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Abstract One hundred twenty-four patients—53 with neuroborreliosis, 48 with erythema migrans, and 23 with Lyme arthritis—were tested in a prospective study for the presence of the DNA of *Borrelia burgdorferi* sensu lato in plasma, cerebrospinal fluid (CSF), urine, and synovial fluid by nested polymerase chain reaction (PCR). Specific DNA was detected using five amplification systems simultaneously: three targeted chromosomal genes encoding 16S rDNA, flagellin, and p66; and two plasmid sequences of OspA and OspC. Patients were examined clinically and by PCR before and after treatment and again after 3 and 6 months. Before treatment, the specific DNA was detected in 78 patients (62.9 %). Forty-one neuroborreliosis patients were DNA-positive (77.4 %), with CSF positivity in 26 patients, urine in 25, and plasma in 16. Twenty-six erythema migrans patients were DNA-positive (54.2 %), with plasma positivity in 18 cases and urine in 14. Eleven Lyme arthritis cases (47.8 %) were DNA positive (six in urine, five in plasma, and four in synovial fluid). The frequency of PCR positives was comparable in CSF and urine, and it was lower by approximately 50 % in plasma. Specific DNA was also found in a significant number of patients in later testing periods: 48 patients after treatment, 29 patients after 3 months, and 6 patients after 6 months. The prolonged PCR positivity was not explainable by persistent infection according to the clinical manifestations of the disease. Possible explanations of the problem are discussed.

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Abbreviations

AI_{Bb}	Specific antibody index
CSF	Cerebrospinal fluid
EIA	Enzyme immunoassay
EM	Erythema migrans
ISEM	Immunosorbent electron microscopy
LA	Lyme arthritis
LD	Lyme disease
NB	Neuroborreliosis
PCR	Polymerase chain reaction
WB	Western blotting

Introduction

The serological tests enzyme immunoassay (EIA) and Western blotting (WB) are basic methods used routinely for the diagnosis of Lyme borreliosis (Busson et al. 2012). Because the antibody response is only an indirect proof of infection, other methods are being researched. Cultivation is of low sensitivity, and therefore considerable hopes were vested in the polymerase chain reaction (PCR) technique in the early 1990s. Although many procedures have been tested, widely applicable methods have not yet been found (Lebech 2002; Nolte 2012). PCR has been tested in our workplace, and it was noted that the positivity of PCR in urine persisted relatively long after antibiotic treatment in a considerable number of patients. No clinical parallel was recorded in the literature, and we have not been able to satisfactorily explain the result. DNA was detectable by PCR in 17 patients out of 57 at the end of treatment, and the positivity persisted in 14 % of patients after 3 months and in 1 % after 6 months. Moreover, the positive result of PCR did not correlate with the clinical outcome, which was favorable. These results have already been described previously by us (Pícha et al. 2005), and we decided

to verify the experiment in another prospective study in patients with different clinical forms of Lyme disease (LD) and PCR arrangement. Results from the initial phase of this second study (PCR examined before and after antibiotic treatment) have been in part published previously (Pícha et al. 2008). The results from the follow-up (3 and 6 months after therapy) supplemented with new patients are submitted here.

Material and methods

Patients

One hundred twenty-four patients with clinically manifested disease were included in the study: 53 with neuroborreliosis (NB), 48 with erythema migrans (EM), and 23 with Lyme arthritis (LA). The criteria for inclusion were current symptoms of LB and positivity in laboratory tests as seen below. Patients were examined clinically by a dermatologist (skin forms), neurologist (NB), infectious disease specialist, and orthopedist (LA) before and after antibiotic treatment and again 3 and 6 months later. The laboratory testing was performed concurrently. Plasma and urine were tested at each stage. Cerebrospinal fluid (CSF) was tested prior to therapy for NB cases. Synovial fluid was examined only in selected patients, at the doctor's discretion.

The clinical status (objective findings) was assessed at the time of enrolment on a 1 to 5 scale ("1" normal objective status; "5" the most severe). Later, the objective clinical progress was evaluated with another 1 to 5 scale at 3 and 6 months. A "1" represented complete recovery, while "5" reflected an unchanged clinical finding. Treatment response was compared to the severity of initial findings. Subjective complaints of patients were recorded separately (the main clinical data of the patients are summarized in Table 1).

