Molecular Detection of Persistent *Borrelia burgdorferi* in the Urine of Patients with Active Lyme Disease

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Current diagnostic tests for Lyme disease (LD) are dependent upon the host serologic response and are insensitive early in infection and, possibly, following antibiotic therapy. We cloned a library of *Borrelia burgdorferi* 297 DNA and studied one clone, Ly-1, for its potential in diagnostic and pathogenic studies. Using pulsed-field electrophoresis, we demonstrated that Ly-1 is of chromosomal origin and estimated that the *B. burgdorferi* chromosome is approximately 1,100 kb in size. The 3.7-kb Ly-1 clone hybridizes with geographically diverse strains of *B. burgdorferi*. No cross hybridization occurs with DNA from human cells, *Escherichia coli*, *Staphylococcus aureus*, *Clostridium difficile*, or the closely related *B. hermsii*. We used a dot blot assay to detect 10 pg of *B. burgdorferi* DNA. We partially determined the nucleotide sequence of Ly-1 and used it to select and synthesize oligonucleotides for use in the polymerase chain reaction (PCR). Two different primer pairs were found to amplify DNA from nine geographically diverse isolates. We could detect 10 fg (<10 molecules) of *B. burgdorferi* or less than five spirochetes added to human urine. Finally, we were able to use the PCR to detect *B. burgdorferi* DNA in the urine of four of eight patients with suspected active LD (three with arthritis and one with neurologic manifestations), all of whom responded to antibiotic treatment. In contrast, those patients who were PCR negative either had inactive disease or had been appropriately treated and did not respond to additional antibiotics, and all four control urine specimens were PCR negative. We conclude that *B. burgdorferi* DNA can be sensitively detected by the PCR with the primers and methods we describe and that the urinary tract is a site of persistent infection in some cases of human LD, an observation of potential diagnostic and pathogenic importance.

Lyme disease (LD) is an increasingly important public health problem throughout the world and is endemic in the northeastern, northwestern, and midwestern United States (6). It is caused by the spirochete *Borrelia burgdorferi*, which is transmitted to human and animal hosts by *Ixodes* ticks (4, 31). Acute disease in humans may result in a characteristic skin rash, Erythema migrans, and/or nonspecific flulike symptoms. Affected individuals often do not notice or recall the original bite (30), and early disease is also seldom recognized in infected livestock and pets (5, 18). If unrecognized and untreated, up to half of those infected develop more serious clinical manifestations, including neurologic and cardiac abnormalities and arthritis (29). These later manifestations of LD usually require parenteral antibiotic therapy (11, 33, 34) but are effectively prevented by early oral antimicrobial treatment (30, 32). Therefore, accurate diagnosis and early therapy are of critical importance.

Isolation of the spirochete from clinical specimens has proven difficult and insensitive. Currently available diagnostic tests for LD measure the host antibody response. Unfortunately, these serologic assays have significant problems with both sensitivity and specificity (29). Taken together, these difficulties lead to major problems for clinical laboratories, physicians, and veterinarians, particularly in endemic areas. An improved or complementary diagnostic test for LD would be invaluable to clinicians and could also contribute to improving our understanding of the pathogenesis and manifestations of human and veterinary infections.

We developed a molecular probe for *B. burgdorferi* chromosomal DNA. This report describes the origin, nature, and specificity of the Ly-1 DNA clone, part of its nucleotide sequence, its use in the polymerase chain reaction (PCR) to detect <10 DNA copies or less than five organisms in human urine in laboratory reconstructions and, for the first time, the detection of *B. burgdorferi* DNA in the urine of patients with LD. (This research was presented in part at the meeting of the American Federation for Clinical Research, Washington, D.C., April 1989, at the IVth International Conference of Lyme Borreliosism, Stockholm, Sweden, June 1990, and at the Thirteenth Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, Ga., October 1990.)

MATERIALS AND METHODS

Culture methods and strains. *B. burgdorferi* and *B. hermsii* strains were grown to maximal densities in BSK II medium (1). Organisms were enumerated by dark-field microscopy with a Petroff-Hauser chamber. The origins of the *B. burgdorferi* strains were as follows: 297, human cerebrospinal fluid, Conn.; 10293, avian, Conn.; 20047, *Ixodes ricinus*, France; IFT, *I. pacificus*, Calif.; P/GU, human skin, Munich, Federal Republic of Germany; MM1, mouse, Minn.; NCH-1, human skin, Wis.; K48, *I. ricinus*, Czechoslovakia; G25, *I. ricinus*, Sweden; and CT-2, *I. dammini*, Wis. The *B. hermsii* strain used was from the tick *Ornithodoros hermsii* in the western United States (HS1, ATCC 35209).

