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Detection of *Borrelia burgdorferi* Using the Polymerase Chain Reaction

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Segments of the *ospA* gene of *Borrelia burgdorferi* were amplified by the polymerase chain reaction (PCR). Oligonucleotide primers used in the reaction flank a 309-base-pair segment within the *ospA* gene. After 35 cycles of amplification, the product could be detected by agarose gel electrophoresis or dot hybridization with a ^{32}P-labeled probe. This segment was amplified in all strains of *B. burgdorferi* tested, but it was not detected in other bacterial species. An additional primer pair which has a broad specificity for conserved 16S rRNA sequences that are present in eubacteria amplified a 215-base-pair fragment in all organisms tested. The sensitivity of PCR for the detection of *B. burgdorferi* in clinical samples was evaluated by seeding blood and urine specimens with *B. burgdorferi* and subjecting them to amplification. We were able to detect 10 organisms per ml of blood or urine, using PCR with dot hybridization detection. DNA extraction is not required for sample preparation. Blood and urine specimens were obtained from canines with clinical and serologic evidence of Lyme disease and subjected to PCR analysis. Of 17 clinical specimens from 15 animals, one blood specimen showed reactivity in the PCR.

Borrelia burgdorferi, the causative agent of Lyme disease, was first isolated from ticks (10) and subsequently from humans (34), rodents (1, 2), and some wild and domestic animals (9, 11). The organism is transmitted to humans and animals by infected *Ixodes* ticks (10). The white-tailed deer is the primary reservoir of the adult tick, and the white-footed mouse, *Peromyscus leucopus*, serves as one of the primary reservoirs for the larval form (23).

Infection in humans and some domestic animals leads to one or more of a variety of manifestations, including dermatopathy, cardiopathy, neuropathy, and arthropathy (28, 35). Since the spirochete has been identified in clinical specimens from infected tissues and body fluids (7, 35), these manifestations are presumed to result, at least partially, from virulence factors of the persisting spirochete in the infected organ. However, it is relatively difficult to demonstrate the presence of the spirochete in infected tissues (34). Animal experiments have shown that spirochetes can be recovered from blood up to 2 weeks after infection (21). However, the presence of spirochetes in heart blood several months after infection (15) and the apparent hematogenous spread of infection suggest a long-term, and perhaps intermittent, spirochetemia. The inability to recover spirochetes from blood and affected organs routinely may be due to a paucity of organisms present in infected material. Some investigators have successfully demonstrated spirochetes directly by microscopic examination of blood and urine specimens from infected animals (9, 24, 32). However, microscopic studies are impractical for screening large numbers of specimens and may not be sufficiently sensitive for the routine detection of spirochetes in infected material.

To facilitate the understanding of *B. burgdorferi* infection and the role of the spirochete in pathogenesis, we sought to improve on current methods of detection. In order to assay available clinical and research samples for *B. burgdorferi* with a high sensitivity and specificity, we adapted the in vitro gene amplification technology, the polymerase chain reaction (PCR), for this purpose. Using this system, Saiki et al. (29) have amplified the concentration of specific nucleic acid sequences as much as 10^6-fold. This technique has since been applied to the detection of specific DNA sequences of a number of pathogens, including human immunodeficiency virus type 1 in human blood (22), human papillomavirus in paraffin-embedded tissue (31), and a number of human DNA sequences for the diagnosis of genetic and malignant disorders (17, 18). Recently, Olive (27) reported the detection of enterotoxigenic *Escherichia coli* in partially purified human stool specimens using PCR.

In the present study, the detection of a sequence within the outer membrane protein gene (*ospA*) (16) of *B. burgdorferi* by PCR is described. The specificity and sensitivity of this technique as applied to *B. burgdorferi* detection are evaluated, and the detection of *B. burgdorferi* in the blood of an infected canine is described.

