LONG TERM AND REPEATED ELECTRON MICROSCOPY AND PCR DETECTION OF *BORRELIA BURGDORFERI SENSU LATO* AFTER AN ANTIBIOTIC TREATMENT

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SUMMARY

The diagnosis of Lyme disease in 18 patients has been proved by detection of Borrelia burgdorferi sensu lato when using immunoelectron microscopy or detecting its nucleic acid by PCR in the plasma or the cerebrospinal fluid. The positive results occurred in the plasma or in the cerebrospinal fluid in the period of 4-68 months after an antibiotic treatment. The typical clinical manifestations of Lyme disease were observed in 9 patients and non-specific symptoms in another 9 patients. According to presented results we can recommend repeated examination using PCR of the plasma and other biological specimens in the individuals with persistent or recurring complaints after an acute form of Lyme disease and its antibiotic treatment. Also examination of the cerebrospinal fluid with non-specific symptoms and simultaneously displayed pathology electroencephalogram and/or magnetic resonance imaging findings can be advantageous.

Key words: *Borrelia burgdorferi,* persistence, polymerase chain reaction (PCR), immunoelectron microscopy (IEM), therapy

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INTRODUCTION

After a description of an epidemic of arthritis by Steer in 1975, and the causal organism by Burgdorfer in 1981, the clinical manifestations known already for a long time were assigned to Lyme borreliosis (1). The disease pathogenesis has been known only partly but a persistence capability in the infected organism was expected. In some cases a chronic disseminated stage was practically the first clinical manifestation of the infection existing already for many years, or relating the manifestations of the other organ involvement which resulted months and even years after erythema migrans (2,3). The microbiological detection of *Borrelia burgdorferi sensu lato* (*B.b.s.l.*) in the affected tissues was successful only exceptionally. Therefore, a series of theories have appeared about a possibility of the organ damage through immune mechanisms of the host organism without presence of *B.b.s.l* (4). Such mechanism was expected especially in a chronic affection of the nerve tissue and the joints (5,6).

A growing detection of the DNA of borrelia using PCR, and the results obtained in the experimental infection of animals, and through the examination of biological material of humans with Lyme disease resulted in a conclusion that borrelia persistence plays significant role in pathogenesis of the chronic and late stages of Lyme disease (7, 8). The borreliae DNA in the experiment was demonstrated in the encephalic tissue of the non-human primates for months after the infection, or in the synovial fluid of the infected dogs after an antibiotic therapy (9,10). In humans, *B. burgdorferi* DNA sequences were revealed by PCR in blood, skin, cerebrospinal fluid, synovial fluid and in urine. Moreover, in the patients with Lyme disease the detection of the borreliae DNA has been reported either in healthy skin locations after months or years after erythema migrans or in the synovial fluid

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The Ethics committee of the University hospital, Hradec Králové, approved study documents and from each subject informed consent has been obtained. Study has been conducted according to the last recommendation of ICH.

Abbreviations used: PCR – Polymerase Chain Reaction, EEG – Electroencephalogram, EM – Erythema Migrans, MRI – Magnetic Resonance Imaging, ELISA – Enzyme-Linked Immunosorbent Assay, *B.b.s.l. – Borrelia burgdorferi sensu lato*, LD – Lyme Disease, IEM – Immunoelectron Microscopy, CSF – Cerebrospinal Fluid

Table 1. *Detection of Borrelia burgdorferi sensu lato or its DNA: method, type of sample, previous and follow-up antibiotic treatment, time interval between positive results*