Cloning of Ly-1. Early-passage *B. burgdorferi* 297 was grown to a maximal density, and the cells were washed, suspended in 50 mM Tris (pH 8)–50 mM EDTA–20 mM NaCl with proteinase K (0.2 mg/ml) for 2 h at 60°C, and treated with RNase A (20 mg/ml) for 45 min at 37°C. The DNA was isolated by phenol-chloroform extraction and ethanol pre-
cipation. The DNA was digested with EcoRI (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and ligated to the EcoRI site of pUC18 with T4 ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). From the resultant library of clones, one which did not cross hybridize with human DNA was named Ly-1 and used in the studies reported here.

Southern and dot blot hybridization studies. For Southern blotting, spirochetal DNA from B. burgdorferi 297, P/GU, 20047, and IPT was prepared as described above. Human leukocyte DNA and bacterial DNA from Escherichia coli DH5, Staphylococcus aureus Wood (ATCC 10832), and Clostridium difficile (from a clinical isolate provided by Stuart Johnson, Minneapolis Veterans Administration Medical Center) were prepared by lysis with lysozyme (Bethesda Research Laboratories, Schlein & Schuell, Inc., Keene, N.H.). For dot blot analysis, DNA diluted in water and denatured in 0.4 M NaCl plus 0.015 M sodium citrate was cleaved, eluted from the plasmid, and nick translated with [α-32P]dCTP (21) for hybridization with a Ly-1 DNA probe. DNA was fractionated with 50% formamide-10% dextran sulfate, denatured with 0.15 M sodium bromide, sonicated, and precipitated with 1.5 M NaCl. For dot blot analysis, DNA was cleaved, eluted from the plasmid, and nick translated with [α-32P]dCTP to a specific activity of >10^8 dpm/μg. The probe (10^6 dpm/ml) was hybridized to the membranes as described previously (21) but under stringent conditions (50°C with 50% formamide-10% dextran sulfate-1× Denhardt's solution-4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]-0.1% SDS) 100 μg of sheared, sonicated, denatured salmon sperm DNA per ml-0.5 mg of heparin per ml. The blots were washed extensively prior to autoradiography with four washes of 30 min each at 65°C in 0.1% SDS and, respectively, 1× SSC, 0.5× SSC, 0.25× SSC, and 0.1× SSC.

Pulsed-field electrophoresis. Whole organisms at 10^6/ml were directly embedded in 1% low-melting-point agarose (Bethesda Research Laboratories) in 0.1 M EDTA-0.01 M Tris (pH 7.5)-0.02 M NaCl. The gel was solidified in glass tubes on ice, and the solid plugs were removed and incubated at 50°C for 24 h in the same buffer with 1% Sarkosyl and 1 mg of proteinase K per ml added. The agarose plugs were sliced and loaded directly into the troughs of a 1% horizontal agarose gel, which was subjected to CHEF (constant-clamped homogeneous electric field) pulsed-field electrophoresis (8) for 24 h at 135 V with 3-min pulses. After electrophoresis, electrotransfer of the DNA to a nylon membrane (Zeta-probe; Bio-Rad) was performed at 35 V in 0.5× TBE buffer (21) for 16 h with a Transblot (Bio-Rad) apparatus. The blot was hybridized as described above with a [α-32P]dCTP-nick-translated Ly-1 probe.

DNA sequencing. Plasmid DNA was isolated by lysis with alkaline and lysozyme (21), and sequencing was performed with supercoiled double-stranded DNA by the method of Chen and Seeburg (7). Plasmid DNA was annealed to an M13 sequencing primer complementary to either strand of pUC18 in the presence of [α-35S]dATP at 37°C for 15 min. Standard dideoxy sequencing reactions were performed with aliquots of the annealed template primer mixtures, with a 20-min sequencing reaction and a 15-min chase with excess unlabeled dideoxy nucleotide triphosphates. The DNA was fractionated on 5 to 8% polyacrylamide gels and autoradiographed.

