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ARTICLE

C6 peptide ELISA test in the serodiagnosis of Lyme borreliosis in Sweden

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Abstract The aim of this study was to evaluate the synthetic C6 peptide test as a first-line test in a two-tiered scheme for *Borrelia* serology in a clinically well-characterized population of patients with Lyme borreliosis in Kalmar County, Sweden. The study population consisted of a prospective group (n=200), a control group (n=255), and a retrospective group (n=29). The test panel consisted of the Immunetics Quick ELISA C6 Borrelia assay kit (Immunetics, Cambridge, MA, USA), the Virotech *Borrelia burgdorferi* ELISA (Genzyme Virotech, Rüsselsheim, Germany), and the Liaison Borrelia CLIA (DiaSorin, Saluggia, Vercelli, Italy). Seroprevalence among 200 healthy blood donors was significantly lower in the C6 test (8%) compared to the Virotech ELISA (14%) and the Liaison CLIA (12%). In convalescent sera (2–3 months and 6 months post infection) from 158 patients with erythema migrans, the seropositivity in the C6 test was also significantly lower compared to both the Virotech ELISA and the Liaison CLIA. Serosensitivity in the acute phase of erythema migrans and other clinical manifestations of borreliosis did not differ significantly between the C6 test and the Virotech ELISA or the Liaison CLIA. Overall, a positive C6 test seems to correlate well with acute borreliosis. Cross-reactivity was lower in the C6 test in sera positive for Epstein-Barr virus infection as compared to the Virotech ELISA. This study supports the use of the C6 test as a screening test for borreliosis, in endemic areas.

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Introduction

Lyme borreliosis (LB) is a common tick-borne disease caused by *Borrelia burgdorferi* sensu strictu, *Borrelia garinii*, and *Borrelia afzelii*. The incidence of clinically detected LB around the coasts and lakes in southern Sweden has been calculated as an average of 69/100,000/year, with considerable variations between counties [1]. The incidence in Kalmar County has been reported to be as high as 160/100,000/year [1]. LB may present as a number of different clinical conditions. In Europe, the most frequent manifestation is erythema migrans, followed by neuroborreliosis, arthritis, acrodermatitis, borrelial lymphocytoma, and carditis. Neuroborreliosis may also present with a number of different symptoms. Therefore, clinical diagnosis of LB is not always obvious.

Clinical data have been used in combination with a number of laboratory tests, of which serological analysis is of central importance. Assessment of the immune response is usually performed with enzyme-linked-immunosorbent assays (ELISAs) using different antigens. Serological analysis has proven difficult to interpret in the southeast of Sweden because of high seroprevalance, genetic diversity of the infectious agent, incomplete immunological response in the early stages of disease, and cross-reactions with other diseases and disorders [2–4].

In order to improve the serological diagnosis of LB, a number of different native and recombinant borrelial

proteins have been evaluated [5–7]. A synthetic peptide, C6, based on the immunodominant conserved region IR_6 of *B. burgdorferi* VlsE protein (variable membrane protein-like sequence, expressed), has proved promising. Antibody response to all three subspecies was strong [8]. Data from previous studies imply a high sensitivity, even in early stages of infection, as well as high specificity [8, 9]. It has also been shown that the antibody response to IR_6 wanes rapidly after antibiotic treatment [10]. Serodiagnostic kits based on the VlsE protein have now been commercially introduced.

The purpose of the present study was to evaluate the performance of the C6 peptide test in screening for borreliosis in an area of northern Europe with a high incidence of LB and a high seroprevalence in the healthy adult population.

Materials and methods

The material consisted of three groups of patients: a prospective group (n=244), a control group (n=255), and a retrospective group (n=29). The prospective group was recruited in a clinical study in Kalmar County in Sweden from May through December 2003. Inclusion criteria were as follows: erythema migrans >5 cm in diameter; lymphocytoma; acrodermatitis; neurological symptoms such as meningitis, cranial nerve palsy, sensorial and/or motorial radiculitis for <30 days; subacute mono- or oligoarthritis; myocarditis; and patients for whom borreliosis was a possible diagnosis and for whom *Borrelia* serology would have been ordered in the investigation anyway.

