Original Article

Polymerase Chain Reaction Detection of Lyme Disease

Correlation With Clinical Manifestations and Serologic Responses

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The authors have developed a simple, nested polymerase chain reaction (PCR) assay for amplification of an outer surface protein A (OspA) gene fragment of *Borrelia burgdorferi* using rapid temperature cycling and ethidium bromide detection on agarose gels, and applied it to the diagnosis of Lyme disease in humans. With denaturing and annealing temperature spikes instead of holds, cycle times were less than 20 minutes for a 30-cycle amplification. Using this rapid cycle PCR technique, as few as 5 spirochetes per mL of phosphate buffered saline were detected. In addition, *B burgdorferi* DNA was detected from spirochetes that had been spiked into one of several types of human body fluids including serum, synovial fluid, and cerebrospinal fluid (CSF). A number of clinical samples, which had been tested for Lyme immunoglobulin M (IgM) and immunoglobulin G (IgG) antibody were also examined. In 29 serologic positive samples (14 IgG and IgM positive, 9 IgM alone and 6 IgG alone), *B burgdorferi* DNA was not detected. In contrast, nine serum samples and one synovial fluid from patients with definite clinical features of Lyme disease were found to be negative by EIA and Western blot analysis for IgG and IgM antibody, but contained *B burgdorferi* DNA, as detected by PCR. Polymerase chain reaction analysis of serum and synovial fluid may be of significant diagnostic value in Lyme disease, especially in the absence of a serologic response in early, partially treated and seronegative chronic disease. This is the first study to report an association between PCR positivity and the absence of a serologic response to Lyme borreliosis. (Key words: Polymerase chain reaction; *Borrelia burgdorferi*; Serum; Rapid cycle amplification) Am J Clin Pathol 1996;105:647–654.

Lyme disease is caused by the tick-borne spirochete *Borrelia burgdorferi*.[1] There is great difficulty associated with making a definitive diagnosis of this disease for several reasons. First, manifestations of Lyme disease are varied and rarely clear cut. Generally, the presence of the skin rash, erythema migrans (EM), is the best indicator of infection. However, this type of rash is lacking in 20% to 40% of the cases.[2] Musculoskeletal, neurologic, and cardiovascular involvement occurs in Lyme disease, but may be present in a variety of combinations and generally appears later in the course of the disease.[2] Therefore, Lyme disease can be very misleading and difficult to diagnose in the early stages, particularly if EM is not present. A second major problem is associated with the immune response to *B burgdorferi*. Many affected individuals do not mount an immune response until after the organism has reached the joints, central nervous system, or the heart. Even when an immune response is present, it may be variable and difficult to interpret.[3] The third problem lies in the inability of the available serologic tests to eliminate cross reactivity with other spirochetes and to be sensitive enough to detect low levels of antibody.[4,5]

Because of the problems associated with detecting and interpreting an immune response, the use of the polymerase chain reaction (PCR) to detect the DNA from *B burgdorferi* seems promising. We have developed a nested PCR assay using rapid cycle DNA amplification techniques to detect the outer surface protein A (OspA) gene, which is located on a 49-kb plasmid contained within the spirochete.[6] The OspA gene has been shown to be highly specific for *B burgdorferi*.[7,8] The nested PCR assay we used makes use of a rapid air thermocycler to amplify the products.[9,10] Nocton and colleagues[11] have performed PCR for OspA sequences on the joint fluid of

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TABLE 1. OLIGONUCLEOTIDE PRIMER SEQUENCES FOR *BORRELIA BURGDORFERI* OspA GENE

	Primer Name	*Position Base No.*	*Sequence 5'-3'*
Outer set	Bb-1	152–177	TGAAAAAATATTTATTGGGAATAGGT
	Bb-2	340–316	TAAGCTCAAGCTTGTCTACTGTTGC
Inner set	Bb-3	202–224	AAGCAAAATGTTAGCAGCCTTGA
	Bb-4	295–268	CTTTGTTTTTTTCTTTGCTTACAAGAAC

patients with chronic Lyme arthritis and have found *B burgdorferi* in 85% of the patients. We hypothesized that it might be possible to make an accurate diagnosis of early or chronic Lyme disease by examining serum or plasma samples. Our preliminary data, on a limited number of cases, suggest that PCR can be positive in such samples, especially if they are obtained before a serologic response to *B burgdorferi* is present. To our knowledge, this is the first study in which a negative correlation between serology and PCR results has been observed.

