Chapter 4

High sensitivity and specificity of C6-peptide ELISA on CSF in Lyme neuroborreliosis patients

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Abstract

LNB is a serious but treatable disease. Diagnosing LNB poses a challenge to clinicians and improved tests are needed. C6-peptide ELISA is frequently used on serum but not CSF. Data about sensitivity of C6-peptide ELISA in CSF in patients suffering from LNB has been conflicting.
Serum-CSF pairs from 59 LNB patients, 36 Lyme non-neuroborreliosis cases, 69 infectious meningitis/encephalitis controls and 74 neurological controls were tested in a C6-peptide ELISA.
Using the optimal cut-off of 1.1, sensitivity of the C6-peptide ELISA for LNB patients in CSF was 95% and specificity was 83% in the Lyme non-neuroborreliosis patients, 96% in the infectious controls and 97% in the neurologic controls
These results suggest that C6-peptide ELISA has a high sensitivity and good specificity for diagnosing Lyme neuroborreliosis patients in CSF. The C6-peptide ELISA can be used on CSF in a clinical setting to screen for LNB.

Introduction

Lyme neuroborreliosis (LNB) is the neurologic manifestation of an infection with the tick-borne spirochete *B. burgdorferi* sensu lato (sl). LNB can present with many neurological signs, varying from facial nerve paralysis and Bannwarth's syndrome to a range of neurological disorders [114, 559]. Diagnosing Lyme neuroborreliosis poses a challenge to clinicians. Detecting *B. burgdorferi* sl directly by culture or by PCR from cerebrospinal fluid only yields a maximum sensitivity of about 50% [235]. A standard for diagnosing LNB is determining intrathecal specific antibody index (AI), despite the fact that the sensitivity of AI has been reported to vary from 48-92% [354, 356].
A peptide of interest for diagnosing LB has been the immunoreactive peptide C6 (IR6), a highly conserved peptide among different *B. burgdorferi* sl [339].
C6-peptide is the sixth invariable region of the VlsE protein. The vls locus consists of 15 silent vls cassettes and the gene for the VlsE lipoprotein. By application of unidirectional recombination events VlsE can display antigenic variation [57]. The C6-peptide has been shown to be an immunodominant peptide [341]. IgG antibodies to C6-peptide have been shown to be detectable as early as two weeks post-infection and antibodies wane over time after treatment [339, 347]. Sensitivity and specificity of C6 ELISA in serum has been reported to be equal, if not superior, to 2-tier testing in North American patients [234, 310]. C6-peptide serology has been implicated to have a high sensitivity in LNB patients, varying from 67 to 100% [345, 560]. The commercially available C6-peptide ELISA has only been validated for serum samples. Data on performance of the C6-peptide ELISA performed on CSF for diagnosing LNB is limited and conflicting [360-362]. The aim of this study was to determine whether a C6-peptide based ELISA can be used on CSF samples to diagnose early and late LNB patients, using a large cohort of well-defined patients and controls.

Materials and methods

Selection of clinical specimens and control samples

Patients and controls from the time period between January 2004 and October 2009 were identified retrospectively using the laboratory information management system from the Leiden University Medical Center (Leiden), OLVG Hospital (Amsterdam), IZORE Center for Infectious Diseases (Leeuwarden), Academic Medical Center Amsterdam (Amsterdam), and the Isala clinic (Zwolle). Cerebrospinal fluid (CSF)-serum pairs from 59 LNB patients were included. Criteria for diagnosing LNB patients were four of the following five

LNB criteria: 1; detection of *B. burgdorferi* antibodies in serum, 2; CSF pleocytosis (>5/µl), 3; absence of other evident cause of meningitis, 4; evidence of intrathecal production of specific *B. burgdorferi* antibodies 5; objective neurological complaints with favorable outcome after treatment [414]. Thirty-six CSF-serum samples were available from Lyme borreliosis (LB) patients that did not have LNB according to the applied algorithm. The LB patients' group consisted of 12 recent erythema migrans (EM) patients, 21 Lyme arthritis patients and 3 acrodermatitis chronica atrophicans (ACA) patients. CSF and serum samples were available from 69 patients with other infectious diseases, 62 CSF-serum pairs were collected from neurological inflammatory diseases and 12 CSF-serum samples were collected from patients with neurological complaints including dizziness, headache and fatigue without evident diagnosis and trauma patients (See table 1). Additional data was collected for all patient groups; age at presentation, sex, duration of illness (>6 months was classified as late LNB), CSF findings at diagnosis (intrathecal leukocytes and erythrocytes/µl, percentage mononuclear cells, glucose level, total protein, IgG and albumin). For LNB patients the clinical presentation, duration of complaints and report of an EM was documented.

