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DETECTION OF *BORRELIA BURGDORFERI* DNA BY POLYMERASE CHAIN REACTION IN SYNOVIAL FLUID FROM PATIENTS WITH LYME ARTHRITIS

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Abstract *Background.* *Borrelia burgdorferi* is difficult to detect in synovial fluid, which limits our understanding of the pathogenesis of Lyme arthritis, particularly when arthritis persists despite antibiotic therapy.

Methods. Using the polymerase chain reaction (PCR), we attempted to detect *B. burgdorferi* DNA in joint-fluid samples obtained over a 17-year period. The samples were tested in two separate laboratories with four sets of primers and probes, three of which target plasmid DNA that encodes outer-surface protein A (OspA).

Results. *B. burgdorferi* DNA was detected in 75 of 88 patients with Lyme arthritis (85 percent) and in none of 64 control patients. Each of the three OspA primer–probe sets was sensitive, and the results were moderately concordant in the two laboratories (kappa = 0.54 to 0.73). Of 73 patients with Lyme arthritis that was untreated or treated with only short courses of oral antibiotics, 70 (96 percent) had positive PCR results. In contrast, of 19 patients who received either parenteral antibiotics or long courses of oral antibiotics (≥1 month), only 7 (37 percent) had positive tests ($P<0.001$). None of these seven patients had received more than two months of oral antibiotic treatment or more than three weeks of intravenous antibiotic treatment. Of 10 patients with chronic arthritis (continuous joint inflammation for one year or more) despite multiple courses of antibiotics, 7 had consistently negative tests in samples obtained three months to two years after treatment.

Conclusions. PCR testing can detect *B. burgdorferi* DNA in synovial fluid. This test may be able to show whether Lyme arthritis that persists after antibiotic treatment is due to persistence of the spirochete. (N Engl J Med 1994;330:229-34.)

LYME DISEASE is caused by the tick-borne spirochete *Borrelia burgdorferi*.[1] Weeks to months after the onset of disease, approximately 60 percent of untreated patients begin to have brief, intermittent attacks of arthritis that may recur for several years.[2] A small percentage of these patients have continuous arthritis for one year or longer, a condition we have termed "chronic Lyme arthritis."[2,3] Eventually, both intermittent and chronic Lyme arthritis resolve, even in untreated patients.[2,4]

Lyme arthritis can usually be treated successfully with either a one-month course of doxycycline or amoxicillin or a two-week course of intravenous ceftriaxone or penicillin.[5-7] However, a small percentage of patients have persistent arthritis despite multiple courses of oral and intravenous antibiotic therapy.[5-7] In these patients there is an increased frequency of HLA-DR4 or, secondarily, HLA-DR2 in association with antibody reactivity to outer-surface proteins A and B (OspA and OspB) of the spirochete.[8,9] It has been unclear whether this treatment-resistant course results from persistent infection or from postinfective immune-mediated phenomena. The ability to demonstrate the presence or absence of *B. burgdorferi* in the joint would improve our understanding of the pathogenesis of Lyme arthritis.

B. burgdorferi can be cultured readily from the skin lesions of erythema migrans,[10] but it has been difficult to detect the spirochete in joints; it has been recovered from the synovial fluid of only two patients with Lyme arthritis.[11,12] With immunohistologic techniques, spirochetal forms have been seen in synovial tissue,[13] but this method of detection has been inconclusive. The polymerase chain reaction (PCR) has recently been used to amplify and detect *B. burgdorferi* DNA in cultured spirochetes,[14,15] *Ixodes dammini* ticks,[16,17] infected animals,[18,19] and patients with Lyme disease.[20-33] In these studies, DNA sequences have been successfully detected in blood,[20,21,30,32] cerebrospinal fluid,[22-25,32,33] urine,[21,25,28,32] skin,[29-31] and (in eight cases) synovial fluid.[21,26,27,32] Thus, the PCR assay is capable of amplifying and detecting *B. burgdorferi* DNA, but its value as a reliable diagnostic test, particularly in synovial fluid, is not yet clear.

