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Identification of *Borrelia burgdorferi* and *B. hermsii* Using DNA Hybridization Probes

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Fragments of plasmid DNA from *Borrelia burgdorferi* and *B. hermsii* were cloned and tested for specificity as hybridization probes to identify these two species of pathogenic spirochetes. Three fragments from the 49-kilobase-pair linear plasmid of *B. burgdorferi* were tested: a 500-base-pair (bp) *Hin*dIII fragment (probe 49A), a 445-bp *Pst*I-*Hin*dIII fragment (probe 3G), and a 320-bp *Hin*dIII fragment (probe 16H). When hybridized to purified DNA or whole spirochetes, all of the probes distinguished *B. burgdorferi* from the other species examined, including *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. coriaceae*, *B. crocidurae*, and *B. anserina*. Probe 49A was the most useful, however, hybridizing with all strains of *B. burgdorferi* originating from both North America and Europe while not cross-hybridizing with *B. hermsii*. A 790-bp *Hin*dIII fragment of *B. hermsii* DNA hybridized with DNA and whole spirochetes of this species and also with *B. parkeri*, confirming the close relatedness of these two species. These probes provide a new method of identifying these *Borrelia* species once the organisms have been grown in culture.

The genus *Borrelia* includes many species of tick-borne pathogenic spirochetes that infect humans and a variety of other animals throughout many regions of the world (12). Within the United States, at least six described *Borrelia* species are established, including the causative agents of Lyme disease (*Borrelia burgdorferi*), tick-borne relapsing fever (*B. hermsii*, *B. parkeri*, and *B. turicatae*), fowl spirochetosis (*B. anserina*), and the agent suspected of causing epidemic bovine abortion (*B. coriaceae*) (3). With the recognition of Lyme disease in Connecticut in 1975 (33), the discovery of its causative agent, *B. burgdorferi*, in 1981 (8, 17, 32), and the fact that in the United States Lyme disease has become the most prevalent arthropod-borne disease of humans (9), many clinical and research laboratories have become involved with its diagnosis and the identification of this and other species of spirochetes.

In the past, *Borrelia* species have been identified by their arthropod host and geographical place of origin (10). More recently, guanine-plus-cytosine composition of total genomic DNA has been used to examine species and their taxonomic relationship within the genus (15). Currently, *B. burgdorferi* and *B. hermsii* are identified most often by reactivity to monoclonal antibodies specific for epitopes associated with outer surface proteins unique to these species of spirochetes (7). However, since antigenic changes in outer surface proteins of *B. hermsii* and *B. burgdorferi* can occur when these borreliae are cultured in artificial media (28, 35), such changes could interfere with the proper identification of the spirochete, depending on the particular monoclonal antibody used for identification.

The use of specific sequences of DNA as probes for the recognition of infectious agents is becoming a valuable alternative to problematic immunological identification assays (36). Given the increasing demand for reagents to correctly identify *Borrelia* species and the potential for antigenic changes in cultured spirochetes, we have been investigating the potential use of DNA probes for the detection and identification of *Borrelia* sp. In a preliminary abstract, the feasibility of using DNA probes for the identification of *B. burgdorferi* was examined (27). Herein, we demonstrate the utility of these probes and describe additional DNA restriction fragments for the specific identification of *B. burgdorferi* and *B. hermsii*, the two most prevalent *Borrelia* spp. that infect humans in North America.

