine et al. (2) reported that in the northeastern United States the white-footed mouse, *Peromyscus leucopus*, serves as the primary vertebrate host for *I. scapularis* larvae and nymphs, thus it is the primary reservoir host for *B. burgdorferi*. In the southeastern U.S., *Dermacentor variabilis* larvae and nymphs infest *P. leucopus* most often. Schwan et al. (8) reported that white-footed mice remain persistently infected with *B. burgdorferi*. This information indicated that infected mice should be seropositive for antibodies against *B. burgdorferi* long after initial exposure and elevated antibody titers should be detectable weeks or even months after infection.

In order to ascertain whether *B. burgdorferi* possibly exists in western Kentucky, a study was initiated to examine the serum of white-footed mice for antibodies against *Borrelia* sp. The study was also designed to attempt culture of spirochetes from tissue samples of white-footed mice. Additionally, ticks removed from mice were analyzed by indirect fluorescent antibody technique (IFA) for the presence of *Borrelia* sp.

Materials and Methods

Bacterial Strain.—The *B. burgdorferi*–Guilford strain used in this study was obtained from Dr. Gary Mullin, Dept. of Entomology, Auburn University, Alabama 36849. The Guilford strain is a high-passage isolate of *B. burg-*

* Corresponding author.

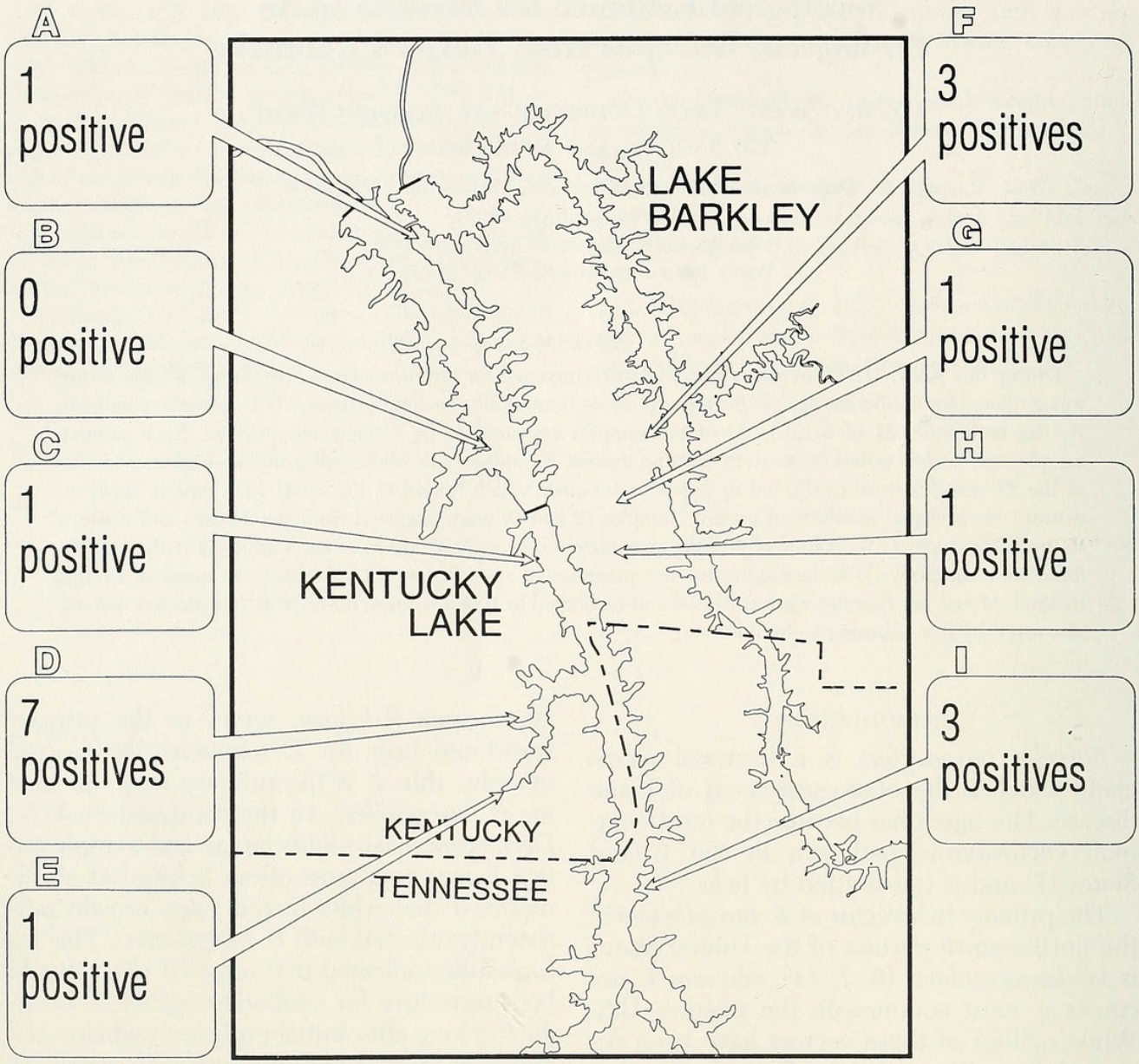

FIG. 1. Sample localities from LBL and TVA recreation areas: a total of 200 *P. leucopus* were collected from TVA public use areas on the western shore of Kentucky Lake: B (Jonathan Creek), C (White Sands Beach), D (Wildcat Creek), and E (Blood River). The positive samples were found in localities C, D, and E. A total of 112 *P. leucopus* were collected from the eastern shore of Kentucky Lake (LBL areas A, F, G, H, and I). Mice with positive sera were found in each of these five localities.

dorferi originally isolated by Alan Steere from *Ixodes* in Guilford, Connecticut.

Animal Capture.—Between 30 June 1990, and 29 August 1991, 185 *P. leucopus* were live-trapped at 4 sites along the western shore of Kentucky Lake in Calloway and Marshall counties, Kentucky. The 4 sites (Fig. 1), Blood River (BR), Wildcat Beach (WC), White Sands Beach (WS), and the Boy Scout Camp on Jonathan Creek (JC) were selected as areas of high human activity and potential human exposure to *B. burgdorferi*. Each site included campgrounds, beaches, picnic areas, and/or natural areas and trails that concentrate recreational activities on TVA-owned lands. Additionally, 127 mice from the eastern shore of

Kentucky lake at Land Between the Lakes (LBL) recreation area were examined (Fig. 1).