MATERIALS AND METHODS

Bacterial strains and cultivation. Several strains of *B. burgdorferi* were used: strain B31 (ATCC 35210), the prototype strain which was isolated from *Ixodes dammini* (10); *B. burgdorferi* IRS (ATCC 35211) (5), which was isolated from *Ixodes ricinus* (European); strain SH-2-82, an isolate from a naturally infected *I. dammini* tick (30) which was kindly provided by Tom Schwan, Rocky Mountain Laboratories, Hamilton, Mont.; human blood isolates CDC 005, CDC 031, and CDC 033; European tick isolate CDC 008; human skin isolate CDC 029; and cerebrospinal fluid isolate CDC 030, which were generously supplied by Susan Stolz, University of Wisconsin-Madison Medical School, Madison; human cerebrospinal fluid isolate Boutin, which was originally obtained from G. Baranton, Pasteur Institute, Paris, France. Other spirochetes tested were *Borrelia coriaceae* (ATCC 43381) (19); *Borrelia hermsii* (ATCC 35209), *Borrelia parkeri*, and *Borrelia turicata*, which were kindly provided by Russell Johnson, University of Minnesota Medical School, Minneapolis; *Treponema denticola* ATCC 33520; and *Leptospira interrogans* serovar *canicola* ATCC 23470. The

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following isolates from human infections were identified by routine microbiological assays in our clinical microbiology laboratory and were tested for PCR reactivity: *E. coli*, *Klebsiella pneumoniae*, *Streptococcus viridans*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Streptococcus pyogenes*.

All *Borrelia* species were maintained in BSK II medium (6) at 35°C, and spirochetes were enumerated in a Petroff-Hausser counting chamber. All other organisms were grown on conventional plated media, and colonies were suspended in filtered (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) 0.15 M phosphate-buffered saline (PBS; pH 7.3) at a concentration of approximately 3×10^8 cells per ml (McFarland no. 1 standard) and diluted 1:3,000 in BSK II medium.

DNA extraction and Southern hybridization. Plasmid DNA was purified from *B. burgdorferi* B31 and IRS as described previously (3). Plasmids were separated by electrophoresis in 0.2% agarose gels (SeaKem GTG Agarose; FMC Corp., Marine Colloids Div., Rockland, Maine) and transferred to a nylon membrane (Schleicher & Schuell, Inc., Keene, N.H.) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (26). The membrane was baked at 80°C and prehybridized (45°C for 1 h) in 50% formamide–5× SSC–4× Denhardt solution (10× Denhardt solution is 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, and 0.2% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.])–100 μg of denatured and sonicated herring sperm DNA per ml. PCR-amplified *B. burgdorferi* B31 DNA was labeled with ^{32}P by using a random primed labeling kit (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) and hybridized to the membrane at 45°C for 16 h in the prehybridization solution. The blot was washed first in 1× SSC–0.1% sodium dodecyl sulfate for 30 min at room temperature, and the membrane was then transferred to 0.1× SSC–0.1% sodium dodecyl sulfate for 1 h at 65°C (26). The membrane was dried and exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen.

Preparation of samples for PCR analysis. (i) Bacterial strains. Approximately 10^5 organisms of each strain were suspended in 1 ml of BSK II medium and centrifuged at 12,800 × *g* for 8 min. The resulting supernatant was decanted, and the pellet was washed twice with 1 ml of filtered PBS. The pellet was suspended in 100 μl of filter-sterilized distilled water and boiled for 10 min. PCR was carried out with 10 μl of the boiled suspension.

(ii) Blood. A 4-day-old culture of *B. burgdorferi* B31 (10^8 cells per ml in BSK II medium) was serially diluted (10-fold) in EDTA-anticoagulated whole blood. The tubes were centrifuged at 800 × *g* for 15 min, and 1 ml of plasma was removed. The plasma was centrifuged at 12,800 × *g* for 8 min, the supernatant was decanted, and the pellet was washed twice with filtered PBS. The pellet was suspended in 100 μl of sterile filtered distilled water and boiled for 10 min. The PCR was performed with 10 μl of the boiled suspension.

(iii) Urine. Urine was seeded with *B. burgdorferi* B31; and tubes were centrifuged at 12,800 × *g*, washed, and boiled, following the same procedure described above for blood. The PCR was performed with 10 μl of the boiled suspension.

(iv) Clinical specimens. Blood and urine specimens were obtained from 15 canines that had clinical and serological evidence of Lyme disease and 5 normal, healthy canines. Specimens were prepared for PCR analysis as described above.

