LABORATORY METHODS

Contribution to laboratory diagnostics of neuroborreliosis

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Abstract

Background: **Neuroborreliosis affects peripheral and central nervous system.**
Objectives: **Point out on possibilities of laboratory diagnostics of neuroborreliosis.**
Subjects and methods: **During 1997–2001 we tested 666 pair samples of CSF and serum from 661 patients with different neurological diagnosis by ELISA, Westernblot, PCR, completed by biochemical and cytological investigations.**
Results: **We confirmed intrathecal specific IgG antibodies production by AI in 14 cases (2.1 %) of total 666 samples tested. From those in 7 cases there were present also IgM antibodies in CSF. We found borderline AI values in 3 cases (0.5 %) and isolated intrathecal production, antibodies present only in CSF, in 1 case (0.15 %). There were normal AI values found in 25 cases (3.8 %). Specific antibody possitivity by WB method was detected only in one case. DNA positivity by PCR was detected in one CSF from 43 samples during 2 years period.**
Conclusions: **The microbiological test results should not be used in isolation but used in correlation with the biochemical and cytologic tests and also with clinical symptoms and epidemiological data to produce an overall clinical diagnosis.** ***(Tab. 7, Fig. 1, Ref. 21.)***
Key words: neuroborreliosis, CSF, antibody index (AI), westernblot, PCR

Lyme borreliosis (LB) is an inflamatory disease that affects many organs in human body. In our climatic connditions *Borrelia garinii* and *Borrelia afzelii* are the most frequent causative agents of human diseases. Transmission is realized mostly by ticks species *Ixodes ricinus* (ixoid ticks). The involvement of nervous system is possible in any phase of the illness. The common clinical symptoms are: cephalea, meningitis, head nerves paresis, polyradiculoneuritis with motoric and sensoric dysfunctions, or encephalitis manifestation too (Burgdorfer et al, 1982; Steere et al, 1983; Pachner et al, 1985). Involvement of nervous system is associated with malfunction of other organs, mostly joints and muscles aparatus and cardiovascular system (Bartunek et al, 1996; Dlouhy, 1996). The laboratory diagnostics of neuroborreliosis is based on clinical manifestations supported by laboratory tests and epidemiological data. Cytological, biochemical and imunochemical investigations and vizualization techniques are contributions to diagnostics of neuroborreliosis (NB) but the microbiological diagnostic has the most important signification. In clinical practise it is common to use the detection of specific intrathecally produced IgG antibodies by ELISA method, completed by IgM antibody detection. Confusing results of investigations are completed by westernblot (WB). Direct detection methods as cultivation, histology, electronmicroscopy methods, hybridization and PCR are used rarely. The aim of this work is to assign on possibilities of detection of specific and intrathecally produced antibodies by antibody index and limitation of this method. On the same examples we present the need of complete solution and evaluation of microbiological, biochemical and cythologic investigations in the laboratory diagnostics of neuroborreliosis.

Materials and methods

Since 1997 to 2001 we tested 666 pair samples of CSF (cerebrospinal fluid) and serum from 661 patients with different neu-

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Acknowledgment. Thanks to all physicians from Hospital and Health centre in Trnava for information about patients and collaboration.

Tab. 1. Review of patients with positive and equivocal results of specific antibody index (AI) in CSF and serum.

No.	Patient		Dg.	Serum specific Ab_{Bb}		CSF specific Ab_{Bb}		Laboratory result
	Age (yrs)	sex		IgM	IgG [U/ml]	IgM	AI_{Bb} IgG	
1.	55	F	G05	neg	94	neg	4,7	intrathecal production of antibodies
2.	9	F	G51	pos	74	pos	9,6	
3.	13	M	G98	eqv	11	pos	5,0	
4.	69	M	A69	pos	124	pos	14,0	
5.	11	F	A69	pos	14	neg	7,5	
6.	7	M	G05	pos	16	eqv	5,2	
7.	67	F	G00	pos	140	pos	5,2	
8.	75	F	G98	neg	47	neg	2,5	
9.	23	M	G01	neg	67	neg	9,0	
10.	59	M	G61	eqv	55	neg	2,6	
11.	43	F	G98	neg	85	neg	12,8	
12.	60	M	A69	pos	430	pos	2,0	
13.	57	M	G98	neg	77	neg	4,0	
14.	49	F	G98	pos	210	neg	1,5	suspect intrathecal production of antibodies
15.	43	F	G35	neg	89	neg	1,5	
16.	33	M	G35	eqv	6	neg	N1	
17.	40	F	G05	neg	neg	pos	N2	isolated intrathecal production of antibodies