MATERIALS AND METHODS

Oligonucleotide Primers

Gene sequences from 20 OspA isolates were identified in GENBANK and 2 sets of primers were chosen with 91% homology to all sequences (Table 1). Primer BB-1 is paired with primer BB-2 to form an outer primer set that produces a PCR product that is 189 base pairs (bp) in length. The nesting primer set is made up of primers BB-3 and BB-4 and amplifies a 93 bp product. Primers were synthesized by phosphoramidite, solid phase chemistry (Pharmacia LKB-Gene Assembler Plus; Pharmacia Biotech, Uppsala, Sweden).

PCR Reaction Mixture

One μL of sample DNA was mixed in a U-bottom microtiter dilution tray with nine μLs of a reaction mixture containing 0.50 mM of each primer, 200 mM of each deoxynucleotide triphosphate (Pharmacia Biotech, Uppsala, Sweden), 50 mM tris-hydrochloride, 3 mM magnesium chloride, 2.5 mg/mL of BSA, 0.5% Ficoll, 1 mM tartrazine, and 0.4 Units of Taq polymerase (Amplitaq, Perkin-Elmer Cetus, Norwalk, CT) in sterile distilled H_2O. Reagents were from Sigma (St. Louis, MO) unless otherwise specified.

Product Amplification

The above reaction mixture was drawn from the microtiter tray into a 10 μL glass capillary tube (0.56 mm internal diameter). After the ends of the capillary were sealed the tube was placed in a Model 1605 rapid air thermocycler (Idaho Technology, Idaho Falls, ID), where it was temperature-cycled for 40 cycles. By using sharp temperature spikes instead of holds at denaturation and annealing, cycle times for DNA amplification can be reduced from 4 to 6 minutes per cycle down to 30 seconds.[12] Nesting was performed by aspirating 1 μL of amplified product from the tip of the capillary with the use of a clean pipet tip attached to a dedicated pipettor. This 1 μL from the first amplification was mixed in a sterile microtiter dilution tray with 9 μL of a new amplification mixture containing the nested primers. For nesting, the extension time was lowered to 1 second and the samples were run for 30 cycles. Amplified product was resolved by running 10 μL on 1.5% agarose gels (SeaKem agarose, FMC Corporation, Rockland, ME). Gels were run at 80 V for 30 to 45 minutes, in a solution of trizma base, boric acid, EDTA (TBE) with 0.5 mg/mL ethidium bromide. DNA was visualized on a UV transilluminator at 300 nm and compared to molecular weight markers (50-1000 bp), (Bio Ventures, Murfreesboro, TN). The total time for amplification, nesting, and gel electrophoresis was approximately 2 hours.

Because of the problems associated with contamination and false positivity, we incorporated strict guidelines for maintaining a sterile working environment and unidirectional work flow between three distinctly separate work stations. Sterile disposable equipment and dedicated pipettors were used for every reaction, and reaction mixtures were made up by adding the sample DNA as the last component.

Samples were run in duplicate along with positive controls made from whole blood inoculated with approximately 400 spirochetes per mL and negative controls from a known negative donor.

PCR Protocol

Our primer sets were optimized[13,14] by running a series of reactions using 1, 2, 3, 4, and 5 mM magnesium chloride at annealing temperatures varying from 40 °C. to 60 °C. The final optimized protocol for the primary am-

plification was as follows: denaturation at 94 °C for less than 1 second, annealing at 47 °C for less than second, and elongation of 72 °C for 5 seconds. For the secondary or nested amplification, all of the cycling parameters were the same except for the elongation time that was lowered to 1 second.