Oligonucleotide synthesis and gene amplification. Oligonucleotide synthesis was performed with an Applied Biosystems DNA synthesizer by standard methods (16). PCR primers were purified by polyacrylamide gel electrophoresis, eluted, and chromatographed with Sephadex.

For laboratory reconstructions, samples of urine were prepared by being boiled for 5 min. Washed B. burgdorferi cells were resuspended in water and boiled for 5 min prior to direct amplification by the PCR. For patient urine samples (previously stored at -20°C), 500 μl of boiled urine was adjusted to 0.5% SDS-10 mM Tris (pH 8)-5 mM EDTA and proteinase K (0.2 mg/ml) was added. The samples were incubated for 3 h at 65°C and extracted with phenol-chloroform and then with chloroform. Yeast tRNA (0.1 mg/ml) was added, and the DNA was ethanol precipitated and resuspended in 50 µl of distilled water. For each PCR reaction, 20 µl of the resuspended DNA was added as a target.

The PCR reaction (25) was set up with a final mixture containing each deoxynucleoside triphosphate at 200 μM, 1.5 to 2.5 mM MgCl_2 (see Results), 10 mM Tris (pH 8.3), 0.01% (wt/vol) gelatin (Sigma, St. Louis, Mo.), and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) as well as 44 pmol of each primer in a volume of 100 μl and 2.5 μl of light mineral oil (Sigma). Target specimens and positive and negative controls were added through the mineral oil as the final step. No-target controls contained all reaction components, including Taq polymerase, but no added DNA or clinical material. All samples were prepared with positive-displacement pipettes with disposable pistons (Gilson Instruments, Woburn, Mass.) in a containment facility never exposed to B. burgdorferi, concentrated samples of B. burgdorferi DNA, or final PCR products. The PCR mixture was placed in a Perkin-Elmer Cetus thermal cycler, heated for 5 min at 94°C, and subjected to 39 cycles of 1 min of denaturation at 94°C, 2 min of annealing at 50°C, and 2 min of extension at 72°C. A final cycle was identical, except for 6 min of extension at 72°C. PCR products were analyzed by electrophoresis of 5 μl of the PCR mixture on 3% NuSieve agarose (FMC, Rockland, Maine) minigels in TBE buffer for 1.5 h at 80 V and staining with ethidium bromide. For Southern hybridization, DNA was electrotransferred to Zeta-probe membranes as described above.

For identification of PCR products fixed to the nylon membranes, internal oligonucleotides were radiolabeled by poly(A) tailing with terminal dideoxynucleotidyl transferase (TdT) (9). In brief, 140 μCi of [α-32P]dATP (NEN, Wilmington, Del.; activity 5,000 Ci/mmole) was dried down to a volume of 7 μl, 10 to 20 ng of the oligonucleotide was added in a volume of 2 μl, and 3 μl of 5× tailing buffer (Bethesda Research Laboratories) and 3 μl (45 U) of TdT (Bethesda Research Laboratories) were added for 1 h at 37°C. The tailed oligonucleotide was purified on a Sephadex G50 column. The specific activity of the probe was typically 10^8 dpm/μg. After prehybridization for 2 to 4 h at 55°C in a solution consisting of 6× SSC, 40 mM Tris (pH 7.4), 10 mM sodium P0, 0.5% SDS, 0.3 mg of sonicated, sheared salmon sperm DNA per ml, 0.1 mg of poly(A) per ml, 0.1 mg of proteinase K per ml, 0.5 mg of heparin (Sigma) per ml, and 5× Denhardt's solution, 2×10^6 dpm of the probe per ml was added, and the mixture was hybridized for 4 to 16 h at 55°C. Following hybridization, the filters were washed two to three times in 6× SSC-0.5% SDS, first at 20°C and then at 55°C, air-dried, and exposed to XAR film with two Cronin intensifying screens.
EcoRI-cleaved DNA to contains cleaved human sii (lane 4), 59, chromosome XIII, 260-kb radiolabeled Ly-1 chromosome (C) of preparations chromosome cerevisiae were localized (B). (B) shown). 2. FIG. 1. FIG. 1.

FIG. 1. (A) Pulsed-field electrophoretic gel of B. burgdorferi. Shown from left to right are 10⁶ cells of strain 297 and two different preparations of S. cerevisiae chromosomes. The B. burgdorferi chromosome (C) migrated slightly slower than did 1,050-kb S. cerevisiae chromosome XVI. Also shown are 1,300-kb S. cerevisiae chromosome XIII, 260-kb S. cerevisiae chromosome I, and the visible B. burgdorferi plasmid (P). The S. cerevisiae chromosomes were localized by hybridization with specific cloned probes (not shown). (B) Southern blot of the same gel hybridized to the radiolabeled Ly-1 clone.