Patients with NB were included in the study when CSF antibody synthesis was proven either by positivity of the antibody index CSF/serum (AI_{BbIgG}; in 42 patients) or by its equivalent (solitary IgM and/or IgG positivity in CSF; in 11 patients). This fact necessarily influenced some results of the study (percentage of antibody-positive patients). The aim of this criterion was to make diagnosis maximally credible.

Patients with skin LB were enrolled when typical EM was manifested and verified by a dermatologist. Specific antibodies were not chosen as the criteria for inclusion because they are only sparsely detected in EM (this correlates with our results: of 48 patients, 42 and 43 were seronegative for IgM and IgG, respectively, before treatment; 31 were seronegative all the time).

The joint involvement group was composed of 23 patients with acute and chronic forms of the disease (duration 3 weeks–24 months). The type of clinical involvement is displayed in Table 1; 18 patients suffered from monoarthritis (17 of the knee and 1 of other joint), and five patients had involvement in more than one joint. Borreliosis was diagnosed by seropositivity in 12 cases (five patients had manifested EM, and nine reported one or more tick bites). The other LA patients demonstrated positivity in joint fluid: DNA positivity (four); positivity of spirochetes in immunosorbent electron microscopy (ISEM) and antibodies in synovial fluid in parallel (two); DNA and ISEM (three); and antibodies in synovial fluid (two). Diseases with duration longer than 6 months were classified as chronic LA (10 patients out of 23).

Eleven NB patients (20.7 %) were lost after 6 months due to lapses in clinical follow-up. Twenty-one EM patients dropped out (43.7 %), and in LA, ten did not complete all check-ups. The clinical manifestations of patients in NB and EM did not differ substantially between those who attended all the check-ups and those who did not. In LA, the proportion of dropped acute forms was higher (three chronic and seven acute diseases).

All patients were questioned about tick bites during the follow-up because reinfection was considered, and three responded positively. None had displayed additional EM.

PCR testing for negative controls was performed in 39 patients; all of them were examined in plasma and urine and 20 in CSF—each sample was tested with all primer sets. The patients were enrolled when borreliosis had been excluded according to history, clinical status, and negative serology. In all, six positive signals in plasma, two in urine, and none in CSF were obtained.

All patients were treated with antibiotics after the samples had been taken. Choice of antibiotics was left to medical discretion. All NB cases were treated with benzyl penicillin (20 mil. units) or ceftriaxone (2 g) per day for 3 weeks. In EM, deoxymykoin (27 patients), amoxycilin (16), benzyl penicillin (2), and azithromycin (3) were used for the duration of 2–3 weeks. In LA, deoxymykoin (20 patients), benzyl penicillin (1), and azithromycin (2) were used for 3–4 weeks.

Detection of specific antibodies was performed using EIA commercial kits for *Borrelia afzelii* IgM/IgG and *Borrelia garinii* IgM/IgG. All results were confirmed by WB (TestLine Clinical Diagnostic, Brno, Czech Republic). Antibodies were examined in serum in all study periods and in CSF or synovial fluid when punctures had been performed.

Concentration of IgG and albumin was measured by immunoturbidimetry (Vitalab Eclipse, Darmstadt, Germany). CSF/serum albumin–globulin quotient (Q_{alb}/Q_{IgG}) demonstrated the condition of the blood–CSF barrier function.

Specific antibody index CSF/serum (AI_{BbIgG}) was established by the method of Kaiser and Lücking (1993). Values of AI_{Bb} greater than 1.5 were considered indicative for the intrathecal synthesis of specific antibodies.

ISEM visualization of *Borrelia* was performed in the laboratory of the National Institute of Health in Prague, Czech Republic (Hulínská et al. 1999).