MATERIALS AND METHODS

***Borrelia* strains and cultivation.** The spirochetes used in this study came from the following sources. *B. burgdorferi* B-31 (ATCC 35210), the prototype strain, originated from *Ixodes dammini* from Shelter Island, N.Y. (8, 17, 32). *B. burgdorferi* HB-19 was isolated from human blood in Connecticut (5, 32). *B. burgdorferi* ECM-NY-86 (30), NY-1-86, and NY-2-86 were isolated from erythema migrans lesions of three human patients in New York in 1986. *B. burgdorferi* Sh-2-82 was isolated from two naturally infected *I. dammini* ticks collected on Shelter Island, N.Y., in 1982 (30). *B. burgdorferi* CA-2-87 was isolated from a pool of eight adult *I. pacificus* ticks collected in Tulare County, Calif., in 1987 (30). *B. burgdorferi* JD-1 originated from a naturally infected nymphal *I. dammini* tick collected at Crane's Beach, Ipswich, Mass. (24). *B. burgdorferi* G-1 and G-2 were isolated from human cerebrospinal fluid in the Federal Republic of Germany (14). *B. hermsii* HS1 serotype C (ATCC 35209) originated from *Ornithodoros hermsi* collected near Spokane, Wash. (35). *B. hermsii* FG was isolated at Rocky Mountain Laboratories in April 1987 from the blood of an 8-year-old boy from Seattle, Wash. *B. coriaceae* Co53 (ATCC 43381) originated from *O. coriaceus* collected in California (16, 19). *B. parkeri*, *B. turicatae*, *B. anserina*, and *B. crocidurae* were in the Rocky Mountain Laboratories bacterial pathogen collection.

Live borrelial cultures were maintained in BSK-II medium (1) at 34°C and passaged twice a week. Numbers of spirochetes were determined by dark-field microscopy by using the Stoenner (34) counting method.

Purification of plasmid-enriched DNA. DNA was purified from all borrelial strains as described previously (2), except that cesium chloride-ethidium bromide gradient ultracentrif-

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ugation was not done. DNA concentration and purity were determined by UV A_{260} and A_{280} (20). Each sample was examined in a 0.2% agarose gel stained with ethidium bromide to determine its plasmid profile.

Generation of DNA probe. Recombinant plasmids pTRH44 and p7.1 were provided by Alan G. Barbour, University of Texas Health Science Center, San Antonio. pTRH44 contains the *ospA* gene of *B. burgdorferi* (13), while p7.1 contains the 5′ portion of variable major protein 7 (*vmp7*) gene of *B. hermsii* and adjacent upstream sequences (25). For probes to *B. burgdorferi*, we subcloned two fragments of the *ospA* gene from pTRH44: a 445-base-pair (bp) *Pst*I-*Hin*dIII fragment (probe 3G) and a 320-bp *Hin*dIII fragment (probe 16H). Another 500-bp *Hin*dIII fragment (probe 49A), which resulted from a restriction endonuclease digest of total plasmid-enriched DNA, was also cloned and screened for its specificity to *B. burgdorferi*. For a probe to *B. hermsii*, we subcloned a 790-bp *Hin*dIII fragment (probe E4) from p7.1. Each of the fragments was ligated into cloning vector pUC19 or pUC13 cut previously with the appropriate endonucleases (Bethesda Research Laboratories, Gaithersburg, Md.) and treated with alkaline phosphatase. *Escherichia coli* DH5-α (Bethesda Research Laboratories) was transformed with the recombinant plasmids and screened for the appropriate DNA inserts. Plasmid DNA from *E. coli* was purified by a method described previously (11), except that cells were lysed by being heated at 68°C for 30 min. The lysate was phenol-chloroform extracted, and the DNA was precipitated in ethanol (20). Recombinant plasmids were then digested with the appropriate endonucleases and electrophoresed in 0.7% agarose gels, and the fragments of interest were recovered by elution with a UEA electroeluter (International Biotechnologies, Inc., New Haven, Conn.) as recommended by the manufacturer. The purified fragments were nick translated by using a commercial kit (Bethesda Research Laboratories) and labeled with [α-^{32}P]dCTP as recommended by the manufacturer. The unincorporated isotope was separated from the DNA by centrifugation in a Mini Spin Column (Worthington Diagnostics, Freehold, N.J.) as recommended by the manufacturer. Probes were labeled to a specific activity of 10^7 to 10^8 cpm/μg of DNA. Immediately before hybridization, labeled probes were denatured in 0.1 N NaOH at 37°C for 10 min.