FIG. 2. (A) Southern blot of EcoRI-cleaved DNA from B. hermsii (lane 1) and B. burgdorferi 20047 (lane 2), IPT (lane 3), P/GU (lane 4), and 297 (lane 5) hybridized to the Ly-1 clone. Lane 6 contains cleaved human leukocyte DNA, and lane 7 contains radiolabeled molecular weight (M.W.) markers. (B) Southern blot of EcoRI-cleaved DNA from B. burgdorferi 297 (lane 1) and C. difficile, S. aureus, and E. coli (lanes 2 to 4, respectively) hybridized to the Ly-1 clone.

FIG. 3. Dot blot hybridization to the radiolabeled Ly-1 clone of 10-fold serial dilutions of DNA from (top to bottom) B. burgdorferi 297 and P/GU, B. hermsii, C. difficile (C. diff.), S. aureus, Ly-1 itself, human leukocyte DNA (Hum.), and 297 DNA plus 2.5 µg of human DNA (Hum + 297).

RESULTS

Chromosomal Origin of the Ly-1 clone. Because B. burgdorferi contains multiple plasmids (3), we wished to determine whether Ly-1 was of chromosomal or plasmid origin. For this purpose, we performed CHEF pulsed-field electrophoresis of strain 297 DNA from whole organisms embedded in agarose; electrophoresis was followed by electrotransfer to a nylon membrane and hybridization with the twice-eluted nick-translated Ly-1 probe. The probe only hybridized to a very high-molecular-weight band corresponding to chromosomal DNA, slightly above the position of 1,050-kb Saccharomyces cerevisiae chromosome XVI and well above the position of the apparent plasmid seen on the original gel (positions noted on Fig. 1). Since extremely large (e.g., >50-kb) supercoiled plasmids as well as chromosomal DNA could migrate slowly in pulsed gels, we also performed prolonged stationary-field electrophoresis. Under such conditions, Ly-1 hybridized only to the major mass of chromosomal DNA (data not shown). Thus, on the basis of these studies, we believe that Ly-1 is of chromosomal origin and estimate that the B. burgdorferi chromosome is approximately 1,100 kb in size.

Specificity and sensitivity of hybridization with the Ly-1 clone. To determine whether Ly-1 was specific for B. burgdorferi, we used the eluted nick-translated Ly-1 clone as a probe in Southern hybridization against EcoRI-cleaved DNA from B. burgdorferi strains and from various other bacterial species (Fig. 2A). Ly-1 hybridized strongly to a 3.7-kb EcoRI fragment in B. burgdorferi DNA from strains 297 (lane 5) and 20047 (lane 2), and an isolate from I. pacificus (lane 3). A clear strain-specific polymorphism was seen with the German isolate P/GU. Ly-1 hybridized strongly to P/GU DNA, but the major band was at 1.8 kb.
FIG. 4. DNA sequence of the plus and minus ends of the Ly-1 clone and primers used in experiments.
(lane 4). The higher-molecular-weight bands present in some lanes reflected partial digestion of the genomic DNA, as evidenced by their disappearance following more extensive digestion (e.g., 297, Fig. 2B, lane 1). In contrast, Ly-1 did not hybridize in Southern blots to DNA from the closely related spirochete B. hermsii (Fig. 2A, lane 1), to human genomic DNA (Fig. 2A, lane 6), or to DNA from C. difficile, S. aureus, or E. coli (Fig. 2B, lanes 2, 3, and 4, respectively).

Given the specificity of hybridization observed, we next asked whether Ly-1 could sensitively detect B. burgdorferi DNA in a dot blot assay. The nick-translation Ly-1 probe detected 100 pg of bound DNA from strain 297 and from the most distantly related strain, P/GU (Fig. 3). This level of sensitivity was maintained in the presence of over a 1,000-fold excess of human DNA. In addition, the Ly-1 probe again did not cross hybridize to human DNA or to DNA from C. difficile, S. aureus, or B. hermsii.

