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EXPERIMENTAL INFECTION OF NORTHERN BOBWHITE QUAIL WITH BORRELIA BURGDORFERI

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ABSTRACT: Four-week-old northern bobwhite quail (Colinus virginianus) were inoculated subcutaneously with 10⁶ organisms from a low passage culture of Borrelia burgdorferi. Blood was collected weekly for culture, antibody detection, and immunoblot analysis. Three weeks post-inoculation, viable spirochetes were isolated from the blood of one bird, but not from kidney, spleen, liver, or heart; all infected birds from which preinfection antibody titer had been established, had antibodies by the enzyme-linked immunosorbent assay (ELISA). The inoculated birds did not show clinical signs of disease and there were no detectable gross or histopathologic lesions. Borrelia burgdorferi was detected in sections of kidneys on fluorescent antibody tests. Using a polymerase chain reaction (PCR) analysis to detect Borrelia burgdorferi DNA in tissue samples, the expected PCR product (DNA) of 246 base pairs was visible on agarose gel stained with ethidium bromide. The identity of the PCR product was confirmed by slot blot hybridization with Borrelia burgdorferi specific DNA probe. Thus, these birds sustained infections for at least 3 weeks without clinical signs and may play a role in the transmission of Borrelia burgdorferi.

Key words: Borrelia burgdorferi, northern bobwhite quail, Colinus virginianus, serology, pathology, polymerase chain reaction.

INTRODUCTION

Lyme borreliosis, a disease affecting both man and animals, is caused by the spirochete Borrelia burgdorferi and is transmitted by several species of Ixodid ticks (Burgdorfer, 1984). In the northeastern United States where the disease is enzootic, the primary vector is Ixodes scapularis (formerly I. dammini) which feeds on many mammals, most importantly the white-footed mouse (Peromyscus leucopus) and the white-tailed deer (Odocoileus virginianus). The tick also has been associated with many species of ground feeding birds, but the role of birds in the transmission of the disease is not clearly understood. Their feeding and migratory habits of birds present them as potential hosts, both as passive carriers of the tick and as reservoirs of the spirochete. Errant birds from northern Europe (Shwartz, 1987), where the disease has been recognized since the turn of the century, may have been a means of introduction of the disease by infected ticks or spirochetes to the northeastern United States. Avian transmission also may explain the three geographically separate areas of endemic Lyme borreliosis determined within a few years of its initial discovery in this country. These regions include the shores of Long Island sound, Cape Cod, Massachusetts, and the state of Wisconsin (USA) (McLean et al., 1993).

Our objective was to determine the pathogenicity of B. burgdorferi in birds. We experimentally inoculated northern bobwhite quail (Colinus virginianus) with B. burgdorferi to establish infectivity, immune response and pathological changes, and to attempt to detect spirochetes in blood and tissues by bacterial culture, the fluorescent antibody (FA) test, and polymerase chain reaction (PCR) techniques.

MATERIALS AND METHODS

The northern bobwhite quail was selected as the experimental subject for this study because it is a woodland, ground dwelling species and is commercially available. Thirty 4-wk-old northern bobwhite quail (Ronald Urban, Eastford, Connecticut, USA) were allowed to acclimate for a period of 2 wk before starting the experiment. Each bird was individually identified. The experimental infection study started in early October 1990 and ended in December 1991.

The B. burgdorferi culture used to infect 24
birds was derived from a tick foregut isolate of third passage #25550-3, which was provided by Dr. John Anderson, Department of Entomology, Connecticut Agricultural Station, New Haven, Connecticut. The cultures were grown and maintained in Barbour-Stoenner-Kelly (BSK) media (Barbour, 1984). Pre-infection blood samples were collected from the wing veins of the birds. Twenty-four birds were inoculated subcutaneously with approximately 10^6 organisms of the above low-passage B. burgdorferi suspended in 7 ml of BSK media. Blood was collected weekly and approximately 0.25 ml was cultured in 7ml of BSK media containing 20 mg/ml rifampicin (Sigma Chemical Company, St. Louis, Missouri, USA). The tubes were incubated at 34 °C and wetmounts of the cultures were checked weekly for 3 mo under darkfield microscopy (Model BH-2, Olympus Optical Company, Tokyo, Japan). Six birds were inoculated subcutaneously with 0.2 ml of BSK media for use as negative controls.