Hybridization procedures. Hybridizations were done on GeneScreen Plus membranes as recommended by the manufacturer (Dupont, NEN Research Products, Boston, Mass.). The membrane was presoaked in 0.5 M Tris (pH 7.55) for 30 min and then placed in a 96-well blot manifold (Bio-Rad Laboratories, Richmond, Calif.) and tightened under vacuum. Serial dilutions of DNA or whole spirochetes in 0.125 N NaOH–0.125× SSC (1× SSC in 0.15 M sodium chloride plus 0.015 M sodium citrate) were loaded in wells and pulled onto the membrane with vacuum. The membrane was air dried (37°C) and then treated with 0.5 N NaOH twice for 2 min each time, neutralized with 1 M Tris (pH 7.6) twice for 2 min each time, and air dried. The membrane was prehybridized with 50% formamide–6× SSC– 5× Denhardt solution (20)–0.5% sodium dodecyl sulfate–0.1% sodium pyrophosphate–100 μg of denatured salmon sperm DNA per ml for 24 h at 42°C. Hybridization was performed under the same conditions for 16 h. Following hybridization, the membrane was washed twice for 5 min each time in 2× SSC at room temperature, twice for 15 min each time in 2× SSC–1% sodium dodecyl sulfate at 65°C, and twice for 15 min each time in 0.1× SSC at 65°C. Membranes were exposed to Kodak X-Omat film at −70°C with an intensifying screen for 2 to 72 h, as indicated in the figure legends, and developed with a Kodak X-OMAT M20 processor.

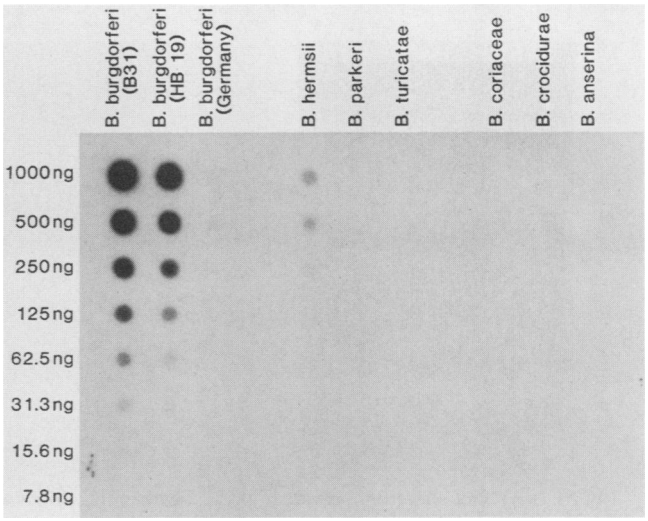

FIG. 1. *B. burgdorferi* probe 16H hybridized with eight twofold serial dilutions of standardized concentrations of plasmid-enriched DNAs of seven *Borrelia* species. Film was exposed to the membrane for 7 h.

Southern blot analysis. Plasmids of *B. burgdorferi* and *B. hermsii* were separated by electrophoresis in 0.2% agarose gels. The gels were then depurinated twice for 15 min each time in 0.25 M NaCl, denatured in 1.5 M NaCl–0.5 M NaOH for 1 h, and neutralized in 1 M Tris hydrochloride (pH 8.0)–1.5 M NaCl for 1 h. DNA was then transferred onto GeneScreen Plus membranes for probing (31). Following transfer, the membranes were treated identically to the dot blots described above, except that they were exposed to film for 5 to 7 days.

RESULTS

Two fragments of DNA were subcloned from *ospA* of *B. burgdorferi* and examined for hybridization specificity. These fragments were thought to be ideal candidates for diagnostic sequences because *ospA* encodes for an outer surface protein (OspA) unique to the Lyme disease spirochete. Probe 3G, a 445-bp *Pst*I-*Hin*dIII fragment, hybridized strongly with plasmid-enriched DNA and with whole spirochetes of two North American strains of *B. burgdorferi* (B31 and HB19) but not with a strain from the Federal Republic of Germany (G1; data not shown). Probe 16H, a 320-bp *Hin*dIII fragment, showed the same pattern of hybridization with plasmid-enriched DNA (Fig. 1) and with whole spirochetes (data not shown), although its reactivity with the German strain (G1) was very weak. Probes 3G and 16H also hybridized weakly with *B. hermsii* but not with the other five *Borrelia* species tested under the hybridization conditions used in this study.

