JOURNAL OF CLINICAL MICROBIOLOGY, July 1991, p. 1523–1526
0095-1137/91/071523-04$02.00/0

Vol. 29, No. 7

Detection of the Lyme Disease Bacterium, *Borrelia burgdorferi*, by Using the Polymerase Chain Reaction and a Nonradioisotopic Gene Probe

DARLA J. WISE† AND TERRY L. WEAVER*

Biology Department, State University of New York College at Fredonia, Fredonia, New York 14063

Received 26 November 1990/Accepted 2 April 1991

A 419-bp region of the flagellin gene sequence of *Borrelia burgdorferi* was used as a target for the polymerase chain reaction. With a nonradioactively labeled gene-specific probe, sensitivity to as few as 1 to 10 spirochetes was observed. The targeted gene fragment was conserved in the American and European strains of *B. burgdorferi* tested and among several other pathogenic borreliae.

Lyme disease is a multisystem disorder caused by the spirochete *Borrelia burgdorferi* (8, 29). The symptoms associated with the early stages of Lyme disease are rather general (e.g., fever and malaise) and make diagnosis difficult (29). Furthermore, the immune response early in Lyme disease is unpredictable, which renders antibody detection via enzyme-linked immunosorbent assay of limited diagnostic value (10, 13). However, early detection and treatment reduce the probability of the more severe complications associated with later stages of Lyme disease (28).

Use of the polymerase chain reaction (PCR) has recently attracted much interest as a genetic approach to microbial diagnostics (1, 11, 21, 30). Rosa and Schwan were the first to apply the PCR to detection of *B. burgdorferi* (25). Of the 18 strains of *B. burgdorferi* examined, 17 were detected and no reactivity was associated with the other *Borrelia* species tested. The outer surface protein A gene has also been used as a PCR target for detection of *B. burgdorferi* (23, 24). This gene is located on a linear plasmid associated with the spirochete (4). In a study by Nielsen et al., amplification products were identified with a radioactively labeled oligonucleotide probe selected from the outer surface protein A gene (23).

We chose to evaluate the flagellin gene as a PCR target for detection of *B. burgdorferi*. The flagellin gene sequence was known; it was located on the chromosome and had not been previously examined with a PCR-based detection method. Furthermore, we wanted to examine the sensitivity of a nonradioisotopic detection system when coupled with PCR for detection of *B. burgdorferi*. The nonradioisotopic detection method has advantages over radioisotopic methods in that problems of expense due to a short half-life, storage, and disposal associated with radioactively labeled probes are avoided (19). In addition, biotinylated probes have been determined to have sensitivities approaching those reported with radioactively labeled probes.

Two strains of *B. burgdorferi*, the American prototype strain (ATCC 35210) and an isolate from Switzerland (ATCC 35211), were obtained from the American Type Culture Collection, along with cultures of *B. hermsii* (ATCC 35209) and *Treponema pallidum* (ATCC 27087). Cultures of *B. anserina*, *B. coriaceae*, *B. hermsii*, *B. parkeri*, and *B. turicatae* were provided by Thomas Schwan, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases. The *B. burgdorferi* strains were maintained in BSKII medium (2). The culture of *T. pallidum* was reconstituted in 0.5 ml of BSKII medium and used immediately. Other cultures were used as received.

The target for PCR amplification was located within the *B. burgdorferi* flagellin gene sequence (12). Two primers, both 18-mers, were selected from a portion of the gene sequence conserved in both the American and European strains of *B. burgdorferi*. Primers 1F (5′ GCATTAACGCTGCTAATC) and 2F (5′ TTGCAGGCTGCATTCCAA) were located at nucleotides 50 to 67 and 451 to 468, respectively, on the basis of the published flagellin gene sequence (12). Also selected from the flagellin gene sequence was a 20-mer probe (nucleotides 300 to 319) for detection of the amplified product by hybridization. The probe was selected such that it did not hybridize with either of the primer sequences, and both ends were biotinylated (B): 5′ BCACATATTCAGATGCAGA CAB. The oligonucleotide primers and probe were synthesized by Genesys Biotechnologies, Inc., Woodlands, Tex.