Clinical data was recorded in a study protocol. Cerebrospinal fluid (CSF) was collected from patients with suspected neuroborreliosis. For patients in the prospective group, erythema migrans, borrelial lymphocytoma, and acrodermatitis chronica atrophicans were clinically diagnosed on the basis of history and physical examination. Only a typical rash resembling erythema migrans with a diameter >5 cm was considered erythema migrans. In the same group (prospective patients), neuroborreliosis was defined as a positive serum-CSF antibody index, i.e. as determined by measurement of intrathecally produced anti-*Borrelia* antibodies of class IgM or IgG or both. Sera were collected at the first consultation, at 2–3 month, and at 6 months, consecutively, for all patients except children <15 years of age, from whom sera were collected at the first visit and 2–3 months later. Patients with haematological malignant disease, HIV infection, immunosuppressive treatment, or ongoing antibiotic treatment were excluded from the study.

In the prospective group, 200 of 244 patients completed the study. Forty-four patients did not complete the study protocol because they either exited the study or were unavailable for collection of follow-up serum samples. Seventeen of these 44 patients were under the age of 15. Of these 44 patients, 23 could be classified as having erythema migrans at inclusion. Of the remaining 21 patients, seven suffered from facial palsy, three had sensory radiculitis, and two had arthritis. The remaining nine patients could not be classified. Patients were classified according to clinical data and, in case of suspected neuroborreliosis, analysis of CSF. Baseline data from the prospective group are shown in Table 1. No cases of Lyme arthritis or carditis could be identified among the prospective group. Thirty-one patients had symptoms or clinical signs consistent with LB and were therefore included in the study, but a clinical diagnosis could not be determined.

The control group consisted of regional blood donors (n=200) and patients with conditions known for cross-reactivity in LB serological tests. To assess cross-reactivity, sera from 55 patients collected during 2004 were analyzed: 22 from patients with serological evidence of ongoing Epstein-Barr virus (EBV) infection, 23 from rheumatoid factor-positive patients, and ten from patients with positive syphilis serology. Ten sera positive in syphilis serology were divided into two separate groups: five sera were positive both for the *Treponema pallidum* particle agglutination (TPPA) test and the rapid plasma reagin (RPR) card test, i.e. consistent with active infection, while five were positive only in the TPPA test, an indication of healed syphilis infection.

The retrospective group consisted of patients with disseminated LB. Sera from 20 cases of neuroborreliosis (collected during 2004), six cases of acrodermatitis chronica atrophicans (collected between 2001 and 2003), and three cases of Lyme arthritis (collected between 1999 and 2003) were analysed. The sera from retrospective cases of neuroborreliosis were selected according to the same criteria as the prospective group. Acrodermatitis chronica atrophicans was identified clinically by dermatologists and by typical IgG Western blot reactivity using the Virotech EcoBlot [11]. Finally, Lyme arthritis was defined as a clinical case of arthritis with a positive PCR result from synovial fluid using 16S rRNA partial sequencing [12].

Sera from the retrospective group and sera from patients with conditions known for cross-reactivity were identified and collected from the serum collection at the routine diagnostic laboratory at the County Hospital in Kalmar, Sweden.

All sera were analysed by the Immunetics Quick ELISA C6 Borrelia assay kit (IQC6) (Immunetics, Cambridge, MA, USA) [13], the Virotech Borrelia burgdorferi ELISA IgG/IgM test kit (VT) (Genzyme Virotech, Rüsselsheim, Germany) [14], and the Liaison Borrelia IgM (310890) IgG (310880) chemiluminescence immunoassay (Li CLIA) (DiaSorin; Saluggia, Italy) [15]. CSF and paired serum samples were analysed by the IDEIA Lyme Neuroborre-

Table 1 Baseline data of the prospective group of 200 patients

Parameter		No. (%) Unknown	Total (n=200)
Median age in years (range)	54.5 (4–85)		
No. (%) of females/males	121 (60)/79 (40)		
Tickbite, no. (yes/multiple/suspected/none)	83/37/53/21	6 (3%)	
No. with antibiotic treatment: pcV/DOX/CFT/none	149/29/3/15	4 (2%)	
Previous LB, no. (%)	55 (28%)	7 (4%)	
Manifestation			
Erythema migrans, no. (%) of females/males	97 (80%)/61 (77%)		158 (79%)
Lymphocytoma			2 (1%)
Neuroborreliosis			6 (3%)
Facial palsy			3
Nonspecific symptoms			3
Acrodermatitis			3 (1.5%)
Possible LB			31 (15.5%)
Arthritis			2
Facial palsy			4
Oculomotorius affection			1
Nonspecific symptoms, no. (%) of females/males	16 (13%)/8 (10%)		24

pcV penicillin V, *DOX* doxycycline, *CFT* ceftriaxone

liosis kit (DakoCytomation, Cambridgeshire, UK) [16]. All tests were performed and interpreted according to the manufacturers' instructions.