The serum samples from 29 birds, 24 infected and 5 controls, were tested in triplicate by enzyme-linked immunosorbent assay (ELISA) as described by Magnarelli et al. (1984). The B. burgdorferi antigen was derived from the culture (25550-3) used to infect the birds and was prepared by washing the organisms three times with 0.1% merthiolate in phosphate buffer saline (PBS), pH 7.2, centrifuged at 10,000 × G for 30 min each time. The organisms were resuspended in PBS, and standardized spectrophotometrically to an absorbance of 0.025 at 650 nm (A650 = 0.025). Microtiter plates (Dynatech Labs, Alexandria, Virginia, USA) were coated with 50 μl/well (A405 = 0.01) of antigen or 50 μl of the PBS with merthiolate (1:10,000) and incubated for 20 hr at 4 °C. The plates were then used for the ELISA or stored at −70 °C for up to 1 mo. We added 200 μl/well of 0.5% horse serum as a blocking solution, incubated the plates for 1 hr at 37 °C, and washed them three times in PBS containing 0.05% Tween 20 (Sigma Chemical Company) solution. A two fold serial dilution of bird serum starting at 1:40 was made in PBS containing 0.05% Tween 20, 5% horse serum, and a 1% solution of dextran sulfate (Sigma Chemical Company). Sixty microliters of each dilution was added in triplicate, containing either antigen or PBS (control), followed by a 45 min incubation at 25 °C and washed. Horse-radish peroxidase conjugated with goat anti-chicken IgG containing both the heavy chain and light chain (1:400) (Kirkegaard and Perry Laboratory Inc. Gaithersburg, Maryland, USA) was added to each well. Incubation was carried out for 45 min at 37 °C and washed three times with PBS. We added 60 μl/well of ABTS (2,2'-azino-di-(3-ethylbenthiazoline sulfonate)) per-oxidase substrate (Kirkegaard and Perry Lab) to the wells, incubated them for 50 min at 37 °C, and read the plates on a Microplate Reader (Dynalect Lab) at 410 nm. The absorbance of the control wells was subtracted from the absorbance of the corresponding antigen coated wells to obtain a net value.

We compared the third passage of B. burgdorferi BB(25550-3) with the BB reisolated from the blood of infected birds 3 wk post-inoculation with an immunoblot analysis. The organisms were grown in 25 ml BSK medium for 36 to 48 hrs at 37 °C. The cells were centrifuged at 15,000 × G for 25 min and washed four times in PBS. Protein concentration was estimated by the Bradford method (Bio-Rad, Richmond, California, USA). One-hundred micrograms of borrelia cell protein was mixed in 100 μl of sample buffer (2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris pH 6.8, 0.001% bromphenol blue) (Sigma Chemical Company), which was boiled for 5 min along with a molecular weight marker (Bio-Rad); samples were subjected to electrophoresis using a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Laemmli, 1970). Electrophoresis was done at 50 mA at 4 °C until the bromphenol blue dye reached 1 cm from the bottom of the gel. The gels had been pre-equilibrated in transfer buffer (48 mM Tris-Base) (Sigma Chemical Company), 39 mM glycerol (Sigma Chemical Company), 20% (v/v) methanol pH 8.3 and 0.037% SDS for 30 min, and transferred onto nitrocellulose membrane (Bio-Rad) at 70 volts for 2.5 hr using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) as described by Towbin et al. (1979). The membranes were stained with Ponceau S (Sigma Chemical Company) to view the banding patterns. The membranes were cut in strips for immunoblotting, blocked with 2% bovine serum albumin and 1% calf serum in saline, shaken for 1 hr at 25 °C and washed three times (10 min each) in washing buffer (0.4% Tween 20 in PBS). Antisera (1:100 dilution) from three infected birds and from non-infected birds were incubated for 1 hr at 25 °C. Alkaline phosphatase labeled- affinity purified goat anti-chicken immunoglobulin G (IgG) (H+L) (Kirkegaard and Perry Lab Inc.) diluted 1:2500 with blotting buffer was incubated for 2 hr at 25 °C by rotating gently. The strips were washed briefly in PBS and then allowed to reacted with BCIP/NBT phosphatase substrate (Kirkegaard and Perry Lab) until bands appeared. The reaction was stopped by washing with distilled water. The strips were carefully blotted with filter paper and stored in a dry dark place at 25 °C.

