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Analysis of Relapsing Fever Spirochetes from the Western United States

Tom G. Schwan, Ph.D.,* Kenneth L. Gage, Ph.D., and Joe Hinnebusch, Ph.D.

Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana

Tick-borne relapsing fever occurs in scattered foci throughout much of the western United States. *Borrelia hermsii, B turicatae,* and possibly *B. parkeri,* are the etiological agents and these spirochetes are maintained in zoonotic cycles involving rodents and soft ticks of the genus *Ornithodoros.* Five isolates of *B. hermsii* were examined by numerous molecular and genetic techniques and compared to other species of *Borrelia.* Plasmids in the isolates of *B. hermsii* were similar except for one (YOR-1) from northern California. This isolate also differed from the others of *B. hermsii* by not having DNA amplified by the polymerase chain reaction (PCR) when using primers specific for variable major protein (Vmp) genes 7 and 21. These results demonstrate that the repertoire of Vmp genes differs among isolates of *B. hermsii.* DNA probes based on *vmp* sequences also hybridized to *B. coriaceae,* the suspected agent of epidemic bovine abortion disease of cattle, suggesting that this spirochete may also cause a relapsing phenomenon.

Key words: *Borrelia hermsii,* tick-borne-relapsing fever, ticks

INTRODUCTION

Tick-borne relapsing fever of humans in the western United States is presently known to be caused by two or three closely related species of *Borrelia* (*B.*) spirochetes transmitted by Argasid ticks in the genus *Ornithodoros* (*O.*) (1). The specific status of these bacteria, which includes *B. hermsii, B. parkeri,* and *B. turicatae,* is based on their apparent vector specificity for different species of ticks (2). In 1942, Davis identified *B. hermsii* and *B. parkeri* as new species of *Borrelia* based on laboratory studies in which these spirochetes were transmitted only by the ticks *O. hermsi* and *O. parkeri,* respectively (3). Brumpt had named *B. turicatae* 9 years previously based on this spirochete's specificity to *O. turicata* (4). While some have questioned the validity of using vector specificity as the sole criterion for establishing species of *Borrelia,* very little work has been done since the studies of Davis to address the genetic relatedness of the recognized species of relapsing fever spirochetes in North America (5) and to re-examine the issue of vector specificity and spirochete distribution in nature using newer molecular techniques. Such efforts were no doubt hindered by the lack of an artificial culture medium until 1971, when Kelly described a liquid broth that allowed him to successfully maintain *B. hermsii in vitro* continuously for 8 months while the spirochete retained its infectivity in mice (6). Subsequent improvements of this medium have now allowed for the successful cultivation of many species of *Borrelia,* providing the opportunity for molecular and genetic studies of these spirochetes (7). Over the last 15 years, significant advances have been made toward understanding the structural properties of the relapsing fever spirochete's genome and defining, in part, the genetic mechanisms that control antigenic variation (8–15). However, nearly all of the recent molecular and genetic studies have been restricted to one strain (HS1) of *B. hermsii* isolated from one adult *O. hermsi* tick collected from eastern Washington. Other isolates of *B. hermsii* and the other species of relapsing fever spirochetes remain virtually unstudied.

One of our long-term goals is to understand the mechanisms responsible for the apparent specificity of the different species of relapsing fever spirochetes for different species of *Ornithodoros* ticks, as described by Davis many years ago (3). Another goal is to examine the antigenic behavior of *B. hermsii* during infection in its tick vector. As a prerequisite, we need to first develop molecular diagnostic techniques that will rapidly identify these spirochetes to their currently accepted taxon and to examine isolates within each species to identify conserved and variable molecular and genetic determinants. Toward these objectives, we recently developed both a DNA hybridization probe (16) and a monoclonal antibody (17) that rapidly identified *B. hermsii* from the other species of *Borrelia* currently known to exist in North America. In this report, we examine three uncharacterized isolates of *B. hermsii* from California and compare them with other *Borrelia* spp.

