M. E. Bayer, L. Zhang, M. H. Bayer

Borrelia burgdorferi DNA in the Urine of Treated Patients with Chronic Lyme Disease Symptoms. A PCR Study of 97 Cases

Summary: The presence of *Borrelia burgdorferi* DNA was established by PCR from urine samples of 97 patients clinically diagnosed as presenting with symptoms of chronic Lyme disease. All patients had shown erythema chronica migrans following a deer tick bite. Most of the patients had been antibiotic-treated for extended periods of time. We used three sets of primer pairs with DNA sequences for the gene coding of outer surface protein A (OspA) and of a genomic sequence of *B. burgdorferi* to study samples of physician-referred patients from the mideastern USA. Controls from 62 healthy volunteers of the same geographic areas were routinely carried through the procedures in parallel with patients' samples. Of the 97 patients, 72 (74.2%) were found with positive PCR and the rest with negative PCR. The 62 healthy volunteers were PCR negative. It is proposed that a sizeable group of patients diagnosed on clinical grounds as having chronic Lyme disease may still excrete *Borrelia* DNA, and may do so in spite of intensive antibiotic treatment.

Introduction

Lyme disease is caused by the spirochete *Borrelia burgdorferi* and is transmitted to humans and other vertebrate hosts by *Ixodes* ticks. The disease has been classified to occur in three stages with an early stage revealing an erythema chronica migrans which provides a diagnostic basis for the infection [1, 2]. However, erythema chronicum migrans is only recognized in approximately half, or even less, of the patient population. In the second stage, disseminated infection occurs within days, weeks or years in untreated (and treatment-resistant) patients who may develop arthritis, cardiac and neurologic abnormalities. The third stage is considered to begin months or years after infection, with time periods of varying length of latency. Neurological involvement in this stage of persistent infection may occur as encephalopathy, memory loss, peripheral sensory disturbances and a variety of other symptoms. Definitions of clinical criteria characteristic for early and late stages, as well as treatment courses, have been proposed [1, 2]. In the majority of cases the diagnosis is made on clinical grounds with the support of laboratory findings. However, in the absence of an observed erythema chronicum migrans, the onset of clinically recognized symptoms places many patients likely in stage two with late manifestations or in stage three. The clinical diagnosis of Lyme disease is often supported by detection of characteristic serologic responses. Serologic data provide information with potentially high sensitivity and specificity although significant inter-laboratory procedural differences and test variabilities have been found [3]. Characteristic serological patterns develop during early and late stages of the disease and reflect the changing expression of antigenic determinants of the bacterium such as surface proteins (OspC, OspA, B) and other proteins [4–7] including flagellar epitopes [8, 9]. However, a group of infected patients may remain seronegative for months, or fail to develop a complete antibody profile [1, 6–9] even after a more prolonged course of the disease. Since only a portion of clinically diagnosed Lyme disease patients fulfills the current serological criteria used for positivity [4–6], the search for direct evidence of the bacterium or its components becomes desirable and necessary. Such tests include culturing of *B. burgdorferi* which has been acquired from skin [9, 10], blood [11, 12], joint fluid [13], and urine [12, 14]. Other direct tests include the microscopic visualization of spirochaetes [15], indirect immunofluorescence [16] as well as immune capture methods [17] for detection of antigens or membrane vesicles of *B. burgdorferi*. Unfortunately, culturing has a low rate of success and the other tests listed above are labor intensive and often still in the investigative phase. The detection of *B. burgdorferi*-specific DNA by use of the polymerase chain reaction (PCR) has proved its value as a sensitive diagnostic tool in cases of active Lyme disease. PCR has revealed *B. burgdorferi* sequences [9, 18–20] in biopsies of patients' erythema chronicum migrans [10] and in cerebrospinal [19, 21, 22] and synovial fluid [13] in patients with chronic Lyme disease. The excretion of *Borrelia* DNA in the urine has been reported for patients with active Lyme disease [23, 24] and neuroborreliosis [21, 22, 25]. Criticism of PCR focused often on the reliability of the methodology, in part due to inhibitors of the reaction, but mainly due to the potential of carryover of amplified DNA into other samples. However, carryover is avoidable with proper handling during routine procedures and can be recognized by the use of appropriate controls.

Received: 20 September 1995/Revision accepted: 17 July 1996

Prof. *M. E. Bayer*, M.D., *Lanmin Zhang*, M.D., *Margret H. Bayer*, Ph.D., Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111, USA.

Our study used PCR to determine whether *B. burgdorferi* DNA is present in the urine of a physician-selected group of chronic patients. All patients had originally presented with an erythema chronicum migrans and were subsequently under medical care because of continuing, often gradually worsening Lyme disease-like symptoms. Physicians of various medical specialties had asked us to perform such PCR tests to assist them in their evaluation of whether chronic Lyme disease was the cause of the present illness or whether an additional disease might be responsible or might be overlaying its symptoms on a patient's Lyme disease background.

We used three primer pairs as well as nested primers in the search for the presence of DNA sequences of the outer surface protein A (OspA) [18, 20] and of a chromosomal sequence of *B. burgdorferi* [23]. Our data show that a high percentage of this group of patients excretes *B. burgdorferi*-specific DNA and does so after periods of prolonged antibiotic treatment.