1	2	3	4	5	6	7	8	9
Patient's No.	Sex, age at the 1st detection	Type of antibiotics used for the treatment and time period in months prior to the 1st detection	Method of 1st detection	Type of sample, 1st direct detection of B.b.	Type of antibiotics between the 1st and 2nd detection	Method of the 2nd detection	Type of sample, 2nd detection of B.b.	Time period in months between 1st and 2nd detection
1	F., 63		IEM	CSF	PEN	PCR	plasma	33
2	F., 22	ROX / 4	IEM	CSF	PEN, PEN	PCR	CSF	32
3	M., 62	DOX/ 7	IEM, PCR	plasma	PEN, CTR, DOX, CTR	PCR	plasma	37
4	M., 51		IEM	CSF	CTR, AZI, CTX	IEM	plasma	16
5	F., 43	DOX / 10	IEM	CSF	CTX	IEM	CSF	10
6	F., 32	DOX / 12	IEM	CSF, plasma	CTR	IEM	plasma	17
7	M.,14		IEM	CSF	CTR	IEM	plasma	25
8	M., 14		IEM	CSF, plasma	CTR	IEM	CSF	13
9	F., 12		IEM	CSF	CTR	IEM	plasma	18
10	M., 64		IEM	CSF	PEN	PCR	plasma	68
11	M., 35	DOX / 16	PCR	plasma	PEN	PCR	plasma	7
12	M., 36		PCR	plasma	CTR, AZI	PCR	plasma	15
13	F., 39	DOX / 9	IEM	plasma	PEN, CTR, CTX	PCR	plasma, CSF	64
14	F., 61		PCR	plasma	CTR, AZI	PCR	plasma	7
15	M., 22		PCR	CSF	PEN	PCR	CSF	4
16	F., 29		PCR	plasma	PEN	PCR	plasma	9
17	F., 23	DOX / 12	PCR	plasma	PEN	PCR	plasma	6
18	M., 27		PCR	CSF	PEN, DOX	PCR	plasma	5

Note: PEN – penicilin G; CTR – ceftriaxon; DOX – doxycyklin; AZI – azitromycin; CTX – cefotaxim; ROX – roxitromycin; F – Female; M – Male; CSF – Cerebrospinal Fluid; IEM – Immunoelectron Microscopy; PCR – Polymerase Chain Reaction

of the patients who had already been treated with antibiotics for diagnosis of Lyme arthritis (2, 3).

Bacteremia or detection of the borrelia DNA in the plasma among patients with chronic forms of Lyme disease have been also described.

Objective. In this paper we present our results of repeated *B.b.s.l.* detection using immuno-electron microscopy or detection of *B.b.s.l.* DNA by PCR in patients 4-68 months after antibiotic treatment of Lyme disease.

MATERIAL AND METHODS

Particular methods for immuno-electron microscopy, PCR and also evaluation of immunoblot analysis have been already described (2,11).

DNA Isolation and PCR

Whole blood (5 ml) and cerebrospinal fluid were collected in tubes with citrate for PCR and IEM investigation. The buffy coat on top of the red cells was collected separately. The DNA was extracted using QiaAmp columns (QIAGEN Tissue DNA isolation kit, QIAGEN Inc., CA, USA) according to the manufacturer's instructions. The cells (buffy coat) or synovial biopsy material were prepared with proteinase K added to a DNA lysis buffer and incubated for 1 hour at 55 °C. DNA was extracted with phenol/chloroform and the aqueous layer was precipitated with isopropanol. The DNA pellet was rinsed with 70 % ethanol and resuspended in water.

The primers used for amplification were selected according to Marcone and Garon (19), who defined phylogenetically significant target sequences, based upon 16S rRNA signature nucleotide analysis, i.e. the LD primers known to amplify all species associated

with LB to generate an amplification product of 357 bases. The BB primer set, BG and VS461 primer sets generate amplification products of 169, 574 and 519 bp and differentiate between *B. garinii* and *B. afzelii*. The other primers used were selected specifically to target a 600 bp or 448 bp region of the OspA gene (Table 1) and a 110 bp region of the OspC gene (kindly donated by B.J. Luft, SUNY). All PCR components were obtained from the GeneAmp kit and used as recommended by the supplier (Perkin-Elmer Cetus). PCRs were performed with a PTC-200 DNA Engine (MJResearch, Inc. Massachusetts, USA) thermocycler by denaturing for 1 min at 94 °C, followed by 35 three-step cycles recommended. Control samples included DNA extracted from control patients, two blank control samples with 5 µl of water substituted for DNA, and positive control samples with 1.5 and 15 pg of total *B. garinii* DNA (strain M192), of *B. afzelii* DNA (strain Kc90), also together with negative control samples from patients with rheumatoid arthritis and four healthy donors. All positive samples were tested by at least two different primer sets. Ten percent of the products amplified by PCR were analyzed by horizontal electrophoresis on 1.5 % agarose gels using standard electrophoresis conditions with molecular size markers pUC18 HAEIII, and PCR Low ladders 100 bp (Sigma).