Statistical analyses were performed using an exact test corresponding to McNemar's paired chi-squared modification, first proposed by Liddell (1983) for paired measurements [17], and Fisher's exact two-tailed test for nonpaired measurements (Statistica 6.0). $P<0.05$ was considered significant. An exact test has been used to calculate confidence intervals for proportions using the binomial distribution. The study was approved by the regional ethical board of Linköping University, Sweden.

Results

The combined diagnostic results from the prospective, retrospective, and control groups are presented in Table 2. All cases of erythema migrans were included prospectively. A total of 26 cases of neuroborreliosis consisted of 20 retrospective cases and six prospectively collected cases, with paired CSF and serum samples defined by positive intrathecal anti-*Borrelia* antibody production. Nine cases of acrodermatitis consisted of six retrospective cases and three prospective cases. Three samples from patients with PCR-verified Lyme arthritis were collected retrospectively. Sera from 31 patients in whom a clinical diagnosis could not be determined were all collected prospectively and are presented in Table 2 as possible cases of LB. Sera from all cases of EBV-, rheumatoid factor- and syphilis-positive sera were collected retrospectively. All sera from blood donors were collected prospectively.

Serum was not sufficient for one analysis by the Li CLIA in the group of patients with erythema migrans in the acute phase.

Cases of neuroborreliosis were sorted on the basis of pleocytosis of $\geq 20\times10^6$ leukocytes/l in CSF and are separately presented in Table 2. In all cases of neuroborreliosis with pleocytosis, the cell count was dominated by mononuclear cells (data not shown). Serum was not sufficient for one analysis by the Li CLIA in the group of patients with neuroborreliosis with pleocytosis.

Serosensitivity based on the IQC6 test did not differ significantly for different clinical conditions of LB as compared to the combined interpretation of the IgM and IgG VT ELISA or Li CLIA.

For 22 sera with evidence of EBV infection, 18% were positive in the IQC6 test as compared with 55% in the VT ELISA. Of the 23 rheumatoid factor-positive sera, 26% were cross-reactive in the IQC6 test and 22% in the VT ELISA. In five patients with serological evidence of ongoing syphilis infection, 80% showed reactivity in the IQC6 test, whereas equivocal reactivity was detected in 60% with the VT ELISA. No reactivity in these five sera was detected in the Li CLIA. In 200 blood donors from our region, seropositivity varied from 8 to 14% in the different assays, with the IQC6 test showing the lowest figures with no equivocal results. The proportion of equivocal test results for the different assays varied from 0 to 5% in the IQC6 test as compared to 0–60% and 0–23% in the VT ELISA and Li CLIA, respectively. Seropositivity rates in the different immunoassays over time in patients with erythema migrans treated with antibiotics (145/158 pcV-treated, 13/158 doxycycline-treated) are shown in Table 3. The combined IgM and IgG interpretation

Table 2 Serological results of the prospective, retrospective, and control groups, taken together

	Diagnostic result	IQC6 IgM+IgG % (95%CI)	Virotech ELISA IgM+IgG % (95%CI)	Liaison CLIA IgM+IgG % (95%CI)
Type of clincal cases				
Erythema migrans in acute phase (n=158)	+	34 (26–43)	35 (27–43)	37 (29–45)
	+/–	3	11	5
Neuroborreliosis regardless of pleocytosis (n=26)	+	88 (69–98)	88 (69–98)	80 (59–94)
	+/–	0	8	4
Neuroborreliosis with pleocytosis (n=20)	+	95 (75–100)	95 (75–100)	79 (54–94)
	+/–	0	5	5
Neuroborreliosis without pleocytosis (n=6)	+	67 (22–96)	67 (22–96)	83 (35–100)
	+/–	0	17	0
Acrodermatitis (n=9)	+	89 (51–100)	100 (71–100)	67 (29–93)
	+/–	0	0	0
Lyme arthritis (n=3)	+	67 (9–100)	67 (9–100)	67 (9–100)
	+/–	0	0	0
Possible LB (n=31)	+	45 (27–64)	42 (24–61)	45 (27–64)
	+/–	0	6	0
Cross-reactivity				
EBV positive (n=22)	+	18[a] (5–41)	55[a] (32–76)	0 (0–13)
	+/–	5	18	23
RF positive (n=23)	+	26 (10–49)	22 (7–44)	22 (7–44)
	+/–	0	13	4
TPPA positive, RPR negative (n=5)	+	20 (0–72)	60 (14–95)	20 (0–72)
	+/–	0	0	0
TPPA positive, RPR positive (n=5)	+	80 (28–100)	0 (0–46)	0 (0–46)
	+/–	0	60	0
Population background				
Blood donors (n=200)	+	8[b] (4–13)	14[b] (9–21)	12[b] (7–18)
	+/–	0	10	7