Materials and Methods

Patient population: The majority of patients lived in areas endemic for Lyme disease – in New Jersey, Pennsylvania, Delaware and New York and presented with symptoms defined as late manifestations of Lyme disease (stages two and three [1, 2]). Practising physicians selected the patients for this study largely on clinical grounds.

Background information was collected from the physicians and their patients by means of questionnaires and telephone affirmations pertaining to date of tick bite, erythema chronicum migrans, onset and character of clinical symptoms, start and type of antibiotic treatment, dosage and length of treatment and present health status. An erythema chronicum migrans at the site of a tick bite had been observed in all cases. It should be reemphasized that this study deals with clinically chronically ill patients and does not represent a typical cross section through the overall population of treated Lyme disease patients. Serological data are not referred to in this study, as they were not complete in the majority of cases. This fact was due to the patients' repeated switching of physicians and hospitals and the resulting incomplete access to records.

Specimens: Incoming specimens were handled in a sterile hood in a separate laboratory. Centrifuges and rotors were operated in sterile hoods, routinely cleaned, and UV-sterilized. Tubes and pipettes were discarded after use, buffers used only once. Frozen samples of first morning urine were treated in parallel with PCR-negative control urines as follows: after centrifugation at 10,000 × g, pellets were resuspended in 1 × diluted SSC (20 × : sodium citrate [88 g/l], 3 M NaCl), and washed again in the same buffer.

Isolation of DNA: The sediments were resuspended in 0.2 M Na-acetate pH 7.0 and 0.25% SDS with 10 µl of 0.2 mg/ml Proteinase K (Sigma, St. Louis, MO), incubated for 2 h at 50°C, extracted with phenol chloroform and precipitated in 3 M sodium acetate (pH 5) and cold ethanol, washed in 70% ethanol, centrifuged, dried, and stored in 50 mM Tris-HCl, 1 mM EDTA at 4°C. The DNA concentration, determined by UV absorption with thymus DNA as standard and adjusted to 2 µg/ml, was used in a 50 µl PCR reaction mixture. In a number of experiments we also extracted specimens by boiling in 5% Chelex 100 Resin for 10 min [26] in sets parallel to phenol extracted specimens.

Primers: Oligonucleotide primers were chosen from highly conserved regions of the OspA gene, GeneBank accession number X14407 of *B. burgdorferi* [18], synthesized on an Applied Biosystems DNA synthesizer and purified through OPC cartridges (Applied Biosystems, Foster City, CA). Selected from the OspA sequences were primer A = (5′-AAGTACGATCTA-ATTGCAACAG) and primer B = (5′-TTCCTTCTTTAACCA-CCAATGT) which are located from nucleotide number 301 to 322 and from 700 to 679 of the complementary strain, respectively. Furthermore, OspA primer sets, reported by *Guy* and *Stanek* [19] were used as nested primers with the primer pair C = (5′-GAGCTTAAAGGAACTTCTGATAA) and D = (5′-GTATTGTTGTACTGTAATTGT) (nucleotide 334–356 and from 894–874 respectively, plus the second primer pair E = (5′-ATGGATCTGGAGTACTTGAA) and F = (5′-CTTAAAG-TAACAGTTCCTTCT) (nucleotide 362–381 and 713–693). All primer pairs including the nested primer pairs were used in parallel sets. A further primer pair was synthesized with the genomic DNA sequence of a clone (Ly-1) [23] with the + end 5′-GAAATGGCTAAAGTAAGCGGAAT, nucleotide 16 to 38 (primer 01) and 5′-TCTGTAAACTAATCCCACCTAAAA, nucleotide 240 to 217 (primer 02) of the complementary strand. Hybridization with OspA sequences was performed with a 400 bp probe (see below). In addition, we often employed in parallel an oligoprobe of 18 nucleotides with the sequence 5′-GAGCAGACGGAACCAGAC. The probe for the genomic sequence was 5′-TATTTTAGATGAGTATGGTT. The oligoprobes were γ32P end-labeled with T4 polynucleotide kinase with a specific activity of $1-2\times10^3$ cpm/µl.

Probe subclone and sequencing: The 400 pb probe was cloned according to *Chiba* et al. [27] by adding to the primers A and B of OspA extraneous nucleotides (5-GAGCTC-3′) which comprise SstI cleavage sites at their 5′ ends. After subcloning the PCR fragment of OspA into pBluescript SK(–) plasmid (Stratagene, La Jolla, CA), purification in phenol-chloroform and ethanol and preparation of pBluescript SK(1) and SstI cleaving, the PCR product was recovered from 0.8% agarose gel, ligated to the cleaved pBluescript SK(–) with T4 DNA ligase (Gibco BRL, Gaithersburg, MD), transfected in Jm83 cells and grown overnight; its DNA was purified according to *Sambrook* et al. [28]. Sequencing of subclones by dideoxy-nucleotide chain-termination and Sequenase 2.0 system (USB, Cleveland, OH) revealed the amplified 400 pb portion of the Osp*A* gene to be identical to the published sequence of OspA. This subclone was used as a routine probe during the study. A α32P Prime-It Random Primer Kit (Stratagene) was used for labeling according to the instructions by the manufacturer. For PCR, 25 ng of DNA template, 10 µl random oligonucleotide primers plus H_2O were used in a total of 34 µl. Subsequent to boiling (5 min), cooling, centrifugation, 10 µl of 5 × primer buffer containing dATP, dGTP, and dTTP were added to the supernatant plus 5 µl labeled nucleotide ([α-^{32}P dCTP], 3000 Ci/m mole) and 1 µl T7 DNA polymerase. Incubation (37°–40°C, 30 min) was followed by addition of 2 µl Stop mix (Stratagene) and purification (Centre-Sep columns, Princeton Separations Inc., Adelphia, NJ). Radioactive counts of approximately 2×10^6 dpm were used in each hybridization procedure.