An oligonucleotide corresponding to and internal sequence among the OspA primers was selected as a probe and labelled with digoxigenin-11-dUTP using the random primed method (Boehringer Mannheim) for southern blots. PCR products were transferred from the agarose gels to a nylon membrane by using standard blotting conditions (Bio-Rad, Trans-blot SD cell for semi-dry electrophoretic DNA transfer). Following transfer, the membranes were washed, DNA-fixed, denatured and neutralized as recommended. Southern blots were prehybridized for 2 h and then hybridized for 12 h at 42 °C in 50 % formamide-5x SSC (5 x 0.15 M NaCl plus 0.015 M sodium citrate) – 0.1 % N laurylsarcosine – 5 % blocking agent. The bound probe was visualized with digoxigenin antisera conjugated to alkaline phosphatase. Colour development was produced with 4-Nitro Blue Tetrazolium in substrate buffer (Bio-Rad).

In order to simplify procedures for testing, large numbers of samples were measured using the solid phase sandwich hybridization system with a specific oligonucleotide covalently linked to microtiter wells and with a specific biotinylated revelation probe (Biocode, Prob-Lyme, Belgium). The presence of borrelia DNA in a sample to that mined by relating the optical density (OD = 450 nm) of the unknown sample to that of the cut-off value, which had been defined as 3.5 x standard deviation of 4 negative controls + average of 4 negative controls. For the inhibitor test, each specimen was spiked with genomic DNA of *E.coli* and related primers recommended by PerkinElmer Cetus.

Electron Microscopy

Synovium biopsy specimens, blood and synovial fluid, CSF and cultures were studied by electron microscopy using negative staining techniques, immunosorbent electron microscopy and sectioning techniques.

Tissue samples were fixed in 4 % paraformaldhyde with 1.5 % glutaraldehyde in 0.1 M cacodylate buffer, washed, dehydrated and embedded into Epon 812 and for immunode into Lowicryl K4M resin (Polyscience, Warrington, PA). Semithick and semithin sections were cut using ultracut Reichert and LKB equipment and stained by the modified Bosma technique with amylase pretreatment or processed sections (Lowicryl K4M) with monoclonal antibodies (Mab) and secondary goat anti--mouse anti-bodies conjugated with peroxidase (Sigma) for detection of borrelial structures in semithick sections under the light microscope or with uranyl acetate and lead citrate for ultrastructural examination under the Jeol PC XII electron microscope.

The samples of blood plasma and synovial fluid were assayed by the immunocapture of immunosorbent method as described previously. The blood samples of healthy donors spiked with different amounts of Borrelia and reference blood samples from mice prepared for the electronmicroscopy served as positive controls, respectively.

Serology

IgG and IgM titres to the Lyme spirochete were determined by ELISA using standard conditions as recommended by the supplier (Test-Line Ltd, CR). The 98th percentile of absorbance values for the controls was used as the cut-off level. In ELISA with sonicated antigens, a positive value was defined as 0.73 for IgG and 0.850 for IgM. In the IgM capture ELISA (Dako Diagnostika GmbH), a positive value was 0.380.

Western blot with commercial *Borrelia garinii* Western blot IgG and IgM kits (Biowestern Ltd, CR) was performed, or WB IgM was prepared "in-house" and standardized as recommended. Sonicated antigens (strains M192 and Kc90) were separated in 10.5 % polyacrylamide gel (acrylamide-bisacrylamide 37.5: 1, Bio-Rad) and transferred to a nitrocellulose sheet (4.5 µm, Sigma) by a semi-dry blotter (Transblot SD Semi-dry cell transfer, Bio-Rad). The strips were incubated with 1:200 dilutions of sera or fluids after a blocking step, washed and incubated with swine anti-human IgM conjugated to peroxidase under standard conditions.