Restriction Digest

To ensure that the final DNA product contained the correct sequence, we used the restriction enzyme Bst71 I (Promega, Madison, WI), which recognizes a 5 base sequence within the inner primer product. One unit of the restriction enzyme was allowed to locate the template sequence of the 93 bp product and cut it during a 16-hour incubation at 37 °C. This cut produces fragments at 27 and 66 bp.

Specificity of our primers was checked by attempting to amplify spirochete DNA from buccal swabs obtained from five normal donors. DNA isolation and PCR for these samples was done simultaneously with the positive and negative controls described above. Genomic DNA in each sample was verified by amplifying the D1S80 locus.

DNA Isolation Techniques

Four different methods of DNA isolation were evaluated. The first method was to simply boil the samples. In the second method, samples were spun at 16,000 × g for 15 minutes and the pellet was digested with proteinase K (Sigma) in sodium dodecyl sulfate (SDS) digestion buffer (0.2M trizma base, 0.05M EDTA and 1% SDS, pH 8.0). This was followed by a standard phenol/chloroform extraction and ethanol precipitation. DNA was diluted in 1X Tris EDTA (TE) buffer to an A260 reading of 1.0 and boiled for 5 minutes. In the third method, samples were again spun at 16,000 × g for 15 minutes and the pellet was digested with 5 μL of proteinase K mixed with 45 μL of a Tween 20 digestion buffer (0.02M trizma base, 0.05M EDTA and 1% Tween 20, pH 8.5). Following a 1-hour incubation at 55 °C, the samples were boiled for 5 minutes. The fourth method made use of a solid phase DNA isolation kit (IsoQuick; Microprobe, Garden Grove, CA), which uses guanidine thiocyanate and nuclease adsorption with an extraction matrix. Twenty μg of glycogen were added to each sample as a DNA carrier. Purified DNA from this method was resuspended in 50 μL of RNase free water. One μL of DNA from all samples was used to perform PCR.

Bacteria and Sample Spiking

Spirochetes of the N40 isolate, passage 5, were cultured in BSK-II (Sigma) media at 30 °C and counted using dark field microscopy. Spirochetes were diluted in PBS from 400 spirochetes per mL down to 5 spirochetes per mL. Diluted spirochetes were then added to either PBS alone, or a variety of human body fluids. The purpose for using spirochetes diluted in PBS was to determine the optimal sensitivity limits for the assay without the possible interference of any non-*B burgdorferi* debris to affect the PCR. Spirochetes were also spiked into the human body fluids for the purpose of determining the sensitivity limits for each specific sample type. For sample spiking experiments, we chose to use whole blood, plasma, serum, synovial fluid, CSF, and urine from normal donors. Spirochetes were added to each human sample type in varying concentrations and incubated overnight at 30 °C. so as to allow the spirochetes to bind to any cellular structures. The spirochetes do not undergo significant cell division under these conditions. DNA was then extracted using proteinase K in Tween 20 digestion buffer. Spirochetes diluted in PBS did not undergo overnight incubation and were processed shortly after inoculation.

Clinical Samples

Specimens used in this study were obtained from a variety of suspected Lyme disease patients. A majority of the samples were sent as serum to our reference laboratory for general serological testing by enzyme immunoassay and/or Western blot (MarDx Diagnostics, Carlsbad, CA). It was assumed that samples that were considered Lyme serologically positive by either EIA and/or Western blot testing were from patients who currently exhibited or were recovering from Lyme type disease (ie, erythema migrans, chronic arthritis, or neurologic impairment). Generally, those patients who presented with positive IgG results alone would be considered as having past exposure to *B burgdorferi*. Whereas, those patients who exhibited positive IgM or IgG and IgM would be considered to have more recent or acute infection and be in the earlier stages of disease. We assumed that those samples that tested negative by serology and PCR were obtained from patients who were affected by some type of Lyme-associated illness based on the fact that they were sent to us specifically for Lyme testing. Even patients whose samples were sent to us merely to rule out Lyme disease must be considered potential candidates for having the disease. Therefore, every patient included in this study most likely had symptoms that were at least suggestive of Lyme disease. Unfortunately, we do not know whether the symptoms were suggestive of early or late disease. Because ours is a reference laboratory receiving specimens from other medical centers or regional laboratories, clinical data on

the patients, including symptoms, timing of the sample, and physical findings, are almost never available, even after repeated requests. However, we were able to get clinical data on all 10 samples with positive PCR results after an exhaustive effort.