PCR. Gene-Amp reagents (Perkin Elmer Cetus, Norwalk, CT) were used in a thermal cycler (MiniCycler, MJ Research Watertown, MA).

2 µg DNA from urine or *B. burgdorferi* B31 cultures were amplified in 50 µl mixtures (PCR buffer II, Perkin Elmer Roche, Branchburg, NJ: 15 mM $MgCl_2$, 200 µM of each of the deoxynucleotide tri-phosphates and of the primer pairs [1 µM] and Amplitaq DNA polymerase).

Amplification: 35 cycles, denaturation at 94°C, 1 min; annealing at 56°C, 2 min; extension at 72°C, 2 min. The nested primer pairs C and D were used in 32 cycles, followed by pair E and F with 25 cycles; annealing at 54°, 1 min, extension at 72°C, 1 min. After electrophoresis of the PCR product in 1.5% agarose gel (containing Tris boric acid, EDTA, plus ethidium bromide) the fluorescence bands were recorded, the gels were denatured in 0.25 M HCl, followed by 1.5 M NaCl, 0.5 M NaOH, and neutralized in 0.5 M Tris pH 7.4, 8 M NaCl. Transfer to Hybond N^+ membrane (Amersham, Arlington Heights, IL) with transfer buffer (20×SSC) [20], was followed by one rinse and cross-linking with 0.12 J of UV or paper-drying and baking at 80°C for 2 h. To prevent DNA contamination by product carry-over, the PCR instrumentation (cycler and ancillary equipment) was operated in rooms separate from the areas where buffer preparation and vial preparation took place. Stoppered micropipette tips and disposable centrifuge tubes were used throughout. All re-useable equipment was UV-treated after each run; glassware was acid cleaned. Parallel to our standard procedures, the amplification product [29] was inactivated with isopsoralene (IP) (4′-aminomethyl 4.5′-dimethylangelicin, HRI Associates, Inc.). The PCR product of urine from ten patients and DNA-free controls was tested with IP concentrations of between 10 and 50 µl/ml and irradiated for 60 min with long wave UV of 25 µW/cm². The results of the IP-inactivated samples were indistinguishable from those of the non-crosslinked parallel tests of the same material.

Hybridization: Pre-hybridization: 6 h in 50% formamide, 5×SSC, 0.5% sodium dodecylsulfate (SDS), denatured salmon sperm DNA, 5×Denhardt's reagent (containing 5 g Ficoll type 400, [Pharmacia, Piscataway, NJ], 5 g polyvinyl pyrrolidine, 5 g bovine serum albumin [Fract. V, Sigma, St. Louis, MO] and 50 ml H_2O). Hybridization with the α-^{32}P-labeled probes in hybridization buffer [28], incubation for 12 h at 42°C, washing in 2×SSC (plus 0.1% SDS) and shaking for 30 min at 22°C, further washing for 30 min in 0.1×SSC + 0.1% SDS at 42°C and drying of the membrane; subsequently exposure to Kodak X-OMAT Imaging film at −70° for 2−4 h.

PCR controls: DNA samples of cultured B31, corresponding to DNA of 100−200 *B. burgdorferi,* were routinely run in the Southern blots together with molecular weight standards (Gibco BRL, Gaithersburg, MD); to guard against false positives, two (most often more) lanes per gel (Figure 1) contained samples from healthy volunteers. PCR was repeated whenever a question arose regarding the presence of very faint bands or unusual band positions. Forty patient samples were repeated at least once, using the primer pair A, B.

PCR sensitivity: When samples of normal urine or buffer were "spiked" with *B. burgdorferi* DNA, serial dilutions of the DNA extracts revealed, after hybridization to the corresponding probe, the presence of *B. burgdorferi* DNA of ten (or fewer) cells. The nested primer pairs CD and EF most often showed an enhanced sensitivity over that of the primer pair AB. The signal from the genomic sequence was, in general, less frequent than that of the OspA primer pair AB.

Bacterial cultures: B. burgdorferi strain B31 (ATCC35210) was grown at 35°C in BSK-H medium [30] supplemented with 0.6% rabbit serum R-7136 (both from Sigma) to densities of 10^6−10^7/ml as counted by phase light microscopy. The cells were washed 3× in PBS containing 5 mM $MgCl_2$, and the presence of OspA and OspB antigens was established by Western blotting with affinity purified anti-*B. burgdorferi* IgG, anti-OspB IgG (Kierkegaard and Perry, Gaithersburg, MD) and anti-OspA IgG (a gift from *A. Barbour*).