MATERIALS AND METHODS

Borrelia Strains and Cultivation. Borrelia hermsii HS1 (ATCC 35209) serotype C originated from *O. hermsi* collected near Spokane, WA (18). *Borrelia hermsii* FG was isolated at Rocky Mountain Laboratories in April 1987 from the blood of a human with relapsing fever in Washington. *Borrelia hermsii* CON-1, MAN-1, and YOR-1 were isolated from the blood of humans with relapsing fever in California (19). *Borrelia coriaceae* CO53 (ATCC 43381) was isolated from *O. coriaceus* from California (20, 21). *Borrelia parkeri, B. turicatae,* and *B. anserina* were isolated from *O. parkeri, O. turicata,* and a domestic chicken, respectively, and were in the Rocky Mountain Laboratories bacterial pathogen collection, *Borrelia burgdorferi* B31 (ATCC 35210) was isolated from *Ixodes scapularis* (= *dammini*) from Shelter Island, NY (22). Live borrelial cultures were maintained in BSK-II medium (23) at 34°C and passaged twice a week.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis. Whole-cell lysates of *Borrelia* were prepared as described (24). The Laemmli buffer system (25) was used with a Vertical Gel Electrophoresis System (Bethesda Research Laboratories-Gibco, Gaithersburg, MD) following the instructions of the manufacturer. After electrophoresis, lysates were transferred to nitrocellulose in a Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Laboratories, Richmond, CA) using the Towbin buffer system. The *Borrelia* genus-specific monoclonal antibody H9724 (26) was used at a dilution of 1:50. Membranes were prepared and examined by ^{125}I-protein A radiography, as described previously (24).

DNA Purification. Total DNA was purified from 500-mL stationary phase cultures of the various borreliae, as described previously (27). A technique that enriches for plasmid DNA was also used for some of the DNA preparations (28). All samples were precipitated in cold 95% ethanol, washed twice in 70% ethanol, suspended in TE (10 mM Tris [pH 7.6], 1 mM EDTA), and quantified by UV A_{260}.

DNA Analysis. Profiles of plasmid DNA were examined by agarose gel electrophoresis using a Mini-Sub DNA Cell (Bio-Rad Laboratories), as described previously (29). Briefly, undigested samples of DNA were electrophoresed in 0.3% agarose gels with TBE buffer (90 mM Tris, 90 mM boric acid, 20 mM EDTA) to separate plasmids. This was accomplished by electrophoresing for 5 minutes at 50 V and continuing for 16 hours at 12 V and staining with ethidium bromide.

DNA Probes. Three DNA probes were used to examine DNA samples of borreliae in either dot blot or Southern blot hybridizations. Probe E4 was described previously (16) and is a cloned 570-bp *Hin*dIII fragment that contains the 5′ end of the variable major protein 7 (*vmp7*) gene and the adjacent upstream sequence of the expression locus of *B. hermsii* HS1. Probes V-7 and V-21 were 890-bp amplification products of the polymerase chain reaction (PCR) described below, using primers unique to the *vmp7* and *vmp21* genes, respectively. Probes were nick translated by using a commercial kit (Boehringer Mannheim Biochemical, Indianapolis, IN) and labeled with [alpha-^{32}P]dCTP following the instructions of the manufacturer. Unincorporated isotope was separated from the labeled DNA by centrifugation in a Mini Spin Column (Worthington Diagnostics, Freehold, NJ). Probes were denatured by heating to 95°C for 5 minutes and cooled on ice until use.

DNA Hybridization Procedures. Serial twofold dilutions of borrelial DNA were applied directly to membranes using a 96-well dot blot manifold (Bio-Rad Laboratories), as described previously (16). Borrelial plasmids separated by low percentage agarose gel electrophoresis were transferred to membranes by the method of Southern (30), as described previously (16). Dot blot hybridizations were done on GeneScreen Plus membranes and Southern blots were done on GeneScreen Plus membranes (Dupont, NEN Research Products, Boston, MA) following the instructions of the manufacturer. Hybridization conditions, buffer, and membrane washes were described previously (16). DNA hybridization patterns were determined by exposing Kodak X-Omat film to the membranes at −70°C with an intensifying screen and development with a Kodak X-OMAT M20 processor.

Polymerase Chain Reaction (PCR). Nucleotide sequences for primers were chosen from sequences published for the *vmp7* and *vmp21* genes (11) and were as follows: *vmp7,* 5′-TGT-GAG-ATG-TGT-TGG-GAT and 5′-ACT-GGC-TCT-TGT-GAA-CCT; and *vmp21,* 5′-TTT-CAG-ATA-CAT-TAG-GCT and 5′-CCT-GCT-GGT-TTT-GGA-TCT. The predicted size of both amplification products is 890 bp. Oligonucleotide primers were synthesized with a SAM One DNA Synthesizer (Biosearch, San Rafael, CA). Taq DNA polymerase and PCR reagents (Perkin Elmer-Cetus, Norwalk, CT) were used with an automated DNA thermal cycler (Perkin Elmer-Cetus) to amplify DNA for 35 cycles under the following conditions: 94°C for 1 minute; 37°C for 30 seconds; 60°C for 3 minutes. After amplification, 10 μL of the total 100-μL reaction were examined in 0.7% agarose electrophoresis gels stained with ethidium bromide. DNA samples were also PCR amplified with primers based on a chromosomal target specific to relapsing fever spirochetes from North America that was described previously (31).