Sherman live traps baited with peanut butter or bird seed were set and checked daily. Captured animals were transported to laboratory facilities at Murray State University.

Animal Handling.—Following transport, mice were held for a period of at least 3 days over water-filled containers lined with double-sided tape in order to collect engorged ticks. Any ticks collected were placed into vials and held at 4°C for analysis by IFA (see below). At the end of the holding period, animals were sacrificed by etherizing, bled, and ears removed. Blood was allowed to clot, and serum was collected, frozen at −80°C, and analyzed by immunoblotting. Ear punch biopsies were performed on each mouse as described below. Ear tissue remaining from the mice was stored frozen at −80°C.

Cultivation of Borrelia burgdorferi.—The medium for cultivation was the standard Barbour-Stoenner-Kelly (BSK) described by Barbour (1). The ear-punch biopsy method of Sinsky and Piesman (10) was initially used to culture spirochetes from captured wild mice. During the study this method was modified as follows. A pie-shaped wedge was clipped from the ear and soaked in 10% bleach for 10 min followed by a 10-min soak in 70% ethanol. The tissue was minced, added to 100 µl of BSK in a microcentrifuge tube and incubated at 32°C for 2 weeks in a candle jar. The culture was checked twice (7 days and 14 days) under a phase contrast microscope for the presence of spirochetes. With each new batch of media, positive controls were cultured by inoculating fresh media with a 10 µl aliquot of viable spirochetes (Guilford) and incubating at 32°C.

Indirect Fluorescent Antibody (IFA) Testing.—This test was modified from a procedure described by Luckhart et al. (3). Ticks were dissected, and the contents mixed with one drop of sterile water on the surface of a clean slide. This was allowed to air dry, and fixed by mild heating. Fifty µl of polyclonal rabbit anti-*B. burgdorferi* serum (1/50) was added to the tick smear, and the slide was incubated for 30 min at 37°C in a moist chamber. The slide was washed once in phosphate buffered saline (PBS) for 10 min and air dried. To the dried slide was added 1 drop of goat anti-rabbit gamma globulin (1:50 dilution) labeled with fluorescein isothiocyanate (Sigma, St. Louis, Missouri). Incubation proceeded for 30 min at 37°C in a moist chamber. The slide was washed once with PBS as above, and rinsed with distilled water. The slide was air dried and observed using a fluorescence microscope. Positive controls consisted of placing a drop of live spirochetes from BSK culture on a slide instead of a tick smear.