Table 1 Clinical syndrome, diagnosis, and subjective symptoms of patients at the beginning and after 6 months

Diagnosis	Number	Objective findings	Number	Subjective symptoms Before treatment	Number	Subjective symptoms after 6 months	Number
Neuroborreliosis							
Meningoradiculitis	17	Facial monoparesis	18	Paresis	38	Paresis	12
Meningomononeuritis	17	Facial diparesis	6	Radicular pains	16	Headache	6
Subacute encephalitis	7	Oculomotor paresis	1	Headaches	16	Fatigue	9
Polyneuritis (acute)	4	Radiculopathy and peripheral neuropathy	22	Fatigue	8	Sensitive symptoms	8
Mononeuritis (facial paresis)	2	Multiple CNS involvement	4	Arthralgias	10	Arthralgias	5
Aseptic meningitis	2	Normal	6	Sensitive symptoms	12	Radicular pains	2
Subacute poly and mononeuritis	4			Others: sleep and sphincter disturbances, myalgias, hypogeusia, backaches, vomiting		Others: sleep disturbances, myalgias, hypogeusia, backaches, depression	
Total	53		NR		NR		NR
Erythema migrans[a]							
		Erythema annulare	31	Arthralgias	21	Arthralgias	5
		Erythema homogeneous	10	Headaches	25	Headaches	7
		Erythema iridiformis	5	Fatigue	28	Fatigue	4
		Erythema other type	2	Others	15	Others	10
Total	48		NR		NR		NR
Arthritis[b]							
		Knee	17	Arthralgias	23	Arthralgias	7
		Shoulder	5	Fatigue	13	Headaches	5
		Ankle	3	Headaches	9	Fatigue	4
		Other joint	3				
Total	23		NR		NR		NR

NR not relevant

[a] Form of EM: 40 times solitaire, five times multiple

[b] Additional joint pathology: osteoarthrosis, nine; psoriatic arthritis, one; Bechterew's disease, one

DNA isolation from body fluids (plasma, urine, CSF, synovial fluid) was extracted using the commercial kit QIAamp DNA Mini Kit (QIAGEN G.m.b.H., Hilden, Germany) modified by increasing the amount of sample to be processed (0.5 mL of plasma, 1 mL of CSF, 1 mL of synovial fluid, and 15 mL of urine). Fresh biological material was centrifuged (5,000×*g*/10 min), the supernatant was discarded, and the pellet was dissolved in a 180-μL ATL buffer. Then the material was processed according to the manufacturers' instructions. Elution volumes were 50 μL.

Primers

Each biological fluid was tested using the five primer sets (East Port, Prague, Czech Republic): two targeted plasmid genes encoding OspA and OspC proteins, three primer sets amplified chromosomal genes encoding 16S rDNA, flagellin, and p66 protein. Sequences were designed according to the database on www.ncbi.nlm.nih.gov/GenBank (Table 2). Positive controls for Eli DNA of *B. burgdorferi* s.l. were used for testing the specificity of these primers (Elisabeth Pharmacon, Brno, Czech Republic).

Nested PCR

Each PCR consisted of one test tube with DNA aliquot and primer set. Amplifying systems and technical conditions of all five systems were the same. The first reaction was as follows: 14 min at 92 °C (enhanced denaturation of polymerase); 9 cycles (30 s) with denaturation at 92 °C; elongation of primers started at 59 °C and temperature decreasing by 1 °C at each cycle down to 51 °C (30 s); and extension at 72 °C (30 s). Afterwards, 50 cycles of PCR followed: denaturation at 92 °C (20 s), annealing at 50 °C (20 s), and extension at 72 °C (20 s). Conditions of the second amplification were identical, and only the number of cycles was reduced to 35.

Sequencing

The PCR products were sequenced using a Thermo Sequenase Dye Terminator Cycle Sequencing Kit with an ABI 377 DNA sequencer (GE Healthcare/AP Czech, Czech Republic). All of the tested samples specific for 16S rDNA were sequenced and provided positive correlation with the original sequence.

Negative controls containing all of the reagents but lacking template DNA were routinely processed exactly as described above to monitor for contamination with borrelial DNA. They were negative in all experiments. Positive control Eli DNA *B. burgdorferi* s.l. was included in all experiments.