A small panel of 10 plasma and serum samples, sent to us by Boston Biomedica (West Bridgewater, MA) was also included in this study. This panel was tested by nine laboratories using four different serologic methodologies. Nine of the samples were selected to demonstrate IgG and/or IgM reactivity.

It was necessary to verify that our methods of DNA isolation could be used to isolate minimal quantities of genomic DNA so that we could feel confident in reporting negative results for Lyme PCR. For this, we used 10 sera obtained from 10 different blood donors. Because serum lacks a significant cellular population, we added approximately 25 cultured lymphocytes to each sample, before DNA extraction. After the DNA was isolated each sample was tested for the presence of human genomic DNA by using oligonucleotide primers specific for the beta-globin gene. After testing each sample for the presence of human genomic DNA, we tested each sample using primers for *B burgdorferi*.

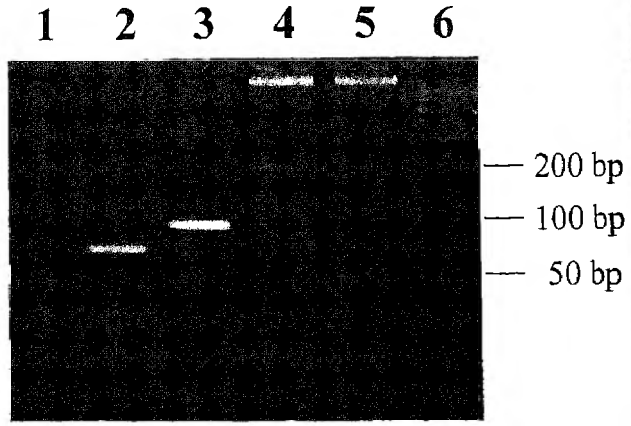

FIG. 1. Product identification using restriction enzyme Bst71 I. Agarose gel electrophoresis of rapid cycle PCR-amplified products from N40 isolate of *Borrelia burgdorferi*. Lane 1 (Left lane): negative control that was isolated from uninfected Balb/c mouse heart. Lane 2: inner nested product after digestion with restriction enzyme Bst71 I. Lane 3: inner nested primer product, at 93 bp, from positive sample that was isolated from infected Balb/c mouse heart. Lane 4: 93 bp product amplified from infected Balb/c mouse heart using the inner primer set only without nesting. Lane 5: Outer nonnested primer product, at 189 bp, also isolated from infected Balb/c mouse heart. Lane 6: molecular weight marker (BioVentures).

RESULTS

Confirmation of Primer Specificity

In Figure 1, we amplified and detected the *B burgdorferi* DNA using both the inner and outer primers without nesting (Lanes 4 and 5). By using a nested PCR assay, the bands for our inner nested product (Lane 3) become much more intense and defined in comparison to the nonnested products of Lanes 4 and 5. We identified our final nested primer product by using restriction enzyme Bst71 I. This restriction enzyme was able to recognize a specific sequence within the nested primer product in Lane 3 and make a cut at 8 and 12 bp downstream from the recognition site. The restriction digest worked to produce two new bands at 66 and 27 bp (Lane 2).

