JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1992, p. 2625–2631
0095-1137/92/102625-07$02.00/0

Vol. 30, No. 10

Comparison of Polymerase Chain Reaction and Culture for Detection of *Borrelia burgdorferi* in Naturally Infected *Peromyscus leucopus* and Experimentally Infected C.B-17 *scid/scid* Mice

ERIK K. HOFMEISTER,* RICHARD B. MARKHAM, JAMES E. CHILDS,† AND RAY R. ARTHUR

Department of Immunology and Infectious Diseases, The Johns Hopkins University School of Hygiene and Public Health, 615 North Wolfe Street, Baltimore, Maryland 21205

Received 3 October 1991/Accepted 23 July 1992

Culture and the polymerase chain reaction (PCR) were compared for detection of *Borrelia burgdorferi* infection in wild-caught *Peromyscus leucopus* and experimentally inoculated C.B-17 *scid/scid* (severe combined immunodeficient) mice. PCR targeted highly conserved regions of the *ospA* gene and could detect one to five cultured organisms and 10 to 50 copies of molecularly cloned *ospA* DNA. Organs (kidney, spleen, and urinary bladder) and/or ear biopsy samples were obtained from 108 captured *P. leucopus* mice, and tissues were obtained from 7 experimentally inoculated mice. A simple sample-processing procedure with proteinase K and detergent treatment was used in the PCR analysis. Overall, *B. burgdorferi* was detected in 29 of 108 (27%) *P. leucopus* mice by culture and in 31 of 108 (29%) mice by PCR. As assessed by the kappa statistic, agreement between PCR and culture was high for ear and bladder (kappa = 0.80 and 0.65, respectively) and low for kidney and spleen (kappa = 0.37 and 0.03, respectively). While concordant results were obtained from 98 animals, PCR detected *B. burgdorferi* from 6 additional mice for which cultures were negative and culture detected *B. burgdorferi* from 4 animals which were PCR negative. Further phenol-chloroform extraction of DNA in a limited number of samples improved the sensitivity of PCR compared with that of culture. These results indicate that PCR may be as sensitive as culture for detecting *B. burgdorferi* in ear samples and that PCR analysis is suitable for establishing the infection status of animals in mark-release-recapture studies.

Detection of *Borrelia burgdorferi*, the spirochete which causes Lyme disease (7, 11), by culture or by immunostaining has played an important role in determining the epizootiology of infection in *Peromyscus leucopus* (1, 2), the primary mammalian reservoir (24), and other host species (3, 9, 32). Methods for direct detection of the organism, its antigens, or nucleic acids have proven especially important, since antibody-based assays are in poor agreement with infection status, as determined by culture, in natural populations of *P. leucopus* (22). Additionally, detection of the organism by culture and histopathological methods (6, 15, 29, 34) has been essential in determining the suitability of various animals as experimental models for Lyme disease. The use of the polymerase chain reaction (PCR) has been described elsewhere (14, 17, 18, 21, 23, 25–27, 33) as an alternative to the previously mentioned direct diagnostic methods because of its potentially greater sensitivity in detecting the organism and its facility of use. Recently, the detection of *B. burgdorferi* in the tissues of experimentally infected gerbils by PCR has been reported and compared with in vitro isolation (21), but the relative merits of the two methods for examining field-collected materials remain unexplored.

Most methods for the detection of *B. burgdorferi* have involved sacrifice of the animal and examination of its internal organs, primarily the kidney, spleen, and urinary bladder. Animal sacrifice obviously precludes longitudinal study of infected individuals in field populations. Culturing the organism from blood, a method which does not necessarily require the sacrifice of the animal, has proven to be inefficient in animal models of Lyme disease (6, 12, 15, 34). In contrast, ear punch biopsy samples have been reported to be highly efficient tissues for culturing the organism from experimentally and naturally infected animals (30). Our objective was to develop a PCR assay that could be used for longitudinal study of individual mice infected with *B. burgdorferi*. To establish the utility of these methods, we compared the results of PCR and culture for the detection of *B. burgdorferi* in different tissues obtained from naturally and experimentally infected mice. We describe the development of a specific PCR assay for the *ospA* gene of *B. burgdorferi* and compare the agreement between PCR and culture for the detection of *B. burgdorferi* in tissues obtained from experimentally infected C.B-17 *scid/scid* mice and *P. leucopus* mice captured at a Lyme disease enzootic site in Maryland.

MATERIALS AND METHODS

Specimens. (i) *Borrelia* specimens. Specimens of *B. burgdorferi* tested included the highly passaged strains B-31, Plag 9T-3-1-7, Boutin, and Guilford (provided by D. Malloy, Maryland Medical Laboratory, Baltimore, Md.); low-passage tick isolate 31248 (provided by J. Anderson, Connecticut Agricultural Experiment Station, New Haven, Conn.); and three low-passage field isolates from *P. leucopus* (cultured from mice captured as part of this study). Other culture-adapted members of the genus *Borrelia* that were tested included *B. hermsii*, *B. coriaceae*, *B. parkeri*, *B. turicatae*, *B. anserina*, and *B. crocidurae* (all provided by R. C. Johnson, University of Minnesota Medical School, Minneapolis).

* Corresponding author.
† Present address: Epidemiology Section, Viral and Rickettsial Zoonoses Branch, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, GA 30333.

(ii) Animal specimens. In order to provide DNA from animals known to be free of infection with *B. burgdorferi* for PCR studies, kidney, spleen, bladder, and ear punch biopsy specimens were obtained from six colony-reared *P. leucopus* mice (Peromyscus Stock Center, University of South Carolina, Columbia). To provide tissues from animals of known infection status for PCR and culture studies, kidney, liver, heart, brain, and ear tissues were removed from seven severe combined immunodeficient (SCID) mice (C.B-17 mice deficient in functional B and T lymphocytes [10]) experimentally inoculated with three low-passage strains (cultured from *P. leucopus* mice in Maryland) and the laboratory-adapted B-31 strain of *B. burgdorferi*. For experimental inoculations, cultures were grown to late log phase in BSK II medium (4) at 34°C, quantitated in a Petroff-Hausser counting chamber under dark-field conditions, washed once in sterile phosphate-buffered saline (PBS; pH 7.4), and inoculated into each mouse by an intraperitoneal injection (5.5×10^7 to 7.5×10^7 live spirochetes). The experimental inoculation of each field strain and the B-31 strain was replicated in two SCID mice (with the exception of one field isolate, which was inoculated into only one SCID mouse). Five weeks after inoculation, the mice were observed for signs of arthritis and then sacrificed and tissue samples were removed.