C6-peptide ELISA

All sera and CSF samples were tested in the C6 Lyme ELISA Kit (Immunetics, Boston USA). Preliminary results showed good performance of a 1:5 dilution for CSF. Therefore and for practical reasons all CSF samples were tested in a 1:5 dilution continuing thereafter with the manufacturer's protocol for serum. C6-peptide ELISA was performed on sera according to manufacturer's protocol. The Lyme Index value (LI) was calculated according to the manufacturers' protocol: $Absorbance_{450\text{-}650nm}$ sample/ (($Absorbance_{450\text{-}650nm}$ calibrator) plus 0.3). Samples with LI values < 0.9 are considered negative, 0.9-1.1 equivocal and values≥ 1.1 positive for antibodies against C6-peptide in serum.

Antibody index

All sera and CSF samples were tested with the IDEIA^{TM} Lyme Neuroborreliosis kit according to manufacturer's protocol (OXOID, Cambridgeshire, UK). Antibody index (AI) was calculated as (OD_{csf}/OD_{serum}) * (OD_{csf}-OD_{serum}). The CSF contained IgG or IgM if the OD_{csf} IgG or IgM is > 0.150. The AI was positive when the CSF was positive and the AI_{IgG} or AI_{IgM}≥ 0.3.

Statistical analysis

Statistical analysis was performed using a statistical software package (SPSS for windows, version 17.0). The Student's t-test was used to compare levels of C6-peptide LI between groups. P-values <0.05 were considered significant.

Results

Patient characteristics

All patient serum and CSF samples were tested according to protocol. Patient epidemiologic data are represented in Table 1. The group of LB and LNB patients showed a bimodal distribution of age with a peak in childhood and a peak at 55 years. Of the 59 LNB cases 20% reported an EM at presentation.

	n	Male/Female (%	Mean age yrs (SD)	Mean CSF leukocyte count (/µl CSF) (SD)
Lyme neuroborreliosis (LNB)	59	60/40	39 (24)	135 (159)
Lyme borreliosis (LB)	36	50/50	51 (17)	1(1)
Infectious meningitis/encephalitis controls	69			
T. pallidum	12	83/17	40 (8)	40 (79)
C. neoformans	2	50/50	52 (6)	94 (89)
Bacterial meningitis				
S. pneumoniae	2	50/50	41 (6)	337 (99)
L. monocytogenes	1	0/100	61	1280
M. tuberculosis	1	0/100	4	25
Viral meningitis/encephalitis				
HIV	6	50/50	43 (8)	51 (45)
VZV	11	45/55	51 (23)	130(173)
HSV1	6	33/67	55 (30)	46 (51)
Enterovirus	23	61/39	13 (17)	271 (381)
Parechovirus	3	0/100	0 (0)	1 (1)
TBE	2	50/50	37 (4)	59 (12)
Neurologic controls	74			
Facial nerve paralysis eci	19	66/34	48 (18)	40 (145)
Multiple sclerosis	26	35/65	35 (14)	15 (17)
Polyneuritis/polyneuropathy	16	56/44	45 (17)	17 (22)
ADEM	1	0/100	21	266
Neurologic non-inflammatory controls	12	25/75	47 (13)	4 (6)

Table 1: Epidemiological characteristics of patients and baseline cerebrospinal fluid (CSF) leukocyte count (per µl).