Three weeks post-infection and weekly thereafter four infected birds and one control bird
were killed by cervical dislocation. Prior to their death a blood sample was obtained from a heart puncture. One-half cc of blood was added to 6 ml of BSK media and incubated at 34°C; the remainder was used to harvest the serum for determination of antibody response. The birds were checked for gross lesions; pieces of kidney, liver, heart, skin, and spleen were aseptically removed and transferred into 7 ml of sterile BSK media containing 20 mg/ml rifampicin. Samples of each tissue were also fixed in 10% neutral buffered formalin (Surgipath, Grace Lake, Illinois, USA). Freezing samples for fluorescent antibody assay, histopathology, and PCR was accomplished by adding tissue samples from each bird to a 5 ml Nalgene cup (Nalgene Laboratories, Rochester, New York, USA) containing 1% buffered gluteraldehyde (An-Con Genetics Inc., Melville, New York). They were covered and stored at −20°C.

Tissue sections from kidney, liver, heart, skin, and spleen were fixed in 10% formalin for 24 hr and embedded in paraffin, and 5 μm sections were stained with Steiners Silver (Swisher, 1987) and hematoxylin and eosin (H&E) (Sigma Chemical Co.) (Hrapchak, 1980). Five-micrometer sections of frozen tissue sections (kidney, liver, heart, skin and spleen) from non-infected and infected birds were transferred to poly-L-lysine slides (Sigma Chemical Company) and stored at −70°C. A positive control was created by injecting chicken liver with 10⁴ live B. burgdorferi derived from the same culture which had been used to infect the birds. A direct fluorescent antibody (FA) test was done on frozen tissue sections as described by Hrapchak (1980). Each tissue section was placed on a slide and was dipped into cold acetone and allowed to dry. Two drops of fluorescein isothiocyanate (FITC) conjugated polyclonal antibody (goat-anti-borrelia) (1:40 dilution) (Kirkegaard and Perry Lab) were placed on to the tissue sample and incubated in a moist chamber for 30 min at 37°C. Each slide was washed with PBS and examined under the fluorescent microscope (Olympus model BH-2).

The DNA was isolated from the originally described third passage culture of B. burgdorferi by the procedure of Sambrook et al. (1989). The borrelia cultures were grown in BSK media and centrifuged in 50 ml Nalgene (Fisher Scientific, Medford, Massachusetts) tubes at 7,000 × G for 15 min. The pellet was lysed with 3 ml of a buffer containing 10 mM Tris and 1 mM ethylene-diaminetetra-acetic acid (EDTA) (TE buffer) with SDS added to a 1% final concentration. Ribonuclease A (Bethesda Research Laboratory (BRL), Bethesda, Maryland) was added to a final concentration of 25 μg/ml and incubated for 20 min at 25°C, followed by the addition of 25 μg/ml proteinase K (Sigma Chemical Company) and an incubation of 1 hr at 57°C. The DNA was extracted once with an equal volume of phenol and once with an equal volume of a 1:24 mixture of chloroform and isopropanol. Centrifugation was at 7,000 × G for 5 min. The supernatant was placed in corex tubes (Fisher Scientific) and the DNA was precipitated with 1/10 volume of 3 M sodium acetate. Two volumes of 100% ethanol were added and incubated overnight at −20°C. The DNA extract then was centrifuged at 7,000 × G for 10 min and the pellet was resuspended in 500 μl of TE buffer, pH 7.6. The DNA concentration was estimated spectrophotometrically (Sambrook et al., 1989) and stored at −20°C.