Tissue specimens were also obtained from 108 *P. leucopus* mice captured live in Sherman traps (H. B. Sherman Traps Inc., Tallahassee, Fla.) between July 1990 and January 1992 at two sites in Baltimore County, Md., at which Lyme disease is enzootic. Field-captured mice were either sacrificed (39 animals) or captured, sampled, and released (69 animals). Kidney, spleen, and bladder tissues from 16 mice sacrificed in July 1990 (group 1) were sampled, and, in addition to these organs, ear tissue was obtained from 23 mice sacrificed between March and May 1991 (group 2). Duplicate ear biopsy samples were obtained with sterile disposable biopsy instruments from 69 mice captured and released for longitudinal study between June 1991 and January 1992 (group 3).

Culture. (i) Cultivation and identification of spirochetes. Laboratory-adapted *Borrelia* strains and passages of field isolates were grown in BSK II medium at 34°C. Primary isolates of *B. burgdorferi* from field-collected specimens were incubated at 34°C in BSK-RAP medium (BSK II medium containing 10 μg of rifampin, 4 μg of amphotericin B, and 1,000 μg of phosphomycin per ml [all antibiotics were from Sigma Chemical Co., St. Louis, Mo.]). The quality of BSK II and BSK-RAP media was monitored by inoculating samples of each batch of medium with strains B-31 and 31248 and quantitating viable spirochetes at 4 days postinoculation. All cultures were examined by dark-field microscopy at weekly intervals for spirochetes with morphology consistent with that of *B. burgdorferi*. Cultures not exhibiting growth of spirochetes by 6 weeks were discarded.

Spirochetes from positive cultures were identified as *B. burgdorferi* by immunofluorescence staining (IFA) with monoclonal antibody H5332 specific for OspA (5) (provided by A. G. Barbour, University of Texas Medical School, Austin). The presence of *ospA* sequences in these spirochetes was confirmed by PCR with a pair of *ospA* primers (described below). For IFA, spot slides were prepared from spirochetes harvested from BSK II medium by centrifugation and washed once in PBS. The slides were then reacted with a 1:100 dilution of monoclonal antibody H5332 in PBS, incubated at 37°C for 1 h, washed, reacted with a 1:30 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), and observed for fluorescence at ×400 magnification. For PCR, a 1:100 dilution of spirochetes from those harvested for the preparation of spot slides or a 1:10 dilution of spirochetes taken directly from BSK II medium was placed in a boiling water bath for 10 min. All dilutions of spirochetes were prepared with sterile distilled water. A 10-μl sample containing approximately 10^4 organisms from these preparations was added to the reaction mixture prior to thermal cycling.

Tissue processing. From sacrificed animals, separate 10- to 20-mm^3 samples of tissue were aseptically removed from each sampled organ and placed directly in 8.5 ml of BSK-RAP medium for culture and in 100 μl of tissue extraction buffer (TEB) for PCR studies. TEB contains 50 mM Tris-HCl, 1 mM EDTA, 1% (vol/vol) Laureth 12 (PPG/Mazer Chemicals, Gurnee, Ill.), and 100 μg of proteinase K (GIBCO-BRL, Gaithersburg, Md.) per ml. The instruments used to remove tissue from sacrificed animals were dipped in 90% ethanol and flamed between tissues. Ear biopsies were obtained by first swabbing the surface of the ear with 90% ethanol and, following air drying, biopsying the central area (equidistant from the outer margin of the ear) with a sterile disposable 2-mm skin biopsy punch (Accuderm Inc., Ft. Lauderdale, Fla.). Duplicate ear tissue samples from individual mice were placed separately in 8.5 ml of BSK-RAP medium and TEB. To prevent carryover contamination of ear biopsy samples of released animals, the forceps used in handling ear tissue samples were placed in 10 mM HCl between examinations of animals and rinsed with 90% ethanol prior to use. The efficacy of this decontamination procedure was tested at each capture session by placing the decontaminated forceps in TEB and processing the negative-control TEB sample in the same manner as that used for the ear biopsy samples.

PCR. (i) Oligonucleotide sequences for primers and probes. Published *B. burgdorferi ospA* DNA sequences (8) were examined, and sequences for primers BAE-1 (5′-CTG CAGCTTGGAATTCAGGC-3′) and BAE-2 (5′-ATTTGGT GCCATTTGAGTCG-3′) were selected at nucleotide positions 788 to 807 and 913 to 894, respectively. The expected amplification product of these primers was 126 nucleotide pairs in length. A third sequence, designated BAE-3 (5′-AC TAAAGACCTTGTGTTTAC-3′), was selected at nucleotide position 844 to 863 to be used as a specific probe to identify the *B. burgdorferi ospA* sequences in the amplification products. Additionally, primers BBC-1 and BBC-3 were synthesized corresponding to published sequences for *B. burgdorferi* chromosomal PCR primers which produce an amplification product of 371 nucleotide pairs in length (27). Oligonucleotide primers were synthesized on an Applied Biosystems synthesizer (model no. 380A, Foster City, Calif.) and purified on a reverse-phase high-performance liquid chromatography column.