**DNA sequence analysis of Ly-1.** Through double-stranded DNA sequencing, we obtained 241 bp of sequence at one end of the Ly-1 clone and 283 bp at the other. The *EcoRI*-cleaved end containing most of the pUC18 polylinker (e.g., including the pUC18 *SalI* site) was arbitrarily designated the plus end. The result (Fig. 4) revealed a G+C content of 32%. A DNA sequence homology search performed for both sequences against the bacterial, vector, and human sequences in GenBank revealed no extensive similarities which might prevent the specific use of the sequences in DNA hybridization and in the PCR.

**Sensitivity and specificity of the PCR for detection of B. burgdorferi DNA.** We next synthesized primers to define a 224-bp fragment at the plus end of the clone (679 and 680) and a 176-bp fragment at the minus end of the clone (681 and 682). In addition, internal oligonucleotides 583 and 683 were synthesized for use as probes for the amplified plus- and minus-end sequences, respectively (Fig. 4). The PCR was performed as described above. With each primer pair, we detected 100 pg of the initial target by ethidium bromide staining as amplified bands of appropriate molecular weights (Fig. 5). Southern blots of membranes transferred from gels containing 5 μl from PCR reactions with 100 pg of 297 or *E. coli* DNA and a no-target negative control demonstrated that internal oligonucleotide probe 583 specifically hybridized to the product amplified by 679 and 680 (Fig. 6A), that probe 683 hybridized to the product from primers 681 and 682 (Fig. 6B), that no *E. coli* DNA was amplified by either primer pair, and that the reactions containing a no-target negative control were not contaminated. The efficiency and specificity of the PCR are often affected by the concentrations of both Mg and the primer in the reaction (25). To optimize these variables for our primer pairs, we performed the PCR with Mg concentrations of 0.5 to 3.5 mM in the absence or presence of excess heterologous DNA and with 22 to 220 pmol of...
primers added. Primers 679 and 680 amplified optimally with 2.5 mM Mg, while primers 681 and 682 amplified optimally with 1.5 mM Mg. Optimal amplification of a total of 1 ng of B. burgdorferi DNA occurred with 44 pmol of primers present. At optimal Mg and primer concentrations, no bands resulted from amplification of human DNA, even when a 2,500-fold excess was present (data not shown).

We next determined more extensively the sensitivity and specificity of the primer pairs. Hybridization of electrotransferred blots with the appropriate TdT-tailed oligonucleotide probes showed that as little as 10 fg of 297 DNA could be amplified to yield a detectable product. We observed no amplification of unrelated DNA (S. aureus or E. coli), and controls containing no added DNA showed no hybridization signals (data not shown; see Fig. 8 and 9).

Direct amplification of DNA from spirochetes. Because strains of geographically, genetically, and antigenically diverse spirochetes are clinically encountered (35), we directly amplified DNA from nine B. burgdorferi strains of diverse origin with both primer pairs. All B. burgdorferi strains tested (Fig. 7 for 679 and 680; data not shown for 681 and 682) yielded the appropriately sized amplified products with both primer pairs, although strain P/GU was amplified inefficiently with 681 and 682. B. hermsii yielded no PCR amplification signal.

On the basis of the estimated size of 1,100 kb for the B. burgdorferi chromosome (Fig. 1; 24) and our detection of 10 fg of strain 297 DNA, we calculated that we should be able to detect ≤10 organisms. Because the urinary tract is a potential target of infection (see below), we chose to directly dilute spirochetes in human urine prior to DNA amplification by the PCR. In this manner, we were able to detect as few as 2.5 organisms (Fig. 8, lane 3). Again, it is important to note that simultaneous control PCR reactions containing no target (lanes 1 and 6), 2.5 × 10⁻⁷ organisms (lane 2), and 100 pg of S. aureus (lane 7) were all negative. In addition, control PCR reactions with DNA extracted from the urine of three patients with bacterial urinary tract infections caused by E. coli, S. aureus, and alpha-hemolytic streptococci were all negative (data not shown).