We report here on our evaluation of PCR testing as

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a diagnostic test for the presence of *B. burgdorferi* DNA in synovial fluid. If the test can reliably identify the presence of spirochetes, it may help to determine whether persistent arthritis after treatment is due to active infection or to an immune reaction that persists after the eradication of viable spirochetes.

Methods

Patients

During a 17-year period, samples of synovial fluid were collected from 127 patients with Lyme arthritis who were seen in the Lyme disease clinics at Yale–New Haven Hospital (1975 to 1987) or New England Medical Center (1987 to 1992). The following criteria were used to define Lyme arthritis: brief, intermittent attacks of oligoarticular arthritis, exposure in an area of endemic disease, an elevated antibody response to *B. burgdorferi* on enzyme-linked immunosorbent assay, and the exclusion of other known forms of arthritis. The synovial-fluid samples were divided into multiple aliquots and frozen at −70°C; in most instances, the aliquot used for PCR testing was not opened before this study. Samples from 37 of the patients were collected in heparin, a known inhibitor of PCR amplification[34]; these samples were excluded from the study. Single samples were tested in 61 of the remaining 90 patients, and two to five serial samples were tested in 29. Clinical data collected from the charts of patients with Lyme arthritis were analyzed without knowledge of the results of PCR assays. During the same 17-year period, synovial fluid was also obtained from 69 control patients with other forms of arthritis. Seventeen of these samples were collected, processed, and stored in the same way as those from the patients with Lyme arthritis. Among the control patients, 20 had rheumatoid arthritis; 7 each had gout, osteoarthritis, and degenerative joint disease; 5 had juvenile rheumatoid arthritis; 2 each had pseudogout, psoriatic arthritis, scleroderma, spondyloarthropathy, and Reiter's syndrome; and 13 had other forms of arthritis.

PCR Assay

Synovial-fluid samples from case and control patients were processed in a blinded manner in two separate laboratories according to the following protocol. DNA was isolated from 100 to 200 μl of synovial fluid with a commercially available kit (Isoquick, Microprobe, Bothell, Wash.) according to the manufacturer's specifications, modified by the addition of 20 μg of glycogen to each sample as a carrier during isopropanol precipitation. With each DNA extraction, synovial-fluid samples from case and control patients were processed simultaneously. Filter-barrier pipette tips and a dedicated set of pipettors were used to prepare all samples.

Three separate regions of the *B. burgdorferi* genome were targeted for PCR amplification by four sets of primers and probes (Table 1). Sets 1, 2, and 3 targeted portions of the *B. burgdorferi* plasmid gene encoding OspA, and set 4 targeted a portion of the chromosomal DNA encoding 16S ribosomal RNA.[35] Sets 2 and 3 targeted the same sequence of the OspA gene for amplification, but used different internal probes for detection.

Primers and probes were synthesized on an oligonucleotide synthesizer (Applied Biosystems, Foster City, Calif.), desalted on an oligonucleotide-purification cartridge (Glen Research, Sterling, Va.), and used without further purification. Total DNA was dissolved in 30 μl of water; 5 μl of this solution was added to a PCR mixture containing 50 pM of each primer (final concentration, 1.0 μM), 200 μM of each deoxynucleoside triphosphate, 10 mM TRIS–hydrochloride (pH 8.3), 50 mM potassium chloride, 17.5 mM magnesium chloride, 0.01 μg of bovine serum albumin per microliter, 10 percent glycerol, 0.5 percent Tween-20, and 1.25 units of Taq polymerase (Amplitaq, Perkin–Elmer Cetus, Norwalk, Conn.). Five micrograms of isopsoralen was added to the PCR mixture to inactivate products after amplification,[36] and the total volume was adjusted to 50 μl with water. The mixture was overlaid with one drop of mineral oil. Amplification reactions, which were performed in a thermal cycler (Perkin–Elmer Cetus) stored in a separate laboratory, consisted of 45 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 1 minute. The cycles were preceded by a four-minute phase at 94°C and followed by a final seven-minute extension phase at 72°C. After amplification, samples were immediately exposed to 20 mW of 300-to-400-nm light per square centimeter for 20 minutes to inactivate the products and were stored at −20°C.