Immunoblot Screen.—*B. burgdorferi* cells were grown into late log phase in BSK medium, centrifuged, and washed 3 times in PBS. Cells were resuspended in PBS at approximately 10^8 cells/ml, and 100 µl aliquots were spotted onto a nitrocellulose sheet held by a Vacudot (American Bionetics; Hayward, California) apparatus. The nitrocellulose was dried, and each spot was cut from the sheet and stored at −80°C. The immunoblot procedure was adapted from Towbin (13). Each dot blot was blocked by incubating for 1 hr at room temperature (RT) in a solution of 3% bovine serum albumin (BSA, Sigma) suspended in PBS. The blots were then probed with a 1:200 dilution of serum from each experimental mouse for 1 hr at RT. Blots were subjected to 2 10 min washes with 1% BSA in PBS, then incubated with a 1:1,000 dilution of peroxidase conjugated goat anti-mouse IgG (H + L) (Jackson Immunoresearch; West Grove, Pennsylvania) suspended in 3% BSA-PBS for 1 hr at RT. Each dot blot was washed 3 times (10 min each) in 3% BSA-PBS then developed with a solution containing o-dianisidine (25 µg/ml) and 0.01% H_2O_2 in 10 mM Tris (pH 7.4) for 10–15 min. Positive and negative control sera were included with each test run. Positive serum was obtained by injecting each mouse in a group of *P. leucopus* twice with approximately 10^8 freshly grown spirochetes. One week following the second injection, mice were bled and the serum collected and titrated by the above method. Positive control mouse serum attained a titer in excess of 1:3,200 with the immunoblot method. Negative controls were selected from experimental animals which appeared negative on the initial immunoblot screening. Further titrations of these sera were made at dilutions of less than 1:200 to insure that no antibody was present.

Western Blotting.—Western blotting was used to confirm each positive serum sample from the immunoblotting screen. Whole cell