Enumeration of *B. burgdorferi* was routinely performed by the method of Stoenner (31). For sensitivity studies, appropriate amounts of culture were aliquoted such that 0.5-ml tubes (Sarsted, Newton, N.C.) theoretically contained 10,000, 1,000, 100, 10, and 1 cell, and volumes were adjusted to 10 μl with 0.9% NaCl. For specificity analysis, 2 μl of culture was placed into tubes with 3 μl of 0.9% NaCl. An equal volume of the detergent Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) was added to the cells. The tubes were placed in a boiling water bath for 5 min and then cooled rapidly on ice. PCR reagents were added directly to the entire contents of the tubes. A negative control was prepared with BSKII medium and treated like sample tubes. *B. burgdorferi* DNA was purified as described by Marmur (20). A positive control was prepared with 20 ng of purified *B. burgdorferi* ATCC 35210 DNA as the template. The PCR reagent mixture contained 10 mM Tris hydrochloride, 50 mM $MgCl_2$, 0.01% gelatin, each deoxynucleostide triphosphate at 200 μM, and each primer, 1F and 2F, at 1 μM. All reactions were performed in a TempCycler 50 (Coy, Ann Arbor, Mich.). The temperature profile used was as follows: template denatured at 94°C for 2 min, primers annealed at 55°C for 2 min, and extension at 72°C for 3 min, for a total of 30 cycles. The amplification products were analyzed in 2%

* Corresponding author.

† Present address: Department of Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0442.

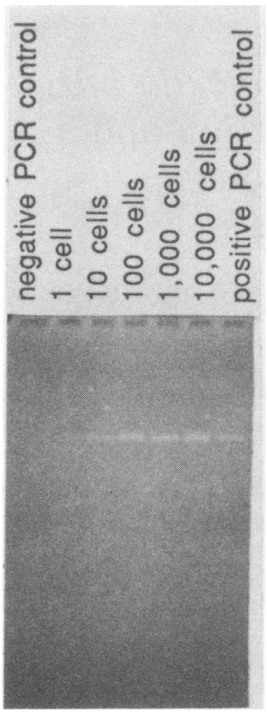

FIG. 1. Electrophoresis of the 419-bp amplification product resulting from the use of *B. burgdorferi* cell extracts derived from 1 to 10,000 cells as templates.

agarose by submarine gel electrophoresis using a standard TAE buffer (19). Ethidium bromide (1 μg/ml) was included in the electrophoresis buffer and gel, and bands were visualized by UV transillumination.

Five-microliter aliquots of the PCR amplification products were heat denatured and directly spotted onto a nitrocellulose membrane (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) which had been wetted in 2× distilled, autoclaved water and briefly soaked in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then blotted between two sheets of Whatman no. 1 chromatography paper. The membrane was then baked at 80°C for 1.5 h, prehybridized for 30 min in a solution of 5× SSC–5× Denhardt's solution–5 mM EDTA, 0.2% sodium dodecyl sulfate (SDS)–0.05% sodium PP_i–50 μg of *Escherichia coli* tRNA (Sigma) per ml. The probe was diluted in the prehybridization solution to 25 μg/ml, and this mixture was used as the hybridization solution. Hybridization was allowed to take place overnight at 42°C. The membrane was washed three times in 0.16× SSC–0.1% SDS, i.e., twice for 5 min each time at room temperature and once for 30 min at 42°C. The membrane was then rinsed once in 1× SSC for 3 min at room temperature and once in 0.1 M Tris (pH 7.5)–0.15 M NaCl for 3 min at room temperature. Hybrids were detected by using the BluGENE Nonradioactive Nucleic Acid Detection System (Bethesda Research Laboratories). The only modification of the protocol suggested by the manufacturer was that the 3% bovine serum albumin wash was performed for 1.5 h at 42°C rather than for 1 h at 65°C.