Comparison of **Borrelia burgdorferi** *DNA Isolation Methods*

Spirochetes were diluted in PBS from 200 spirochetes per mL down to 1 spirochete per mL and DNA was extracted by each of the previously described methods. Boiling the samples followed by PCR amplification of 1 μL of sample failed to demonstrate spirochete DNA at any of these levels. Centrifugation, followed by phenol/chloroform extraction of the pellet and ethanol precipitation, allowed us to detect *B burgdorferi* DNA at the level of 200 spirochetes per mL in PBS. In contrast, by using the proteinase K in Tween 20 digestion buffer and the Isoquick methods, we were able to detect *B burgdorferi* DNA down to 5 spirochetes per mL of PBS. Extraction and PCR analyses were performed 3 to 4 times in these experiments. Because the Tween 20 and IsoQuick methods yielded the same results, we chose to continue our sensitivity experiments using the Tween 20 method of DNA isolation. Figure 2 illustrates the effectiveness of the proteinase K in Tween 20 method for isolating *B burgdorferi* DNA at different concentrations of spirochetes. The positive control is shown in Lane 2 and positive bands are found at 93 bp. From the figure, we can see that DNA was positively identified in the 5, 10, 25, and 50 spirochete samples (Lanes 5 through 8). Conversely, there are no positive bands in the negative control (Lane 3) and in the one spirochete per mL samples (Lane 4). Lower bands shown at approximately 60 bp are probably the result of the formation of primer dimers.

Sample Inoculation Experiments

After determining sensitivity in PBS, we inoculated a variety of human body fluids with *B burgdorferi*. DNA was isolated by the Tween 20 method and subsequently amplified. Samples, which yielded the greatest sensitivity, were plasma, synovial fluid, and CSF. In these sample types, we could detect as few as 10 spirochetes per mL of sample. We detected down to 20 spirochetes per

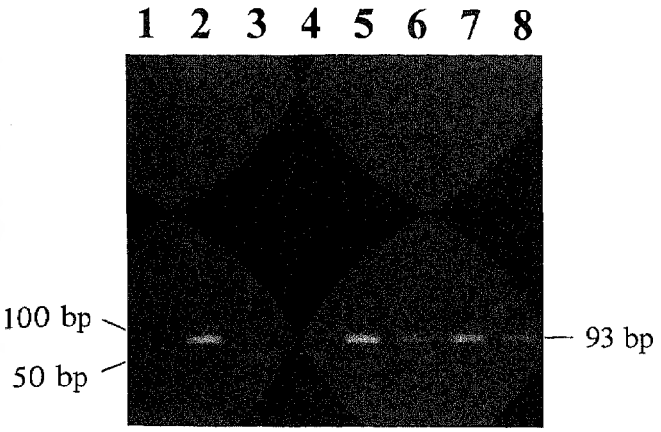

FIG. 2. Sensitivity of spirochete DNA detection using rapid cycle PCR and visualized by agarose gel electrophoresis and ethidium bromide staining. DNA was isolated by proteinase K in Tween 20 digestion buffer from spirochetes spiked directly into PBS. Lane 1 (Left lane): Biomarker low molecular weight marker (BioVentures). Lane 2: positive control isolated from infected Balb/c mouse heart. Lane 3: negative control isolated from uninfected Balb/c mouse heart. Lane 4: is a sample which contained approximately 1 spirochete per mL of PBS. No amplifiable spirochetal DNA was detected in this sample. Lane 5: *Borrelia burgdorferi* DNA amplified from a 5 spirochete per mL sample. Lane 6: *B burgdorferi* DNA amplified from a 10 spirochete per mL sample. Lane 7: *B burgdorferi* DNA amplified from a 25 spirochete per mL sample. Lane 8: *B burgdorferi* DNA amplified from a 50 spirochete per mL sample.

mL in serum samples, 50 spirochetes per mL in urine, and 100 spirochetes per mL in whole blood. Extraction and PCR analyses were performed 2 to 3 times for each body fluid.

Clinical Samples

Table 2 compares results of serology and PCR data from 67 samples. All samples had DNA isolated by both the IsoQuick and proteinase K in Tween 20 methods of extraction. Fourteen of the samples were identified as positive for both IgG and IgM. However, none had detectable *B burgdorferi* DNA by PCR. Nine of the samples were positive for IgM and negative or equivocal for IgG. These nine samples were negative for *B burgdorferi* DNA. Six samples, which were positive for IgG antibodies only, were also negative for *B burgdorferi* DNA. Of the remaining 28 samples with negative PCR results, all had either negative or equivocal antibody results, with the exception of seven that were initially sent to our laboratory for PCR testing and had insufficient sample volumes for serologic testing.