Table 1. Oligonucleotide Primer and Probe Sequences.

Oligonucleotide (Base No.)	Sequence*
Set 1	
Primers	
OspA2 (943–920)	GTTTTGTAATTTCAACTGCTGACC
OspA4 (788–812)	CTGCAGCTTGGAATTCAGGCACTTC
Probe	
OspA3 (906–881)	GCCATTTGAGTCGTATTGTTGTACTG
Set 2	
Primers	
OspA319 (343–319)	CTTTAAGCTCAAGCTTGTCTACTGT
OspA149 (149–173)	TTATGAAAAAATATTTATTGGGAAT
Probe	
OspA6′ (196–216)	GCATGTAAGCAAAATGTTAGC
Set 3	
Primers	
OspA319 (343–319)	CTTTAAGCTCAAGCTTGTCTACTGT
OspA149 (149–173)	TTATGAAAAAATATTTATTGGGAAT
Probe	
OspA6 (165–190)	ATTGGGAATAGGTCTAATATTAGCCT
Set 4	
Primers	
DD02 (1472–1455)	CCCTCACTAAACATACCT
DD06 (1105–1124)	ATCTGTTACCAGCATGTAAT
Probe	
BR01 (1304–1350)	GATTGAAGTCTGAAACTCGACTTCATGAAGT-TGGAATCGCTAGTAAT

*Sequences are shown from 5′ to 3′.

Control samples included with each amplification assay included samples with DNA extracted from control patients, three blank control samples with 5 μl of water substituted for DNA, and a positive control sample with 60 pg of total *B. burgdorferi* DNA (strain 297). Amplification products were stored and analyzed in a separate area, and positive-displacement pipettes with disposable pistons were used to prepare all PCR reagents.

Amplified products (5 μl) were resolved by 4 percent agarose gel electrophoresis (3 percent NuSieve and 1 percent SeaKem, FMC Corporation, Rockland, Me.) at 35 to 100 V for one to three hours. The gel was then stained with ethidium bromide and visualized, washed with water, washed in denaturation solution (1.5 M sodium chloride and 0.5 M sodium hydroxide) for 45 minutes, rinsed again with water, washed in neutralization solution (1.5 M sodium chloride and 0.5 M TRIS [pH 7.5]), and blotted. Blotting was performed overnight on a nylon membrane (Hybond-N, Amersham, Arlington Heights, Ill.) with 0.15 M sodium chloride and 0.015 M sodium citrate (SSC). The membrane was cross-linked with 0.12 J of ultraviolet light.

Membranes were washed in hybridization fluid (5× Denhardt's solution [1× Denhardt's solution is 0.02 percent Ficoll, 0.02 percent polyvinylpyrrolidone, and 0.02 percent bovine serum albumin], 0.75 M sodium chloride, 0.025 M sodium phosphate, 0.005 M ethylenediamine tetraacetate, 0.5 percent sodium dodecyl sulfate, and 100 μg of denatured salmon-sperm DNA per microliter) for four hours at 55°C. An oligonucleotide probe end-labeled with phosphorus-32 was then added for 15 to 17 hours at 55°C. After hybridization, the membranes were washed in 2× SSC and 0.1 percent sodium dodecyl sulfate for 10 minutes, 1× SSC and 0.1 percent sodium dodecyl sulfate for 20 minutes, and 0.2× SSC and 0.1 percent sodium dodecyl sulfate for 30 minutes. Finally, the membranes were exposed to Kodak XAR-5 film for 4 to 72 hours at −70°C. Alternatively, the amplification products were detected by means of a chemiluminescent probe as described elsewhere.[37] DNA bands were rarely seen on ethidium-stained gels; samples were therefore considered positive on the basis of signal detection after hybridization.

Inhibition Assays

The initial samples from each patient with Lyme arthritis in which *B. burgdorferi* DNA was not detected and 62 of the 69 samples from the controls were tested for the presence of PCR inhibitors.