Clinical presentation consisted most frequently of facial nerve paralysis (58%) and meningoradiculitis (27%), the remainder of the cases presented with malaise and headache (10%), meningoencephalitis and a sensation of altered vision with papilloedema. Most patients had an early disseminated LNB (53/59). Four of the six patients with a late LNB presented with a meningoradiculitis with duration between 6 months and 2 years. Two patients had complaints for 6 to 18 months of an altered gait with MRI abnormalities. Ninety-five percent of patients presented with pleocytosis. Only two patients presenting with early LNB, one facial nerve paralysis and one meningoradiculitis, and one patient presenting with late LNB, meningoradiculitis, did not have pleocytosis. These three patients eventually all had antibodies against *B. burgdorferi* in serum and CSF and the AI was positive. Furthermore, they all responded favorably to treatment.
The AI in the IDEIA neuroborreliosis detected anti-Borrelia IgG or IgM in 78% of the LNB patients. IgG AI was positive in 75% and IgM AI in 49% of LNB patients (Table 2).

	Negative (%)	IgM +	IgG +	IgM-index +	IgG-index +	Ig + (%)	Ig-index + (%)	Total n
Lyme neuroborreliosis	1 (2)	52	49	29	44	58 (98)	46 (78)	59
Lyme borreliosis	26 (72)	3	8			10 (38)		36
Infectious controls	61 (88)	7	1	2		8 (12)	2 (3)	69
Neurologic controls	70 (95)	3	1	1		4 (5)	1 (1)	74

Table 2: Results of the antibody index from the IDEIA. Represented are the number of samples that are negative or positive for IgM and IgG against flagellin in CSF and the index calculated as described. Ig and Ig index are the results of the IgM and IgG results combined per patient.

C6-peptide ELISA results on serum

The results for the C6-peptide ELISA are shown in figure 1. C6-peptide antibodies were detected in serum in 98% of the LNB patients with a mean LI value of 8.4 (95% CI 7.7-9.1). The one C6-peptide ELISA negative patient was a young child with an early LNB presenting with a facial paralysis with an elevated CSF leukocyte count (236/µl). In this patient the CSF showed detectable antibodies in the C6-peptide ELISA (LI=8.7) as well as a positive AI in the IDEIA for IgG and IgM. The patients that presented with an early LNB had a comparable LI value in serum as the patients that presented with a late LNB with a respective mean LI 8.3 and mean LI 9.1 (p=0.5). In the non-

neuroborreliosis LB patient group the sensitivity was 97%, mean value LI=6.9 (95% CI 5.6-8.2). In all other controls the C6-peptide seroprevalence was 5%.

C6-peptide ELISA results on CSF

Sensitivity and specificity for the C6-peptide ELISA on CSF are shown in table 3. C6-peptide ELISA on CSF detected antibodies in 95% (56/59) of the LNB patients.