Immunofluorescence assay (IFA). Slides coated with *B. burgdorferi* whole-cell antigen were obtained from Bion, Inc. (Park Ridge, Ill.). Twofold dilutions of serum in PBS were made, and a drop of each dilution above 1:32 was placed on a slide well. The slides were incubated in a moist chamber at room temperature for 60 min. The slides were rinsed, soaked in PBS for 10 min, and gently blotted. A 25-μl volume of flourescein isothiocyanate-labeled sheep anti-dog immunoglobulin (American Qualex, La Mirada, Calif.) was placed on each slide well; and the slides were incubated in a moist chamber at room temperature for 30 min, rinsed, and dried. Slides were covered with carbonate-buffered mounting fluid (pH 9.0) and cover slips and read with a fluorescence microscope (Labolux-12; Leitz/Opto-Meric Div. of E. Leitz Inc., Rockleigh, N.J.) equipped with a mercury light source (HBO-50).

Enzymatic amplification. PCR was run as described previously (29) on a Thermal Cycler (Perkin Elmer-Cetus, Norwalk, Conn.). Oligonucleotide primers were synthesized on an automated DNA synthesizer (Cyclone; MilliGen Biosearch, Burlington, Mass.). Each 10-μl sample was amplified for 35 repeated cycles of denaturation at 94°C for 30 s, annealing at 55°C for 40 s, and chain elongation at 74°C for 55 s in the presence of heat-stable DNA polymerase from *Thermus aquaticus* (*Taq*; Perkin Elmer-Cetus) and each of the four deoxynucleotide triphosphates (Perkin Elmer-Cetus) in the reaction mixture. Amplification products were analyzed by agarose gel electrophoresis in 1.5% agarose and dot hybridization.

Dot hybridization. PCR nucleic acid products (20 μl) were combined with 10 μl of 3 M NaOH and 80 μl of TE (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]). After 60 min at 65°C, the pH was neutralized by the addition of 100 μl of 2 M ammonium acetate. The samples were applied to a nylon membrane by using a filtration manifold. The filters were dried and hybridized with the *B. burgdorferi* probe as described above.

RESULTS

Amplification of *ospA* sequence in *B. burgdorferi* DNA and analysis of amplification products. The outer membrane protein genes of *B. burgdorferi*, *ospA* and *ospB*, have been sequenced previously (8). We chose to amplify a segment of the *ospA* gene because this gene may be unique to *B. burgdorferi*, based on DNA probe hybridization studies (6), and is widely expressed among various strains of *B. burgdorferi* that have been studied by other investigators (3, 6, 8).

The oligonucleotide primers $ospA_1$ and $ospA_2$ (Fig. 1) span a 309-base-pair (bp) sequence within the *ospA* gene. These primers were used to amplify the *ospA* sequence in purified plasmid DNA from strain B31. The 309-bp amplification product (Fig. 1) hybridized with the 49-kilobase *B. burgdorferi* plasmid (Fig. 2), which is known to be the location of the *ospA* gene (3). This confirmed the expected amplification.

Specificity of amplification. To determine the specificity of the oligonucleotide primers for *B. burgdorferi*, a number of pathogenic organisms were tested for their reactivities in the PCR. An additional primer pair which has broad specificity for conserved 16S rRNA sequences that are present in eubacteria (12) (Fig. 1) was used as an amplification control. This primer pair amplifies a 215-bp fragment. The broadly specific primers gave rise to a 215-bp DNA fragment in all organisms tested, while $ospA_1$ and $ospA_2$ amplified a 309-bp fragment only in strains of *B. burgdorferi* (Fig. 3).

Sensitivity of PCR and dot hybridization detection. To evaluate the sensitivity of PCR, human blood and urine specimens were seeded with *B. burgdorferi* and subjected to