Results

Before the therapy, of the 53 patients with NB, there were 41 PCR-positive (77.4 %); among 48 EM patients, there were 26

Table 2 Nucleotide sequences of primers specific for *B. burgdorferi* sensu lato

Gene	Sequences	Amplicon size (bp)	GenBank
OspA	(A) 5′ ATG AAA AAA TAT TTA TTG GGA A 3′		EU635992.1
	(D) 5′ GAA GTT CCT TTT AGC TCA A 3′		GU320003.1
	(B) 5′ GCA GCC TTG AYG ARA AAA A 3′		GU906888.1
	(C) 5′ TAA GCT CAA GCT TGT CTA CTG TTG C 3′	80	
OspC	(A) 5′ CAC AAT TAA TGA AAA AGA ATA 3′		CP001250.1
	(D) 5′ GCA TTA GAA TCY GTA ATT TTT TT 3′		CP001319.1
	(B) 5′ TT A AGT GCG ATA TTA ATG ACT 3′		GU569091.1
	(C) 5′ ATC YGT AAT T TT TTT ACT TAT TTC 3′	94	
Flagellin	(A) 5′ GAT GAA GCA ATT GCT GTA AAT 3′		HM345909.1
	(D) 5′ TGT AAT AG C ATC AAC TGT GGT T 3′		X69613.1
	(B) 5′ TAT TCA GCT AAT GTT GCA AAT C 3′		HM345905.1
	(C) 5′ AGT TGT AAC ATT AAC AGG AGA 3′	141	
16S r DNA	(A) 5′ CGC TGG CAG TGC GTC TTA 3′		CQ918148.1
	(D) 5′ GAC GCA GAC TCA TCT ACA AG 3′		CQ925712.1
	(B) 5′ CTG CTT AAG CAT GCA AGT CAA AAC 3′		CQ918151.1
	(C) 5′ CTC ATC TAC AAG CGA AGC TT 3′	147	
p66	(A) 5′ CAC CTT TTG AAT TAA AYT TTG G 3′		CP000395.1
	(D) 5′ ATC TAT TGA TGA ATT ATT GAA TGT 3′		M58431.1
	(B) 5′ TCT GTA ATT GCA GAA ACA CCT 3′		CP000013.1
	(C) 5′ GAG TAT GCT ATT GAT GAA TTA TT 3′	70	

(A) and (D) signify outer primers; (B) and (C) signify inner primers; R=A/G, Y=C/T

positive (54.2 %); and in 23 LA patients, there were 11 positive (47.8 %) (Table 3). At the end of therapy, PCR positivity was found in 22 patients with NB (41.5 %), 16 (38.1 %) with EM, and 10 (47.6 %) with LA. After 3 months, PCR positivity was proven in 29 out of 100 examined patients, and after 6 months, there were six patients with positive PCR results among the 76 examined (7.9 %).

The clinical involvement and outcome of subjective symptoms are summarized in Tables 1 and 4. Patients with NB had very mild to moderate involvement (assessed by degrees 1–3) in 50 out of 53 cases, and the result of treatment after 6 months was very good also (26 patients in full recovery and 12 patients at low degrees 2 and 3). Only in four cases was the clinical outcome assessed by degrees 4 and 5 (residual paresis and radicular symptoms).

In EM, most lesions were solitary (45, degree 1), with the rest displaying multiple EM (three cases, degrees 2 and 3). Their outcome was unambiguously favorable.

Joint inflammations were represented mostly by an involvement of the knee (17) with moderate extent (12 cases, degree 3). The recovery of these patients was less favorable than in other organ manifestations (six in full recovery and seven in the other rating groups).

Clinical manifestations of LB were mostly typical (summarized in Table 1 with subjective symptoms). Some clinical forms were diagnostic for LB (EM, Bannwarth's syndrome) or very suspicious (facial palsy). While arthritis of the knee is a typical LA manifestation, it was not possible to exclude the hypothesis that other concomitant joint pathologies (nine osteoarthrosis; Table 1) could interfere with the clinical picture.

Table 3 Results of PCR with the respect to the clinical forms of Lyme borreliosis

	No. of examined patients	No. of positive patients, *n* (%)
Before treatment		
Nerve involvement	53	41 (77.4)
Skin involvement	48	26 (54.2)
Joint involvement	23	11 (47.8)
After treatment		
Nerve involvement	53	22 (41.5)
Skin involvement	42	16 (38.1)
Joint involvement	21	10 (47.6)
After 3 months		
Nerve involvement	49	16 (32.7)
Skin involvement	31	7 (22.6)
Joint involvement	20	6 (30)
After 6 months		
Nerve involvement	42	5 (11.9)
Skin involvement	21	0
Joint involvement	13	1 (7.7)

An additional course of antibiotic treatment was given for two patients in the study; neither of them were DNA-positive at 3 and 6 months.