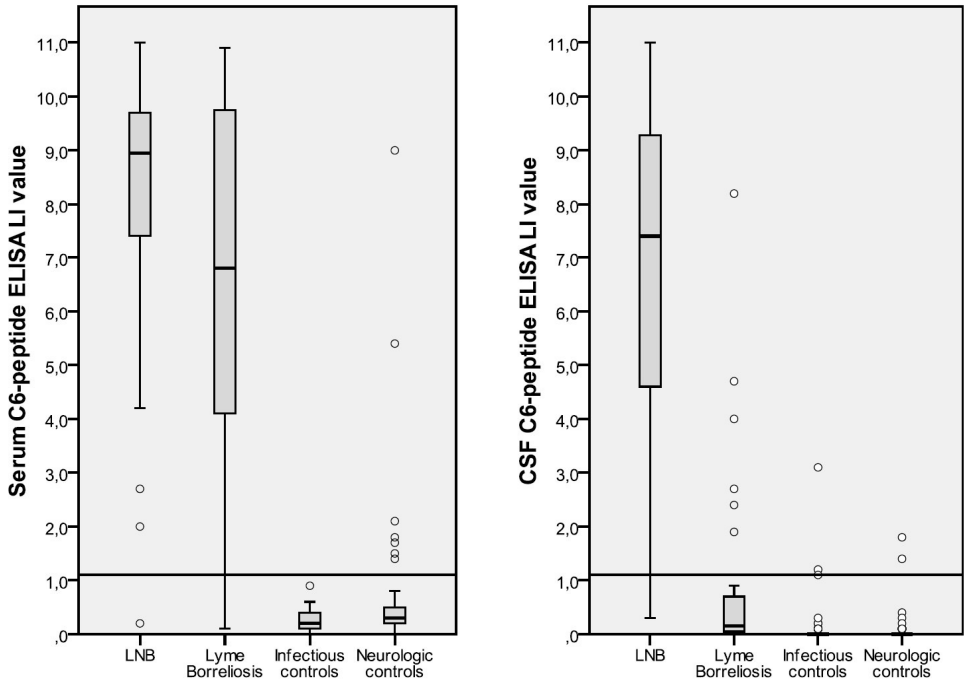

Figure 1: Values for C6 ELISA in serum and CSF. Horizontal lines indicate medians, bars represent interquartile ranges, and lines represent 95% confidence interval and bullets represent outliers. The reference line is located at the cut-off for detection of antibodies (LI=1,1).

The patients that presented with an early LNB had a lower LI value in CSF than the patients that presented with a late LNB with a respective mean of LI 6.6 (95% CI 5.6-7.3) and mean LI 8.6 (95%CI 7.3-10.3) ($p<0.01$). Two patients did not have detectable antibodies in the CSF, a child and an adult. Both had early LNB with facial nerve paralysis at presentation. The adult patient presented with a right facial paralysis but did not have pleocytosis at presentation and a negative AI. Antibodies against C6-peptide were already present in the serum at presentation (LI=9.2). Diagnosis was later substantiated when he presented with bilateral facial paralysis and conclusive CSF serology. The child had

antibodies against C6-peptide (LI=9.4) and IgM was detected in the IDEIA, but both AI were negative in the CSF. At presentation she had a pleocytosis of 56/µl CSF and she responded rapidly to treatment. The third patient, a borderline (LI=0.9) positive patient had a pleocytosis of 31 leukocytes/µl and complaints of dysarthria with a high IgM AI and an elevated IgG AI. Antibodies against C6-peptide in serum were detectable (LI=6.8).

	Anti-C6-peptide negative (%)	**Equivocal (%)**	**Anti-C6-peptide positive (%)**	**Total**
Lyme neuroborreliosis	2 (3)	1 (2)	56 (95)	59
Lyme borreliosis	29 (81)	1 (3)	6 (17)	36
Infectious controls	66 (96)		3 (4)	69
Neurologic controls	72 (97)		2 (3)	74

Table 3: LI values of C6-peptide ELISA in CSF. Samples with LI values < 0.9 are considered negative for antibodies against C6-peptide, 0.9-1.1 equivocal and values≥ 1.1 positive for antibodies against C6-peptide.

Specificity in all controls varied from 83-97% (See table 3). Specificity was high in the infectious and neurologic control group (96% and 97% respectively). In the infectious control group there were no controls with detectable antibodies against C6-peptide in the serum, but three controls had detectable levels in the CSF. These controls were an enterovirus meningitis, a neurosyphilis and an HIV meningitis patient. In the neurologic control group seven patients had detectable antibodies against C6-peptide in serum; these were five MS patients and 2 Guillain-Barré patients. In the CSF two MS patients had low detectable antibodies against C6-peptide (LI=1.4-1.8). In the LB group alone the specificity was 83% (30/36). Values of the C6-peptide ELISA in CSF were significantly higher in the LNB than in the LB cases (mean LI= 6.7 (95% CI 6.0-7.6) and LI=0.8 (95% CI 0.3-1.4) respectively, $p<0.05$). Lowering the LI threshold to 0.5 would enhance sensitivity to 97%, but lower specificity to 63% in LB patients. No effect was seen on the specificity in the other controls.

Discussion and conclusions

In this study we evaluated the C6-peptide ELISA on CSF for diagnosing a LNB infection. We found a high sensitivity as well as a good specificity of the C6-peptide ELISA.