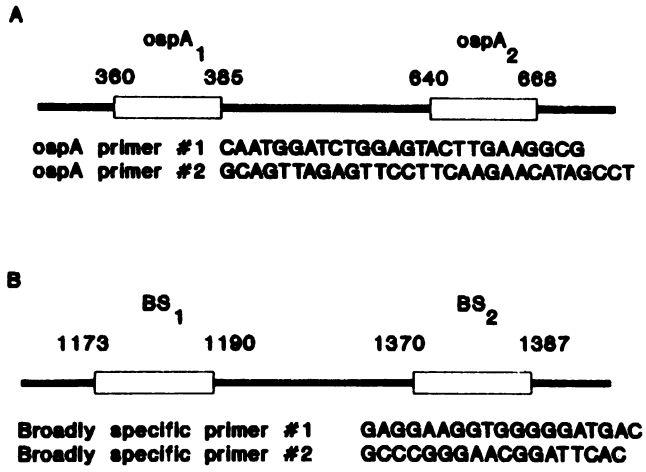

FIG. 1. The oligomer primers used in PCR. (A) The base positions of *B. burgdorferi* are numbered from the previously published sequence (8). (B) Broadly specific primers (BS_1 and BS_2) are those described by Chen et al. (12).

amplification. By using dot hybridization to detect amplification products, 10 organisms per ml of blood or urine could be detected. Results of these experiments are shown in Fig. 4.

Serological analysis. A total of 308 clinical canine specimens which were submitted for serological testing for Lyme disease were evaluated by IFA. Of these 308 serum specimens 76 were reactive in the IFA with titers ranging from 1:64 to 1:16,384.

PCR analysis of clinical samples. Clinical specimens from 15 canines which were diagnosed as having Lyme disease were chosen for PCR analysis on the basis of clinical symptoms and serology (IFA titer, greater than 1:64). Whole blood from 15 canines and two urine specimens were obtained and analyzed by PCR. One blood specimen from an animal with an IFA titer of 1:4,196 was found to be positive (Fig. 5). This result was confirmed by Southern blot analysis (data not shown). All other specimens were negative in the PCR.

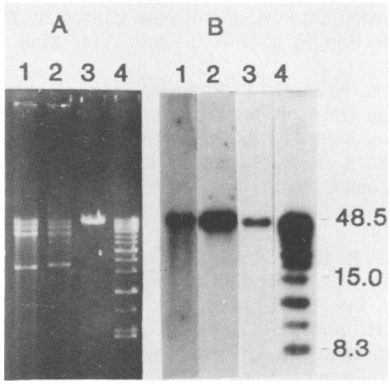

FIG. 2. *B. burgdorferi* B31 (lanes 1) and IRS (lanes 2) plasmids were separated in 0.2% agarose (A) and subsequently transferred to a nylon membrane. The membrane was probed with labeled PCR amplification products of *B. burgdorferi* B31. An autoradiograph is shown (B; lanes 1 and 2). Size markers are ^{32}P-labeled lambda DNA (48.5 kilobases) (lanes 3) and lambda DNA *Hin*dIII digest fragments (lanes 4; molecular sizes are noted on the right in kilobases).

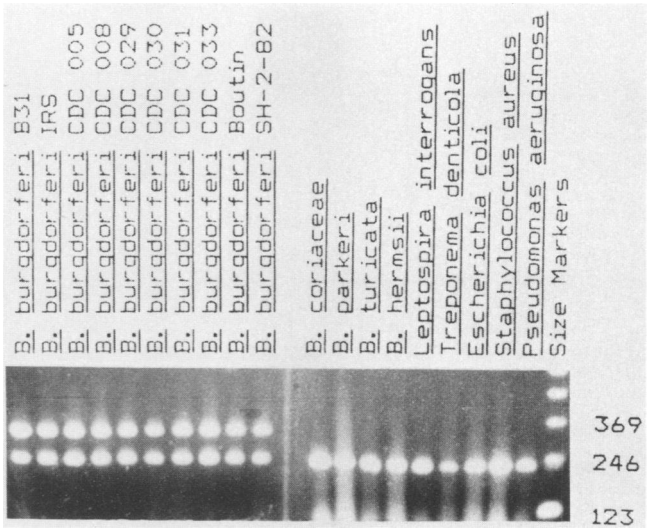

FIG. 3. Agarose gel analysis of PCR products of *B. burgdorferi* strains and other bacteria. Not shown in the figure are other organisms which gave rise to a 215-bp fragment only, as follows: *Streptococcus viridans*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Streptococcus pyogenes*, and *Klebsiella pneumoniae*. Numbers at the right are molecular size markers (in kilobases).