Plasmids of *B. burgdorferi* B31, *B. hermsii* HS1, and *B. hermsii* FG were probed with 3G by Southern blot hybridization. As expected, probe 3G hybridized with the 49-kilobase (kb) linear plasmid of *B. burgdorferi*, which contains the *ospA* gene (Fig. 2). The sequence similarity of probe 3G to *B. hermsii* was localized to a single but different plasmid in the two strains: a 30-kb plasmid in strain HS1 and 26-kb plasmid in strain FG.

In view of the relatively weak reactivity of probes 3G and 16H with the German strain (G1) of *B. burgdorferi* and

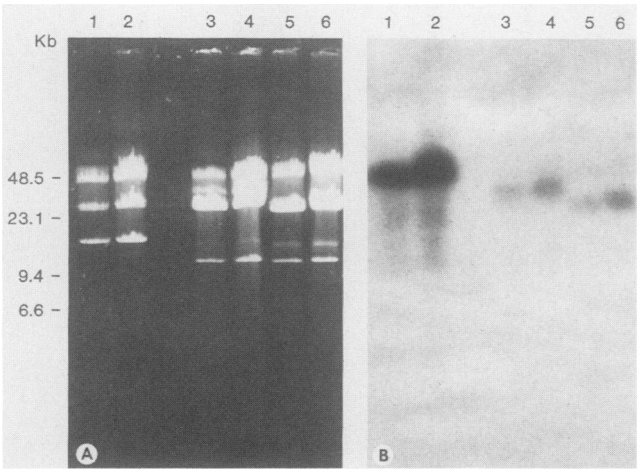

FIG. 2. (A) Gel electrophoresis in 0.2% agarose of *Borrelia* plasmid-enriched DNAs at two concentrations before Southern transfer. Lanes: 1 and 2, *B. burgdorferi* B31; 3 and 4, *B. hermsii* HS1; 5 and 6, *B. hermsii* FG. (B) Southern blot analysis of the same *Borrelia* plasmids hybridized with probe 3G. Note strong signals due to hybridization with the 49-kb linear plasmid of *B. burgdorferi* (lanes 1 and 2) and weak signals resulting from hybridizations with smaller linear plasmids of *B. hermsii* (lanes 3 to 6). Film was exposed to the membrane for 7 days.

because both hybridized with DNA from *B. hermsii*, we screened additional fragments of DNA for a more specific probe. Probe 49A, a 500-bp *Hin*dIII restriction fragment, was cloned from a plasmid-enriched DNA digest of *B. burgdorferi* Sh-2-82. Southern blot analysis of the plasmids from three North American strains and culture passages of *B. burgdorferi* separated in a low-percentage agarose gel demonstrated that probe 49A hybridized only to the 49-kb linear plasmid (data not shown). This probe hybridized with plasmid-enriched DNA of *B. burgdorferi* but not with the other six *Borrelia* species tested, including *B. hermsii* (Fig. 3). In additional tests, this probe hybridized with plasmid-enriched DNAs of all 10 strains of *B. burgdorferi* tested, including eight from various hosts and localities in North

