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High sensitivity and specificity of the C6-peptide ELISA on cerebrospinal fluid in Lyme neuroborreliosis patients

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Abstract

Lyme neuroborreliosis (LNB) is a serious but treatable disease. The diagnosis of LNB poses a challenge to clinicians, and improved tests are needed. The C6-peptide ELISA is frequently used on serum but not on cerebrospinal fluid (CSF). Data on the sensitivity of the C6-peptide ELISA in CSF in patients suffering from LNB have 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. With the optimal cut-off of 1.1, the sensitivity of the C6-peptide ELISA for LNB patients in CSF was 95%, and the specificity was 83% in the Lyme non-neuroborreliosis patients, 96% in the infectious controls, and 97% in the neurological controls. These results suggest that the C6-peptide ELISA has a high sensitivity and good specificity for the diagnosis of LNB patients in CSF. The C6-peptide ELISA can be used on CSF in a clinical setting to screen for LNB.

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Introduction

Lyme neuroborreliosis (LNB) is the neurological manifestation of an infection with the tick-borne spirochete *Borrelia 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 [1,2]. The diagnosis of LNB poses a challenge to clinicians. Detecting *B. burgdorferi* sl directly by culture or by PCR from cerebrospinal fluid (CSF) yields a maximum sensitivity of only about 50% [3]. A standard method for diagnosing LNB is determination of the intrathecal specific antibody index (AI), despite the fact that the sensitivity of the AI has been reported to vary from 48% to 92% [4,5].

A peptide of interest for diagnosing LNB has been the immunoreactive peptide C6, a highly conserved peptide among different *B. burgdorferi* sl [6].

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 [7]. The C6-peptide has been shown to be an immunodominant peptide [8]. IgG antibodies to C6-peptide have been shown to be detectable as early as 2 weeks post-infection, and antibodies wane over time after treatment [6,9]. The sensitivity and specificity of the C6-peptide ELISA in serum have been reported to be equal, if not superior, to those of two-tier testing in North American patients [10,11]. C6-peptide serology has been shown to have high sensitivity in LNB patients, varying from 67% to 100% [12,13]. The commercially available C6-peptide ELISA has been validated only for serum samples. Data on the performance of the C6-peptide ELISA performed on CSF for the diagnosis of LNB are limited and conflicting [14–16]. 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 by use of the laboratory information management system from the Leiden University Medical Centre (Leiden), OLVG Hospital (Amsterdam), the IZORE Centre for Infectious Diseases (Leeuwarden), the Academic Medical Centre Amsterdam (Amsterdam), and the Isala clinic (Zwolle). CSF–serum pairs from 59 LNB patients were included. Criteria for diagnosing LNB patients were four of the following five: (i) detection of *B. burgdorferi* antibodies in serum; (ii) CSF pleocytosis (>5/μL); (iii) absence of other evident cause of meningitis; (iv) evidence of intrathecal production of specific *B. burgdorferi* antibodies; and (v) objective neurological complaints with favourable outcome after treatment [17]. Thirty-six CSF–serum samples were available from Lyme borreliosis (LB) patients who did not have LNB according to the applied algorithm. The LB patient group consisted of 12 recent erythema migrans (EM) patients, 21 Lyme arthritis patients, and three acrodermatitis chronica athrophicans patients. CSF and serum samples were available from 69 patients with other infectious diseases, 62 CSF–serum pairs were collected from patients with neurological inflammatory diseases, and 12 CSF–serum pairs were collected from patients with neurological complaints, including dizziness, headache and fatigue without evident diagnosis, and trauma patients (Table 1). Additional data were collected for all patient groups: age at presentation, sex, duration of illness (>6 months was classified as late LNB), and CSF findings at diagnosis (intrathecal leukocytes and erythrocytes per microlitre, percentage of mononuclear cells, glucose level, total protein, IgG, and albumin). For LNB patients, the clinical presentation, duration of complaints and report of an EM were documented.

C6-peptide ELISA

All sera and CSF samples were tested with the C6 Lyme ELISA Kit (Immunetics, Boston, MA, 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 with the manufacturer's protocol for serum. C6-peptide ELISA was performed on sera according to the manufacturer's protocol. The Lyme index (LI) was calculated according to the manufacturer's protocol: absorbance$_{450–650\ nm}$ sample/[(absorbance$_{450–650\ nm}$ calibrator) + 0.3]. Samples with LI values <0.9 were to be considered negative, those with LI values 0.9–1.1 equivocal, and those with LI values ≥1.1 positive for antibodies against C6-peptide in serum.

AI

All sera and CSF samples were tested with the IDEIA Lyme Neuroborreliosis kit, according to the manufacturer's protocol (Oxoid, Ely, UK). The AI was calculated as (optical density (OD)$_{CSF}$/OD$_{serum}$) × (OD$_{CSF}$ − OD$_{serum}$). The CSF was considered to contain IgG or IgM if the OD$_{CSF}$ IgG or IgM was >0.150. The AI was considered to be positive when the CSF was positive and the AI$_{IgG}$ or AI$_{IgM}$ was ≥0.3.