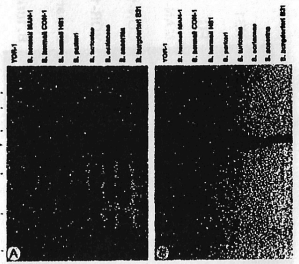

FIG. 1. SDS-PAGE of (A) *Borrelia* whole-cell lysates and (B) Western blot analysis with monoclonal antibody H9724. The 12.5% gel was stained with Coomassie brilliant blue, and molecular weight standards (MWS) are shown on the left. The arrow indicates flagellin, which binds antibody H9724, and was detected by ^{125}I-protein A radiography.

RESULTS

Spirochete cultures designated MAN-1, CON-1, and YOR-1 were each isolated from the blood of human patients clinically ill with relapsing fever in California. These isolates were provided to us by Jane Wong, Microbial Diseases Laboratory, California Department of Health Services, Berkeley, CA. These spirochetes were compared to the prototype strain HS1 of *B. hermsii* and to other species of *Borrelia* known to occur in North America.

Protein profiles of the three new isolates were consistent for relapsing fever spirochetes and grouped together with *B. hermsii* (Fig. 1A). The new isolates also bound monoclonal antibody H9724 (Fig. 1B), which is specific to flagellin of all known members of the genus *Borrelia* tested to date. Reactivity with this antibody identified the new isolates as *Borrelia* ssp. Reactivity with monoclonal antibody H9826 identified the spirochetes MAN-1, CON-1, and YOR-1 as *B. hermsii* (17).

Samples of total DNA from the cultured spirochetes were examined by several techniques and demonstrated that the YOR-1 isolate varied from the other isolates of *B. hermsii.* Agarose gel electrophoresis of total, undigested DNA showed that the plasmid profile of the YOR-1 isolate was distinctly different from four other isolates of *B. hermsii* (Fig. 2). The PCR amplification of a chromosomal target resulted in

*Corresponding author

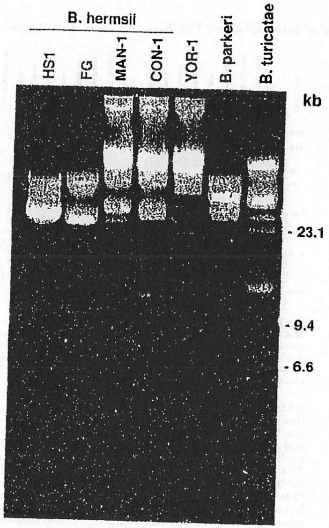

FIG. 2. Agarose gel electrophoresis of *Borrelia* DNA demonstrating the unique plasmid profile of the YOR-1 isolate. Size standards are shown on the right in kilobase pairs (kb).

all *B. hermsii* isolates yielding the predicted 276 base pair product while DNA of *B. burgdorferi* was not amplified (Fig. 3). However, dot blot hybridization using the E4 probe derived from *B. hermsii* HS1 with DNA from 10 borreliae demonstrated again that YOR-1 varied from the other isolates of *B. hermsii* (Fig. 4). This probe bound significantly less under high stringency to YOR-1 than to the other isolates of *B. hermsii*. This same probe was also used following the separation of plasmid DNA in an agarose gel and transfer to a membrane for hybridization (Fig. 5). Of the four typical isolates of *B. hermsii*, this probe hybridized to two plasmids (Fig. 5B). This pattern resulted from the *vmp7* portion of the probe hybridizing to the linear plasmid containing the silent loci of *vmp* genes and from the other part of the probe hybridizing to a different linear plasmid containing the expression locus. Note that for an exposure of 18 hours, very little hybridization was detected with YOR-1 (Fig. 5B). With a longer exposure of 72 hours, hybridization with two distinctly different-sized plasmids in the YOR-1 isolate was seen (Fig. 5C). This is probably due to the presence of sequences homologous to both the expression site and the silent copies of *vmp* genes on these different-sized plasmids in YOR-1.