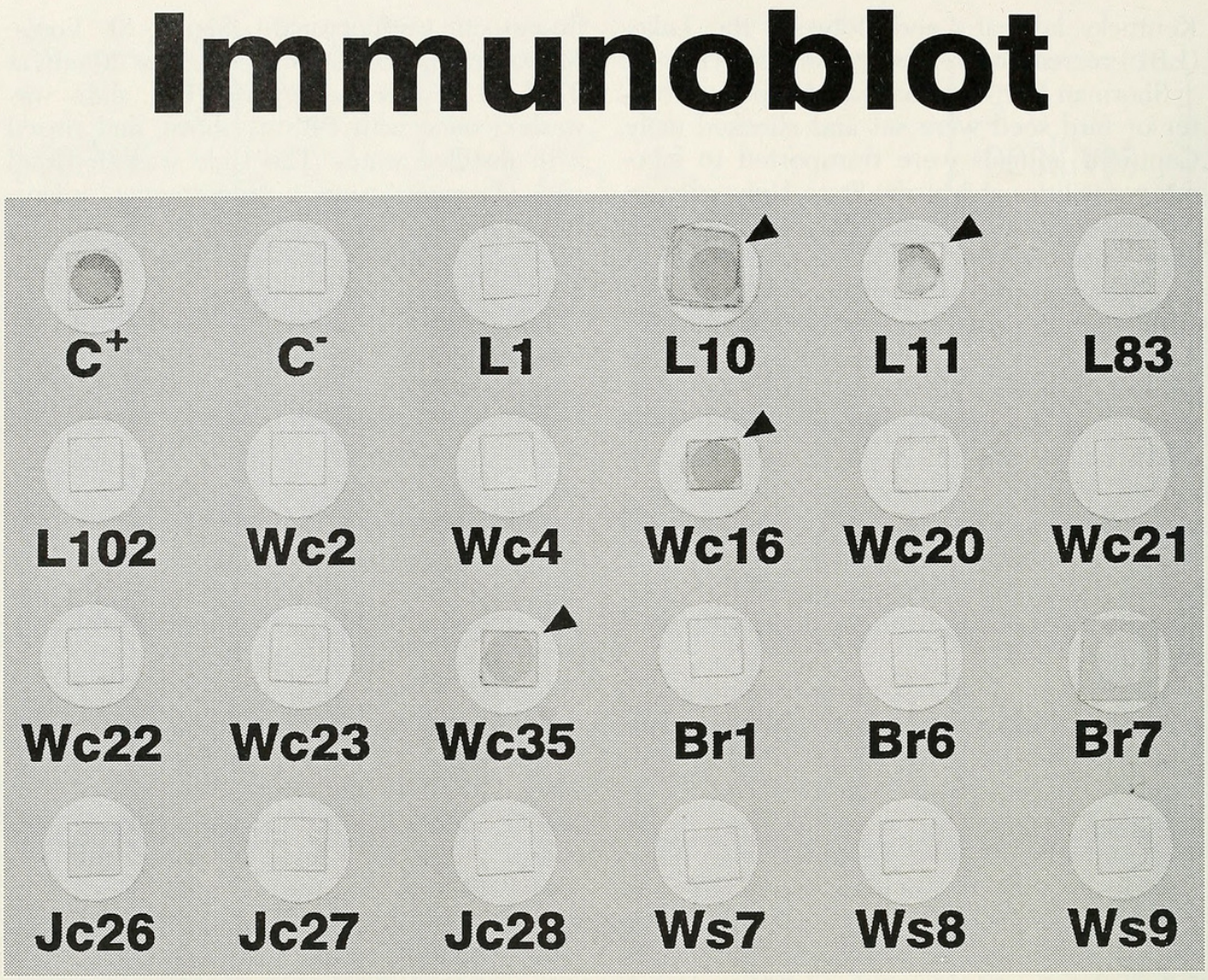

FIG. 2. Immunoblot screen: each serum sample was diluted 1/200 and used to probe whole spirochete antigen spotted on nitrocellulose. Arrows indicate positive reactivity of experimental serum with *B. burgdorferi* antigen. C+ and C− = positive and negative serum samples; L1, 10, 11, 83, 102 = experimental serum taken from mice captured from Land Between the Lakes; WC 2, 4, 16, 20, 21, 22, 23, 35 = experimental serum taken from mice captured from Wildcat Beach; Br 1, 6, 7 = experimental serum taken from mice captured from Blood River; Jc 26, 27, 28 = experimental serum taken from mice captured at Jonathan Creek; Ws 7, 8, 9 = experimental serum taken from mice captured at White Sands Beach.

proteins were extracted from *B. burgdorferi* using the boiling water bath method described by Simpson et al. (9). An aliquot containing 275 μg of protein was added to an SDS gel and subjected to polyacrylamide gel electrophoresis for 4 hr at a constant current of 40 ma. An aliquot containing 50 μg of marker protein (SDS-6; Sigma) was loaded adjacent to bacterial proteins in order to obtain a reference of molecular masses. Following resolution by SDS-PAGE, proteins were electroblotted onto nitrocellulose membranes (0.45 μ) (MFS; Dublin, California) using a Bio-Rad (Richmond, California) *Transblot Electrophoretic Cell* according to manufacturer's instructions. The transferred membranes were dried and cut into 3 mm strips for probing with experimental mouse serum. The probing procedure was the same as described above in the immunoblot technique, again including positive and negative control sera.

RESULTS

Cultivation of Borrelia *from Wild Animal Hosts.*—All attempts to cultivate *B. burgdorferi* from *Peromyscus* tissue were negative, whereas positive control cultures produced high concentrations of viable spirochetes.

Indirect Fluorescent Antibody Testing.—A total of 40 ticks (all *D. variabilis* larvae or nymphs) were collected from 24 *P. leucopus*

Table 1. Immunoblot screen of *Peromyscus* serum.

	Number positive/total number			
	Month* collected			
Sample site	1–4	5–8	9–12	Subtotal
LBL[a]	0/5	8/109	2/13	10/127
BR[b]	1/27	0/10	0/2	1/39
JC[c]	0/15	0/20	0/15	0/50
WC[d]	0/5	9/43	—	9/48
WS[e]	1/5	0/43	—	1/48
Subtotal	2/57	17/227	2/30	
Total				21/312 (6.7%)

* Numbers indicate calendar month.
[a] Land Between the Lakes.
[b] Blood River.
[c] Jonathan Creek.
[d] Wildcat Beach.
[e] White Sands.

during the course of the study. IFA tests were considered negative for all ticks tested.