Detection of B. burgdorferi DNA in urine from patients with LD. We next asked whether we could detect B. burgdorferi in the urine of nine patients with suspected LD. For these studies, extracted DNA (see above) derived from 200 µl of urine was subjected to the PCR with the 679-680 primer pair. The PCR results are shown in the ethidium bromide-stained gel and the corresponding Southern blot in Fig. 9. The patients' clinical characteristics, serologic results, and PCR results are tabulated in Table 1. The experiment was repeated, with identical results. By Southern hybridization, four of eight patients suspected of having active LD were PCR positive (patients 3, 7, 8, and 10; Fig. 9B). One patient with arthritic disease, patient 9, had a very faint hybridization signal seen on the original blots. Because the signal intensity was so low, we considered the PCR result nondiagnostic, although the experiment was repeated with the same result. On the original stained gel, positive amplification of an appropriately sized PCR product was seen for the two patients with the strongest signals, patients 3 and 7 (Fig. 9A). All of the PCR-positive patients were from endemic areas and had illnesses consistent with active LD. Three had predominantly arthritic symptoms, and one (patient 7) had a chronic neurologic process. Three were clearly seropositive, but one was borderline, as determined by an enzyme immunoassay. Three of the four PCR-positive patients responded dramatically to appropriate antibiotic treatment. Patient 7 had longstanding neurologic deficits, which did not change, but experienced subjective improvement.

The four clearly PCR-negative patients were all seropositive, with prior clinical histories consistent with “late” LD. Patients 1, 4, and 5 had received intensive treatment with appropriate parenteral antibiotics. Although they had persistent symptoms, they were PCR negative and did not clinically respond to further antibiotic treatment. Patient 6 had received appropriate therapy for heart block 1 year before the negative urine PCR result was obtained. He was without symptoms and has remained well without further therapy. Finally, urine specimens from all four control patients (patients 2, 11, 12, and 13) and two no-target controls tested at the same time with no DNA or urine added were PCR negative. Thus, four of eight patients suspected of
having active LD versus zero of six patients and no-target controls were clearly PCR positive.

**DISCUSSION**

The studies presented here report the detection of spirochetal DNA in the urine of patients with LD and demonstrate that the urinary tract is a potential site of spirochetal persistence. The ability to study and diagnose an infection with *B. burgdorferi* has been limited by deficiencies of previously available methodologies. Direct cultivation of these slow-growing organisms from blood, cerebrospinal fluid, joint fluid, and heart has been reported but is only successful in a minority of cases (2). The host antibody response may develop quite slowly, and up to one-half of patients with acute LD are seronegative even 2 months after infection (28), although one laboratory has reported better sensitivity with immunoblotting (13) and with an enzyme immunoassay specific for immunoglobulin M (10). Because seroconversion may be blunted by the administration of antibiotics, some individuals have been reported to have late LD without detectable antibodies (12). Finally, significant problems exist with false-positives (20), and there are tremendous interlaboratory variations in the performance and interpretation of the assays (14, 27). Thus, considerable confusion exists in medical and veterinary diagnosis and may lead not only to the tragic failure to recognize and treat LD but also to the expensive and potentially toxic treatment of many individuals who are not infected.

DNA-based methodologies provide a suitable strategy for the detection of an organism which is presently poorly cultivatable. Few studies have been performed to date. Schwan and Barbour reported using subclones of outer surface protein A (OspA) of *B. burgdorferi* in dot blot hybridization (26). In their study, 500 pg of *B. burgdorferi* DNA was detected and significant cross-reactivity with *B. hermsii* was noted. Rosa and Schwan recently reported using a chromosomally derived *B. burgdorferi* B31 DNA sequence in the PCR (24). They detected 17 of 18 *B. burgdorferi* strains; one, a German isolate, failed to be amplified. They noted no amplification of *B. hermsii*, but extensive amplification of eucaryotic sequences occurred when excess human DNA was present. Persing et al. reported the use of the PCR for detecting *B. burgdorferi* in *Ixodes* ticks (23) with sequences from the plasmid-encoded OspA protein of strain B31. The sensitivity of the assay was not reported, and 3 of 10 *B. burgdorferi* isolates tested failed to be amplified with their primers. One, again, was from Germany, but two others were of U.S. origin. Protein electrophoretic studies suggested that the nonamplified strains had alterations in OspA. Thus, as Persing et al. (23) pointed out, the use of sequences from OspA may not be optimal for diagnostic purposes, since such plasmid sequences may not be stably maintained in all strains.