(ii) Amplification. For PCR, tissue specimens in TEB were incubated overnight at 37°C and then placed in a boiling water bath for 10 min. DNA from colony-reared *P. leucopus* was further extracted with phenol-chloroform and precipitated in ethanol (28). TEB alone was utilized as a negative control following every fifth tissue sample and was processed in the same manner as that used for tissue samples. DNA target sequences were amplified in a total reaction volume of 50 μl containing 10 μl of sample DNA, 200 μM (each) the four deoxynucleotide triphosphates, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, and 0.01% [wt/vol] gelatin). The *ospA* amplification reaction mixtures contained 1.0 μM BAE-1 and 0.5 μM BAE-2. The reaction mixtures were overlaid with min-

eral oil prior to the addition of sample DNA and were subjected to 40 cycles of thermal cycling in a DNA thermal cycler (Perkin-Elmer). Thermal cycles of 30 s each at 94°C for denaturation of DNA, 61°C for annealing of primers, and 72°C for product extension were used for *ospA* amplification. Chromosomal amplification was performed as previously described (27). Reaction mixtures were prepared and the samples were added with a set of positive-displacement pipettes that were used only for this purpose. All manipulations were conducted in a laminar flow hood in which PCR product was not handled. Distilled H_2O was used in place of template DNA after every 10th specimen to serve as a negative PCR control.

To standardize the *ospA* PCR assay, known quantities of molecularly cloned *ospA* DNA (pTRH44 [20] provided by A. G. Barbour) were used as templates and amplified by primers BAE-1 and BAE-2. Plasmid pTRH44 was purified and quantitated by standard procedures (28) prior to amplification. The sensitivity of the PCR assay was evaluated by amplifying a dilution series of pTRH44 and *B. burgdorferi* B-31 in TE (10 mM Tris and 0.5 mM EDTA) containing 0.1 μg of *Escherichia coli* DNA (Sigma Chemical Co.) per ml.

(iii) Detection and identification of reaction products. PCR amplification products were detected and identified as *B. burgdorferi*-specific DNA sequences by visualization of bands of the expected size on agarose gels and by Southern hybridization. For visualization on agarose gels, 10 μl of PCR product was electrophoresed on a 4% agarose gel (NuSieve 3:1 Agarose; FMC Bioproducts, Rockland, Maine) containing 0.05% ethidium bromide and examined. The DNA was then transferred from the gels to nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, N.H.) by the technique described by Southern (31). Following Southern transfer, the filters were exposed to UV light for 2 min and allowed to air dry. The filters were prehybridized for 1 h at 42°C with a solution containing 3× SSPE (20× SSPE contains 3.6 M NaCl–200 mM NaH_2PO_4–20 mM EDTA [pH 7.4]), 5× Denhardt's solution (10× Denhardt's contains 0.2% bovine serum albumin–0.2% polyvinylpyrrolidone–0.2% Ficoll), 0.5% sodium dodecyl sulfate (SDS), and 0.25 mg of tRNA per ml. Ten nanograms of oligonucleotide probe BAE-3 per ml, end-labeled with ^{32}P by using T4 polynucleotide kinase (GIBCO-BRL) to a specific activity of 4×10^8 to 6×10^8 cpm/μg, was added, and the solution was incubated for 5 h at 42°C. The filter was washed once for 5 min at room temperature in 3× SSPE, three times for 5 min each at room temperature in 1× SSPE containing 0.1% SDS, and once for 10 min at 55°C in 5× SSPE containing 0.1% SDS. The filter was then exposed to X-Omat XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens for 12 to 24 h at −70°C.

Data analysis. The agreement between PCR and culture was quantitated by calculating kappa (range, −1.0 to +1.0) and its standard error (16). The calculation of kappa, the ratio of chance-corrected observed agreement to chance-corrected complete agreement between two assays, was used because some degree of agreement by chance alone was expected. The one-sided hypothesis test of kappa = 0 versus kappa > 0 was conducted, and calculated agreement between PCR and culture was characterized as excellent (kappa > 0.75), moderate (kappa ≥ 0.40 and ≤ 0.75), or poor (kappa < 0.40) (16). For comparisons of PCR and culture in detecting infection with *B. burgdorferi* in individual mice, a mouse was designated as infected if any tissue was positive by either assay.

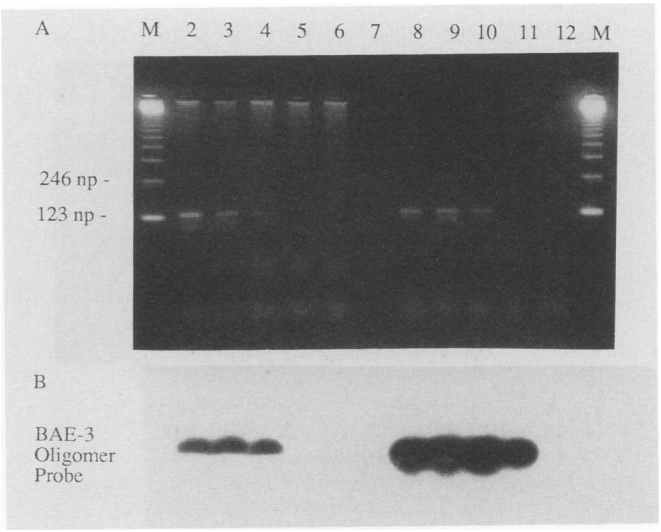

FIG. 1. Sensitivity of PCR for the detection of *B. burgdorferi* molecularly cloned DNA (pTRH44) and *B. burgdorferi* DNA extracted from strain B-31 organisms grown to early log phase in BSK II medium. Amplification products (10 μl) were analyzed by agarose gel electrophoresis (A) and by Southern hybridization with ^{32}P-labeled probe BAE-3 (B). The autoradiographic exposure time in panel B was 18 h. The numbers of copies of pTRH44 added to 50-μl reaction mixtures prior to amplification were 10^3 (lane 2), 10^2 (lane 3), 10 (lane 4), 1 (lane 5), and 10^{-1} (lane 6). Similarly, the numbers of B-31 organisms were 10^3 (lane 8), 10^2 (lane 9), 10 (lane 10), 1 (lane 11), and 10^{-1} (lane 12). Lane M contains 123-bp markers (GIBCO-BRL), and lane 7 does not contain a sample. np, nucleotide pair.