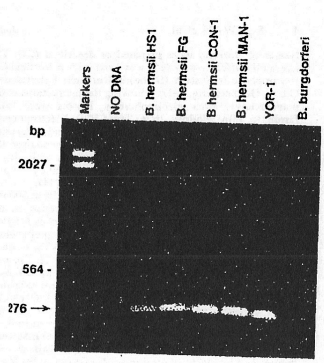

FIG. 3. PCR amplification products of the predicted 276 base pairs from relapsing fever spirochetes but not the Lyme disease spirochete, *B. burgdorferi*. Size standards are shown on the left in base pairs.

Next, we attempted to amplify by PCR an 890 base pair region of either the *vmp7* or *vmp21* genes based on sequences obtained from the prototype strain HS1. While four of the isolates of *B. hermsii* yielded the predicted amplification product using primers specific for *vmp7* (Fig. 6A), no amplification was detected from YOR-1 or the other species of *Borrelia* tested. Attempts to amplify DNA using

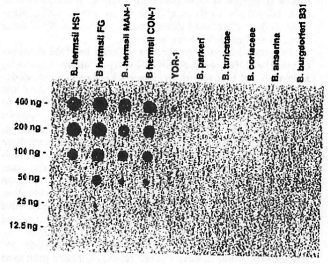

FIG. 4. *Borrelia hermsii* probe E4 hybridized with six twofold serial dilutions of standardized concentrations of total DNA from 10 *Borrelia* isolates.

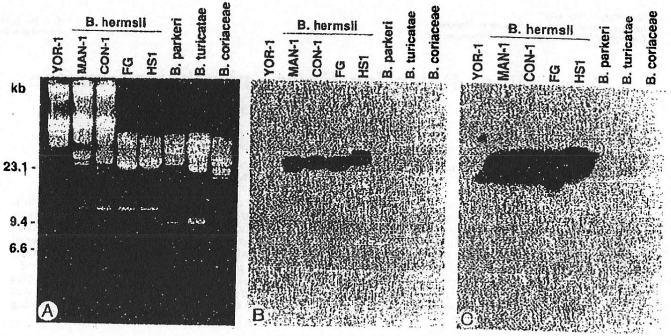

FIG. 5. (A) Plasmid profile and Southern hybridization using *B. hermsii* probe E4 with film exposure of (B) 18 hours and (C) 72 hours. Size standards are shown on the left in kilobase pairs (kb). Note the reduced and unique pattern of hybridization of this probe with DNA from the YOR-1 isolate of *B. hermsii*.

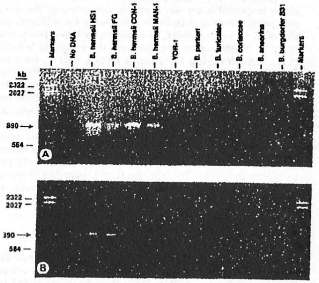

FIG. 6. PCR amplification products using primers specific for *B. hermsii* HS1 (A) *vmp7* gene or (B) *vmp21* gene. Size standards are shown on the left in base pairs. The YOR-1 isolate of *B. hermsii* was not amplified with either set of primers.

primers specific for *vmp21* resulted in only two isolates of *B. hermsii* yielding a product (Fig. 6B). These results suggest that there was most likely sequence variation in the *vmp* genes among the *B. hermsii* isolates.

Our failure to amplify DNA above could have resulted from only minor differences in DNA sequence between the prototype strain and the other isolates. Therefore, we next used the entire 890 base pair fragments of the *vmp7* and

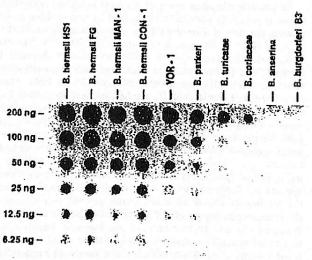

FIG. 7. *Borrelia hermsii* probe V7 hybridized with six twofold serial dilutions of standardized concentrations of total DNA from 10 *Borrelia* isolates. This probe was generated by PCR amplification of *B. hermsii* HS1 DNA using primers specific for the *vmp7* gene and has 890 base pairs.

vmp21 genes amplified from the prototype strain HS1 as probes in dot blot hybridizations (Figs. 7 and 8). The *vmp7* probe (V7) hybridized as strongly to the same four isolates that were also amplified by PCR using *vmp7*-specific sequences (Fig. 7). Weaker hybridization was also detected with YOR-1, *B. parkeri*, *B. turicatae*, and *B. coriaceae*. The *vmp21* probe (V21) hybridized strongest with the two isolates that were also amplified by PCR using *vmp21*-specific primers (HS1 and FG) (Fig. 8). Again, weaker hy-