TABLE 1. Epidemiological characteristics of patient groups and baseline cerebrospinal fluid (CSF) leukocyte count (per μL)

	n	Male/female ratio (%)	Mean age (years) (SD)	Mean CSF leukocyte count (per μL of CSF) (SD)
Lyme neuroborreliosis	59	60/40	39 (24)	135 (159)
Lyme borreliosis	36	50/50	51 (17)	1 (1)
Infectious meningitis/encephalitis controls	69			
Treponema pallidum	12	83/17	40 (8)	40 (79)
Cryptococcus neoformans	2	50/50	52 (6)	94 (89)
Bacterial meningitis				
Streptococcus pneumoniae	2	50/50	41 (6)	337 (99)
Listeria monocytogenes	1	0/100	61	1280
Mycobacterium 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)
Neurological 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
Neurological non-inflammatory controls	12	25/25	47 (13)	4 (6)

ADEM, acute disseminated encephalomyelitis; HIV, human immunodeficiency virus; HSV1, herpes simplex virus 1; TBE, tick-borne encephalitis; VZV, varicella zoster virus; SD, standard deviation.

Statistical analysis

Statistical analysis was performed with a statistical software package (SPSS for Windows, version 17.0). Student's *t*-test was used to compare levels of C6-peptide LI between groups, and p-values <0.05 were considered to be significant.

Results

Patient characteristics

All patient serum and CSF samples were tested according to protocol. Patient epidemiological 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. 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 papillo-oedema. Most patients had early disseminated LNB (53/59). Four of the six patients with late LNB presented with meningoradiculitis of duration between 6 months and 2 years. Two patients had suffered for 6–18 months from an altered gait with magnetic resonance imaging abnormalities. Ninety-five per cent of patients presented with pleocytosis. Only two patients presenting with early LNB, one with facial nerve paralysis and one with meningoradiculitis, and one patient presenting with late LNB, with 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 favourably to treatment.

The AI in the IDEIA neuroborreliosis kit detected anti-*Borrelia* IgG or IgM in 78% of the LNB patients. The IgG AI was positive in 75% and the IgM AI was positive in 49% of the LNB patients (Table 2).

C6-peptide ELISA results on serum

The results for the C6-peptide ELISA are shown in Fig. 1. C6-peptide antibodies were detected in serum in 98% of the LNB patients, with a mean LI of 8.4 (95% confidence interval (CI) 7.7–9.1). The one C6-peptide ELISA-negative patient was a young child with early LNB presenting with 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 who presented with early LNB had a comparable LI in serum to that of the patients who presented with late LNB, with a respective mean LI of 8.3 and 9.1 (p 0.5). In the non-neuroborreliosis LB patient group, the sensitivity was 97%, and the mean LI was 6.9 (95% CI 5.6–8.2). In all other controls, the C6-peptide seroprevalence was 5%.

C6-peptide ELISA results on CSF

The sensitivity and specificity for the C6-peptide ELISA on CSF are shown in Table 3. The C6-peptide ELISA on CSF detected antibodies in 95% (56/59) of the LNB patients. The patients who presented with early LNB had a lower LI in CSF than the patients who presented with late LNB, with respective mean LIs of 6.6 (95% CI 5.6–7.3) and 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 right facial paralysis, but did not have pleocytosis at presentation and had 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 AIs were negative in the CSF. At presentation, she had pleocytosis of 56/μL CSF, and she responded rapidly to treatment. The third patient, a borderline (LI 0.9) positive patient, had 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).

Specificity in all controls varied from 83% to 97% (Table 3). Specificity was high in the infectious and neurological control

TABLE 2. Results of the antibody index from the IDEIA, showing the number of samples that were negative or positive for IgM and IgG against flagellin in cerebrospinal fluid, and the index calculated as described (Ig and Ig index are the results of the IgM and IgG results combined per patient)

	Negative (%)	IgM$^+$	IgG$^+$	IgM index$^+$	IgG index$^+$	Ig$^+$ (%)	Ig index$^+$ (%)	Total
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
Neurological controls	70 (95)	3	1	1		4 (5)	1 (1)	74