One thousand copies (as determined by serial dilution) of the OspA2–OspA4 amplification product made without isopsoralen cross-linking were added to each sample and then amplified with primers OspA2 and OspA4. Samples that yielded no amplification signal were considered inhibitory and were excluded from the study.

Statistical Analysis

The similarity of groups was compared by Fisher's exact test, the distribution of values among the groups was compared by Wilcoxon rank-sum test, and concordance among test results was calculated with kappa values according to the following formula: kappa = (observed agreement − expected agreement) ÷ (1 − expected agreement). All P values are two-tailed.

Results

In tests performed independently in two laboratories, *B. burgdorferi* DNA was detected with at least one primer–probe set in the initial samples of synovial fluid from 75 of the 90 patients with Lyme arthritis. In contrast, *B. burgdorferi* DNA was not found in any of the 69 control patients or in the blank control samples. In inhibition assays, 2 of the 15 negative samples from the patients with Lyme arthritis and 5 samples from control patients still produced negative results, indicating that inhibitors of PCR amplification were present. With these samples excluded, *B. burgdorferi* DNA was detected in the initial sample from 75 of 88 patients with Lyme arthritis but none of 64 control patients (Table 2).

Of the four primer–probe sets used in the two laboratories, the three OspA sets each detected *B. burgdorferi* DNA in 75 to 89 percent of the 75 patients with positive test results (Table 2). Forty-eight patients (64 percent) had positive results with all three OspA sets, whereas 18 patients (24 percent) had positive results with only one of the sets. Set 4, which detected chromosomal DNA encoding 16S ribosomal RNA, was less sensitive than the OspA primer–probe sets; 56 percent of the 75 patients had positive results with this set, and all of them also had positive results with OspA set 3. In laboratory 1, 88 percent of the patients had concordant results with the two OspA primer–probe sets used in that laboratory (kappa = 0.73). Between laboratories 1 and 2, 78 percent had concordant results with the OspA primer–probe sets that targeted the same gene segment (sets 2 and 3) (kappa = 0.54).

Clinical data from the 88 patients with Lyme arthritis were correlated with the PCR results from their initial samples of synovial fluid (Table 3). As compared with the 13 patients with negative results, the 75 patients in whom *B. burgdorferi* DNA was detected in joint fluid had significantly higher white-cell counts in synovial fluid ($P<0.003$), shorter durations of illness ($P<0.02$) and arthritis ($P<0.03$), and a longer duration of arthritis after aspiration ($P = 0.03$). Almost all of those with positive results were untreated or had only received short courses of oral antibiotic therapy (<1 month) before joint aspiration ($P<0.001$).

Of the 88 patients in this study, 45 never received antibiotic therapy. *B. burgdorferi* DNA was detected in the synovial fluid of 43 of these patients (Table 4). All 12 patients from whom serial samples were available for testing had detectable *B. burgdorferi* DNA in their first sample (Fig. 1). In 9 of the 12 patients, including the 3 who had chronic arthritis (one year or more of continuous joint inflammation), all additional samples were also positive months to years later during subsequent episodes of arthritis. In the remaining three patients, *B. burgdorferi* DNA was not detected in the last synovial-fluid sample, and their arthritis resolved within the next few months.

Forty-three of the 88 patients received antibiotic therapy sometime during the course of Lyme disease. Of the 16 patients with pretreatment synovial-fluid samples available for testing, 15 had positive test results (Table 4). In another 12 patients with synovial fluid obtained two months to four years after short courses of oral antibiotic therapy, all samples were also positive. In 19 patients, 4 of whom also had pretreatment samples available for testing, synovial fluid was obtained after either long courses of oral antibiot-

Table 2. PCR Results in Synovial Fluid from Case and Control Patients.*

	Primer–Probe Sets				
	Set 1	Set 2	Set 3	Set 4	Any Set†
	no. of patients				
Lyme arthritis (n = 88)					
Positive PCR results	57	56	67	42	75
Negative PCR results	31	32	21	46	13
Other arthritis (n = 64)					
Positive PCR results	0	0	0	0	0
Negative PCR results	12	12	57	57	64

*Test results from the initial sample from each patient are shown. Samples that contained inhibitors of PCR amplification were excluded. Laboratory 1 tested all initial and serial samples from patients with Lyme arthritis and all identically stored control samples with primer–probe sets 1 and 2. Laboratory 2 tested the initial sample from each patient with Lyme arthritis, 5 of the identically stored control samples, and an additional 52 control samples from their institution with primer–probe sets 3 and 4.