Notes: pos – anti -Borrelia IgM are present
neg – anti-Borrelia IgM or IgG are not present
eqv – equivocal
N1 – AI_{BbIgG} is not determined because of low value IgG in serum and CSF -6 U/ml (patient No. 16)
N2 – AI_{BbIgG} is not determined because detection specific antibody only CSF – from the first punction 8 U/ml, from the second punction 19 U/ml (IgG), IgM positive in the first and the second punction (patient No. 17)
F – female
M – male
Dg – diagnosis (International Classification Diseases, the tenth revision)
IgG [U/ml] > 6 – positive
IgG [U/ml] = 4-6 – equivocal

rological diagnosis, hospitalized in: Department of Neurology, Children's University Hospital, Bratislava, Department of Infectious and Geographical Medicine, 1st Department of Neurology, University Hospital, Bratislava, Department of Neurology, Hospital Malacky, Nové Zámky and Komárno, Department of Neurology and Infectious Medicine, Hospital Trnava. Detections were made parallely in Biochemical Laboratory of Faculty Hospital Bratislava and Serological Laboratory HPL (Bratislava).

Determination of ELISA

We determined specific antibodies IgG and IgM in serum and CSF by ELISA method Enzygnost Borreliosis (Dade Behring Germany). The kit includes antigens prepared from European clinical isolate of *B. afzelii* (Pko tribe). IgG antibodies were tested quantitatively and IgM qualitatively. We used α-method after recommendation of producer for the determination of specific IgG antibodies. This α-method give us single-point quantification IgG antibodies from the measured extintion under test conditions. For the IgG antibody determination, CSF and serum was diluted by buffer containg ultrasonicate antigen from *Treponema phagedenis* to absorb nonspecific antibodies. Dilution was used according to total IgG, so that the final value IgG in CSF resulted to 1 mg/dl and in serum to 2 mg/dl. For the antibody index calculation the recommended value is 10–80 U/ml which is located in linear section of calibration curve (α-method). If these conditions are not fulfilled another dilution is recommended. We diluted CSF 1:2, serum 1:42 for IgM antibody determination (according to kit manual), absorption of rheumatoid factor was included too. We have expressed mathematicaly IgG antibody level by antibody index (AI). Antibody index is the concentration ratio between specific antibodies to total antibodies in CSF and serum. The function of the blood-CSF barrier must be taken into account for the calculation of results. Values of AI less than 1.5 were considered to be normal and it expressed antibody penetration from plasma into CSF. Values of AI from 1.5 to 1.9 were considered as borderline. For borderline findings the confirmatory testing or repeated sampling in suitable time period is recommended. The values of AI 2.0 and more, were considered as positive. Most probably, in this situation the specific antibodies are produced in CSF intrathecally. The absence of specific antibodies in CSF was indicated as negative finding. We interpreted our results (AI) according to recommendations of Enzygnost producer (Dade Behring).

Westernblot (WB)

We used Anti-*Borrelia-garinni*-Westernblot IgM and IgG (Euroimmun, Germany) kits. Antigens were obtained from suit-

able strain of *B. garinni* (Johnson et al, 1984; Lebech et al, 1994). The serum samples for analysis were diluted 1:51 for IgG and IgM, CSF 1:10 for IgG and IgM. These dilutions were made after instruction of producer. The sufficient amount of CSF (min 2.5 ml) is the limiting factor for this investigation.

Borrelia DNA confirmation

During 1999–2000 we examinated 43 CSF samples by PCR method. We used diagnostic kit DNA PCR *Borrelia burgdorferi* sensu lato (Biowestern, Czech Republic). This set specifically detects nucleic acid sequences in the part of OspA gene, localized in linear plasmide (49 kb). These specific parts are present in four genomspecies (*B. burgdorferi sensu stricto, B. garinii, B. afzelii, B japonica*) (Brettschneider et al, 1998). This method is based on two-stage nucleic acid amplification with electrophoretic evalution of its final products.

Detections made parallely in biochemical laboratory:

CSF cytologic testing: counting of the elements in Fuchs-Rosenthal chamber and their differentiation by cytosedimentative method (Sayk, 1954; Sayk, 1962) followed by May-Grünwald a Giemsa-Romanovsky staining (Adam et al, 1998).