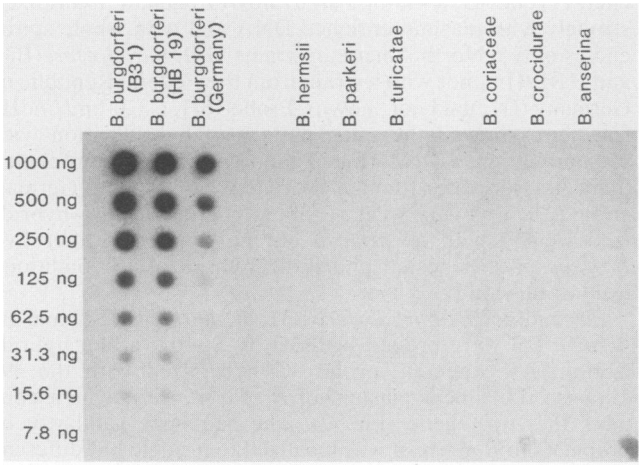

FIG. 3. *B. burgdorferi* probe 49A hybridized with eight twofold serial dilutions of standardized concentrations of plasmid-enriched DNAs of seven *Borrelia* species. Film was exposed to the membrane for 72 h.

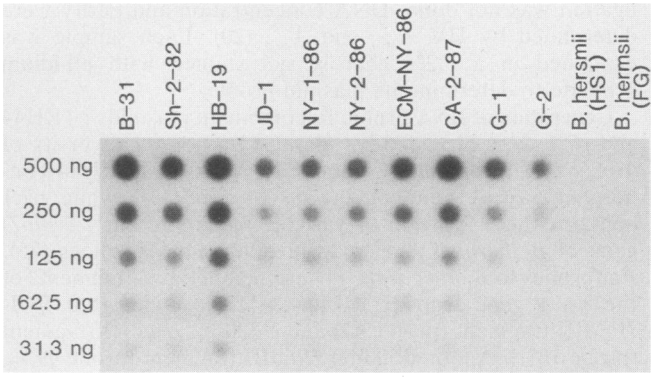

FIG. 4. *B. burgdorferi* probe 49A hybridized with five twofold serial dilutions of standardized concentrations of plasmid-enriched DNAs of 10 strains of *B. burgdorferi* and 2 strains of *B. hermsii*. Film was exposed to the membrane for 48 h.

America and two from the Federal Republic of Germany (Fig. 4). Again, no hybridization with *B. hermsii* was detected, even when the autoradiograph was exposed for up to 72 h.

One DNA fragment of *B. hermsii* was tested as a diagnostic probe for this species. Probe E4, a 790-bp *Hin*dIII restriction fragment, hybridized strongly to both plasmid-enriched DNA (Fig. 5) and whole spirochetes of *B. hermsii* (data not shown). This probe hybridized less strongly to *B. parkeri* but not to the other *Borrelia* species tested. This probe detected 490 pg of plasmid-enriched DNA of *B. hermsii* and 2,800 spirochetes (data not shown), the lowest concentrations of both used in the hybridizations. The probes for *B. burgdorferi* were less sensitive and were unable to detect less than 10,000 organisms (27).

DISCUSSION

An increasing number of investigations have used nucleic acid hybridization probes for detection and identification of

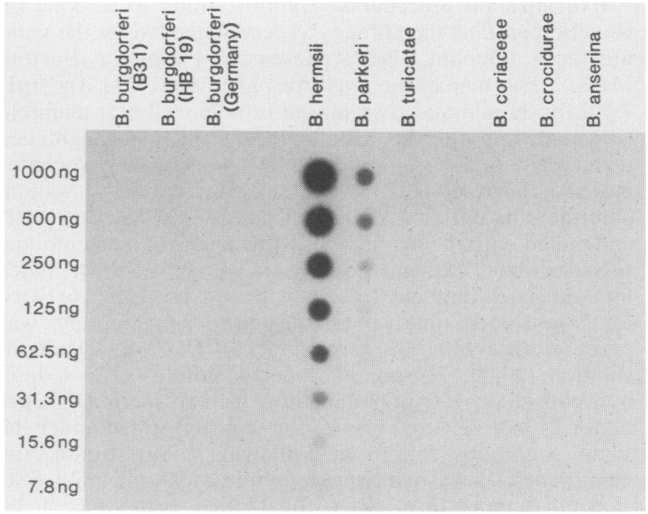

FIG. 5. *B. hermsii* probe E4 hybridized with eight twofold serial dilutions of standardized concentrations of plasmid-enriched DNAs of seven *Borrelia* species. Film was exposed to the membrane for 2 h.