Results

All patients came from areas of high Lyme disease incidence and had been treated with antibiotics for various lengths of time before PCR testing. The antibiotics used were those commonly employed for Lyme disease treatment [31] belonging to a) the beta-lactam group: amoxicillin, ampicillin; b) the cephalosporins: cefotaxime, cefixime, ceftriaxone (i.v.); c) tetracycline, doxycycline; d) macrolides: clarithromycin, erythromycin, ceftriaxone. In almost all cases, the sequence of treatment started with amoxicillin or ampicillin, followed weeks or months later with doxycycline, cefixime and or clarithromycin, eventually with ceftriaxone i.v. More recently, patient treatments were most often started with doxycycline, azithromycin, clarithromycin, followed by ceftriaxone i.v. The age distribution among the patients varied widely with a mean age of 41.5 years, the oldest patient being 72 years, the youngest 4 years old. The ratio of men to women was 1:1.5. Figure 1 shows an agarose gel of PCR products from phenol-extracted patient urines. The nested primers

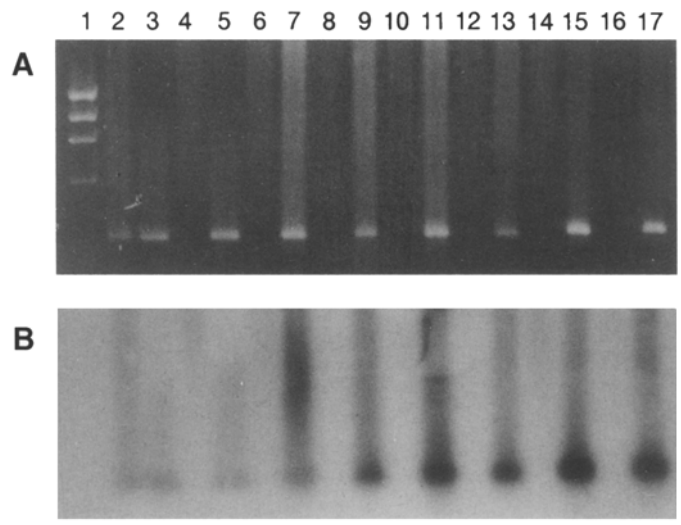

Figure 1: a) Agarose gel electrophoresis of *Borrelia burgdorferi* DNA after amplification of OspA sequences with the nested primer pairs C, D and E, F (see text). Lanes 1 (in both Figure 1 and Figure 2) = marker DNA. (∅X174-HaeIII fragment, BRL. Topdown: 1353, 1078, 872, 603, 310, 281, 271, 235 bp, respectively). Lane 2: *B. burgdorferi* strain B31 DNA. Lanes 3, 5, 7, 9, 11, 13, 15, 17: DNA from patient samples of cases 562, 567, 568, 587, 577, 582, 583, 613 respectively. Lanes 4, 6, 8, 10, 12, 14, 16: DNA from urine of seven healthy controls. b) Hybridization of above sample with 32P 400 bp subclone.

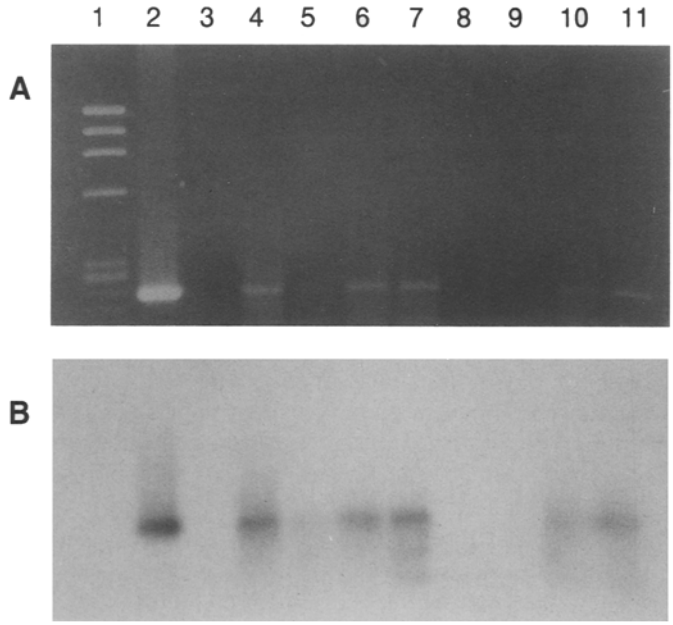

Figure 2: a) Agarose gel electrophoresis of *Borrelia burgdorferi* DNA after amplification of a genomic sequence. Lane 2: *B. burgdorferi* strain B31 DNA, phenol extracted. Lane 3: Target-free control. Lanes 4, 6, 8, 10: phenol-extracted DNA from patients. Lanes 5, 7, 9, 11: DNA extracted with boiling procedure. Patient protocol numbers 445 (lanes 4, 5); 446 (lanes 6, 7); 447 (lanes 8, 9); 448 (lanes 10, 11). b) Hybridization of above samples with 32P oligonucleotides.