Deoxyribonucleic acid was isolated from tissues of infected and non-infected birds, and from chicken liver injected with B. burgdorferi (Rogers et al., 1990). Fifteen milligrams of tissue were mixed in 1 ml of a buffer containing 0.5 M Tris pH 8.0, 0.02 M EDTA, and 0.01 M NaCl (TEN buffer) and incubated for 3 hr at 55°C. Sodium dodecyl sulfate was then added to a final concentration of 1% to each tube and mixed thoroughly. Proteinase K was added to a final concentration of 25 μg/ml and incubated overnight at 50°C. Starting with the phenol extraction, the remainder of the procedure was as before.

The B. burgdorferi primer pairs used in PCR were described by Malloy et al. (1990) and were synthesized by Biotechnical Services of the University of Connecticut, Storrs, Connecticut, as described by Beaucage and Caruthers (1981). The PCR was carried out using reagents from GeneAmp PCR kit (Perkin Elmer Cetus, Norwalk, Connecticut). A reaction volume of 100 μl of PCR mixture contained 100 mM Tris-HCl (pH = 8.3), 500 mM KCl, 200 μM each nucleotide [deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP)] (Perkin Elmer), 0.9 μM of each primer, 2.5 Units AmpliTaq DNA polymerase (Perkin Elmer Cetus), and 2.5 mM of MgCl₂. Samples were overlaid with 50 μl of mineral oil to prevent evaporation. Polymerase chain reaction amplification was performed in a thermal cycler (PHC-2 Dri-Block; Techne, Princeton, New Jersey, USA). The cycling was programmed for denaturing at 94°C for 30 sec, annealing at 55°C for 40 sec, and extension at 74°C for 55 sec for a total of 35 cycles. The final extension was allowed to run for 5 min. Previously isolated spirochetal DNA was amplified with the synthesized primers. Salmonella enteritidis DNA was used as a negative control. Duplicate samples of the B. burgdorferi DNA were run in three concentrations (10 ng/100 μl, 1 ng/100 μl, 0.5 ng/100 μl) and the Salmonella
enteritidis DNA at 10 ng/μl. Twenty-one samples of isolated tissue DNA, which included skin, kidney, heart, spleen, and liver, were amplified. Controls included tissue DNA of a non-infected birds, the B. burgdorferi DNA, and the DNA extracted from the chicken liver injected with the B. burgdorferi. HindIII digested lambda DNA (Sigma Chemical Company) was used as a size marker. The 1% agarose (BRL) gels were run in Tris-borate buffer (Sambrook et al., 1989) at 60 volts and run for 2 hr and stopped when the tracking dye reached the bottom of the gel. The gel was stained with ethidium bromide (1μg/ml) and washed three times with distilled water. The DNA products were seen by ultra-violet illumination at 300 nm and were photographed with a Polaroid camera.

For the use as DNA probe in slot blot DNA hybridization, 200 ng PCR amplified DNA product of B. burgdorferi was digested using 10 units of EcoRI in 20 μl reaction buffer (Sambrook et al., 1989). The digested DNA was heat denatured (95 C, 5 min) and labeled with [alpha ^32P]-dATP using an end labeling kit from Ambion Inc. (Austin, Texas, USA) according to the manufacturer’s instructions. The 1 μg amplified DNA from tissue samples were digested using EcoRI restriction enzyme (Sigma Chemical Company), followed by the addition of six times with a mixture of 0.9 M NaCl and 0.09 M sodium citrate (pH 7.0), (SSC), and denatured by boiling for 5 min. The samples were applied to a Zeta-Probe membrane (Bio-Rad) using the microfiltration unit (slot blot manifold) (Bethesda Research Lab) for binding nucleic acid solution on to a Zeta-Probe membrane (Bio-Rad). The membrane was vacuum dried and baked for 2 hr at 80 C, sealed inside a plastic bag with a prehybridization solution (1 mM EDTA, 0.5 M NaH_{2}PO_{4}, pH 7.2) and incubated for 5 min at 65 C to completely coat the membrane.