Results of specific antibodies examinations before treatment are summarized in Table 5. The most seronegative cases were found in skin borreliosis (36 IgM and 35 IgG negatives). In the NB cases, the antibody types were markedly different (10 IgM and 28 IgG positives; all patients had antibodies in CSF due to inclusion criteria).

Aseptic formula was found in the CSF in most of the NB patients. Ten patients had normal cytological result, and seven of them also displayed normal value of the total CSF protein concentration. Abnormal findings included (number in 1 μL) lymphocytes with an average of 276.87 (range 0–1,115; median 210.00; SD 274.27); monocytes with an average of 14.30 (median 12.00; SD 15.47); and neutrophilic leukocytes with an average of 29.34 (median 2.00; SD 54.41). Concentration of the total protein was in average of 1.09 g/L (0.33–2.98 g/L), median of 0.89, and SD of 0.67 (normal value 0.2–0.6 g/L).

PCR was also found to be positive 3 and 6 months after treatment. After 3 months, 29 patients were positive out of 100 tested (the percentage of positive results was 32.7 % in NB, 22.6 % in EM, and 30 % in LA). Six patients (7.9 %) were found positive after 6 months (five NB and one arthritis). Seventy-six patients of 124 completed the study, and their initial clinical condition did not differ substantially from those that did not attend the final screening.

Better information about clinical and laboratory results evaluating the protracted PCR positivity is in Table 6. All EM PCR-positive patients were seronegative before treatment, and only one became seropositive after 6 months. None of the examined EM patients were DNA-positive at 6 months past treatment. Clinical outcome was excellent in all in the EM group; cluster headaches were diagnosed in one patient (negative lumbar puncture excluded NB).

Among NB patients, all who tested positive for DNA at 6 months had tested positive earlier. One patient had tested positive in just one primer prior to treatment and then tested positive at the end of the study. The rest of the patients who tested positive at the 6-month point had DNA positivity in two or more body fluids. The same is without exception valid for DNA examination after 3 months. Clinical findings in this group did not differ substantially from the rest of the NB group. The single patient with the manifestation classified at severity "4" suffered from severe lumbosacral discopathy (following two surgeries), and this pathology overlapped with borrelial radiculopathy symptoms. The rest of the patients assessed by the outcome "3" comprised patients with polyradiculopathies or polyneuropathies (where subjective symptoms play a role in the assessment). In summary, it was not possible to find clear clinical correlations between the PCR positivity and clinical outcome.

Table 4 Severity of involvement and clinical outcome in patients with Lyme borreliosis

Severity of involvement[a]	No. of patients	Clinical outcome after 3 months[a]	No. of patients	Clinical outcome after 6 months[a]	No. of patients
Nerve involvement					
1	13	1	18	1	26
2	26	2	13	2	8
3	11	3	11	3	4
4	3	4	6	4	1
5	0	5	1	5	3
Total	53		49		42
Skin involvement					
1	45	1	29	1	20
2, 3	3	2, 3	2	2, 3	1[b]
Total	48		31		21
Joint involvement					
1	4	1	6	1	6
2	5	2	6	2	2
3	12	3	4	3	2
4	3	4	2	4	2
5	0	5	2	5	1
Total	23		20		13

[a] Degree; arbitrary units (see text)

[b] One patient with sensitive neurological deficit in previous EM

The same can be concluded in joint LB as far as it concerns the clinical outcome and diagnosis. However, there was one patient who was DNA-positive after 3 months and another after 6 months for the first time. Four of seven patients were DNA-negative prior to therapy.

PCR examination of urine was similar to CSF in sensitivity before treatment in the acute period of the disease (25 positive samples of CSF vs. 24 in urine). PCR in plasma was less sensitive—16 NB cases, 14 EM, and 5 LA tested positive before treatment.

The impact of the concurrently examined PCR targets on the percentage of PCR-positive patients was assessed prior to treatment (Fig. 1). The percentage of the patients reacting only with 16S rDNA primer set was 38.7 %; when the OspA target was added, 52.4 % of the patients were detected. Adding flagellin to these two primers provided 59.7 % positives. The fourth and fifth did not increase the rate of detection substantially (testing for OspC, 61.3 %, and all of them, 62.9 %).