†Values indicate the total numbers of patients who had positive test results with at least one primer–probe set or negative results with all four sets.

Table 3. Clinical Data and PCR Results in Patients with Lyme Arthritis.*

	Positive PCR Test (N = 75)	Negative PCR Test (N = 13)	P Value
Age (yr)	29 (8–67)	38 (3–62)	NS
Sex (M/F)	53/22	6/7	NS
Months before PCR			
First symptoms of illness	19 (1.5–76)	38 (2–222)	<0.02
Onset of arthritis	14 (0.25–74)	26 (0.25–153)	<0.03
Current episode of arthritis	0.75 (0.03–18)	1 (0.25–35)	NS
Months after PCR			
Resolution of current episode†	0.25 (0.03–6)	0.2 (0.03–2.5)	NS
Resolution of last episode†	18 (0.1–113)	2 (0.1–58)	0.03
Latest follow-up	114 (0–198)	18 (2–179)	<0.008
Synovial fluid‡			
White-cell count ($\times 10^{-3}$/mm^3)	16.7 (0.05–110)	5.1 (0.44–16.9)	<0.003
Protein (g/dl)	5.0 (1.7–9.6)	4.1 (3.3–5.3)	NS
Antibiotic therapy (no. of patients)			
None	58	3	<0.001
Short oral courses (<1 mo)	12	0	
Long oral courses (≥1 mo) or parenteral	5	10	

*Correlation is with the test results from each patient's initial sample only. Unless otherwise indicated, values are medians, with ranges in parentheses. NS denotes not significant.

†Four PCR-positive patients and five PCR-negative patients who still had active arthritis at the time of our analysis were excluded.

‡Synovial-fluid data were available for 73 PCR-positive patients and 10 PCR-negative patients.

Table 4. PCR Results According to Antibiotic Treatment.

Antibiotic Treatment	Patients		Samples	
	Total no.*	no. (%) PCR-positive	Total no.	no. (%) PCR-positive
No antibiotics	45	43 (96)	59	54 (92)
Tested before antibiotics	16	15 (94)	26	25 (96)
Tested after short course of oral antibiotics (<1 mo)	12	12 (100)	15	15 (100)
Total, short course or none	73	70 (96)	100	94 (94)
Tested after parenteral or long courses of oral antibiotics (≥1 mo)	19	7 (37)†	26	7 (27)†

*Three patients from whom samples were obtained before antibiotic treatment and one patient from whom the sample was obtained after a short course of antibiotics also had samples obtained after parenteral antibiotic treatment. Thus, for this analysis, the total number of patients was 92 rather than 88.

†$P<0.001$ for the comparison of parenteral or long courses with short courses or no therapy.

ics (doxycycline or amoxicillin for one month or more) or parenteral antibiotics (intravenous or intramuscular penicillin or intravenous ceftriaxone), regimens recommended for the treatment of Lyme arthritis.[38,39] In 7 of the 19 patients, *B. burgdorferi* DNA was detected in samples obtained 1 day to 17 months after the completion of antibiotic therapy. Three of these patients were treated with both oral and intravenous antibiotics, two received three weekly doses of intramuscular penicillin G benzathine, and two were given only oral antibiotics. The median duration of their oral treatment was 37 days (range, 20 to 58), and the median duration of intravenous therapy was 14 days (range, 14 to 20). In the remaining 12 patients, samples obtained one day to four years after antibiotic treatment were all negative. Seven of these patients were treated with intravenous antibiotics, two received intramuscular penicillin, and three were given only oral antibiotics. Their median duration of oral treatment was 48 days (range, 21 to 120), and the median duration of intravenous therapy was 30 days (range, 7 to 44). Although the patients with negative PCR results tended to have been treated longer than those with positive PCR results, the differences were not statistically significant. Of 10 patients who had chronic Lyme arthritis despite multiple courses of antibiotic therapy, 7 had negative test results in all post-treatment samples.