Nine of the serum samples and one synovial fluid sample had *B burgdorferi* DNA present by PCR. None of these samples contained antibody to *B burgdorferi* (Table 3).

Positive samples 1 and 2 were obtained from a 40-year-old man described as an avid hiker who lived in the New England area until 1988 when he moved to the West Coast. In 1989, the patient was diagnosed with Bell's palsy and has had three episodes since that time without etiology. The patient has had no rash or dermatitis and no detectable neurologic or cardiac involvement.

Positive samples 3 and 4 were from a 47-year-old man who had a tick bite followed by a characteristic EM circular rash in the right groin, first noticed in May of 1994. The rash dissipated and reappeared in August of 1994 when the first serum sample was sent for PCR analysis. At that time, no antibiotic therapy had been administered. In September 1994, the rash was suspected to be more likely due to *Tinea corporis*, and the patient was treated with antifungal therapy. It was after this last episode that the second serum sample was sent for Lyme PCR testing.

Positive samples 5 and 6 were from a 40-year-old man who presented with EM in the ankle region 18 months before PCR analysis. No antibiotic treatment was given before PCR testing.

Positive sample 7 was a grossly hemolyzed serum sample. This patient was a 66-year-old woman known to have had rashes consistent with Lyme disease, including a specific EM type rash on the left thigh approximately 4 years before PCR analysis. This patient also complained of neck and hip arthralgias.

Positive sample 8 was from a 51-year-old woman with chronic arthritis of the hips and knees without a history of a Lyme type rash.

Positive sample 9 was from a 10-year-old boy who presented with an insect bite on the scalp and an associated lesion approximately 3 months before PCR analysis. The patient subsequently was diagnosed with Bell's palsy, headaches, and alterations in mentation. Antibiotic therapy was not administered before PCR testing.

Positive sample 10 was from a 31-year-old woman with frequent exposure to numerous farm animals and numerous insect bites. She did not exhibit any type of rash but did experience memory changes, headaches, and arthralgias. This patient had begun antibiotic therapy with doxycycline and ceftriaxone 2 weeks before PCR analysis.

We were unable to detect any *B burgdorferi* DNA from any of the 10 samples included in the Boston Biomedica panel including 9 serology positive and 1 serology-negative serum.

Testing 10 blood donor sera (spiked with a minimal number of cultured lymphocytes) for the presence of the B-globin gene revealed human genomic DNA in

TABLE 2. SEROLOGY VERSUS POLYMERASE CHAIN REACTION (PCR) FOR *BORRELIA BURGDORFERI* SAMPLES TESTED BY LYME ENZYME IMMUNOASSAY OR WESTERN BLOT AND COMPARED WITH LYME PCR

	No. Samples	*Sample Type*	*IgG Serology*	*IgM Serology*	*PCR Result*
	13	Serum	Positive	Positive	Absent
	1	Plasma	Positive	Positive	Absent
Total	14				
	7	Serum	Negative	Positive	Absent
	1	Serum	Equivocal	Positive	Absent
	1	Plasma	Negative	Positive	Absent
Total	9				
	2	Serum	Positive	Negative	Absent
	3	Plasma	Positive	Negative	Absent
	1	CSF	Positive	Negative	Absent
Total	6				
	6	Serum	Negative	Negative	Absent
	2	Blood	Negative	Negative	Absent
	1	Synovial	Negative	Negative	Absent
	2	Urine	Negative	Negative	Absent
	8	CSF	Negative	Negative	Absent
	1	Plasma	Equivocal	Negative	Absent
	1	Serum	Equivocal	Equivocal	Absent
	2	Serum	QNS	QNS	Absent
	4	CSF	QNS	QNS	Absent
	1	Blood	NA	NA	Absent
Total	28				
	9	Serum	Negative	Negative	Present
	1	Synovial	Negative	Negative	Present
Total	10				

QNS = quantity not sufficient for testing; Ig = immunoglobulin; NA = serology results not available; Absent = no *Borrelia burgdorferi* DNA detected; Present = *Borrelia burgdorferi* DNA detected; CSF = cerebrospinal fluid.

all 10 samples. Thus, the negative results for these samples were not the result of failed DNA extraction or amplification. Further testing of these samples using the primers specific for *B burgdorferi* showed that *B burgdorferi* was absent from all of these 10 blood bank samples.