We chose an algorithm defining LNB patients where a LNB patient could either have absence of pleocytosis or absence of intrathecal antibody production in an abundance of other criteria which made LNB evident. C6-peptide serology has a good sensitivity in the Lyme neuroborreliosis patients, varying from 67 to 100% in serum [314, 345, 360, 560]. The lower sensitivities were mainly reported in very early LNB when duration of symptoms was less than 8 days. The sensitivity of C6-peptide ELISA on serum of LNB patients was 98% in this study. Serum from one child with early LNB was negative for anti-C6-peptide antibodies in the ELISA. In previous studies it was demonstrated that patients with LNB can have an early response to the flagellin antigen which can be detectable earlier intrathecally than in serum, leading to reports of seronegative LNB [359, 366]. This finding had not been substantiated for the C6-peptide ELISA until now.
In this study we found 95% sensitivity of the C6-peptide ELISA on CSF for diagnosing LNB. Previously two European publications using the C6-peptide ELISA have determined the sensitivity of the C6-peptide ELISA on CSF and the data has been conflicting. Skarpaas et al used undiluted CSF and a cut-off of OD=0.5, which is comparable to the LI value of 0.9 compared to OD/cut-off standard used in the present kit. This cut-off is comparable to the borderline cut-off in our study. Prospectively, sixty adult LNB patients, defined as clinical LNB, pleocytosis and evidence of intrathecal anti-Borrelia IgG production by ELISA, were tested in the C6-peptide ELISA and a sensitivity of 98% on CSF was found. The C6-peptide ELISA was also performed on CSF from 42 controls in whom the specificity was 88% [360].
Another study used diluted and undiluted CSF with a LI cut-off of 0.5 and 1 [362]. Retrospectively 31 tentative LNB were identified by evidence for intrathecal antibody production by western blot. Twenty-eight LNB patients were identified according to clinical presentation and concurrent clinical response to antibiotic treatment. Sensitivity of the C6-peptide ELISA in these patients was only 61%, which is lower than the previously reported sensitivity and our findings. The low sensitivity found in that study may be explained by inclusion of non-LNB patients in the study group. Clinical data, including CSF findings, were not provided. Furthermore the use of immunoblots to determine intrathecal antibody responses is problematic and can lead to overdiagnosis [300, 376, 378]. In addition, it has been reported that up to 20 percent of patients that have detectable antibodies against *B. burgdorferi* sl and respond to treatment do not have LNB but have other self-limiting conditions [370, 561]. It is likely that the low sensitivity of C6-peptide ELISA that has been reported results from a poorly defined LNB patient group.

Specificity of the C6-peptide ELISA on CSF for detecting LNB was 88% in previously reported studies. In the current control group the specificity varied

from 83-97% with lowest specificity in the LB patient group (83%). In the infectious and neurological control group the specificity was 96% and 97% respectively. Passively acquired antibodies from the serum can be an explanation for the detectable anti-C6-peptide antibodies in the CSF. However, in the infectious control group none of the analyzed controls with antibodies in the CSF had anti-C6-peptide antibodies in the serum. In the neurologic control group only two MS patients had detectable antibodies in the CSF. Production of polyclonal Ig in the CSF due to MS might also be an explanation for the false positive result in these patients. Calculating the C6-peptide AI with the IgM/IgG C6-peptide ELISA was not possible because it was a combined IgM and IgG ELISA. Because no actual AI could be calculated, specificity of the C6-peptide ELISA with CSF will by definition be suboptimal in patients with detectable anti-Borrelia antibodies in the serum because no correction is made for passively acquired antibodies in the CSF.

A shortcoming of this study is that it is a retrospective study which might have accounted for a selection bias. The strength of this study however is the number and wide variety of the controls. Many samples were selected from patient groups from whom the clinical presentation could be mistaken for LNB. The LNB and control groups also included patients from all age groups. Based on the previous publications, the specificity of the C6-peptide ELISA for diagnosing LNB on CSF was insufficiently investigated.
In interpreting serology results a combination of duration of complaints, patient history and knowledge of laboratory parameters, as for instance pleocytosis in LNB, is essential to reach correct diagnosis. In a Lyme endemic region antibodies can be detected in patients who do not suffer from neuroborreliosis. When faced with the clinical situation wherein diagnosis of a LNB seems less probable, with detectable anti-C6-peptide in the CSF, it can be useful to use a more specific assay like calculating a specific AI.
In conclusion, we show a good sensitivity and specificity of the C6-peptide ELISA on CSF. The C6-peptide ELISA is a reliable screening test that can be used in serum and CSF to assist in the diagnosis of LNB.

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