RESULTS

PCR sensitivity and specificity. On the basis of the sensitivity studies, PCR amplification followed by Southern hybridization with BAE-3 detected 10 to 50 copies of pTRH44 and 1 to 5 B-31 organisms prepared in culture (Fig. 1). The lower estimate represents the preamplification number of copies per organisms contained in a 10-μl sample (volume examined after amplification), whereas the higher estimate approximates the entire 50-μl reaction mixture. All culture-adapted laboratory strains of *B. burgdorferi*, the low-passage tick isolate 31248, and the field isolates inoculated into SCID mice were successfully amplified by primers BAE-1 and BAE-2 and yielded positive hybridization signals with BAE-3 (Fig. 2). In contrast, no amplification product (Fig. 2) was seen on agarose gels following the testing of other *Borrelia* species, and the results of Southern hybridization with the BAE-3 probe were consistently negative.

Detection of *B. burgdorferi* by culture. (i) Experimental infections. Our ability to obtain *B. burgdorferi* from mice of known infection status was confirmed by culturing spirochetes from all sampled tissues (kidney, liver, heart, brain, and ear) from all SCID mice experimentally inoculated with field isolates of *B. burgdorferi*. All five mice inoculated with field isolates of *B. burgdorferi* also developed clinical signs of tibiotarsal arthritis consisting of lameness, swelling, and red discoloration of the skin over affected joints, indicating the pathogenic potential of the isolates in this animal. In contrast, spirochetes were not recovered from the two mice inoculated with the B-31 strain of *B. burgdorferi* nor were clinical signs of arthritis observed in these animals.

(ii) Natural infections. Overall, *B. burgdorferi* was isolated in culture from 29 of 108 (27%) captured *P. leucopus* mice.

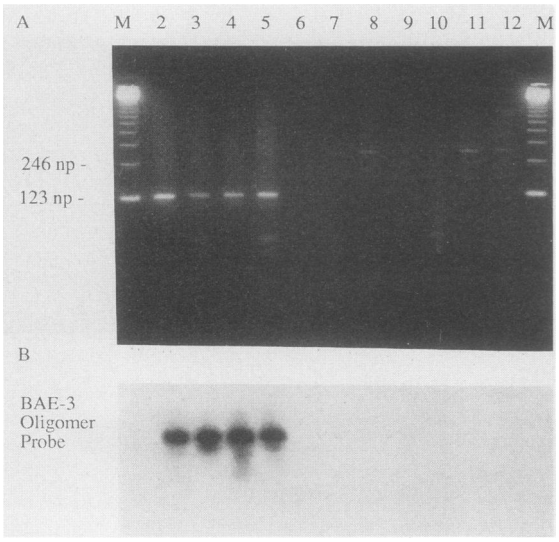

FIG. 2. Detection of *ospA*-coding sequences in DNA extracted from strains of *B. burgdorferi* (lanes 2 to 5) and specificity analysis of oligonucleotide primers BAE-1 and BAE-2 in non-*B. burgdorferi* strains (lanes 7 to 12). Organisms grown to early log phase in BSK II medium and 10 μl of amplification products were analyzed by agarose gel electrophoresis (A) and by Southern hybridization with ^{32}P-labeled probe BAE-3 (B). The autoradiographic exposure time in panel B was 18 h. Lanes: 2, Plag 9T-3-1-7; 3, Boutin; 4, Guilford; 5, 31248; 7, *B. hermsii*; 8, *B. coriaceae*; 9, *B. parkeri*; 10, *B. turicatae*; 11, *B. anserina*; 12, *B. crocidurae*. Lane M contains 123-bp markers (GIBCO-BRL), and lane 6 does not contain a sample. np, nucleotide pair.

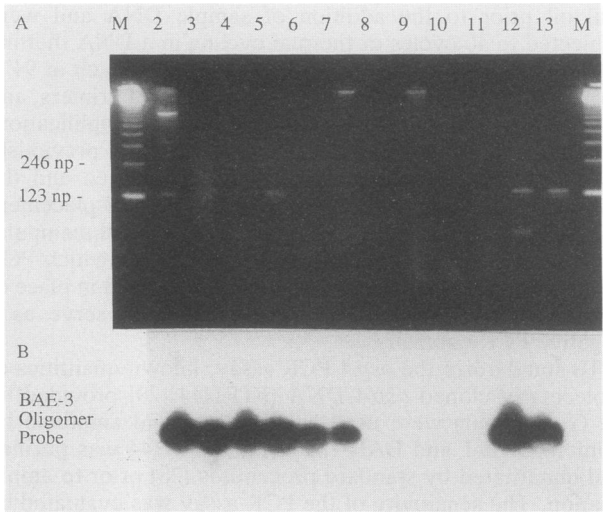

FIG. 3. *B. burgdorferi ospA* sequence in tissue specimens and field isolates from captured mammals by PCR and analysis of 10 μl of amplification products by gel electrophoresis (A) and Southern hybridization with probe ^{32}P-labeled BAE-3 (B). The autoradiographic exposure time in panel B was 18 h. Lanes: 2, 10^3 copies of pTRH44 per 50-μl reaction mixture; 3 to 5, spleen, bladder, and ear tissue from *P. leucopus* 90-18; 6, ear tissue from *P. leucopus* 91-230; 7, kidney tissue from *P. leucopus* 91-234; 8 to 11; kidney, spleen, bladder, and ear tissues from the colony-reared *P. leucopus*; 12, isolate from *P. leucopus* 90-18; 13, isolate from *P. leucopus* 91-230. Lane M contains 123-bp markers (GIBCO-BRL). np, nucleotide pair.