When we examined the sensitivity of our assay, the 419-bp amplification product from as few as 1 to 10 spirochetes was directly visible in the agarose gel (Fig. 1). The 419-bp PCR amplification product was seen in lanes 3 to 6, ranging from 10 to 10,000 cells. A faint band was visible in the one cell

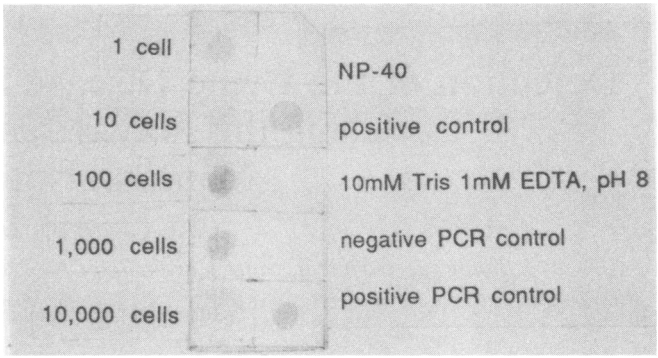

FIG. 2. DNA hybridization results of amplification products generated in the PCR with DNA from various numbers of *B. burgdorferi* cells as templates. The positive control was 5 ng of the biotinylated 20-mer probe. The positive PCR control was the amplification product resulting from 20 ng of purified *B. burgdorferi* DNA used as the template in the PCR. The negative control was the amplification product resulting from BSKII medium used as the template in the PCR. NP-40, Nonidet P-40.

lane on the transilluminator. No amplification was observed in the negative control lane. The lower limits of detection were made more visible by use of hybridization, and single-cell dilutions were readily detected by spot blot (Fig. 2). No reactivity was noted in the various negative control spots, and the 1- and 10-cell spots were clearly visible. As evidenced by visible bands in the agarose gel and visualization

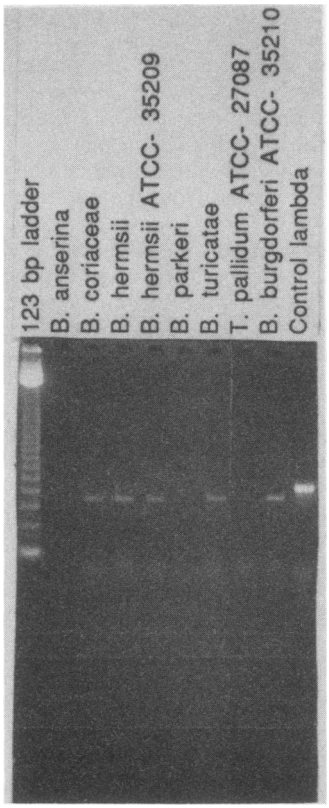

FIG. 3. Electrophoresis of amplified spirochete DNA generated from a variety of *Borrelia* species and *T. pallidum*.

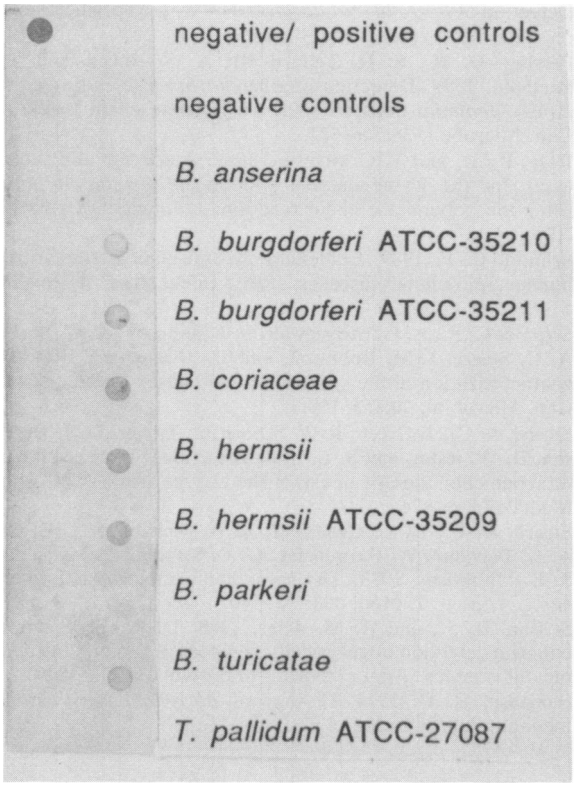

FIG. 4. Hybridization of the flagellin gene probe to spirochete DNA. The first row of dots, on the left below the positive control spot (upper left-hand corner), is a 10-fold dilution of the second row of spots, on the right, except for the control spots themselves. The positive control is 5 ng of the biotinylated 20-mer probe. The negative control spots are Nonidet P-40 (upper right-hand corner), the 500-bp amplified lambda control DNA (second row, left), and the amplification product resulting from BSKII medium used as the template in the PCR (second row, right).

by hybridization, our assay approaches the single-cell detection limit. These sensitivity levels are comparable to those demonstrated by other PCR-based assays for detection of *B. burgdorferi* (23, 25).