disease-causing agents (36). Just a few recent examples include the detection of dengue virus in mosquitoes (23), *Trypanosoma congolense* in tsetse flies (18), various *Bacteroides* species in human clinical isolates (26), *Plasmodium falciparum* in human blood (6), and *Yersinia pestis* in fleas (21). Lyme disease is now the most prevalent arthropod-borne disease in the United States, and the number of human cases each year probably outnumbers the cases of all other arthropod-borne diseases combined. The DNA probes developed in this investigation offer a new method to specifically distinguish *B. burgdorferi* and *B. hermsii* from the other known species of *Borrelia* in the United States.

Developing a probe for plasmid DNA might seem undesirable, since some plasmids of *B. burgdorferi* are lost early during growth in artificial medium (29). However, probes 3G, 16H, and 49A originate from the 49-kb linear plasmid, which is not lost, even after many years of in vitro cultivation (2). Probe 49A had greater DNA similarity with the *B. burgdorferi* isolate from the Federal Republic of Germany than did the probes derived from the *ospA* gene. Furthermore, probe 49A, in contrast with probes 3G and 16H, did not hybridize with *B. hermsii*. These data and the specific reactivity of probe 49A with the 10 North American and 2 German isolates of *B. burgdorferi* tested indicate that this probe is preferred and may prove useful for identifying the Lyme disease spirochete from many wide-ranging geographical locations. However, additional testing of this probe with *B. burgdorferi* from other sources and localities throughout Eurasia is required.

Probes 3G and 16H, on the other hand, may still prove to be useful for identifying *B. burgdorferi*, particularly in areas endemic for Lyme disease but where *B. hermsii* does not occur. The signal detected when these probes were hybridized to *B. hermsii* DNA can also be eliminated by reducing the exposure time during autoradiography or performing the hybridizations at higher stringency.

Probe 49A sequences appear to be more conserved among isolates from both continents, raising the possibility that this 500-bp fragment of DNA is associated with a cellular product that does not vary among isolates from different geographical localities. Based on a published restriction map of pTRH32, which contains approximately 6 kb of the 49-kb linear plasmid, including *ospA* and *ospB* (13), our 500-bp *Hin*dIII fragment is not part of either of these genes.

Probe E4 was derived from *B. hermsii* to serve as a specific probe for this organism, although it hybridized weakly with *B. parkeri* DNA. The extent of DNA similarity between these strains at the locus examined is perhaps not surprising, however, because a study examining DNA homologies between *Borrelia* species concluded that *B. hermsii* and *B. parkeri* are conspecific (15). Nevertheless, none of the other *Borrelia* species tested hybridized to probe E4, and if appropriate hybridization conditions are used, this probe should prove useful as a means of specifically identifying *B. hermsii*.

In the present study, we demonstrated in both dot blot and Southern blot analyses that different portions of the *ospA* gene hybridize to specific plasmids in two distinct strains of *B. hermsii*. In an earlier study in which the entire *ospA* gene was used as a probe to plasmid-enriched DNA of *B. hermsii* digested by restriction endonucleases, no hybridization signals were detected (4). This suggests that the hybridizing sequences detected in the present study are associated with DNA sequences that may not be detected by Southern blot analysis if the plasmid DNA is cut into smaller fragments by endonucleases. It will be of interest to assess the relatedness of the two *B. hermsii* plasmids that share DNA sequences with the *ospA* gene.

We detected hybridization by using a radioactive nuclide label; however, many researchers cannot or choose not to use such isotopes. Also, given the potential hazards of using such material, nonradioactive labels are desirable. Commercial kits are available for chemical labeling of DNA probes with biotin (vitamin H), with detection of hybridization by a chromogenic change resulting from binding of an avidin-enzyme conjugate to the biotin (22). We are currently working on this approach to make the probes discussed herein available to more laboratories.

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