Immunoblot Screen.—Using an immunoblot technique, serum from each mouse was diluted 1:200 and used to probe a whole spirochete antigen preparation spotted on nitrocellulose. Figure 2 illustrates a typical test run with a positive and negative serum control. The positive experimental sera are marked with arrows. Table 1 represents the preliminary results of immunoblot screening by sample site, and approximate time period of the year.

Western Blotting.—Each positive sample from the immunoblot screen was subjected to further analysis by western blotting. Serum samples which possessed specific antibodies to the 39/41 kDa protein of *B. burgdorferi* were considered confirmed positives, since these proteins have been shown to occur in all varieties of *B. burgdorferi* (8, 9, 14) and to specifically mark this species. Figure 3 shows a selection of the confirmation experiments. Each of the 9 experimental serum samples shown in Figure 3 as well as 9 others (not shown) were found to contain antibodies which reacted with the 39/41 kDa protein band. Some additional reactive antibodies were noted, for example: serum sample WC19 (D) reacted with a 56 kDa protein band and LBL100 (H) reacted with 49, 56, and 72 kDa protein bands. The positive serum control possessed antibodies which recognized protein bands at 21, 32, 36, 41, 49, 56, and 72 kDa, whereas the negative serum control failed to bind any *Borrelia* proteins. The geographic locality of each confirmed positive animal from all localities sampled is illustrated on Figure 1.

Discussion

The inability to cultivate infectious spirochetes in this study was disappointing, but not unusual. Luckhart et al. (3) presented evidence of *Borrelia* sp. in tick populations by the indirect fluorescent antibody technique, but were unable to culture the bacterial agent from a large sample of wild rodents. Also, the inability to demonstrate ticks infected with *Borrelia* in our study is not unusual given the small sample size, and the fact that we were unable to collect ticks from any animals which were seropositive for this bacterium.

The dot blot rapid screen test (Fig. 2) proved to be a reliable indicator of the presence of antibodies versus *B. burgdorferi*, since 18 of 21 samples were confirmed by western blot analysis. From Table 1 it is notable that most seropositive animals were collected during the summer months from Wildcat Beach and 2 localities within LBL (Fig. 1). The western blot test (Fig. 3) showed 18 of 21 positive sera which contained antibodies reactive with the 39/41 kDa band. The 41 kDa protein band has been described by other investigators (8, 14), and corresponds to the flagellin antigen of *B. burgdorferi*. The 39 kDa protein band has more recently been described by Simpson et al. (9), and was described by these authors as a marker for infection of animals naturally inoculated with *B. burgdorferi*. It should be noted that these protein bands were resolved on PAGE gels, but the resolution did not extend to nitrocellulose blots. The positive serum control lane (C) in Figure 3 displays a broad band of reactivity in the 40 kDa region which includes both 39/41 kDa bands in the PAGE control (lane A).

All of the confirmed positives showed this broad band of reactivity which is especially evident from samples in lanes F, G, and L. These findings are in accord with other researchers in the southeastern U.S. who have performed serological surveys of wild animal populations to detect antibodies versus *B. burgdorferi*. Magnerelli et al. (4) reported that in Connecticut and other states on the eastern seaboard 51% and 20% of the sera examined (by ELISA) from *Odocoileus virginianus* and *Pro-*