C, D and E, F of the OspA sequence were used. Alternating rows of identically-treated urine from healthy controls served to indicate immediately if product carry-over had occurred. Figure 2 shows the use of the genomic primer pair 01/02. In addition to the phenol-extracted samples in lane 4, 6, 8, 10, DNA was extracted by boiling as shown in lanes 5, 7, 9, 11. Phenol extraction was in general more sensitive and was used routinely throughout the study.

Of the 97 patients, 72 (= 74.2%) tested positive and 25 (= 25.7%) were negative by PCR (Table 1).

With the exception of two cases, patients were off antibiotics for at least one week before sampling for PCR. The two patients under treatment at the time of sample collection were both PCR positive. All patients had been treated for between 3 weeks and 2 months continually and most of them were, after a few weeks' pause, again treated with antibiotics for additional periods of 1–4 months. In treatments that were even longer, cycles of antibiotic treatment and intermittent drug-free periods of a few weeks were repeated several times. In 17 cases, the tests were repeated at least twice over a period of several weeks and months between oral or intravenous treatment courses (amoxicillin, azithromycin and ceftriaxone). In four of these patients, the PCR had become temporarily negative, as determined 1 week after ending the treatment period. However, 4–6 weeks later, all had again become PCR positive.

Table 1: Urine-PCR of 97 antibiotic-treated patients with erythema chronicum migrans.

Sample Group	PCR Positive		PCR Negative	
	No.	%	No.	%
Total of 97 patients	72	74.2	25	25.7
62 healthy volunteers	0	0	62	100

Almost all patients in this study reported to be not well at the present time; most of them were ill and unable to work, including those (approximately 28%) who had shown an initial improvement during earlier antibiotic treatment. Only two patients described their current condition as "healthy", with a remaining few symptoms; their urine PCR, however, remained positive when tested between 6 weeks and 4 months, respectively, following their last antibiotic treatment.

We observed that, on average, the time delay between infection (erythema chronicum migrans) and the late onset of treatment (a median of 8 months) did not affect the outcome of the PCR result (Table 2). We conclude that the two parameters are independent (p = 1.0, Fisher's exact test).

A similar conclusion can be reached for the association of the duration of treatment (median of 2.5 months) and PCR status (Table 3) (p = 0.814, Fisher's exact test). Logistic regression was also applied to the data (from Table 2 and Table 3) to predict PCR status simultaneously. Again, no relation was seen.

A close inspection of the information available to us in relation to the urine PCR of patients testing negative provided the following scenario: of the 25 PCR negative

Table 2: Delay between erythema chronicum migrans and treatment.[1]

	PCR			
	+	–	Total	
Above median	31	11	42	
At or below	31	12	43	(median = 8 months)
Total	62	23	85	

Fisher's exact test gives p = 1.0 (not significant).
[1] Data of 85 patients (= 87.6%) with known delay in treatment.

Table 3: Duration of treatment.[1]

	PCR			
	+	–	Total	
Above median	35	13	48	
At or below	37	11	48	(median = 2.5 months)
Total	72	24	96	

Fisher's exact test gives p = 0.814 (not significant).
[1] Data of 96 patients (= 99%) with known duration of antibiotic treatment.

patients, five had begun antibiotic treatment only 10–16 months subsequent to the onset of an erythema chronicum migrans and were treated for 2–4 months; seven patients were delayed in treatment for 8 months to several years but were treated for time periods of 6 months to 2 years. These numbers, although small, appear to support the hypothesis that even after a late start of treatment an extended antibiotic regimen may cause a favorable outcome of the PCR status.

Controls

Sixty-two healthy adults who lived in areas of Pennsylvania and New Jersey, regarded as endemic for Lyme disease, served as controls. These persons considered themselves as being healthy, had encountered neither joint or neurological symptoms nor persistent headaches, and had never been diagnosed with Lyme disease. Of the 62 persons, none showed a positive PCR. In all cases, the test was double blinded with positive samples interspersed, and was repeated at least twice, with the same result. Furthermore, many of these specimens were used as controls to ascertain the absence of product (DNA) carry-over.

Stringent collection and preparation procedures, including DNA target-free samples, "spiked" urine samples and the samples of healthy controls used in parallel with patients' samples resulted in the absence of false positives within the 3 years of this study. Whenever we employed the post-amplification inactivation of the amplicon [29] in ten patient urines, no differences to parallel samples without amplicon inactivation were observed.

Discussion

The diagnosis for Lyme disease is primarily a clinical diagnosis. Since the erythema chronicum migrans is a significant symptom in the diagnosis of Lyme disease, we included only patients with recorded erythema chronicum migrans in this study. The diagnosis is most often supported by serological profiles [1, 4], such as an early IgM and later IgG production against OspC, and a subsequent anti-OspA and anti-OspB production persisting over longer periods of time, including other *B. burgdorferi*-specific peptide markers [5–7]. Direct evidence for the presence of spirochaetes in active borreliosis has been provided in skin, heart, brain, joints, blood, and CSF, with *B. burgdorferi* persisting in latent forms in human tissues for years [1]. The proof of presence of *Borrelia*-specific DNA by PCR is also an indicator for the persistence of the spirochaete in the patient.