The labeled DNA probe was added with fresh buffer to the Zeta-Probe membranes and allowed to hybridize overnight by shaking at 65 C. The membrane was removed from the bag and washed at 65 C twice for 1 hr in a solution made up of 1 mM EDTA, 40 mM NaHPO_{4}, pH 7.2 and 5% SDS. This was followed by two more washes at 65 C for 1 hr in a solution containing 1 mM EDTA, 40 mM NaHPO_{4}, pH 7.2, and 1% SDS. The membranes were air dried and exposed to X-ray film for 6 hr using an intensifying screen at ~70 C (Sambrook et al., 1989).

**RESULTS**

Using the BSK culture, B. burgdorferi was isolated from the blood of one bird 3 wk post-infection. After weekly examinations of the blood inoculated media, B. burgdorferi was identified in culture 6 wk post-inoculation. The remaining cultures from blood and tissues (kidney, liver, skin, heart and spleen) of the 24 infected and five non-infected birds were negative.

Based on the ELISA analysis, all 24 infected birds from which pre-infection titers had been established, developed antibodies by the end of 3 wk. The pre-infection titers for 21 of the birds was ≤1:80 and for three birds the titers were 1:320. Serumconversion was defined as a four-fold increase in antibody titer, which was not shown by any of the control birds. There was a dramatic rise in geometric mean titers in the first week post-infection, with a slight depression in the third week (Fig. 1). There was a second rise in titers by the fourth week, which remained elevated for the duration of the study period.

The bird sera were tested by the immunoblot method against both the B. burgdorferi strain used to infect the birds and the organism which was re-isolated from an infected host. The protein banding patterns of immune sera from infected birds tested against the protein from the two borrelia cultures were antigenically similar when comparing the bands most
commonly associated with Lyme disease, namely flagellin, OspA and OspB. The lower banding patterns differed, perhaps due to slight variations in conditions at the time of blotting or due to changes which occurred after the spirochete was passed through the bird (Fig. 2).

Five of 24 infected birds had focal hemorrhagic skin lesions at the injection sites and one of these birds had a similar lesion over the keel bone. All other organs in all the birds, infected and non-infected, appeared normal. Histopathologically, a diffuse inflammatory response, corresponding to the focal skin lesions described above, was seen on the H&E slide preparations of fixed skin tissue from these five birds. The lesions were hemorrhagic and had subcutaneous and dermal infiltration of plasma cells and lymphocytes. The Steiner stain slide preparations had a few fragmented borrelia in the skin sections.

In the direct FA test, borrelia were detected in the control preparations, which had been injected with live *B. burgdorferi*. No borrelia were seen on 80 sections of tissues from five non-infected birds. Of the 280 tissue sections examined from 14 infected birds, nine kidney sections from three birds were positive for borrelia.

With the PCR evaluation 21 tissues samples of kidney, liver, spleen, heart, and skin, from 14 infected birds yielded a 246 base pair (bp) DNA product as expected. Representative *B. burgdorferi* infected tissue samples indicating the amplified DNA product of 246 bp on 1% agarose gel are shown in Fig. 3. The slot blot hybridizations had specificity for detecting PCR amplified product from *B. burgdorferi* infected tissue (Fig. 4).

**Discussion**

Northern bobwhite quail experimentally infected with *B. burgdorferi* did not develop evident clinical signs or lesions. The antibody response to *B. burgdorferi*, the isolation of spirochetes from the blood, the FA demonstration of borrelia in nine kidney sections, and PCR amplification of specific DNA from the tissue of the infected birds confirmed the infection. An antibody response developed by the end of the first week post-infection and remained elevated for the 7-wk study period. However, there were no clinical signs of disease, gross or histopathologic lesions, except an inflammatory response at the injection site of five birds. These results were similar to the findings of earlier studies on mallard ducks (*Anas platyrhynchos*) and wild turkeys (*Meleagris gallopavo*) which did not develop clinical signs but had positive cultures and antibody titers (Burgess, 1989). *Borrelia burgdorferi* was isolated from the liver of a veery (*Catharus fuscens*) (Anderson et al., 1986) and it was later shown that larvae of *Ixodes scapularis* (formerly *I. dammini*), which had fed on birds, were infected with the borrelia (Anderson, 1988; Anderson et al., 1990; McLean et al., 1993).