We cloned and partially determined the nucleotide sequence of a chromosomal DNA fragment, Ly-1, of *B. burgdorferi* 297 and found it to be potentially useful in pathogenic and diagnostic studies of LD. On the basis of Southern hybridization with the Ly-1 probe in Southern blots of CHEF pulsed-field gradient electrophoresed DNA.
from whole *B. burgdorferi*, we estimated a size of approximately 1,100 kb for the *B. burgdorferi* chromosome. This estimate is consistent with that of Rosa and Schwann (24). We specifically detected 100 pg (10^2 genomes) of *B. burgdorferi* DNA in dot blot hybridization. The dot blot methodology has potential advantages of economy and the ability to handle large numbers of samples but may not detect the small numbers of organisms likely to be present in most natural infections. Using the PCR and synthetic oligonucleotide primer pairs derived from the Ly-1 sequence, we detected <10 copies of *B. burgdorferi* DNA. The use of our primers in the presence of excess heterologous human DNA did not result in amplification of unrelated human sequences such as that seen in another study (24). By using longer primers, we could increase the annealing and extension temperatures in the PCR and eliminate nonspecific priming and amplification. These modifications have potential advantages in dealing with samples, such as fresh or embedded human and animal tissue specimens, which might contain eucaryotic DNA.

Nine geographically diverse *B. burgdorferi* strains were amplified by both primer pairs, but no amplification of *B. hermsii*, *S. aureus*, or *E. coli* DNA was noted. The only *B. burgdorferi* strain which was amplified inefficiently in our studies was the German isolate, P/GU, with the 681-682 primer pair. This result is of interest when considered with the apparent failure of the chromosomal sequences of Rosa and Schwann (24) to amplify the German G2 isolate and the failure of the OspA sequences to amplify another German isolate. Our Southern blots (e.g., Fig. 2A) suggest a major DNA polymorphism in the P/GU region corresponding to the Ly-1 clone in which only a 1.8-kb EcoRI fragment of strain P/GU hybridizes to the 3.7-kb strain 297 clone while the other strains tested contain the conserved 3.7-kb fragment. We hypothesize that the other 1.9 kb may be deleted from or rearranged in P/GU. Taken together, such findings in three laboratories suggest that a significant sequence divergence has occurred, particularly with some European isolates. Fortunately, our 679-680 primer pair efficiently amplified DNA from all *B. burgdorferi* strains tested, regardless of origin.

We next demonstrated that less than five spirochetes could be directly amplified in human urine. This finding was highly relevant because *B. burgdorferi* antigens have been detected in the urine of experimentally infected animals and in the urine of some human patients with LD (15). Therefore, we next chose to examine stored urine specimens from patients suspected of having LD. Our studies demonstrated that the urinary tract is involved in some cases of LD. We found spirochetal DNA in the urine of four of eight patients with late manifestations of suspected active LD, three with arthritic symptoms and one with central nervous system involvement. The signal intensities of the positive specimens varied from levels corresponding to from <10 fg to >1 pg of initial input DNA in positive controls. Thus, by extrapolation, the equivalent of from approximately <50 to >5,000