PCR allows the identification of fewer than ten DNA sequences per sample [20, 23, 24] and has been shown to operate at the limit of detection with one specific DNA segment [29]. In our study, the lower limit of detection was routinely under ten spirochaetes (per sample of spiked urine). The sensitivity of PCR may vary considerably, possibly due to adsorption of DNA to glass and plasticware, and due to inhibitors in the sample material itself. *Liebling* et al. [24] reported a maximal (100%) PCR sensitivity in urine and in cerebrospinal fluid, lesser sensitivities in synovial fluid, and still less (59%) in serum. Inhibitory activity was also demonstrated in synovial samples from Lyme arthritis patients [13] and in forensic samples [26].

Regarding the test sensitivity, our *in vitro* controls with primer pairs A, B of OspA sequences showed an efficiency of more than 98% from extracts of between 20 and 200 cultured B31 cells. It is possible that a PCR negative patient might at the time of collection excrete none or too few spirochaetes to be detected. Also, a particular urine might contain high amounts of inhibitors. Both instances would result in a negative test. Since PCR positive results may originate from dead microorganisms or fragments thereof, they do not indicate the presence of live *Borrelia* in the sample. Also, ongoing immune processes around persisting antigens at sites distant from both the bladder and the lining of the urinary canal would prevent detection of *Borrelia* DNA in the urine. *Wallach* et al. [12] reported that PCR of blood cultures as well as of serum failed to reveal the presence of *B. burgdorferi* in seven patients with acute Lyme disease, suggesting that spirochaetes and their DNA may circulate intermittently or at a level too low to be detected by culture or PCR. *Malawista* et al. [14] have shown that 15 days after inoculation, tissue of infected mice became negative both *in vitro* and by PCR (probing for OspA and OspB gene sequences) subsequent to ceftriaxone treatment. In contrast, several studies of chronic cases of human Lyme disease revealed that *Borrelia* DNA persisted at various sites after antibiotic treatment [9, 32].

Our results show that of the 97 patients, 74.2% tested positive, whereas 25.7% tested PCR negative. Of the 25 patients that tested negative by PCR, five were treated 10–16 months after the emergence of an erythema chronicum migrans and received antibiotic therapy for 2–4 months; seven patients, with antibiotics starting only after between 8 months and several years, were treated for a total time of 6 months to 2 years. This result seems to support the view that an extended antibiotic treatment may be responsible for the negative outcome of the PCR and may have helped in the elimination of the spirochaetal population even after a much delayed onset of treatment. Nevertheless, it seems to be characteristic for most of the patients in our study that, after antibiotic-free periods of a few months, they had again become increasingly ill with neurological and arthritic symptoms, so that treatment had been resumed.

Karch et al. [33] reported the presence of *B. burgdorferi* DNA in urine samples of thirteen healthy adults with positive serology. PCR became repeatedly negative after treatment of these patients with doxycycline. The authors conclude that excretion of *Borrelia* DNA may occur during asymptomatic infection. It remains to be determined whether the persistent presence of *Borrelia* DNA in the

majority of the PCR positive patients in our study is the driving force or a contributing factor to their current illness.

Patients in the group studied here, including those of the PCR negative group, suffer from Lyme disease-like clinical symptoms which are difficult to distinguish from other diseases [1], such as multiple sclerosis, chronic fatigue syndrome, immune disorders, fibro-myalgia or infection with other microorganisms and viruses. A few of the patients in our study for whom recent serological data were available show an incomplete or negative serological profile for *B. burgdorferi* according to criteria of Western blotting [4, 5]. A further concern has arisen from the discovery that *B. burgdorferi*-positive patients may be also co-infected or superinfected with other tick-born agents such as *Babesia* and *Ehrlichia* strains [34]; specific antibodies to these microorganisms have been found in sera of Lyme disease positive persons [35]. Furthermore, currently uncultivable *Borrelia* species carried by a different tick (*Amblyomma americanum*) have been observed to cause a Lyme disease-like illness [36]. These scenarios create a high degree of complexity of symptoms as well as the potential of an increased severity and duration of illness [37] and heighten the consequences of a persistent presence of *B. burgdorferi* in the patient, a condition that would necessitate repeated broad-based laboratory testing and continued medical attention.

Acknowledgements

This work was supported largely by contributions from the Lyme Disease Association of New Jersey Inc., and also by individual donors and Lyme disease support groups of Abington, PA and Berlin, NJ, and by grant MCB-9206674 of the National Science Foundation (to M. E. B.), and the Institutional Grant CA-06927. We thank Dr. *S. Litwin* (Biostatistics Facility) for the statistical presentations, and acknowledge the help of Dr. *A. Yeung* (DNA Synthesis Facility of the Cancer Center) for providing us with the oligonucleotide probes. We also acknowledge the expert typing of the manuscript by Ms. *Roseanne Diehl.*