The mice were grouped on the basis of tissues sampled as described in Materials and Methods. Culture results showed isolation rates of 38% (6 of 16) in group 1, 35% (8 of 23) in group 2, and 22% (15 of 69) in group 3 (Table 1), suggesting that organs were an important source of parasites. Overall, in order of positive findings by tissues, spirochetes were detected in 11 of 37 (30%) bladder, 10 of 36 (28%) spleen, 22 of 92 (24%) ear, and 5 of 37 (14%) kidney cultures (Table 2). From captured mice in groups 1 and 2 with one or more culture-positive organs, *B. burgdorferi* was detected by culture in 11 of 12 (92%) bladders, 6 of 7 (86%) ears, 10 of 12 (83%) spleens, and 5 of 14 (36%) kidneys.

The identity of the spirochetal isolates as *B. burgdorferi* was established on the basis of IFA and PCR analyses (see below). *B. burgdorferi* chromosomal DNA was successfully amplified by PCR with chromosomal primers BBC-1 and BBC-3 in all field isolates (results not shown). All field isolates were positive for OspA by IFA with monoclonal antibody H5332. Additionally, *ospA* sequences were amplified in all isolates by BAE-1 and BAE-2 primers and specifically hybridized with BAE-3 (as shown for two of the isolates in Fig. 3).

Detection of *B. burgdorferi* by PCR. (i) Experimental infections. Overall, *B. burgdorferi ospA* sequences were detected by PCR in at least two tissues from each of the five SCID mice inoculated with field isolates of *B. burgdorferi*. In SCID mice inoculated with the B-31 strain of *B. burgdorferi*, *ospA* was detected by PCR in the heart and brain tissue of one mouse and in none of the tissues sampled in the second mouse. By tissue, *ospA* sequences were detected by PCR in 2 of 2 (100%) bladder, 5 of 5 (100%) ear, 4 of 5 (80%) heart, 4 of 5 (80%) brain, and 3 of 5 (60%) liver specimens examined from the SCID mice inoculated with field isolates of *B. burgdorferi*. *ospA* was not detected by PCR in any of the tissues removed from the six colony-reared uninfected *P. leucopus* mice (Fig. 3).

TABLE 1. Overall detection of *B. burgdorferi* infection in three groups of field-collected *P. leucopus* mice (n = 108) by PCR and culture[a]

Groups of mice[b]	No. tested	No. positive (%) by:			No. negative (%) by both methods	kappa (SE)
		Both methods	PCR only[c]	Culture only		
1	16	6 (37.5)	0 (0)	0 (0)	10 (62.5)	0.98 (0.129)[d]
2	23	7 (30.4)	2 (8.7)	1 (4.3)	13 (56.5)	0.72 (0.208)[d]
3	69	12 (17.4)	4 (5.8)	3 (4.3)	50 (72.5)	0.71 (0.105)[d]
Total	108	25	6	4	73	

[a] Agreement between PCR and culture, beyond chance, is indicated by kappa values.
[b] Mice were grouped on the basis of tissues sampled. Group 1, kidney, spleen, and bladder; group 2, kidney, spleen, bladder, and ear; group 3, ear only.
[c] Tissues for PCR treated with TEB only.
[d] $P < 0.01$ for the one-sided hypothesis test of kappa = 0 versus kappa > 0.

TABLE 2. Detection of *B. burgdorferi* from different tissues obtained from naturally exposed *P. leucopus* mice (n = 108) by PCR and culture[a]

Tissue examined[b]	No. tested	No. positive (%) by:			No. negative (%) by both methods	kappa (SE)
		Both methods	PCR only[c]	Culture only		
Kidney	37[d]	2 (5.4)	2 (5.4)	3 (8.1)	30 (81.0)	0.37[e] (0.163)
Spleen	36[d]	1 (2.7)	2 (5.6)	9 (25.0)	24 (66.7)	0.03 (0.130)
Bladder	37[d]	7 (18.9)	1 (2.7)	4 (10.8)	25 (67.6)	0.65[f] (0.161)
Ear	92	19 (20.7)	4 (4.3)	3 (3.3)	66 (71.7)	0.80[f] (0.105)
Total	202	29	9	19	145	

[a] Agreement between assays, beyond chance, is indicated by kappa values.
[b] Tissues were obtained from 16 mice sampled for kidney, spleen, and bladder (group 1), 23 mice sampled for those organs plus ear (group 2), and 69 mice sampled for ear alone (group 3).
[c] Tissues for PCR treated with TEB only.
[d] Discrepancy in the number of samples tested and number of animals tested represents contaminated cultures (two kidney, three spleen, and two bladder cultures).
[e] $P < 0.05$ for the one-sided hypothesis test of kappa = 0 versus kappa > 0.
[f] $P < 0.01$ for the one-sided hypothesis test of kappa = 0 versus kappa > 0.

(ii) Natural infections. Overall, *ospA* was detected by PCR in one or more tissues from 31 of 108 (29%) captured *P. leucopus* mice (Table 1). According to sampling group, *ospA* was detected by PCR in 38% (6 of 16) of captured *P. leucopus* mice in group 1, 39% (9 of 23) in group 2, and 23% (16 of 69) in group 3 (Table 1). By tissue, PCR detected *ospA* in 23 of 92 (25%) ear, 8 of 37 (22%) bladder, 4 of 37 (11%) kidney, and 3 of 36 (8%) spleen specimens examined from captured *P. leucopus* mice (Table 2). From captured mice in groups 1 and 2 with one or more culture-positive organ samples, *ospA* was detected by PCR in 6 of 7 (86%) ear, 8 of 14 (57%) bladder, 4 of 14 (29%) spleen, and 3 of 14 (21%) kidney samples. From captured mice in group 3 with culture-positive ear biopsy samples, 12 of 15 (80%) ear samples were also positive by PCR analysis. Additionally, *ospA* was not detected by PCR in any ear biopsy sample obtained from 13 culture-negative mice in group 2.