When we examined the specificity of our assay, the 419-bp amplification product was directly visible in all of the *Borrelia* species pathogenic in mammals both in the agarose gel and by hybridization (Fig. 3 and 4). No amplification product was noted with the avian pathogen *B. anserina*, and only faint reactivity was noted with *B. parkeri*. In DNA homology studies, *B. burgdorferi* has 31 to 59% homology with other borreliae; it is most closely related to *B. hermsii*, with 59% homology, and less related to *B. parkeri*, with 37% homology (16). Thus, the lower reactivity with *B. parkeri* is consistent with the overall DNA homologies. In a recent study, a monoclonal antibody to a flagellar epitope was seen to bind with all of the *Borrelia* species evaluated in our assay, including *B. anserina* and *B. parkeri*, suggesting that the flagellin gene is highly conserved throughout the genus (5). As evidenced by the bands in the agarose gel and the spot blots, our assay also suggests a genus-specific nature of the flagellin gene among the *Borrelia* species pathogenic in mammals. No reactivity with *T. pallidum* in our assay was noted. Immunological testing has demonstrated that borreliae and treponemes have antigens in common, and as a result, patients having antibodies to *T. pallidum* have yielded false-positive results in serological tests for *B. burgdorferi* (14, 18). The N-terminal ends of the *T. pallidum* and *B. burgdorferi* flagellin amino acid sequences have been reported as having 80% homology (9). Even with this degree of homology, however, the stringency of our assay allowed discrimination between these two pathogens.

It has been reported in different studies that European and American strains differ in many ways, such as DNA homology, outer surface proteins, and plasmid composition (3, 6, 7, 17, 22, 27). In addition, it has been reported that European strains are more diverse than American strains (6). Both the European and American strains of *B. burgdorferi* examined were detected in our study.

Our method is a direct, PCR-based, genus-specific method for detection of mammalian pathogenic borreliae. Because human clinical samples were not evaluated and a limited number of strains were examined in this study, any potential application to clinical diagnostics must be considered preliminary. Nevertheless, treatments associated with Lyme disease and other borrelial diseases are similar enough (15) and epidemiologies are different enough (26) that a genus-specific detection method may be both versatile and useful. Furthermore, since the flagellin gene is conserved among the various *Borrelia* species, this method may be suitable for evaluation of a wide variety of *B. burgdorferi* strains and may therefore prove useful for direct detection of the Lyme disease spirochete.

This work was supported by a grant from Clark Laboratories Inc., Jamestown, N.Y.