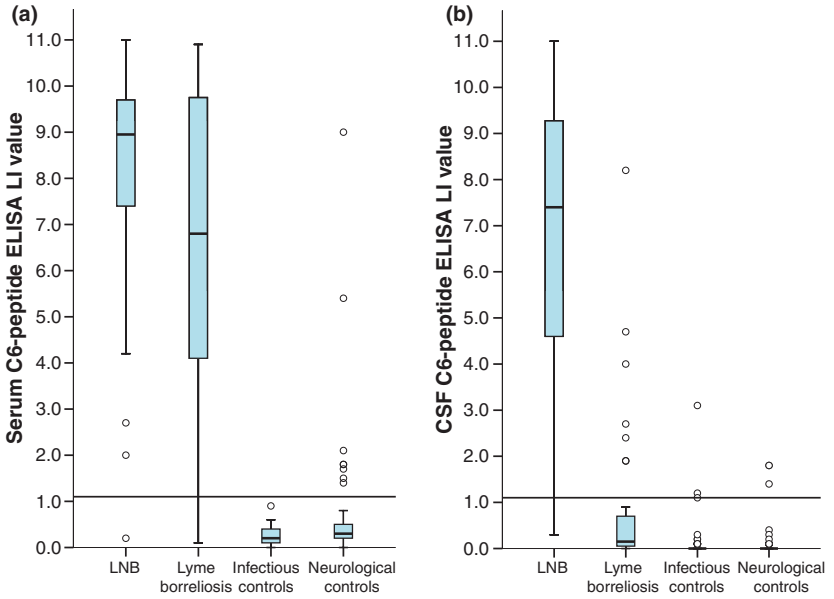

FIG. 1. Values for the C6-peptide ELISA in serum (a) and cerebrospinal fluid (b). Horizontal lines indicate medians, bars represent interquartile ranges, lines represent 95% confidence intervals, and bullets represent outliers. The reference line is located at the cut-off for detection of antibodies (Lyme index (LI) = 1.1). LNB, Lyme neuroborreliosis.

TABLE 3. Lyme index (LI) values of the C6-peptide ELISA in cerebrospinal fluid; samples with LI values <0.9 are considered to be negative for antibodies against C6-peptide, LI values 0.9–1.1 equivocal and LI values ≥1.1 positive for antibodies against C6-peptide

	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
Neurological controls	72 (97)		2 (3)	74

groups (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 patient, a neurosyphilis patient, and a human immunodeficiency virus meningitis patient. In the neurological control group, seven patients had detectable antibodies against C6-peptide in serum; these were five multiple sclerosis (MS) patients and two Guillian–Barré patients. In the CSF, two MS patients had low levels of 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 LIs of 6.7 (95% CI 6.0–7.6) and 0.8 (95% CI 0.3–1.4), respectively, $p < 0.05$). Lowering the LI threshold to 0.5 would increase the sensitivity to 97% in LB patients, but lower the specificity to 63%. No effect was seen on the specificity in the other controls.

Discussion

In this study, we evaluated the C6-peptide ELISA on CSF for diagnosing 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 an LNB patient could either have absence of pleocytosis or absence of intrathecal antibody production in the presence of an abundance of other criteria that made LNB evident. C6-peptide serology has good sensitivity in LNB patients, varying from 67% to 100% in serum [12–14,18]. The lower sensitivities were mainly reported in very early LNB, when the duration of symptoms was <8 days. The sensitivity of the C6-peptide ELISA on serum of LNB patients in this study was 98%. The 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 [19,20]. 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 have 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 as compared with the OD/cut-off standard used in the present kit. This cut-off is comparable to the borderline cut-off in our study. Prospectively, 60 adult LNB patients,

defined as having 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% [14].

Another study used diluted and undiluted CSF with LI cut-offs of 0.5 and 1 [16]. Retrospectively, 31 tentative cases of LNB were identified by evidence for intrathecal antibody production obtained by western blot. Twenty-eight LNB patients were identified according to clinical presentation and concurrent clinical response to antibiotic treatment. The 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 the 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 [21–23]. In addition, it has been reported that up to 20% of patients who have detectable antibodies against *B. burgdorferi* sl and respond to treatment do not have LNB but have other self-limiting conditions [24,25]. It is likely that the low sensitivity of the C6-peptide ELISA reported resulted from a poorly defined LNB patient group.

The 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% to 97%, with the lowest specificity being seen in the LB patient group (83%). In the infectious and neurological control groups, the specificities were 96% and 97%, respectively. Passively acquired antibodies from the serum could explain the detectable anti-C6-peptide antibodies in the CSF. However, in the infectious control group, none of the analysed controls with antibodies in the CSF had anti-C6-peptide antibodies in the serum. In the neurological control group, only two MS patients had detectable antibodies in the CSF. Production of polyclonal Ig in the CSF because of MS might also be an explanation for the false-positive result in these patients. Calculation of 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, the specificity of the C6-peptide ELISA with CSF will, by definition, be suboptimal in patients with detectable anti-Borrelia antibodies in the serum, as 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 led to selection bias. The strength of this study, however, lies in the number and wide variety of the controls. Many samples were selected from patient groups in whom the clinical presentation could be mistaken for LNB. The LNB and control groups also included patients from all age groups. On the basis of 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, e.g. pleocytosis in LNB, is essential for a correct diagnosis to be reached. 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 LNB seems to be less probable, with detectable anti-C6-peptide in the CSF, it can be useful to use a more specific assay, such as calculating a specific AI.

In conclusion, we show 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.

Authors' Contributions

N. van Burgel, A. C. M. Kroes and A. van Dam were involved in the conception and design of the study. Data were acquired by N. van Burgel, A. Brandenburg, H.-J. Gerritsen and A. van Dam. Analysis of data was performed by N. van Burgel and H.-J. Gerritsen. The first draft of the manuscript was designed by N. van Burgel. It was critically revised by A. C. M. Kroes, H.-J. Gerritsen, A. Brandenburg and A. van Dam. The final version for submission was approved by all co-authors.

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Transparancy Declaration

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