Basic biochemical parameters in CSF and serum: nephelometric determination of total protein count (nephelometer BN 100, Dade Behring, Germany) by 20 % TCA, GOD-POD determination of glucose (glucosidase and peroxidase enzyme catalysis, BIOLACHEMA-TEST, Czech Republic) and chloride measurements by the technology of ion-selective electrodes (EasyLyte PLUS).

Immunochemical CSF and serum testing: nephelometric determination of IgG immunoglobulines and albumin count (nephelometer BN 100, Dade Behring, Germany) with calculation by Delpech-Lichtablau (D/L) protein index (Delpech and Lichtblau, 1972).

Results

ELISA. We confirmed intrathecal specific IgG antibodies production by AI in 14 cases (2.1 %) of total 666 samples tested. From those in 7 cases there were also IgM antibodies present in CSF. We set borderline AI values in 3 cases (0.5 %) and isolated intrathecal production, e.g. antibodies present only in CSF, in 1 case (0.15 %). The diagnosis – neuroborreliosis was determined in these patients because the production of specific antibodies in CSF and serum correlated with other laboratory tests and clinical manifestations (Tab. 1). There were normal AI values found in 25 cases (3.8 %), the diagnosis – neuroborreliosis was not confirmed (Fig. 1). Overview of complete diagnostics is shown in patient No. 6 (from Tab. 1) with AI positivity in repeated lumbal punctions (Tabs 2, 3 and 4). Antibody index was not determined in the first punction because of low IgG value in serum (Tab. 2). In the next punctions we can see antibody IgG and IgM dynamics in serum and CSF. Investigation by WB and PCR methods was not provided because of insufficient amount of CSF. Biochemical determination (Tab. 3) confirms intrathecal production of IgG antibodies, disturbation of the blood-CSF barrier which was repared in a time of the second punction. Total protein level in the second punction was significantly lower in comparison with the first punction. Lymfocytary pleocytosis with presence of plasmatic cells completes the characteristic figure in case of neuroberreliosis (Tab. 3). For extention to this case we show brief clinical anamnesis. Child 7 years old was bitten in nape by unknown vector. Child was hospitalized because of cephalea, fever, upper respiratory infection, laquidness, weakness. The patient had high fevers and meningeal symptoms and therefore lumbal punction was performed. Diagnosis was concluded as serosal meningitis. Ceftriaxon was administered to this patient. The child in good condition was sent home with final diagnosis of meningoencephalitis – borrelia etiology.

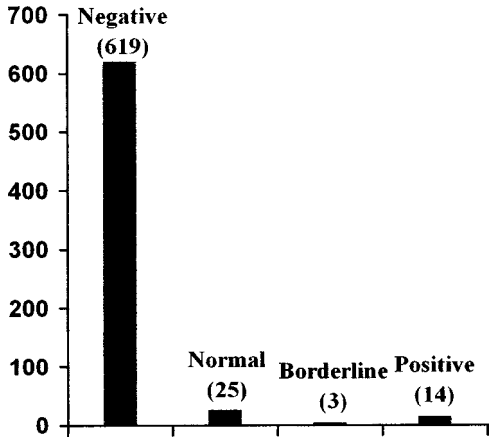

Fig. 1. AI_{BbIgG} values in samples of CSF and serum in 661 patients with various neurological symptoms in 1997—2001. Notes: normal value AI<1.5, bordeline value AI≥1.5<2.0, positive AI≥2.0, negative – not determined specific IgG and IgM in CSF.

WB method

For this method we used pair samples of CSF and serum. In case of positive finding of AI by ELISA method we confirmed their specificity by WB. We present example of WB positivity without detection of antibodies by ELISA (Tab. 5). It was the case of 11 years old boy and tick bite was not found. He was

Tab. 2. Profile of serologic investigations in patient 6.

	Serum (ELISA)		CSF (ELISA)		AI	Serum (WB)		CSF (WB)	
Sampling Date	IgM	IgG	IgM	IgG		IgM	IgG	IgM	IgG
3.8.1999	pos 0,684	eqv 5	pos 0,969	pos 17	N	-	-	-	-
18.8.1999	pos 0,804	pos 16	eqv 0,388	pos 16	5,2	-	-	-	-
8.9.1999	pos 1,545	pos 37	pos 0,553	pos 43	7,3	-	-	-	-
28.10.1999	eqv 0,380	pos 16	neg	pos 20	5,0	OspC p30 p50	p41	-	p41

Notes: Equivocal value for positivity for IgM: 0,402
for IgG: 6 U/ml
AI - ≥ 2,0
N – nondetermined AI for low value IgG in serum
• highly specific antigens – OspC, p 30
• genus-specific antigen – p41
• cross-reacting and undefined antigen – p50

Tab. 3. Profile of biochemical investigations in patient 6.