The group of patients consists of 18 individuals - nine females aged 12 to 63 years, with median 32, and nine males aged 14-64 years (the median 35). Those patients were examined at the Department of Infectious diseases, University Hospital, Hradec Králové during follow-up time period 1991-2000. The enrolment of patients into the study group was conditioned by repeated positivity of IEM or PCR, which was demonstrated in the time period longer than 3 months. This approach allows us to exclude all patients after successful antibiotic treatment, who have had also positive PCR results from killed bacteria. We have chosen a time period of 3 months because it has been demonstrated by Mercier et al. (12) that 5 weeks after an efficient antibiotic treatment of E.M. the PCR examination of urine specimens was negative.

RESULTS

Clinical status of study subjects (altogether 18 patients).

In Table 1 all the important data are summarized, which include basic patient demographic data, type of sample and method of the first and second detection, time period between these two detections (the median 15.5; mean value 21.4) and finally type of antibiotics used for the treatment before and after the first detection.

Table 2. *Total duration (in months) and type of the clinical symptoms*

Patient's number	EM	Arthritis or arthralgia	Headache, fatigue, myalgia, low febrile illness	Neuro-borreliosis	Other	Notes
1	0.5			9		encephalitis, paraparesis of lower extremities after 9 months, intrathecal formation of antibodies
2			62	?		without CSF pleocytosis, progression of abnormal EEG, normal MR, no specific intrathecal antibody production
3		58		2		polyneuropathy
4		98	24	?	24 psychiatric diagnosis	CSF – normal finding, demyelization changes on MR
5		78	1	?		without CSF pleocytosis demyelization changes on MR, abnormal EEG, no specific intrathecal antibody production
6		35	35			
7				1		acute meningoencephalitis
8			87	?	carditis 1.	without CSF pleocytosis, abnormal EEG
9		98	19	?		without CSF pleocytosis, abnormal EEG, demyelization changes on MR
10		2		1		encephaloradiculitis, specific intrathecal antibody production
11	0.5		12			
12		4	4	?		abnormal EEG and MR, without CSF pleocytosis
13	0.5	78	78	?	AV block 1	demyelination changes on MR, abnormal EEG , after 5 years he-micrania, new demyelination foci on MR, without CSF pleocytosis repeatedly, no intrathecal synthesis of specific antibodies
14		17		12		pathological finding in CSF lasting 12 months after encephalitis
15				1		acute meningo-encephalitis, intrathecal synthesis of specific antibodies
16		77	77	?		demyelination changes on MR, without CSF pleocytosis, no intra-thecal synthesis of specific antibodies
17	1	8				
18		7		?		central vertigo without CSF pleocytosis, no intrathecal synthesis of specific antibodies

Note: EEG – electroencephalogram; CSF – cerebrospinal fluid; MR – magnetic resonance imaging; EM – erythema migrans

Two detections of *B.b.s.l.* at the same patient using IEM were obtained 6x, two consecutive detections based on PCR results were obtained among other 8 patients. In remaining 4 cases there was the first detection by IEM, and the second one using PCR.

After the 1st detection of causative agent all patients in our follow-up were treated by penicillin or ceftriaxon. After different time period some of them continued antibiotic treatment by another type of antibiotic as it is indicated in the 6th column.

In Table 2 clinical manifestations in the individual patients are presented in a simplified form. A clinical picture typical for Lyme disease has been present in medical history or at our first examination only in 9 individuals: neuroborreliosis 5x, gonitis 1x, EM 4x (one of these subject with EM developed neuroborreliosis later on). The total duration of clinical manifestations is given in months. The reason for examination of the second half of patients (9 patients) in our department were various combinations of non-specific symptoms like headache, arthralgia, fatigue syndrome, low febrile illness and positive serology results of LD.