Zusammenfassung: *Borrelia burgdorferi* DNA im Urin von Patienten mit chronischer Lyme-Krankheit nach Therapie. PCR-Studie mit 97 Fällen. Bei 97 Patienten mit klinisch verifizierter chronischer Lyme-Krankheit wurde mittels PCR in Urinproben nach *Borrelia burgdorferi*-DNA gesucht. Bei allen Patienten war nach Zeckenstich ein Erythema chronicum migrans aufgetreten. Bei den meisten Patienten war eine langfristige Antibiotikabehandlung erfolgt. Wir verwendeten drei Primerpaare mit DNA-Sequenzen für das Gen, das für das Oberflächenprotein A (OSP A) kodiert und für eine genomische Sequenz von *B. burgdorferi*, um Proben von Patienten zu untersuchen, die von Ärzten aus dem mittleren Osten der USA überwiesen worden waren. Proben von 62 gesunden Freiwilligen derselben geographischen Regionen wurden routinemäßig parallel zu den Patientenproben getestet. 72 der 92 Patienten (74,2%) hatten ein positives Testergebnis, die übrigen waren negativ. Auch die 62 Probanden waren PCR-negativ. Ein beträchtlicher Anteil der Patienten mit klinisch diagnostizierter Krankheit scheidet offensichtlich *Borrelia* DNA aus, auch wenn eine intensive Antibiotikatherapie vorausgegangen ist.

References

1. **Steere, A. C.:** *Borrelia burgdorferi* (Lyme disease, Lyme borreliosis). In: *Mandell, G. L., Bennett, J. E., Dolin, R.* (eds): Mandell, Douglas and Bennett's principles and practice of infectious diseases. Churchill Livingstone, New York 1995, pp. 2143–2155.
2. **Rahn, D. W., Malawista, S. E.:** Lyme disease: recommendations for diagnosis and treatment. Ann. Intern. Med. 114 (1991) 472–481.
3. **Bakken, L. L., Case, K. L., Callister, S. M., Bordeau, N. J., Schell, R. F.:** Performance of 45 laboratories participating in a proficiency testing program for Lyme disease serology. JAMA 268 (1992) 891–895.
4. **Aguero-Rosenfeld, M. E., Nowakoswki, J., Bittker, S., Cooper, D., Nadelman, R. B., Wormser, G. P.:** Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. J. Clin. Microbiol. 34 (1996) 1–9.
5. **Dressler, F., Whalen, J. A., Reinhardt, B. N., Steere, A.:** Western blotting in the serodiagnosis of Lyme disease. J. Infect. Dis. 167 (1993) 392–400.
6. **Engstrom, S. M., Shoop, E., Johnson, R. C.:** Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. J. Clin. Microbiol. 33 (1995) 419–427.
7. **Padula, S. J., Samperi, A., Dias, F., Szczepanski, A., Ryan, R. W.:** Molecular characterization and expression p p23 (OspC) from a North American strain of *B. burgdorferi.* Infect. Immun. 61 (1993) 5097–5105.
8. **Magnarelli, L. A., Fikrig, E., Berland, R., Anderson, J. F., Flavell, R. A.:** Comparison of whole-cell antibodies and an antigenic flagellar epitope of *Borrelia burgdorferi* in serologic tests for diagnosis of Lyme borreliosis. J. Clin. Microbiol. 30 (1992) 3158–3162.
9. **Oksi, J., Uksila, J., Marjamäki, M., Nikoskelainen, J., Viljanen, M. K.:** Antibodies against whole sonicated *Borrelia burgdorferi* spirochetes, 41-kilodalton flagellin, and P39 protein in patients with PCR- or culture-proven late Lyme borreliosis. J. Clin. Microbiol. 33 (1995) 2260–2264.
10. **Berger, B. W., Kaplan, M. H., Rothenberg, I. R., Barbour, A. G.:** Isolation and characterization of the Lyme disease spirochete from the skin of patients with erythema chronicum migrans. J. Am. Acad. Dermatol. 13 (1985) 444–449.
11. **Benach, J. L., Bosler, E. M., Hanrahan, J. P., Coleman, J. L., Habicht, G. S., Bast, T. F., Cameron, D. J., Ziegler, J. L., Barbour, A. G., Burgdorfer, W., Edelman, R., Kaslow, R. A.:** Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. 308 (1983) 740–742.
12. **Wallach, F. R., Forni, A. L., Hariprashad, J., Stoeckle, M. Y., Steinberg, C. R., Fisher, L., Malawista, S. E., Murray, H. W.:** Circulating *Borrelia burgdorferi* in patients with acute Lyme disease: results of blood cultures and serum DNA analysis. J. Infect. Dis. 168 (1993) 1541–1543.
13. **Nocton, J. J., Dressler, F., Rutledge, B. J., Rys, P. N., Persing, D. H., Steere, A. C.:** Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. N. Engl. J. Med. 330 (1994) 229–234.
14. **Malawista, S. E., Barthold, S. W., Persing, D.:** Fate of *Borrelia burgdorferi* DNA in tissues of infected mice after antibiotic treatment. J. Infect. Dis. 5 (1994) 1312–1316.