Discussion

"The persistence of symptoms in Lyme disease patients following antibiotic therapy, and their causes, continue to be a matter of intense controversy" (Embers et al. 2012). It is known from both clinical and experimental practice that spirochetes are capable of persisting in an organism after antibiotic treatment, and that the infection can be chronic or relapsing. Taking into account a high number of infected patients (according to seroprevalence varying between 10 and 20 %) and the generally favorable outcome of correctly and

Table 5 Examination of specific antibodies before treatment

Antibody	Nerve involvement	Skin involvement		Joint involvement	
	No. of patients (serum)	No. of patients (CSF)	No. of patients (serum)	No. of patients (serum)	No. of patients (synovial fluid)
IgM−	29	29	36	15	13
IgM +−	14	6	7	4	3
IgM+	10	22	5	4	1
IgG−	17	4	35	3	7
IgG +−	8	7	8	2	3
IgG+	28	42	5	17	7

+ positive, − negative, +− borderline

Table 6 Results of PCR, serology, and main clinical manifestations in all patients PCR-positive after 3 and 6 months

Serology		PCR				Diagnosis	Range of involvement	Clinical outcome after 3 months[a]	Clinical outcome after 6 months[a]	CSF cell count per 1 μL[f]	CSF protein g/L	Note
Before treatment	After 6 months	Before treatment	After treatment	After 3 months	After 6 months							
Neuroborreliosis												
+	+	F	−	−	U	Bannwarth's syndrome	3	2	1	212/8	2.98	
−	+	F, U	U	U	−	Aseptic meningitis	1	1	1	285/20	1.66	
+	−	F, P	−	P	−	Aseptic meningitis	2	1	1	240/7	1.17/886[b]	
+	+	F, P	U	P, U	−	Distal polyneuropathy	1	3	2	1/0	0.6	
+	+	F, U	P	U	−	Neuritis n. III	3	4	1	2/0	0.43	Normal CSF; 11,7 times elevated AI[c]
+	+	F, P	−	−	P	Aseptic meningitis	2	1	1	67/20	0.33	
+	−	F, U	−	P	U	Aseptic meningitis	2	1	1	136/24	0.53	
+	−	F	P, U	U	−	Bannwarth's syndrome	2	3	2	211/9	1.09	
+	−	F	P, U	P	−	Bannwarth's syndrome	3	2	1	145/16	2.09	IgG+IgM+ only in CSF
+	−	P, U	U	U	−	Polyneuropathy	2	3	3	2/0+	0.48	
+	+	F, P, U	−	P	−	Chronic compressive LS dicopathy complicated by borrelial infection	4	4	3	1/0	0.55	Normal CSF; 2 times elevated AI[c]; assessment of clinical outcome complicated by heavy compressive radiculopathy
+	+	P, U	U	U	−	Polyradiculopathy	3	4	4	1/10	0.62	
+	−	F, P	P, U	P	P, U	Polyneuropathy	2	3	2	2/0	0.89	IgG+ in CSF; complete recovery after 12 months
−	−	F	P, U	P	−	Facial palsy	2	1	1	1/0	0.33	IgG+ in CSF twice
+	−	F, P	−	P	−	Facial palsy	2	1	1	6/0	0.3	IgG+ in CSF
+	+	F, P, U	P, U	P, U	U	Aseptic meningitis	1	3	2	52/3	0.69	
+	−	F	P	P, U	−	Polyradiculopathy	3	4	3	11/0	0.5	IgM+ in CSF
Erythema migrans												
							Manifestation					
−	−	−	−	U	−	EM	Homogeneous; diam. 30 cm	1	1			Completely seronegative
−	−	P	−	P	−	EM	Homogeneous 9×3 cm	1	1			Completely seronegative
−	−	U	P	P	−	EM	Circular 10×15 cm	2	1			Completely seronegative
−	−	P	P	U	ND	EM	Circular 10×5.5 cm	1	1			Completely seronegative

Table 6 (continued)