Agreement between PCR and culture on detection of *B. burgdorferi*. (i) Experimental infections. The concordance between assays was 100% when PCR and culture were compared for the detection of *B. burgdorferi* in individual SCID mice experimentally inoculated with field isolates of the organism. By tissue, we observed 100% concordance between the assays in bladder and ear specimens removed from experimentally inoculated SCID mice. Culture, however, detected *B. burgdorferi* in liver and brain specimens removed from one SCID mouse, heart from a second mouse, and brain from third mouse, whereas PCR tests for those samples were negative. As mentioned previously, heart tissue from one animal inoculated with B-31 was positive by PCR but negative when cultured.

(ii) Natural infections. The relative agreement between PCR and culture in detecting infection with *B. burgdorferi* in captured *P. leucopus* mice in the three sampling groups is shown in Table 1. Excellent agreement between assays was observed in group 1 (kappa = 0.98). However, only moderate agreement between assays was observed in groups 2 and 3 (kappa = 0.72 and 0.71, respectively). PCR detected *ospA* in six additional *P. leucopus* mice from which the organism was not isolated (two mice by two or more organ samples and four mice by ear sample alone), whereas the organism was isolated from four animals in which PCR was negative (one mouse by spleen and bladder samples, and three mice by ear sample alone) (Table 1). Complete agreement between PCR and culture was observed for ear samples obtained from mice in group 2 (data not shown).

The relative abilities of PCR and culture to detect *B. burgdorferi* in different tissues removed from naturally infected *P. leucopus* are shown in Table 2. Overall, more infections were detected by culture (48 total) than by PCR (38 total), and this finding was consistent for all tissues, with the exception of ear tissue. The kappa value was significantly greater than 0 in comparisons of PCR and culture for kidney, bladder, and ear tissue; however, estimates of the standard error of kappa were large. The degree of agreement between assays, as measured by kappa, was excellent for ear tissue (kappa = 0.80), moderate for bladder and kidney, and poor for spleen. In tissues in which discrepant results were observed, PCR alone detected *ospA* in 9 samples (5 organ and 4 ear samples) compared with 19 samples (16 organ and 3 ear samples) that were positive for *B. burgdorferi* by culture alone.

(iii) Discordant results between PCR and culture. To further investigate the discordant results between PCR and culture, we determined the contribution of the minimal tissue-processing method to the potential loss of sensitivity by PCR. DNA was extracted by phenol-chloroform followed by precipitation in ethanol from tissues that were culture positive but PCR negative. Tissues in which sufficient sample was available included liver (n = 2), heart (n = 1), and brain (n = 1) from experimentally inoculated SCID mice and kidney (n = 3), spleen (n = 9), bladder (n = 4), and ear (n = 3) from field-collected *P. leucopus* mice. Upon retesting these samples by PCR, DNA extraction procedures resulted in detectable *ospA* sequences in one additional liver and heart specimen from SCID mice, one additional kidney and spleen specimen from naturally infected mice, and two additional ear specimens.

To investigate whether false-positive PCR assays contributed to the discordant results, we examined the individual capture histories and assay results from the four *P. leucopus* mice which were PCR positive and culture negative. Multiple-organ samples from two mice in group 2 were positive by PCR for *B. burgdorferi* following phenol-chloroform extraction and retesting. We concluded that these results indicated real infections. Three of the remaining four mice with discordant *ospA* PCR results (group 3) were positive only by Southern hybridization but were negative by band visualization on agarose gels. Reamplification of the original ear biopsy digest from these three mice resulted in detection of *ospA* in only one sample, indicating either a low copy number of DNA template in the PCR or false-positive

results. The fourth mouse, which was positive for *ospA* on agarose gel and by Southern hybridization, was recaptured as part of a longitudinal study and resampled by ear biopsy 8 weeks after the first capture. At that time, *B. burgdorferi* was detected by both *ospA* PCR and culture, indicating that the first PCR result was probably specific.

DISCUSSION

This report describes the development and preliminary application of a PCR-based assay for detection of infection with *B. burgdorferi* in *P. leucopus* mice captured in a Lyme disease enzootic area. When the results by PCR are compared with the standard of culture of the spirochete, we conclude that in longitudinal studies, in which individual *P. leucopus* mice are repetitively captured and sampled, PCR examination of ear tissue may be nearly as sensitive, but not as specific, as culture for detection of *B. burgdorferi*. Our data also indicate that PCR of organ tissue, minimally processed in TEB alone, was generally less sensitive than culture for detection of the spirochete. However, in studies in which captured mice are sacrificed and multiple organs are sampled, PCR may be as sensitive and specific as culture for the determination of overall infection with *B. burgdorferi* in individual mice. We conclude that both PCR and culture may be required to detect all mice infected with *B. burgdorferi*.

The *ospA* gene of *B. burgdorferi* has been the amplification target in a number of published reports (14, 18, 23, 25, 26) in which PCR was utilized to detect *B. burgdorferi*. We selected nucleotide sequences to be used as oligonucleotide primers from a region of *ospA* likely to be highly conserved between *ospA* and *ospB*, on the basis of the published homology between the amino acid sequences of the OspA and OspB proteins (8). With the exception of Nielsen et al. (25), the PCR primers described in previous reports (14, 18, 23, 26) amplified *ospA* sequences falling in regions less likely to be highly conserved. While a lack of sensitivity for detecting *ospA* by PCR has been reported elsewhere for some isolates (14, 26), we found PCR primers BAE-1 and BAE-2 to be highly sensitive for the laboratory-adapted strains of *B. burgdorferi* and for the field isolates of the organism that were examined. The analytic sensitivity of PCR with BAE-1 and BAE-2 used for B-31 grown in culture observed in this study (1 to 5 spirochetes) was similar to that observed in other published reports with *ospA* as an amplification target (14, 23) but more sensitive than that reported by Nielsen et al. (25) (50 fg = approximately 45 spirochetes). The sensitivity which we observed is comparable to that reported utilizing PCR primers designed to amplify flagellin (33) and chromosomal (17, 27) gene sequences. Additionally, our results are in agreement with those of others (14, 18, 23) who have found *ospA* primers to be specific for *B. burgdorferi*.