15. **DeKoning, J., Bosma, R. B., Hoogkamp-Korstanje, J. A. A.:** Demonstration of spirochetes in patients with Lyme disease with a modified silver stain. J. Med. Microbiol. 23 (1987) 261–267.
16. **Magnarelli, L. A., Meegan, J. M., Anderson, J. F., Chappeli, W. A.:** Comparison of indirect fluorescent antibody test with an enzyme-linked immunosorbent assay for serological studies of Lyme disease. J. Clin. Microbiol. 20 (1984) 181–184.
17. **Dorward, D. W., Schwan, T. G., Garon, C. F.:** Immune capture and detection of *Borrelia burgdorferi* antigens in urine, blood, or tissues from infected ticks, mice, dogs and humans. J. Clin. Microbiol. 29 (1991) 1162–1170.
18. **Nielsen, S. L., Young, K. K. Y., Barbour, A. G.:** Detection of *Borrelia burgdorferi* DNA by the polymerase chain reaction. Mol. Cell. Probes 4 (1990) 73–79.
19. **Guy, E. C., Stanek, G.:** Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. J. Clin. Pathol. 44 (1991) 610–611.
20. **Rosa, P. A., Schwan, T. G.:** A specific and sensitive assay for the Lyme disease spirochete *Borrelia burgdorferi* using the polymerase chain reaction. J. Infect. Dis. 160 (1989) 1018–1029.
21. **Huppertz, H. I., Schmidt, H., Karch, H.:** Detection of *Borrelia burgdorferi* by nested polymerase chain reaction in cerebrospinal fluid and urine of children with neuroborreliosis. Eur. J. Pediatr. (1993) 414–417.
22. **Keller, T. L., Halperin, J. J., Whitman, M.:** PCR detection of *Borrelia burgdorferi* DNA in cerebrospinal fluid of Lyme neuroborreliosis patients. Neurology 42 (1992) 32–42.
23. **Goodman, J. L., Jurkovich, P., Kramber, J. M., Johnson, R. C.:** Molecular detection of persistent *Borrelia burgdorferi* in the urine of patients with active Lyme disease. Infect. Immun. 59 (1991) 269–278.
24. **Liebling, M. R., Nishio, M. J., Rodriguez, A., Sigal, L. H., Jin, T., Louie, J. S.:** The polymerase chain reaction for the detection of *Borrelia burgdorferi* in human body fluids. Arthritis Rheum. 36 (1993) 665–675.
25. **Lebech, A. M.,Hansen, K.:** Detection of *Borrelia burgdorferi* DNA in urine samples and cerebrospinal samples from patients with early and late Lyme neuroborreliosis by polymerase chain reaction. J. Clin. Microbiol. 30 (1992) 1646–1653.
26. **Walsh, P. S., Metzger, D. A., Higuchi, R.:** CHELEX 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10 (1991) 506–513.
27. **Chiba, A., Takahashi, T., Nau, M. M., D'Amico, D., Curiel, D. T., Mitsudomi, T., Buchhagen, D. L., Carbone, D., Piantadosi, S., Doga, H., Reissman, P. T., Slamon, D. J., Holmes, E. C., Minna, J. D.:** Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. Oncogene 5 (1990) 1603–1610.
28. **Sambrook, J., Fritsch, E. F., Maniatis, T.:** Molecular cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989, Vol. 3.
29. **Rys, P. N., Persing, D. H.:** Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. J. Clin. Microbiol. 31 (1993) 2356–2360.
30. **Barbour, A. G.:** Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. 57 (1984) 521–525.
31. **Preac Mursic, V.:** Therapy of *Lyme borreliosis*. Antibiotic susceptibility of *Borrelia burgdorferi in vitro* and *in vivo*. In: *Weber, K., Burgdorfer, W.* (eds.): Aspects of *Lyme borreliosis*. Springer-Verlag, Berlin 1993, pp. 301–311.
32. **Preac Mursic, V., Weber, K., Pfister, W., Wilske, B., Gross, B., Baumann, A., Prokop, J.:** Survival of *Borrelia burgdorferi* in antibiotically treated patients with Lyme borreliosis. Infection 17 (1989) 355.
33. **Karch, H., Huppertz, H.-I., Böhme, M., Schmidt, H., Wiebecke, D., Schwarzkop, A.:** Demonstration of *Borrelia burgdorferi* DNA in urine samples from healthy humans whose sera contain *B. burgdorferi*-specific antibodies. J. Clin. Microbiol. 32 (1994) 2312–2314.
34. **Telford III, S. R., Dawson, J. E., Katavolos, P., Warner, C. K., Kolbert, C. P., Persing, D. H.:** Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc. Natl. Acad. Sci. USA 93 (1996) 6209–6214.
35. **Magnarelli, L. A., Dumler, J. S., Anderson, J. F., Johnson, R. C., Fikrig, E.:** Coexistence of antibodies of tick-borne pathogenes of babesiosis, ehrilichiosis and Lyme borreliosis in human sera. J. Clin. Microbiol. 33 (1995) 3054–3057.
36. **Barbour, A. G., Maupin, G. O., Teltow, G. J., Carter, C. J., Piesman, J.:** Identification of an uncultivable *Borrelia* species in the hard tick *Amblyomma americanum:* Possible agent of a Lyme disease-like illness. J. Infect. Dis. 173 (1996) 403–409.
37. **Krause, P. J., Telford III, S. R., Spielman, A., Sikand, V., Ryan, R., Christianson, D., Burke, G., Brassard, P., Pollack, R., Peck, J., Persing, D. H.:** Concurrent Lyme disease and babesiosis. JAMA 275 (1996) 1657–1660.