Serology		PCR				Diagnosis	Range of involvement	Clinical outcome after 3 months[a]	Clinical outcome after 6 months[a]	CSF cell count per 1 μL[f]	CSF protein g/L	Note
Before treatment	After 6 months	Before treatment	After treatment	After 3 months	After 6 months							
−	−	P	−	P	ND	EM	Circular 9×8 cm	1	ND			Last examination after 3 months; completely seronegative
−	−	U	U	U	−	EM	Circular 16×12.5 cm	1	1			Completely seronegative; lumbar puncture excluded NB
−	+	P	P, U	P	−	EM	Homogeneous	1	1			IgG+, IgM+after therapy, lately seronegative
Arthritis												
+	+	−	U	P, S	−	Knee	3	1	1			
+	−	U, S	−	S	−	Knee	4	3	1			
+—[d]	−	−	−	−	S	Knee	4	3	1			Seronegative at 6th months
+	+	−	−	P	−	Knee	4	2	2			Intermittent articular pains after 6 months
+	+	U	−	U	−	Shoulder	3	2	1			
+[d]	+[e]	P	P, U	U	ND	Knee	3	2	2			Intermittent artricular pains after 6 months
+	−	−	P, U	P	−	Knee and ankle successively	4	3	1			

PCR in plasma and urine were tested in all patients in all periods

+ positive, − negative, *ND* examination not done, *P*+ plasma positive, *U*+ urine positive, *F*+ CSF positive, *S*+ synovial fluid positive

[a] Arbitrary unit

[b] Artificial erythrocytes

[c] AI: IgG-specific antibody index CSF/serum

[d] Antibodies in synovial fluid

[e] Examinations after 3 months

[f] Mononuclear leukocytes/neutrophillic leukocytes

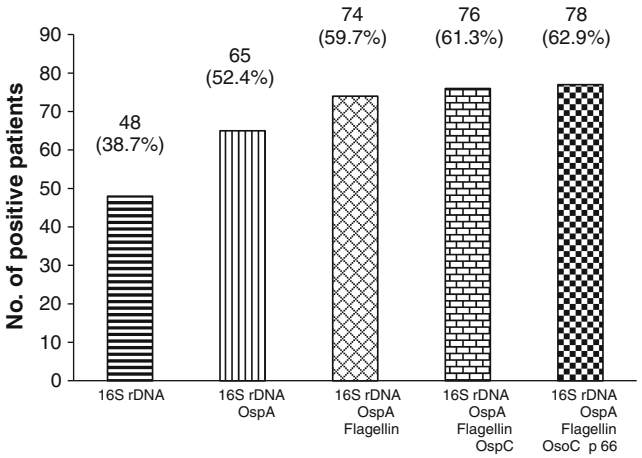

Fig. 1 Frequence of DNA-positive patients using different numbers of primers

successfully treated patients, it seems that the frequency of chronic and relapsing forms of infection is low. The other problem is that the patients with chronic form of disease are often the most complicated. The presence of viable borreliae after antibiotic treatment was proven repeatedly, for instance, by Embers et al. (2012) in experiments with rhesus macaques. Similarly, the specific DNA and viable (but not locally dividing) spirochetes were found in mouse tissues after therapy (Hodzic et al. 2008). Spirochetes can survive in tissue reservoirs, and though they are difficult to localize, they have been repeatedly proven to be present in antibiotically treated or untreated carriers. For example, the spirochete DNA persisted in joints and their immediate vicinity in mice (Yrjänäinen et al. 2010) and also in humans after antibiotic treatment (Priem et al. 1998). And last but not the least, Strle et al. (1995) were able to prove that viable spirochetes persisted in former ECM skin lesions 2 months to 3.5 years after clinical manifestation.

The specific DNA alone was also found in animals after treatment, but that this fact has not been considered a sign of either active infection or the viability of spirochetes is shown by Varde et al. (1999), Iyer et al. (2012), and others. Whether the DNA persistence is related to persisting clinical symptoms or chronic infection is not resolved. The proof of living spirochetes is crucial for answering these questions.

It has been published that PCR in synovial fluid often becomes negative soon after antibiotic treatment (Nocton et al. 1994). But the fact that the specific DNA has been proven in synovial tissue of PCR joint fluid and culture-negative patients (Priem et al. 1998) raised the hypothesis of spirochetes persisting in small numbers in joint compartments. Consideration of whether these spirochetes are dead or alive and which role they play in pathogenesis of articular inflammation has led to introduction of the terms antibiotic-refractory and antibiotic-responsive LA (Steere and Angelis 2006). Each of these concepts reflects different pathophysiology—infective and/or immunopathological processes. Possible mechanisms leading to the generation of the immune-mediating arthritogenic stimulus are presented in the "amber theory" of LA (Wormser et al. 2012). During or after successful suppression of infection, nonviable spirochetes or spirochetal debris are enmeshed in a host-derived fibrinous and collagenous intraarticular matrix. This material may then enter the joint space where it can cause either immune inflammation, or occasional positivity of PCR. Because extracellular collagen has been known for a long time for its spirochetal affinity to the skin or other tissue (e.g., Cabello et al. 2007), this theory could also play a role in other forms of LB.