	CSF					Serum		D/L	Area No.
Samplin Date	Total Proteins (mg/l)	Glykorhachia (mmol/l)	Chlorides (mmol/l)	IgG (g/l)	Albumin (g/l)	IgG (g/l)	Albumin (g/l)		
3.8.1999	1020	2,51	119,9	0,198	0,982	12,340	45,56	0,747	4
18.8.1999	500	2,78	120,0	0,055	0,278	9,877	44,20	0,885	5
8.9.1999	410	2,80	120,6	0,050	0,240	11,170	44,87	0,840	5
3.10.1999	300	2,81	121,1	0,038	0,161	12,080	42,44	0,830	5

Notes (Tabs 3 and 6):
Normal values: total proteins – 150,0 - 430,0 mg/l; chlorides – 120,0 – 128,0 mmol/l; glykorhachia – 2,5 – 4,5 mmol/l; IgG in CSF – up to 0,040 g/l; D/L (Delpech-Lichtblau – protein quotient) - > 0,7 – intrathecal IgG production

Area: graphical demonstration of CSF proteins profile
4 – disorder of the blood-CSF barrier with encreased permeability for big molecule proteins with intrathecal production of IgG
5 – isolated intrathecal IgG production without disorder of the blood-CSF barrier

Tab. 4. Continuation of biochemical investigations and profile of cytologic investigations in patient 6.

	Biochemical tests		Cytologic tests		
Sampling Date	Oligoclonal composition gama-globulins	Gama-globuins (ELFO) in relative %	No. of cells: Mononuclears	No. of cells: Polynuclears	Differenciation of cells
3.8.1999	present	21,5	99 / 3	13 / 3	monocytes 10% lymphocytes 58% plasmatic cells 17% lymphoplasmatic cells 15%
18.8.1999	present	17,4	54 / 3	9 / 3	-
8.9.1999	present	15,3	38 / 3	5 / 3	-
3.10.1999	present	14,1	17 / 3	3 / 3	monocytes 20% lymfocytes 63% plasmatic cells 8% lymphoplasmatic cells 9%

Notes: (Tabs 4 and 7):
normal: non present oligoclonal composition
normal gama-globulins values by ELFO: 11,4 rel. %
normal element values: in to 15 / 3
normal CSF sediment : monocytes 10 – 30 %, lymphocytes 70 – 90%
(Seyko´s chamber, May-Grünwald stain to 100 cells)

Tab. 5. Profile of serologic investigations of 11-years old boy.

	Serum (ELISA)		CSF (ELISA)		Serum (WB)		CSF (WB)	
Sampling Date	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
19.10.1999	negat	negat	negat	negat	p41	OspA OspC p18 p39 p41	negat	p18 p39 p41 p83

Notes:
• highly specific antigens – OspA, OspC, p18, p39, p83
• genus-specific antigen – p41

hospitalized because of high fever and intensive headache. On the basis of CSF screening the patient's state was evaluated as serosal meningitis with the presence of lymphocytary pleocytosis and plasmatic cells (Tab. 7). Intrathecal IgG production and disorders of the blood-CSF barrier were confirmed by biochemical tests (Tabs 6 and 7). Serological tests (WB) supported the diagnosis of meningitis – borrelia etiology.

PCR method

We detected positivity of borrelia DNA in one case (2.3 %) of 63 years old women with sensitive polyneuropathy. The results of biochemical and cytological tests were normal and specific antibodies by ELISA and WB were not found. Infectological and neurological investigations didn't confirm the diagnosis of neuroborreliosis. Other samples of CSF were negative by PCR. The diagnosis – neuroborreliosis was not determined and the intrathecal production of specific antibodies was not proved in these patients.

Discussion

The aim of our work was to find the asset of several laboratory methods for diagnostics of neuroborreliosis. It is necessary to confirm intrathecal origin of specific antibodies (Bojar, 1996; Duniewicz et al, 1999). AI (antibody index) is the most sensitive indicator of intrathecally produced specific antibodies (Reiber et al, 1991; Felgenhauer et al, 1992). AI allows to distinguish nonpathological – from plasma coming fraction of specific antibodies and pathological – from CNS coming fraction of specific antibodies using Q_{lim} formula. Polyspecific imunological response must be taken into account (Reiber et al, 1991). Antibodies penetrating from plasma into CSF (AI≤1.4) were found in 25 patients. We received values AI between 0.6–1.3. Positive values of AI (AI>2.0) were detected in 14 patients and borderline AI (1.5<AI<1.9) in 3 patients. The same authors presented values of AI higher than 1.4 as pathological. Values of AI 0.7–1.3 are considered to be normal (Reiber, 1995; Reiber, 1998). Another authors use different reference values for AI in dependence of the method used (Wilske et al, 1986; Hučková et al, 1999).