Overall, we performed serology and PCR testing on 67 samples from patients that had suspected Lyme disease.

TABLE 3. SEROLOGY RESULTS AND CLINICAL FEATURES OF *BORRELIA BURGDORFERI* POLYMERASE CHAIN REACTION (PCR) POSITIVE PATIENTS

Sample	*Sample Type*	*ARUP IgG EIA*	*ARUP IgM EIA*	*ARUP Western Blot*	*PCR Result*	*Symptoms*
1	Serum	0.21	0.07	Negative	Present	Bell's palsy
2	Serum	0.06	0.07	Negative	Present	Bell's palsy
3	Serum	0.22	0.16	Negative	Present	EM following tick bite
4	Serum	0.37	0.14	Negative	Present	EM following tick bite
5	Synovial	0.00	0.00	Negative	Present	EM, 2–3 cm pigmented lesions on leg Rash in ankle region, lesions with clearing
6	Serum	0.36	0.39	Negative	Present	EM, 2–3 cm pigmented lesions on leg Rash in ankle region, lesions with clearing
7	Serum	0.30	0.19	Negative	Present	EM on left thigh Neck and hip arthralgias
8	Serum	0.10	0.21	Negative	Present	Chronic arthritis in hips and knees
9	Serum	0.65	0.90	Negative	Present	Insect bite with lesions on scalp Bell's palsy, headaches, alterations in mentation
10	Serum	0.22	0.77	Negative	Present	High exposure to farm animals with subsequent tick bites Memory changes, headaches, and arthralgia

EM = erythema migrans; EIA = enzyme immunoassay; ARUP = Associated Regional and University Pathologists; Ig = immunoglobulin.
Enzyme immunoassay results are negative for values below 0.91. Samples 1 and 2 are from the same patient, before treatment, with a 1-month time difference. Samples 3 and 4 are from the same patient, before treatment, with a 1-month time difference. Samples 5 and 6 are also from the same patient, before treatment, with a 1-month time difference.

Among these 67 samples, we detected nine positive (13%) samples by PCR from seven patients. These nine samples were repeatedly positive. Thirty-four of the total 67 samples were submitted specifically for Lyme PCR testing from 29 patients. The remaining 33 samples were sent for Lyme serology testing and selected at random for PCR analysis. Of the 34 samples submitted for PCR testing, 9 sera and 1 synovial fluid sample were positive by PCR, but negative by serology. These nine samples came from seven patients exhibiting characteristic Lyme features (ie, erythema migrans, arthritis, and neurologic involvement). Three patients had positive PCR results for *B burgdorferi* from more than one sample. Seven (24%) of the 29 patients suspected of Lyme disease, who had samples sent specifically for PCR analysis were found to have *B burgdorferi* DNA present. In contrast, 30 of the total 67 samples were positive or equivocal for either IgM, IgG, or both, and yet none of these 30 samples were found to contain *B burgdorferi* DNA. We believe that this lends evidence to the fact that the presence of antibody has some bearing on the outcome of the PCR, most likely because the antibody has the ability to eliminate the already low numbers of spirochetes present in the serum. This does not suggest that the spirochete may not still be present in higher numbers in other tissues or fluids such as joint fluid, the heart or skin.