Altogether, of 73 patients with Lyme arthritis who were untreated or treated with short courses of oral antibiotics before testing, 70 (96 percent) had positive PCR results. In contrast, of 19 patients who received either parenteral antibiotics or long courses of oral antibiotics, only 7 (37 percent) had positive test results after treatment ($P<0.001$). In the 29 patients for whom serial samples were available, all pretreatment samples were positive. Once post-treatment samples became negative, all subsequent samples remained negative.

Discussion

We present evidence that PCR is a useful method for detecting *B. burgdorferi* DNA in synovial fluid from patients with Lyme arthritis. The main concern about this technique is that minute contamination may produce false positive results. To ensure that contamination did not influence our results, control samples of joint fluid were collected and stored like case samples, DNA extraction and PCR preparation were performed in a dedicated room, all PCR products were inactivated with isopsoralen after amplification, multiple blank control samples were included with each group of samples tested, and the samples were tested in a blinded manner in two separate laboratories. In both laboratories, nearly all joint-fluid samples from untreated patients with Lyme arthritis contained detectable *B. burgdorferi* DNA, most post-treatment samples did not, and all control samples were negative. This distribution would be extremely unlikely had the samples been contaminated.

In both laboratories, the sensitivity of each of the three OspA primer–probe sets was high (75 to 89 percent), and the results among these sets were moderately concordant. In contrast, the primer–probe set that detected chromosomal DNA was less sensitive. This discrepancy has been observed consistently in all our studies of *B. burgdorferi* detection in synovial fluid.[35] Multiple copies of OspA DNA segments may be contained within spirochetes, and these targets may therefore be more easily detected. Alternatively, spirochetes may not always be present in synovial fluid but may be capable of shedding OspA segments into the fluid from the surrounding synovium. Membrane vesicles containing extrachromosomal DNA are shed from the surface of the spirochete,[40,41] and they have been postulated to be mediators of DNA transfer between organisms.[42] A third possibility is that plasmid DNA may persist in synovial fluid after the death of the spirochete. In our study and others,[21,24] however, OspA DNA was detected primarily in untreated patients with clinically active disease. After antibiotic treatment, the PCR results were usually negative, which would not be expected if OspA DNA persisted after the spirochete had been killed. Likewise, in studies of experimental *B. burgdorferi* infection in mice, PCR results were almost always negative within two to four weeks after treatment with ceftriaxone (unpublished data). Another example of this phenomenon is the clearance of viral DNA from cerebrospinal fluid within one to four weeks after acyclovir treatment in patients with herpes simplex encephalitis.[43] Thus, it seems likely that the detection of OspA DNA in joint fluid indicates the presence of viable spirochetes. Further studies aimed at detecting potentially more labile spirochetal RNA molecules[44] may help to confirm this hypothesis.

Since joint effusions resolved in most patients during the course of antibiotic therapy, it was usually not possible to obtain samples of synovial fluid after treatment. Of the patients with persistent effusions after one month of oral antibiotics or two weeks of intravenous antibiotics, approximately one third still had positive PCR results, suggesting that the spirochete may not have been eradicated. However, none of the