We have not evaluated the antibiotic treatment efficacy; an explanation is presented in the discussion.

Assessment of clinical symptoms was terminated to 31 December 2000. The question marks designate cases in which the time concerning the beginning and duration of the disease is impossible to assess.

A possible progression of Lyme disease concerning involvement of other organs after the first detection and an adequate treatment we have found in 6 cases once in the form of carditis, once AV block, 1x paraparesis of the lower extremities, 1x arthritis of large joints, 1x psychiatric symptomatology, 1x a severe combined polyneuropathy. The mentioned clinical manifestations

might have even another etiology than Lyme disease, but that has not been ascertained.

Determination of specific antibodies was based on a 2–test approach, using ELISA and Western blot (11,13,). The results are presented in Table 3, the ELISA results are given in the time of the first and second demonstration, Western blot is presented by one value. In time of the first detection the antibodies were determined using ELISA method in the IgM or IgG classes in 11 patients. In seven individuals with a negative detection of specific antibodies using ELISA method, they were detected using the western blot method. During the second detection ELISA in 17 of the sick patients did not prove antibodies.

If the examination of intrathecal production of specific antibodies was carried out, the result is presented in Table 2. In some cases with a normal cytological finding in CSF the examination was not performed, as a positive PCR or IEM result had not been expected.

Table 3. *Antibody examination results at the first and second detection of Borrelia burgdorferi sensu lato DNA*

Patient's number	IgM ELISA 1st/2nd detection	IgG ELISA 1st/2nd detection	IgM-western blot	IgG- western blot
1	neg./ neg.	neg./ neg.	neg.	pos.
2	pos./ neg.	neg./ neg.	pos.	pos.
3	neg. /neg.	neg. /neg.	pos.	pos.
4	pos. /neg.	pos. /pos.	neg.	pos.
5	pos. /neg.	neg. /neg.	pos.	neg.
6	pos. /neg.	neg. /pos.	neg.	pos.
7	neg. /neg.	pos. /neg.	neg.	pos
8	neg. /neg.	pos. /neg.	neg.	pos.
9	pos. /pos.	neg. /neg.	neg.	pos.
10	pos./neg.	neg. /neg.	neg.	pos.
11	pos. /pos.	neg. /neg.	pos.	pos.
12	neg. /neg.	neg. /neg.	neg.	pos.
13	neg. /neg.	pos. /pos.	neg.	pos.
14	neg. /neg.	pos. /pos.	neg.	pos.
15	neg. /neg.	neg. /neg.	neg.	pos.
16	neg. /neg.	neg. /neg.	pos.	neg.
17	neg. /neg.	neg. /neg.	pos.	pos.
18	neg. /neg.	neg. /neg.	neg.	pos.

DISCUSSION

Interactions of *Borrelia burgdorferi* with an infected organism are partially known. After inoculation of causative agent into the skin erythema migrans may occur. In part of the infected persons bacteremia occurs, which is not related to EM occurrence. Frequency of those bacteremia in all the infected individuals is unknown, in humans with EM it is present in up to 1.2 % of cases (14). Borrelia may invade the target organs and induce clinical manifestations of an early-disseminated stage. Late stages of Lyme disease occur due to the action of *B.b.s.l.*, which is in the involved organ present probably for months or years. A remission of the clinical signs may not mean elimination of the causative agent, even after an antibiotic treatment (15).

PCR results are very often positive in the synovial tissue of the affected joints, less frequently in the synovial fluid (16,17). The detection of *B.b.s.l.* in the chronic dermal lesions is frequent, but it has a little practical meaning for diagnosis establishment (18). Very different are PCR results in detection of borrelia DNA in the cerebrospinal fluid in the sick patients with neuroborreliosis. Positive results obtained by this method ranged from 12% to 46%.

A direct detection of *B.b.s.l.* using immunoelectron microscopy is a demanding method used mainly in the experimental studies.