In determining the infection status of field-captured *P. leucopus* mice by culture, our observation that the bladder was the most efficient organ for obtaining *B. burgdorferi*, followed by the spleen and kidney, was in accord with a previous report, according to which the bladder and the spleen were the most efficient organs for culturing spirochetes from wild-caught *P. leucopus* mice (13). However, the proportion of spleen and bladder cultures positive for the organism in infected mice observed in that study was 57% each, compared with our findings, which were 77 and 85%, respectively. Additionally, in our field-captured mice with one or more culture-positive organ samples, the frequency of culture-positive ear samples (86%) was comparable to that previously reported from wild-caught *P. leucopus* mice (85.7%) which were prescreened for infection with *B. burgdorferi* by xenodiagnosis (30).

When we compared the results of PCR and culture for detection of *B. burgdorferi* in tissues obtained from captured *P. leucopus* (Table 2) and experimentally infected SCID mice, we observed that PCR of tissues minimally processed in TEB alone was consistently less sensitive than culture of organ and ear samples. In contrast, Lebech et al. (21), using a tissue digestion step followed by phenol-chloroform extraction of the DNA, reported an increase in sensitivity in the ability to detect *B. burgdorferi* in kidneys, spleens, and urinary bladders obtained from experimentally infected gerbils by PCR compared with that of culture. Our results also indicate that addition of a phenol-chloroform extraction step increases sensitivity. In our study of infected mice, the low level of agreement between PCR for *ospA* and culture, as quantitated by kappa, of kidney and spleen samples may have been affected by the DNA extraction method. Our method allowed known inhibitors of *Taq* polymerase activity (e.g., hemoglobin) (19) to be added to the PCR. Repeat testing by PCR of phenol-chloroform-extracted DNA from a small number of culture-positive and PCR-negative samples only slightly improved the sensitivity of PCR in organ tissue but resulted in a marked improvement in the sensitivity of PCR in ear samples obtained from mice in group 2. In this study, we chose minimal tissue processing in TEB alone in order to limit cross-contamination of samples during the tissue-processing steps. However, it appears that tissue digestion in TEB followed by phenol-chloroform extraction may be necessary for optimal sensitivity of the PCR assay of both organ and ear tissues.

It is difficult to conclusively establish that a new technique, such as PCR, may be more sensitive than an accepted technique, such as culture, for detection of *B. burgdorferi* in infected mice. However, the sensitivity of culture of ear tissue appears to be approximately 85% in naturally infected *P. leucopus* prescreened for infection with *B. burgdorferi* by xenodiagnosis (30) and, in the present study, by culture of internal organs. Because PCR has the ability to detect nonviable *B. burgdorferi*, we were unable to determine whether the detection of *ospA* sequences in culture-negative tissue samples was due to the presence of organisms or due to false-positive results. We believe that detection of *ospA* sequences on retesting by PCR in the PCR-positive, culture-negative tissues from mice in groups 2 and 3 indicated true-positive results but did not necessarily indicate detection of viable organisms. Only through longitudinal study of individual animals exposed to the organism can this question be better resolved. In this regard, we detected *B. burgdorferi* by both PCR and culture in one mouse from group 3, 8 weeks after obtaining the initial PCR-positive culture-negative ear sample. However, because the animal was potentially exposed to infected ticks after the first ear sample was obtained, we cannot rule out the possibility that the infection had been acquired during the intervening period.

While the observed differences in agreement between PCR and culture on detection of infection with *B. burgdorferi* between study groups was not significant (Table 1), the variation in agreement between assays may be influenced by seasonal variation in the ability to detect *B. burgdorferi* in organs obtained from mice. Complete agreement between PCR and culture was observed (kappa = 0.98) in group 1 mice, which were sacrificed in July immediately after the period of peak nymphal *Ixodes dammini* activity. In contrast, only moderate agreement was observed in group 2 mice (kappa = 0.72), sacrificed between March and early

May, prior to the period of nymphal *I. dammini* activity. Therefore, the intensity of *B. burgdorferi* transmission certainly varied over the period during which the two sets of samples were gathered.