While PCR results confirmed DNA in all tissues tested, the FA test showed that only a few intact spirochetes may have been present. Culture of *B. burgdorferi* from tissues resulted in the recovery of spirochetes from the blood of only one bird. The re-isolation of borrelia from the blood at 3 wk post-infection was ascertained to be the same organism as the original culture by immunoblot analysis (Fig. 2).

Based on the presence of antibodies, as detected by ELISA, there appeared to be an active infection in the experimentally inoculated birds. Sixteen of 24 infected birds developed antibodies within 1 to 3 wk post-infection, and later on all of the infected birds had antibodies. None of the control birds developed antibodies. The persistence of elevated titers throughout the 8-wk study period could suggest either an active infection or the persistence of *B. burgdorferi* antigens, as is seen in post antibiotic treatment of both human and animals with borrelia infection (Golightly et al., 1990; Appel, 1990). The immunoblot tests support the ELISA data. The sera of the three infected birds on which immunoblot tests were done reacted to proteins...
specific to *B. burgdorferi*. The bands detected in the immunoblots, corresponded to bands previously determined to be associated with *B. burgdorferi*, especially flagellin (41Kd), Osp A, and Osp B (Craft et al., 1986; Schwan et al., 1989; Zoller et al., 1991).

The PCR amplification showed *B. burgdorferi* DNA in several tissues and indicated the presence of a disseminated in-

**FIGURE 2.** Western immunoblot analysis showing antibody reaction of sera of one bobwhite quail against *Borrelia burgdorferi* cultures. A. Culture used to infect bird; B. culture recovered from infected bird; C. control. Molecular-mass values are given in daltons.

**FIGURE 3.** Representative agarose gel electrophoresis showing polymerase chain reaction amplified *Borrelia burgdorferi* DNA from tissues of experimentally infected bobwhite quail. Lane 1: Hind III digested lambda DNA marker; 2: kidney (non-infected bird); 3 and 4: skin from bird 61; 5: kidney from bird 80; 6 and 7: kidney from bird 81; 7 and 8: heart from bird 81; 9: kidney from bird 82; 10: liver from bird 78; 11: spleen from bird 77; 12: kidney from bird 77; and 13: liver injected with *Borrelia burgdorferi* used as a positive control.

Infection. The amplified DNA was confirmed by the slot blot DNA hybridizations. The results were consistent with similar studies done by others in which *B. burgdorferi* was detected in the similar tissue specimens from infected gerbils (*Meriones unguiculatus*) (Lebeche et al., 1991; Wise and Weaver, 1991) and by Goodman et al. (1991) in which the borrelia was identified in the urine of human patients with the disease.

The presence of the DNA was not necessarily evidence of an active infection as the DNA of dying or dead spirochetes would be detectable by PCR. Based on the recovery of the *B. burgdorferi* at 3 wk post-infection and the presence of FA-positive borrelia in the viscera, we believe that the borrelia were able to survive for that period of time, and that birds are susceptible to infection by *B. burgdorferi*. Thus, the birds ability to carry ticks and to infect ticks in new areas makes them potentially important in the spread of this disease. Migrating birds feed in different regions along their flyways, a factor which increases the possibility of either disseminating already infected ticks or of infecting local ticks present in non-endemic areas. Because *Ixodes scapularis* remains attached to the host for a period of 2 to 6 days (Piesman et al., 1991), the dispersion of ticks could be widespread. At the time of the northern migration in the spring, nymphal activity has begun and any infected immature ticks have maintained spirochetes over the winter (Burgdorfer, 1984). The potential exists that migrating birds transport infected ticks, or carry the spirochete, or both. In the northeast, larval feeding period begins in August and extends into the fall. This time period coincides with the fall migration of birds, which would allow the dispersion of ticks southward from endemic areas.

In order to determine the frequency with which birds may disseminate the infection, additional research is required to show both the duration of active avian borreliosis and the extent to which the spirochetes replicate in birds. While this information would be important, findings of a short infection period would not dramatically lessen the bird’s role.

**LITERATURE CITED**


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