REFERENCES

1. **Abbott, M. A., B. J. Poiesz, B. C. Byrne, S. Kwok, J. J. Sninsky, and G. D. Ehrlich.** 1988. Enzymatic gene amplification: qualitative and quantitative methods for detecting proviral DNA amplified in vitro. J. Infect. Dis. **158**:1158–1169.
2. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57**:71–75.
3. **Barbour, A. G.** 1988. Plasmid analysis of *Borrelia burgdorferi*, the Lyme disease agent. J. Clin. Microbiol. **26**:475–478.
4. **Barbour, A. G., and C. F. Garon.** 1987. Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. Science **237**:409–411.
5. **Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schrumpf, and S. L. Tessier.** 1986. A *Borrelia*-specific monoclonal antibody binds to a flagellar epitope. Infect. Immun. **52**:549–554.
6. **Barbour, A. G., R. A. Heiland, and T. R. Howe.** 1985. Heterogeneity of major proteins in Lyme disease borreliae: a molecular analysis of North American and European isolates. J. Infect. Dis. **152**:478–484.
7. **Barbour, A. G., S. L. Tessier, and S. F. Hayes.** 1984. Variation in a major surface protein of Lyme disease spirochetes. Infect. Immun. **45**:94–100.
8. **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.
9. **Coleman, J. L., and J. L. Benach.** 1989. Identification and characterization of an endoflagellar antigen of *Borrelia burgdorferi*. J. Clin. Invest. **84**:322–330.
10. **Dattwyler, R. J., D. J. Volkman, B. J. Luft, J. J. Halperin, J. Thomas, and M. G. Golightly.** 1988. Seronegative Lyme disease: dissociation of specific T- and B-lymphocyte responses to *Borrelia burgdorferi*. N. Engl. J. Med. **319**:1441–1446.
11. **De, B. K., and A. Srinivasan.** 1989. Multiple primer pairs for the detection of HTLV-I by PCR. Nucleic Acids Res. **17**:2142.
12. **Gassmann, G. S., M. Kramer, U. B. Gobel, and R. Wallich.** 1989. Nucleotide sequence of a gene encoding the *Borrelia burgdorferi* flagellin. Nucleic Acids Res. **17**:3590.
13. **Golightly, M. G., J. A. Thomas, and A. L. Viciana.** 1990. The

laboratory diagnosis of Lyme borreliosis. Lab. Med. **21**:299–304.

14. **Hansen, K., P. Hindersson, and N. S. Pedersen.** 1988. Measurement of antibodies to *Borrelia burgdorferi* flagellum improves serodiagnosis in Lyme disease. J. Clin. Microbiol. **26**:338–346.
15. **Johnson, R. C.** 1989. Isolation techniques for spirochetes and their sensitivities to antibiotics in vitro and in vivo. Rev. Infect. Dis. **11**(6):S1505–S1510.
16. **Johnson, R. C., F. W. Hyde, and C. M. Rumpel.** 1984. Taxonomy of the Lyme disease spirochetes. Yale J. Biol. Med. **57**:79–87.
17. **LeFebvre, R. B., G. C. Perng, and R. C. Johnson.** 1989. Characterization of *Borrelia burgdorferi* isolates by restriction endonuclease analysis and DNA hybridization. J. Clin. Microbiol. **27**:636–639.
18. **Magnarelli, L. A., J. F. Anderson, and R. C. Johnson.** 1987. Cross-reactivity in serological tests for Lyme disease and other spirochetal infections. J. Infect. Dis. **156**:183–188.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 156. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
21. **Melchers, W. J. G., R. Schift, E. Stolz, J. Lindeman, and W. G. V. Quint.** 1989. Human papillomavirus detection in urine samples from male patients by the polymerase chain reaction. J. Clin. Microbiol. **27**:1711–1714.
22. **Milch, L. J., and A. G. Barbour.** 1989. Analysis of North American and European isolates of *Borrelia burgdorferi* with antiserum to a recombinant antigen. J. Infect. Dis. **160**:351–353.
23. **Nielsen, S. L., K. K. Y. Young, and A. G. Barbour.** 1990. Detection of *Borrelia burgdorferi* DNA by the polymerase chain reaction. Mol. Cell. Probes **4**:73–79.
24. **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.
25. **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.
26. **Schmid, G. P.** 1989. Epidemiology and clinical similarities of human spirochetal diseases. Rev. Infect. Dis. **11**(6):S1460–S1469.
27. **Schmid, G. P., A. G. Steigerwalt, S. E. Johnson, A. G. Barbour, A. C. Steere, I. M. Robinson, and D. J. Brenner.** 1984. DNA characterization of the spirochete that causes Lyme disease. J. Clin. Microbiol. **20**:155–158.
28. **Steere, A. C., J. Green, R. T. Schoen, E. Taylor, G. J. Hutchinson, D. W. Rahn, and S. E. Malawista.** 1985. Successful parenteral penicillin therapy of established Lyme arthritis. N. Engl. J. Med. **312**:869–874.
29. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. **308**:733–740.
30. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54**:2185–2191.
31. **Stoenner, H. G.** 1974. Biology of *Borrelia hermsii* in Kelly medium. Appl. Microbiol. **28**:540–543.