DISCUSSION

Lyme disease is now the most common tick-borne infection in the United States and Europe (13, 33). The study of this disease is hampered by the fact that recovery of *B. burgdorferi* from infected body fluids and tissues is difficult. The diagnosis of Lyme disease is generally based on clinical criteria and the results of serological tests. However, symptoms are not pathognomonic, and serological test results are frequently ambiguous. In the early stages of Lyme disease, serology may be negative or equivocal because of a poor or delayed immune response (4, 14). Cross-reactive antibodies

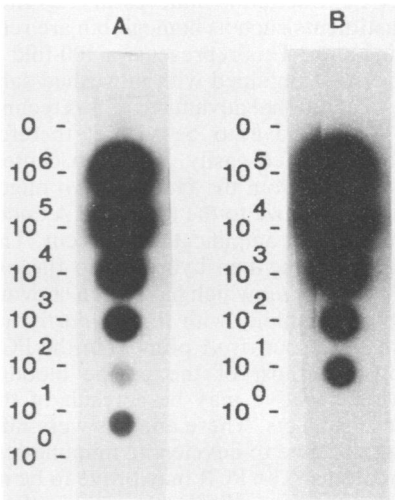

FIG. 4. Dot hybridization of PCR products of blood (A) and urine (B) specimens seeded with *B. burgdorferi* B31. Samples were amplified with the primers $ospA_1$ and $ospA_2$. The number of organisms per milliliter is indicated. The well corresponding to 10^2 cells per ml in panel A was faint because an insufficient sample was applied to the dot hybridization membrane.

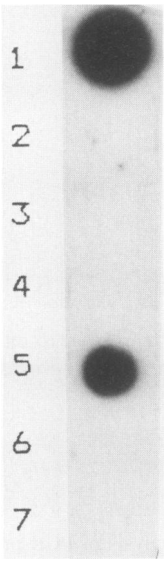

FIG. 5. Dot hybridization of PCR products of canine blood and urine specimens. Specimens were prepared and subjected to amplification as described in the text by using the primers $ospA_1$ and $ospA_2$. The samples were as follows: 1, *B. burgdorferi* boiled cell pellet; 2 through 4, blood specimens from canines with clinical and serological evidence of Lyme disease showing no reactivity in the PCR; 5, blood specimen from a canine with clinical and serological evidence of Lyme disease showing reactivity in the PCR; 6 and 7, blood specimens from normal, healthy canines.

may cause false-positive results in patients with other spirochetal infections (25).

The highly sensitive and specific DNA amplification system is potentially an excellent tool for the identification of the Lyme disease agent in clinical samples as well as in vectors and animal reservoirs. This system is being applied widely to the detection of pathogens in blood and body fluids, as well as in tissue samples. We were able to detect 10 organisms per ml of seeded blood or urine using the PCR followed by dot hybridization detection of amplification products. DNA extraction is unnecessary, as the inhibitory effects of constituents such as hemoglobin are removed by a simple washing step. This represents a 100-fold increase in sensitivity over that obtained with unwashed samples (data not shown). An additional advantage of this technique is that spirochetes do not have to be viable; therefore, stored specimens can be tested easily. The high degree of specificity of PCR results from the fact that two oligonucleotide sequences must hybridize to the target DNA under stringent conditions in order for amplification to occur. The resulting amplified product must then hybridize to the *ospA* probe. Other species of *Borrelia* which have been shown to possess up to 59% DNA homology with *B. burgdorferi* (20) did not show reactivity with our *ospA* primers in the PCR.

Our finding that most of the canine blood and urine specimens were negative may be a result of the stage of disease in these subjects. These animals were infected for a sufficient length of time to develop an immune response and to exhibit symptoms. The PCR may prove to be more useful in detecting the presence of the organism in the very early stages after infection, when there is a greater likelihood of spirochetemia. This technique could also be highly useful in confirming Lyme disease since it could readily be applied to the detection of the spirochete in cerebrospinal fluid, synovial fluid, and tissue from skin lesions. The protocol described here eliminates the need for DNA extraction and thus is adaptable to epidemiological investigations such as the identification of *B. burgdorferi* in infected material from arthropod vectors and animals as well as *B. burgdorferi* isolates cultured from infected specimens.

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