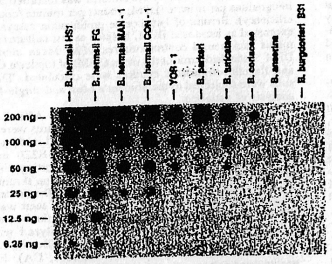

FIG. 8. *Borrelia hermsii* probe V21 hybridized with six twofold serial dilutions of standardized concentrations of total DNA from 10 *Borrelia* isolates. This probe was generated by PCR amplification of *B. hermsii* HS1 DNA using primers specific for the *vmp21* gene and has 890 base pairs.

bridization was detected with all of the samples except *B. anserina* and *B. burgdorferi*. These results demonstrated that, although YOR-1 was not amplified by PCR using primers specific for either *vmp7* or *vmp21*, homologous sequences to these two variable membrane protein genes are present in its genome. These probes also identified homologous sequences to the variable major protein genes in *B. parkeri*, *B. turicatae*, and *B. coriaceae* (Figs. 7 and 8). The former two species are also relapsing fever spirochetes and the presence of genes involved with antigenic variation was not surprising. To date, only a single isolate of *B. coriaceae* (CO53 used in the present study) exists, and nothing is known concerning its behavior during infection in mammals. Our data suggest that *B. coriaceae* has genes homologous to *vmp* genes of *B. hermsii*, and therefore, this spirochete might be a relapsing fever spirochete and have the ability to alter its cell surface to avoid the host's immune response.

DISCUSSION

We have begun to identify molecular and genetic differences among a small group of relapsing fever spirochetes. All five isolates examined were identified as *B. hermsii* by protein profiles, PCR amplification of a conserved chromosomal target, and their reactivity with genus and species-specific monoclonal antibodies. Yet, among these five isolates, some obvious differences were apparent. Each varied slightly in plasmid profile. The YOR-1 isolate, however, displayed a plasmid pattern unique and strikingly different from the other *Borrelia* ssp. examined. In addition, dot blot or Southern blot hybridizations with three DNA probes originating from the prototype strain HS1 demonstrated that there were differences in two *vmp* genes between YOR-1 and the other four isolates of *B. hermsii*. The PCR amplification using primers based on sequences specific to the *vmp7* gene also separated YOR-1 from the other isolates of *B. hermsii*. Picken also identified slight sequence variation in the flagellin gene of YOR-1 compared with the prototype strain HS1 of *B. hermsii* (32). The PCR amplification based on sequences specific to the *vmp21* gene also demonstrated differences among the other four isolates, with only strains HS1 and FC being amplified. This was the first attempt, and admittedly only a start, to compare genes responsible for antigenic variation among isolates of *B. hermsii*. A much larger analysis is currently underway in our laboratory using additional isolates recently acquired from human patients residing in other localities. All identified sequences of *vmp* genes of *B. hermsii* to date have originated from a single strain, HS1. Now we have begun to see a pattern of variation among a small sample of five isolates. The *vmp7* gene was highly conserved in four of the five isolates while *vmp21* was contained in only two of the five isolates. Although YOR-1 genes are not identical to *vmp7* nor *vmp21* genes of other isolates, it does have homologous *vmp* genes including a sequence that is homologous to the expression site. The identification of YOR-1 as *B. hermsii* was based on reactivity with monoclonal antibody H9826 (17) but has been confirmed by sequencing its 16S rRNA and the fact that in our laboratory only *O. hermsi*, but not *O. parkeri* or *O. turicata* was capable of transmitting this spirochete (Gage, Marconi, and Schwan, unpublished observations).

SUMMARY

Relapsing fever spirochetes and their tick vectors are found in numerous foci throughout much of the western United States (33). The mechanisms for restricted maintenance and transmission of each of the currently recognized species of *Borrelia* by their respective species of *Ornithodoros* tick are unknown. In this investigation, we have begun to identify and analyze differences among isolates *B. hermsii*. Despite considerable variation in genetic and molecular determinants among isolates, unpublished studies in our laboratory have shown that these spirochetes are transmitted only by *O. hermsi*. Thus, they share the determinants responsible for vector specificity described by Davis so many years ago (3). Our studies will continue in an attempt to identify the mechanisms that restrict these spirochetes to specific species of ticks.

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Reprint requests: Tom G. Schwan, Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, MT 59840.

Present address for Kenneth L. Gage: Bacterial Zoonoses Branch, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, P.O. Box 2087, Fort Collins, CO 80522.

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