<table>
<thead>
<tr>
<th>Patient</th>
<th>Residence</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Clinical historya</th>
<th>Serologic result</th>
<th>PCR result</th>
<th>Clinical follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arizona</td>
<td>21</td>
<td>F</td>
<td>Myalgias, headaches persisting after treatment</td>
<td>+ (I)</td>
<td>ND</td>
<td>No response to retreatment</td>
</tr>
<tr>
<td>2</td>
<td>Arizona</td>
<td>43</td>
<td>F</td>
<td>Control</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Minnesota</td>
<td>27</td>
<td>F</td>
<td>Tick exposure, polyarthralgias, headaches improved with TCN but recurring Arthritis, difficulty concentrating, some improvement with IV therapy for 6 wk</td>
<td>Borderline (E)</td>
<td>+ (I)</td>
<td>Resolved with 4 wk of PO TCN</td>
</tr>
<tr>
<td>4</td>
<td>Connecticut</td>
<td>?</td>
<td>F</td>
<td>Arthritis, difficulty concentrating</td>
<td>+ (I)</td>
<td>-</td>
<td>No response to retreatment</td>
</tr>
<tr>
<td>5</td>
<td>Connecticut</td>
<td>28</td>
<td>F</td>
<td>Flulike illness, encephalitis, no response to IV CTX for 6 wk</td>
<td>+ (I)</td>
<td>-</td>
<td>No response to retreatment</td>
</tr>
<tr>
<td>6</td>
<td>Minnesota</td>
<td>28</td>
<td>M</td>
<td>EM, heart block, improvement with IV PCN and steroids</td>
<td>+ (I, E)</td>
<td>-</td>
<td>Well without further treatment</td>
</tr>
<tr>
<td>7</td>
<td>Wisconsin</td>
<td>50</td>
<td>M</td>
<td>Tick bites, chronic central nervous system disease recurring 4 mo after 1 wk of IV PCN</td>
<td>+ (I, E, WB)</td>
<td>+</td>
<td>Some improvement with CTX, lost to follow-up</td>
</tr>
<tr>
<td>8</td>
<td>Minnesota</td>
<td>44</td>
<td>M</td>
<td>EM, migratory polyarthritis</td>
<td>+ (E)</td>
<td>+</td>
<td>Resolved after 4 wk of IV CTX</td>
</tr>
<tr>
<td>9</td>
<td>Minnesota</td>
<td>48</td>
<td>F</td>
<td>Possible EM, aseptic meningitis, no treatment; fatigue, arthritis years later</td>
<td>Borderline (E)</td>
<td>±</td>
<td>Arthritis but not fatigue improved with DOX for 4 wk</td>
</tr>
<tr>
<td>10</td>
<td>California</td>
<td>34</td>
<td>F</td>
<td>EM, severe arthralgias 2 mo later</td>
<td>+ (I, E)</td>
<td>+</td>
<td>Resolved with 4 wk of PO PCN, well 1 yr</td>
</tr>
<tr>
<td>11</td>
<td>Minnesota</td>
<td>37</td>
<td>M</td>
<td>Control</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>Minnesota</td>
<td>31</td>
<td>M</td>
<td>Control</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>Minnesota</td>
<td>38</td>
<td>M</td>
<td>Control</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Abbreviations: F, female; M, male; CTX, ceftriaxone; PCN, penicillin; DOX, doxycycline; TCN, tetracycline; ND, not done; EM, erythema migrans; I, indirect fluorescent antibody; E, Enzyme immunoassay; WB, Western blot; ±, borderline signal intensity (see the text); IV, intravenous; PO, oral.

b At time of sampling.
organisms per ml may have been present initially in the urine specimens. All patients with positive urine PCR results, including two (patients 3 and 8) who had been previously treated, responded to appropriate antibiotic treatment. The four patients with negative urine PCR results had already received appropriate and intensive treatment, and those three whose symptoms were persistent failed to respond to additional antibiotics. One possible explanation for these findings is that the PCR-positive patients had persistent active infections present and thus responded to treatment, while the PCR-negative patients had symptoms not directly related to spirochetal persistence. These results, while preliminary, suggest that the detection of spirocheturia by the PCR not only may be diagnostically useful but also may help to identify patients whom further antibiotic treatment will benefit. Because our data concern a small, highly selected group of referral patients, prospective and controlled studies will be needed in other clinical settings to determine the true clinical sensitivity, specificity, and utility of PCR detection of *B. burgdorferi* in urine.

The studies reported here and those reviewed above support the potential utility of the PCR in the study and diagnosis of LD. Significant issues remain to be addressed before this technology can be generally useful for diagnosis. First, the greatest asset of the PCR, its extreme sensitivity, is also its greatest potential drawback. False-positives have been a significant problem in most laboratories (19). For this reason, we always use multiple simultaneous negative controls for all PCRs involving clinical studies. Next, despite our findings, it is not yet fully known in which tissues and/or body fluids spirochetes can be detected and with what sensitivity they can be detected at various stages of infection. Blood is a likely candidate for the acute phase of infection, given its clinical accessibility and the evidence for spirochetemia in acute clinical (22) and experimental (17) LD and other borrelial infections, but spirochetemia is unlikely to be present in late disease. Although seldom available, synovial fluid and cerebrospinal fluid have occasionally yielded spirochetes in cultures (2) and, when clinically involved, are excellent potential candidates for DNA detection. Finally, our results and the reported finding of antigenuria (15) make urine a potentially important target for the detection of spirochetes. In conclusion, the primers that we have described, with their advantages of amplifying a broad range of isolates without amplifying heterologous DNA, and our finding that *B. burgdorferi* can be detected in the urine of some patients with treatable late LD provide potentially powerful approaches to improving the detection and diagnosis of *B. burgdorferi* infections. Studies with such techniques should also be extremely useful in better defining the pathogenesis of human, veterinary, and experimental infections and, in particular, the nature of spirochetal persistence.

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**REFERENCES**