DISCUSSION

The objective of this study was to determine if a nested, rapid cycle amplification technique could be used to detect *B burgdorferi* DNA in clinical samples, including serum, and determine the relationship between the serologic status of the patient and PCR positivity. Using primers to the OspA gene we were able to detect *B burgdorferi* DNA by amplifying an outer primer product at 189 bp and finally an inner nested product at 93 bp. Positive identification of *B burgdorferi* DNA was performed using DNA isolated from infected Balb/c mice and whole blood samples spiked with spirochetes. Restriction enzyme Bst71 I digestion confirmed the identity of the OspA gene. Lebech and Hansen[7] and Leibling and colleagues[8] have also indicated that the OspA gene is specific for *B burgdorferi*.

We were able to determine the *in vitro* sensitivity of our test by spiking various numbers of spirochetes into PBS and isolating the DNA using the four different methods described above. Adding the spirochetes to PBS greatly reduced the possibility of any inhibitors in the PCR reaction. Figure 2 illustrates that we were able to detect as few as five spirochetes using the proteinase K in Tween 20 DNA isolation method. Similar results were also found for the IsoQuick method of extraction. One advantage to using the IsoQuick method is that the final sample is generally cleaner with a pure DNA product. The advantage to using the proteinase K in Tween 20 method is that sample isolation takes place in only one microcentrifuge tube, which minimizes sample loss. These data suggest that either method of DNA isolation gives sensitive, comparable results.

Through inoculating a variety of human body fluids with *B burgdorferi* spirochetes, we were able to establish which sample types had inhibitory effects on the PCR reaction. By adding spirochetes in varying concentrations to each of the different sample types and allowing them to incubate, we hoped to simulate what might be seen *in vivo*. Spirochetes could be detected in each of the sample types with a sensitivity of down to 10 spirochetes in the plasma, synovial fluid, and CSF. We could only detect down to 100 spirochetes in whole blood samples. Leidtke and colleagues[15] have indicated that the presence of hemoglobin may have inhibitory effects on PCR in whole blood samples.

Our methods of DNA isolation were capable of extracting DNA from 10 of 10 blood bank donor serum samples spiked with a low number (25 cells) of lymphocytes. Further testing of these samples for the presence of *B burgdorferi* organisms showed that all samples were negative for this organism. This observation illustrates that the DNA isolation techniques that we have described are capable of isolating available DNA in 100% of the samples. Therefore, samples that do not have bands present after gel electrophoresis are in fact actual negative samples and are not due to failure of the DNA isolation technique. One must keep in mind that a negative result can be because of concentrations of spirochetes below the limit of detection, or lack of any spirochetes in the patient samples.

Leibling and colleagues[8] have shown that it is possible to detect the OspA gene of *B burgdorferi* in serum and other samples using PCR. In results similar to ours, these authors could detect as few as 10 spirochetes per mL of fluid using a phenol/chloroform extraction method. We believe that one of the most critical points of our study is that, using the DNA isolation methods and PCR techniques described previously, *B burgdorferi* DNA was repeatedly detected in serum samples (9 of 22 samples, or 41% of serum samples sent specifically for Lyme PCR). It is also important that all of the samples which contained *B burgdorferi* DNA, as detected by PCR, were negative for antibody by EIA and Western blot. It is a known fact that the level of spirochetes (for most patients) contained in blood are low and it appears, at least in the cases we have presented in this study, that once an immune response is mounted spirochete DNA appears

to be eliminated from the serum. Although the sensitivity of the test may not be high, finding *B burgdorferi* DNA in serum almost certainly establishes the diagnosis of borreliosis in the patients. Moreover, we think this finding may be important in the dissemination and, therefore, the pathogenesis of the disease. We realize that the general sensitivity of PCR testing may never achieve an acceptable level to be useful as a routine screening test for infection. However, it may be extremely useful in the unusual seronegative patient with disease compatible with that caused by *B burgdorferi*.

In conclusion, we believe that this type of testing may be of significant value in the detection of early Lyme disease or disease in which an adequate serologic response has not developed. We strongly suggest performing PCR on duplicate samples as low copy numbers are likely present, especially in serum. We also suggest testing skin biopsies from typical lesions, synovial fluid, and CSF along with serum of suspected patients. A positive test has great significance in establishing the etiology of a Lyme-like illness, whereas a negative PCR result certainly does not exclude *B burgdorferi* infection.

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