To distinguish acute, chronic and recovering disease using AI is not possible in isolation. Therefore, it is necessary to evaluate it in the context with clinical symptoms and other CSF parameters. Combined analysis includes specific AI and other basic CSF parameters. The sensitivity and specifity of this analysis was 80 % and 98 % (Tumani et al, 1995). In one case of our group of patients (clinicaly confirmed neuroborreliosis) we repeatly detected isolated intrathecal production – IgM and IgG were present in CSF. Bojar made a mention of possibility of isolated synthesis also (1996). We couldn't detect AI in these cases. AI can not be determined because of low values of specific antiobodies in CSF or serum, too. The only AI positivity of specific antibodies is not the criterion for beginning, continuation or repeating of antibiotic therapy. This AI positivity can be detected many years (10–15) after successful treatment of neuroborreliosis (Reiber, 1995). The expressive decrease of pleocytosis including activated B lymphocytes and normalisation of the blood-CSF barrier are auxiliary laboratory criteria for the success of antibiotic therapy (Felgenhauer, 1998). Only in one case 4 punctions of CSF were made during 3 months (Patient 6). Higher values of AI persisted over this period. The therapy was successful – disturbance of the blood-CSF barrier returned to normal, pleocytosis and the level of total protein fell down.

In comparison to ELISA method, WB is more sensitive and specific. WB allows detection of antibodies to individual borrelia antigens (Hulínská, 1997). It can be used to distinguish from the non-specific intrathecal antibodies (Tumani et al, 1995), and in case of negative finding by ELISA. The method of WB was succesfully used in a case of 11 years old patient. In case of this patient was the IgM and IgG positivity found in serum and IgG positivity in CSF by WB method. Detection of specific antibod-

Tab. 6. Profile of biochemical investigation of 11-years old boy.

	CSF					Serum		D/L	Area No.
Sampling Date	Total Proteins	Glykorhachia (mmol/l)	Chlorides (mmol/l)	IgG (g/l)	Albumin (g/l)	IgG (g/l)	Albumin (g/l)		
28.10.1999	501	3,5	120,1	0,075	0,356	10,04	38,12	0,799	4

Tab. 7. Continuation of biochemical investigations and profile of cytologic investigations of 11-years old boy.

	Biochemical tests		Cytologic tests		
	Oligoclonal composition gama-globulins	Gama-globulins (ELFO) in relative %	No. of cells		Differenciation cells
Sampling Date			Mononuclears	Polynuclears	
28.10.1999	present	20,4	58 / 3	4 / 3	monocytes 30% lymphocytes 50% plasmatic cells 8% lympfoplasmatic cells 10% Non –segmented elements 2%

ies by ELISA was negative. These experiences are presented also by Schwarzová et al (2001). In patients with clinicaly confirmed diagnosis of neuroborreliosis was borrelia detected in CSF by cultivation method. WB was positive and ELISA was negative. PCR advantages of direct detection of active borreliosis are: time saving, specifity for species and higher sensitivity than in using other methods. Detection of chromozomal DNA *B. burgdorferi* signify presence of death or alive spirocheates. The positivity of DNA in correlation with acute clinical manifestations definitely supports the diagnosis. Detection of plasmid DNA *B. burgdorferi* in CSF means plasmid excretion in to body fluids or means death and degradation of borrelia. In later phase of neuroborreliosis it is problematic to evaluate positive findings in both types of PCR. Biodegradation time of borrelia DNA is not known in the organism, and autoimmune process can play role too. PCR has methodological problems too: antigen variability of European borrelia, high contamination risk, cross reactivity of DNA sequences, presence of inhibitors in body fluids (Hulínská et al, 1999). In 63 years old woman we detected DNA PCR Bb positivity but without clinical proof of neuroborreliosis. This positivity should be due to cross reactivity, contamination or other reason. On the basis of our experience we come to the conclusion that the detection of intrathecally produced specific antibodies by AI has high significance and the microbiological test results should be used in correlation with the biochemical and cytologic tests and also with clinical symptoms and epidemiological data to produce an overall clinical diagnosis.

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Received November 19, 2002.
Accepted February 27, 2003.