The treatment recommendations for acute forms of LB are relatively settled and summarized in national guidelines (Wormser et al. 2006; Bathe and Schwartz 2011; Mygland et al 2009). The majority of clinicians do not recommend continuing antibiotic therapy beyond the limit of 4 weeks (Stanek et al. 2012). On the other hand, recommendations for chronic LB are not generally accepted. This confusion is exacerbated by poor clinical and laboratory definition of this condition. Moreover, it is necessary to mention that there are some researchers considering the risk of spirochete persistence so serious that they recommend prolonged or long intermittent antibiotic treatment (e.g., Stricker 2007). The slow resolution or persistence of LB symptoms has led to the formation of clinical units of chronic borreliosis and post-Lyme syndrome. Their existence, pathogenesis, diagnosis, and treatment are the subject of discussions (Feder et al. 2007; Wormser and Shapiro 2009; Lantos 2011).

Consequently, the main aim of this study is to demonstrate the phenomenon of the persistence of PCR positivity after antibiotic treatment under the conditions of this clinical and prospective study. To expand upon our previous study, the examination of other targets and bodily fluids was added. The performed experiments cannot solve the core issue: whether the positive DNA implies the slow extinction of infection or the slow shedding of spirochete DNA. PCR does not differentiate the origin of the DNA; the origin can only be hypothesized. The specific DNA can originate from dead borreliae, and thus it could be slowly leaked out from involved tissues in this scenario. The abovementioned amber theory could be a contributing factor. Good clinical outcomes recorded in our patients would support this explanation. The second possibility is that the *Borrelia* replication (already primarily slow) merely decelerates after antibiotic treatment. This hypothesis cannot be excluded because it is well documented that spirochete replication can be asymptomatic for weeks or months (Straubinger 2000). Reinfection and chronic infection are completing the picture, but they do not seem to be frequent enough to explain DNA persistence in this study. It would be desirable to repeat these experiments, because they can result in clinical consequences. First of all, verification of DNA persistence can contribute to long-lasting discussions on

repeated or prolonged antibiotic treatment and chronic infection in LD. Secondly, although PCR is not usually used for the laboratory testing of antibiotic treatment efficacy, the misinterpretation of these results can lead (and in clinical practice often leads) to over-treatment and over-examination.

PCR is not accepted as the routine clinical diagnostic tool, and this study does not want to change this. But it should be emphasized here that the presented results support the idea that if PCR were indicated for clinical use, then parallel usage of two or three primers can help to improve sensitivity. It was not possible to calculate the correct diagnostic sensitivity of the applied PCR due to the inclusion criteria.

However, comparison with some reviews (Lebech 2002; Dumler 2003; Wilske et al. 2007) where the referred diagnostic sensitivity of PCR under clinical conditions usually varies between 20 and 50 % depending on body fluid and the method, the 62.9 % of positive results reached in our study prior to treatment could seem to be relatively high. But this percentage is affected by the high number of primers examined in parallel.

Examination of the DNA in urine is not accepted unambiguously in clinical practice. Some authors point to variability and low sensitivity of PCR in this body fluid (due to methodical variability, presence of DNA polymerase inhibitors, etc.; Rauter et al. 2005). Our results from both the previous and this recent study have been optimistic, but they are obtained under experimental conditions. Although rare, references exist which correspond to our positive experience and support the use of this modification of the PCR test in clinical practice (Aberer et al. 2007; Bergmann et al. 2002).

In conclusion, this study wants, above all, to point to the phenomenon of the PCR positivity in LB early after antibiotic treatment and some possible consequences.

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Ethical standards This study was approved by the hospital ethical committee. All enrolled patients expressed their agreement by signing a patient's informed consent.

Conflict of interest The authors confirm that they have no conflicts of interest.

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