The findings of this study are of considerable practical importance, since PCR analysis for *ospA* in DNA from ear biopsy samples will permit prospective analyses of individual reservoir animals in the field. The agreement between PCR and culture was the highest (kappa = 0.80) with ear tissue, indicating the validity of PCR as a detection method. In addition to demonstrating a comparable sensitivity to culture, PCR permitted a more rapid determination of infection than culture of ear tissue. For most longitudinal field studies, simple DNA extraction procedures (TEB) may be suitable to determine the infection status of individual animals. If the goal of the study is to document all infections, then organ sampling may be necessary when both culture and PCR, with additional phenol-chloroform extraction, are performed.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant R55 A130042 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Anderson, J. F., P. H. Duray, and L. A. Magnarelli.** 1987. Prevalence of *Borrelia burgdorferi* in white-footed mice and *Ixodes dammini* at Fort McCoy, Wis. J. Clin. Microbiol. **25:** 1495–1497.
2. **Anderson, J. F., R. C. Johnson, and L. A. Magnarelli.** 1987. Seasonal prevalence of *Borrelia burgdorferi* in natural populations of white-footed mice, *Peromyscus leucopus*. J. Clin. Microbiol. **25:**1564–1566.
3. **Anderson, J. F., L. A. Magnarelli, W. Burgdorfer, and A. G. Barbour.** 1983. Spirochetes in *Ixodes dammini* and mammals from Connecticut. Am. J. Trop. Med. Hyg. **32:**818–824.
4. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57:**521–525.
5. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect. Immun. **41:**795–804.
6. **Barthold, S. W., K. D. Moody, G. A. Terwilliger, P. H. Duray, R. O. Jacoby, and A. C. Steere.** 1988. Experimental Lyme arthritis in rats infected with *Borrelia burgdorferi*. J. Infect. Dis. **157:**842–846.
7. **Benach, J. L., E. M. Bosler, J. P. Hanrahan, J. L. Coleman, G. S. Habicht, T. F. Bast, D. J. Cameron, J. L. Ziegler, A. G. Barbour, W. Burgdorfer, R. Edelman, and R. A. Kaslow.** 1983. Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. **308:**740–742.
8. **Bergstrom, S., V. G. Bundoc, and A. G. Barbour.** 1989. Molecular analysis of linear plasmid-encoded major surface proteins, OspA and OspB, of the Lyme disease spirochete *Borrelia burgdorferi*. Mol. Microbiol. **3:**479–486.
9. **Bosler, E. M., J. L. Coleman, J. L. Benach, D. A. Massey, J. P. Hanrahan, W. Burgdorfer, and A. G. Barbour.** 1983. Natural distribution of the *Ixodes dammini* spirochete. Science **220:**321–322.
10. **Bosma, G. C., M. Fried, R. P. Custer, A. Carroll, D. M. Gibson, and M. J. Bosma.** 1988. Evidence of functional lymphocytes in some (leaky) scid mice. J. Exp. Med. **167:**1016–1033.
11. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216:**1317–1319.
12. **Burgess, E. C., T. E. Amundson, J. P. Davis, R. A. Kaslow, and R. Edelman.** 1986. Experimental inoculation of *Peromyscus* spp. with *Borrelia burgdorferi*: evidence of contact transmission. Am. J. Trop. Med. Hyg. **35:**355–359.
13. **Callister, S. M., W. A. Agger, R. F. Schell, and K. M. Brand.** 1989. Efficacy of the urinary bladder for isolation of *Borreliaburgdorferi* from naturally infected, wild *Peromyscus leucopus*. J. Clin. Microbiol. **27:**773–774.
14. **Debue, M., P. Gautier, C. Hackel, A. Van Elsen, A. Herzog, G. Bigaignon, and A. Bollen.** 1991. Detection of *Borrelia burgdorferi* in biological samples using the polymerase chain reaction assay. Res. Microbiol. **142:**565–572.
15. **Duray, P. H., and R. C. Johnson.** 1986. The histopathology of experimentally infected hamsters with the Lyme disease spirochete, *Borrelia burgdorferi* (42251). Proc. Soc. Exp. Biol. Med. **181:**263–269.
16. **Fleiss, J. L.** 1981. Statistical methods for rates and proportions. John Wiley & Sons, New York.
17. **Goodman, J. L., P. Jurkovich, J. M. Kramber, and R. C. Johnson.** 1991. Molecular detection of persistent *Borrelia burgdorferi* in the urine of patients with active Lyme disease. Infect. Immun. **59:**269–278.
18. **Guy, E. C., and G. Stanek.** 1991. Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. J. Clin. Pathol. **44:**610–611.
19. **Higuchi, R.** 1989. Rapid, efficient DNA extraction for PCR from cells or blood. Amplifications **1989:**1–3.
20. **Howe, T. R., F. W. LaQuier, and A. G. Barbour.** 1986. Organization of genes encoding two outer membrane proteins of the Lyme disease agent *Borrelia burgdorferi* within a single transcriptional unit. Infect. Immun. **54:**207–212.
21. **Lebech, A. M., P. Hindersson, J. Vuust, and K. Hansen.** 1991. Comparison of in vitro culture and polymerase chain reaction for detection of *Borrelia burgdorferi* in tissues from experimentally infected animals. J. Clin. Microbiol. **29:**731–737.
22. **Magnarelli, L. A., J. F. Anderson, K. E. Hyland, D. Fish, and J. B. McAninch.** 1988. Serologic analyses of *Peromyscus leucopus*, a rodent reservoir for *Borrelia burgdorferi*, in northeastern United States. J. Clin. Microbiol. **26:**1138–1141.
23. **Malloy, D. C., R. K. Nauman, and H. Paxton.** 1990. Detection of *Borrelia burgdorferi* using the polymerase chain reaction. J. Clin. Microbiol. **28:**1089–1093.
24. **Mather, T. N., M. L. Wilson, S. I. Moore, J. M. C. Ribeiro, and A. Spielman.** 1989. Comparing the relative potential of rodents as reservoirs of the Lyme disease spirochete (*Borrelia burgdorferi*). Am. J. Epidemiol. **130:**143–150.
25. **Nielsen, S. L., K. K. Y. Young, and A. G. Barbour.** 1990. Detection of *Borrelia burgdorferi* by the polymerase chain reaction. Mol. Cell. Probes **4:**73–79.
26. **Persing, D. H., S. R. Telford III, A. Spielman, and S. W. Barthold.** 1990. Detection of *Borrelia burgdorferi* infection in *Ixodes dammini* ticks with the polymerase chain reaction. J. Clin. Microbiol. **28:**566–572.
27. **Rosa, P. A., and T. G. Schwan.** 1989. A specific and sensitive assay for the Lyme disease spirochete *Borrelia burgdorferi* using the polymerase chain reaction. J. Infect. Dis. **160:**1018–1029.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Schaible, U. E., M. D. Kramer, C. Museteanu, G. Zimmer, H. Mossmann, and M. M. Simon.** 1989. The severe combined immunodeficiency (*scid*) mouse. J. Exp. Med. **170:**1427–1432.
30. **Sinsky, R. J., and J. Piesman.** 1989. Ear punch biopsy method for detection and isolation of *Borrelia burgdorferi* from rodents. J. Clin. Microbiol. **27:**1723–1727.
31. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
32. **Telford, S. R., III, T. N. Mather, G. H. Adler, and A. Spielman.** 1990. Short-tailed shrews as reservoirs of the agents of Lyme disease and human babesiosis. J. Parasitol. **76:**681–683.
33. **Wise, D. J., and T. L. Weaver.** 1991. Detection of the Lyme disease bacterium, *Borrelia burgdorferi*, by using the polymerase chain reaction and a nonradioisotopic gene probe. J. Clin. Microbiol. **29:**1523–1526.
34. **Wright, S. D., and S. W. Nielsen.** 1990. Experimental infectio of the white-footed mouse with *Borrelia burgdorferi*. Am. J.Vet. Res. **51:**1980–1987.