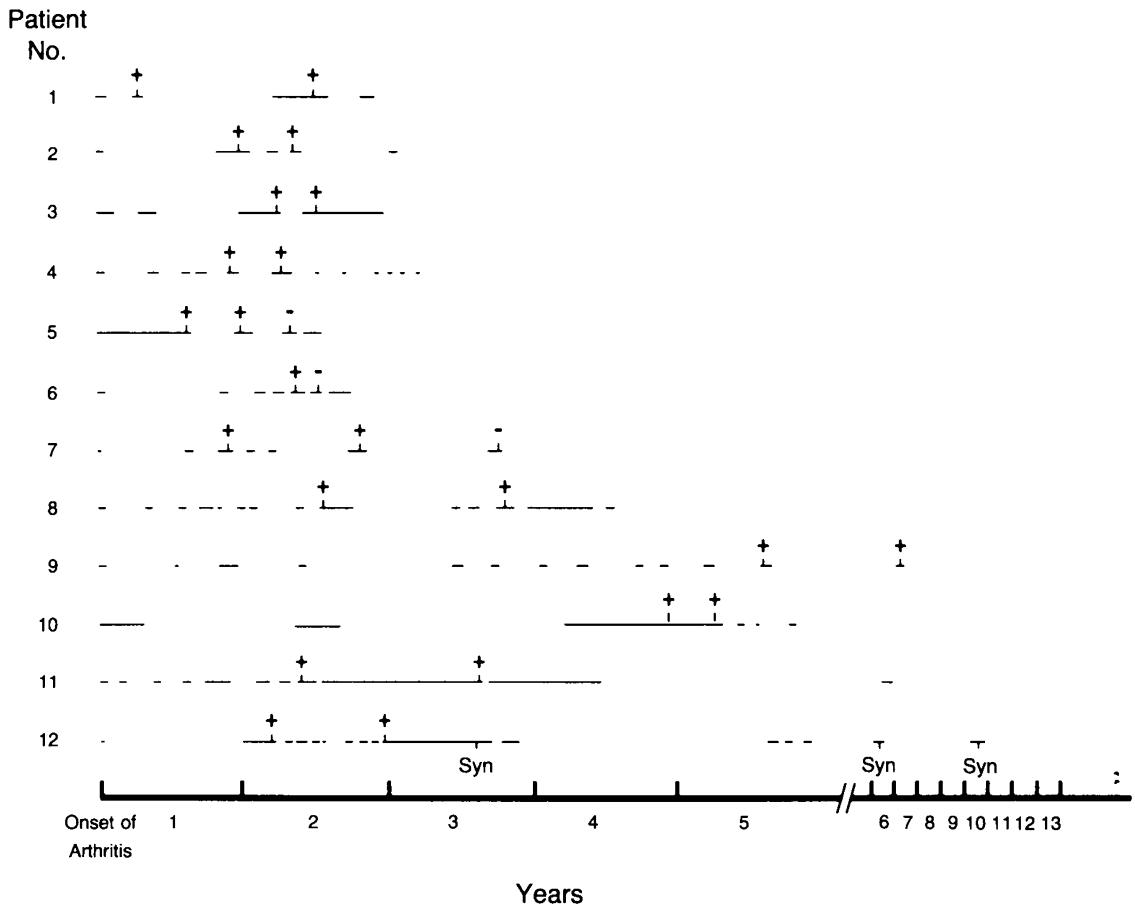

Figure 1. Natural History of Lyme Arthritis and PCR Results in 12 Untreated Patients.
The horizontal lines indicate periods of arthritis. Patients 10, 11, and 12 had chronic Lyme arthritis, defined as one year or more of continuous joint inflammation. The results of PCR testing are indicated above the horizontal lines, with plus and minus signs denoting positive and negative results, respectively. Syn denotes synovectomy.

patients with positive PCR results after treatment had received more than two months of oral antibiotics or three weeks of intravenous antibiotics. Most of the patients who had chronic arthritis despite multiple courses of antibiotic therapy had negative PCR results in all post-treatment samples. In these patients, the lack of response to antibiotics, the negative PCR results, and the association of this syndrome with immunogenetic and immune markers[8,9] suggest that genetically susceptible persons may continue to have arthritis for months or even several years after the eradication of viable spirochetes from synovial fluid.

Despite the identification of *B. burgdorferi* as the cause of Lyme disease in 1982, it has been practically impossible to demonstrate the presence of the spirochete in synovial fluid. The PCR test of synovial fluid in Lyme arthritis now shows promise and may fill the role that culture serves in detecting common bacterial pathogens in septic arthritis. In addition, PCR results may prove useful when therapeutic decisions are made for patients with persistent Lyme arthritis despite multiple courses of antibiotic therapy.

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