A recurrent detection of *B.b.s.l.* in the blood or in CSF using electron microscopy in the individuals in our study group gives evidence for persistence of the causative agent after therapy with antibiotic in 6 patients. In the remaining 12 patients the interpretation of the results of a repeated PCR positivity or a combined detection by electron microscopy and PCR is more difficult. Rate for DNA elimination of borrelia or their fragments after *B.b.s.l.* devitalisation is unknown. It is evident from the PCR method, that it is not possible to consider automatically the DNA detection of borreliae in the plasma as a detection of bacteremia. Considering the above-mentioned results of the immunoelectron microscopy it is probable that in many of our patients bacteremia has occurred even in case of the PCR positivity.

A repeated positivity of PCR or PCR positivity after previous IEM positivity in the time period of many months or years we consider as a sign of the causative agent's persistence in the organism. We consider the presence of *B.b.s.l.* or its DNA as intermittent in the plasma because among the positive findings at different time points there were one or more examinations with negative outcome. The number of that negative examination wasn't the same in all patients in a comparable time period. A failure of curative effect of antibiotics we found in 7 individuals before the first detection, and in all those prior to the second detection. Antibiotics were administered in the recommended doses. After the first detection of *B.b.s.l.* or DNA there followed therapy with i.v. formulation of antibiotic. Other antibiotics were administered in seven individuals in the interval within the second positivity of IEM or PCR.

For a curative effect we can't consider remission of the clinical manifestations, which is very often spontaneous, but mainly elimination of *B.b.s.l.* It is possible to state from the mentioned findings that in some individuals even a repeated treatment does not provide elimination of *B.b.s.l.* If the dominant complaints are markedly lower or non-specific, a post-Lyme syndrome diagnosis is often established. A repeated examination proving a possible *B.b.s.l.* persistence may have its meaning in these patients.

It is difficult to interpret positive *B.b.s.l.* findings in the cerebrospinal fluid together with a normal number of cells and biochemical parameters. In that case there are question marks "?" used in the column of Table 2 for neuroborreliosis. We assume that in these cases an affection of the nervous system might occur, because there were abnormal EEG and/or MR in eight of 9 patients who previously suffered from another clinical form of LD. As an abnormal EEG we consider the curve with the theta and delta waves and in MR occurrence of inflammatory changes in the white matter. An assessment of the individual cases is difficult because either EEG or MR in those sick was not performed in the past, and certainly these pathological findings are not specific for Lyme disease only.

A possible progression of Lyme disease concerning involvement of other organs after the first detection and an adequate treatment we have found in 6 cases. The mentioned clinical manifestations might have even another etiology than Lyme disease, but that has not been assessed within the frame of wider diagnostic procedures.

The results of antibody examination confirm experience with a problematic value of the specific antibodies against *B.b.s.l.* using ELISA kits, especially in chronic ill individuals.

CONCLUSION

Persistence of *B.b.s.l.* in the infected organism after a relevant antibiotic treatment has not been exceptional. In some individuals any of the recommended antibiotic treatment and regimen doesn't lead to elimination of *B.b.s.l.* A capability of the *B.b.s.l.* to persist in the macroorganism even after a repeated antibiotic therapy is necessary to further investigate not only from the view of particular properties of borreliae, but also from the view of the immune response of macroorganisms. It is not fully explicit whether the persistence must result in development of a clinically manifest disease in any individual person. An intermittent bacteremia detected months after the infection points to a possibility of affection of the organs, which hadn't been affected during "primary" bacteremia.

The detection of *B.b.s.l.* or its DNA in the cerebrospinal fluid and current absence of a classical inflammatory signs represent necessity to examine such material using PCR to detect DNA of *B.b.s.l.* together with examination of intrathecal production of antibodies against borreliae. This procedure should be also done in individuals who experienced neuroborreliosis with continuing non-specific symptoms. As appropriate we consider an examination of the cerebrospinal fluid using PCR method also in the patients with pathological findings on EEG or MRI, and concomitant presence of antibodies against borreliosis in the serum or with any other preceding manifestations of Lyme disease.

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