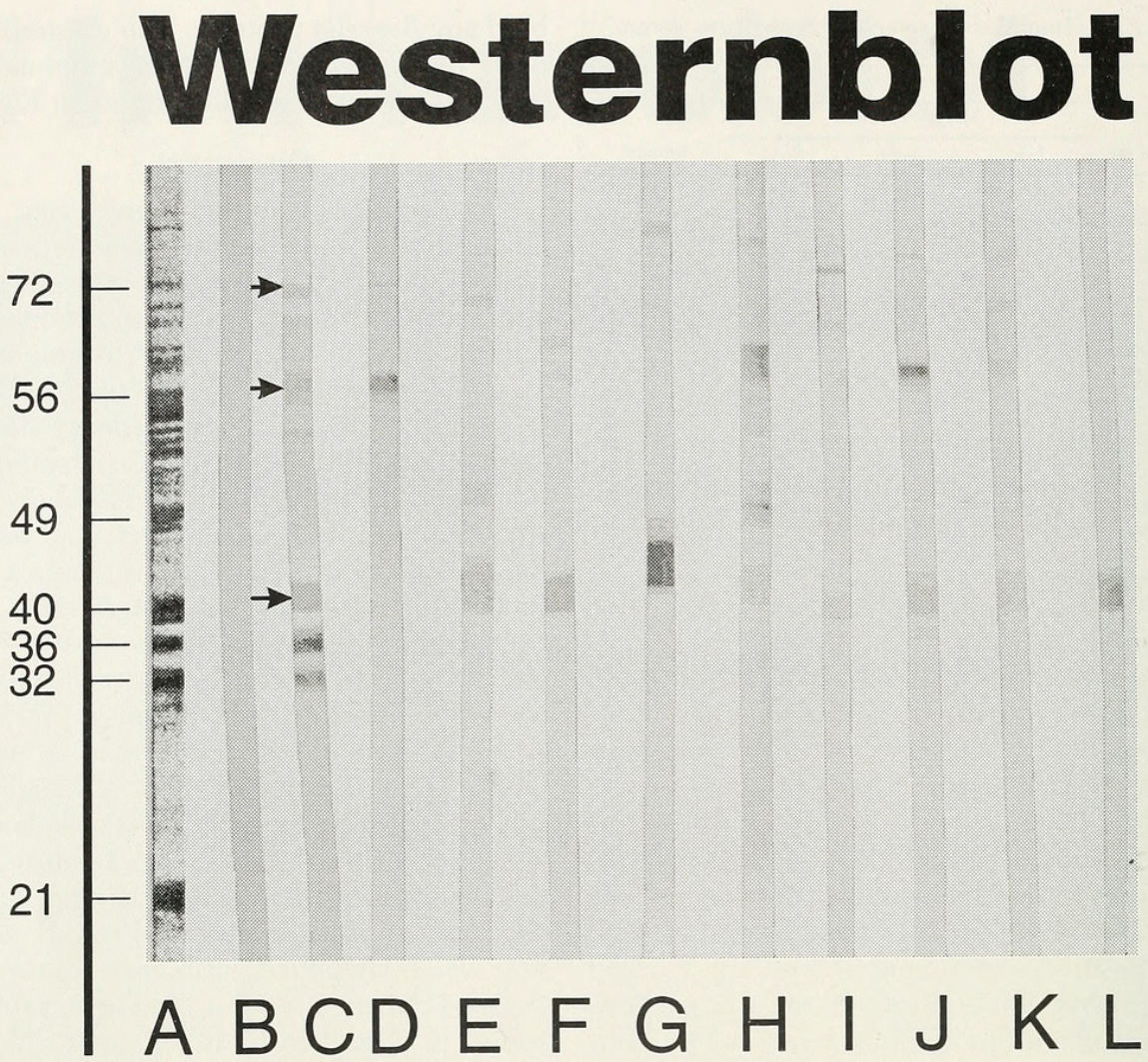

FIG. 3. Western blotting: selected positive serum samples from the immunoblot screen were used to probe whole cell proteins of *B. burgdorferi* subjected to PAGE and blotted to nitrocellulose. Lane A—whole cell *B. burgdorferi* proteins resolved by SDS-PAGE; B—negative *P. leucopus* control serum; C—positive *P. leucopus* control serum; D–L—immunoblot strips probed with serum collected from mice WC19 (D),WS1 (E),WC35 (F),BR16 (G),LBL100 (H),WC45 (I),WC47 (J),LBL6 (K), and LBL7 (L). Arrows indicate reactive proteins of 39/41 kDa, 56 kDa, and 72 kDa in the control lane.

cyon lotor, respectively, contained antibodies to *B. burgdorferi*. In another ELISA study by Magnarelli et al. (5), several localities in the eastern and southeastern U.S. were surveyed for anti-*Borrelia* antibodies in the serum of rodents. Positive *Peromyscus gossypinus* sera were obtained from each of the 7 southeastern states surveyed. The rates of positivity ranged from a high of 38% in South Carolina to a low of 15% in Alabama. From a total of 535 *P. gossypinus* serum samples tested, 27% showed antibodies to *B. burgdorferi*. We conclude that preliminary evidence from our study indicates a low level of infectivity by *Borrelia* sp. exists among *P. leucopus* populations located in western Kentucky. Further studies will focus on identification of which tick species may harbor *B. burgdorferi* in western Kentucky and document the prevalence of the bacterium within potential vector populations.

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