To compare the Virotech ELISA and the Liaison CLIA with the IQC6 test, a combined interpretation of the IgM and IgG Virotech and the Liaison results is shown. If either IgM or IgG or both were positive, the combined interpretation was positive. If either IgM or IgG was equivocal while the other was negative, or if both were equivocal, the combined interpretation was equivocal. Confidence intervals are shown for nonpaired proportions, while statistical analyses have been made using paired measurements.

IQC6 Immunetics quick ELISA C6 Borrelia assay kit, *EBV* Epstein-Barr virus, *RF* rheumatoid factor, *TPPA Treponema pallidum* particle agglutination, *RPR* rapid plasma reagin

[a] IQC6 test seropositivity significantly lower than combined interpretation of Virotech ELISA (p=0.021; paired measurement)

[b] IQC6 test seropositivity significantly lower than combined interpretation of both Virotech ELISA and Liaison CLIA (p=0.0026 and p=0.012, respectively; paired measurements)

of the VT ELISA and the Li CLIA is shown, with a serum result considered positive if either IgM or IgG or both were positive. Serum collected at 6 months from the group of erythema migrans patients was not sufficient for one analysis in the VT ELISA or for five analyses in the Li CLIA.

Discussion

Our results suggest that the single-well combined IgM/IgG IQC6 test performs at least as well as both the two-well IgM and IgG VT ELISA used in our laboratory and the recently introduced IgM and IgG Li CLIA as the first step in a two-tiered testing strategy for LB. In this study, the seropositivity in the IQC6 test seemed to be more strongly correlated with ongoing borreliosis than either the VT ELISA or the Li CLIA. Seroprevalence in healthy blood donors was significantly lower in the IQC6 test as compared with both the VT ELISA and the Li CLIA. In convalescent sera (collected at 2–3 and 6 months) from patients with erythema migrans treated with antibiotics, a significantly lower seropositivity for the IQC6 test was noted in comparison with both the combined IgM and IgG interpretation of the VT ELISA and the Li CLIA. The IQC6 test also showed lower cross-reactivity in sera with evidence of EBV infection as compared with the VT ELISA. No significant difference in serosensitivity regarding erythema migrans in the acute phase or any other

Table 3 Serological results over time in 158 patients with erythema migrans treated with antibiotics

	Diagnostic result	IQC6 test IgM+IgG %, (95%CI)	Virotech ELISA IgM+IgG %, (95%CI)	Liaison CLIA IgM+IgG %, (95%CI)
Acute phase	+	34 (26–43)	35 (27–43)	37 (29–45%)
	+/−	3	11	5
2–3 months	+	30[a] (22–38)	44[a] (35–52)	44[a] (36–53%)
	+/−	1	11	4
6 months	+	19[b] (13–27)	32[b] (24–40)	32[b] (24–41%)
	+/−	1	11	7

To compare the Virotech ELISA and the Liaison CLIA with the IQC6 test, a combined interpretation of the IgM and IgG Virotech and Liaison results is shown. If either IgM or IgG or both were positive, the combined interpretation was positive. Confidence intervals are shown for nonpaired proportions, while statistical analyses have been made using paired measurements

IQC6 Immunetics Quick ELISA C6 Borrelia assay kit

[a] IQC6 test seropositivity significantly lower than combined interpretation of both Virotech ELISA and Liaison CLIA at 2–3 months ($p=0.0016$ and $p<0.001$, respectively; paired measurements)

[b] IQC6 test seropositivity significantly lower than combined interpretation of both Virotech ELISA and Liaison CLIA at 6 months ($p=0.0043$ and $p<0.001$, respectively; paired measurements)

clinical manifestations of LB could be determined comparing the IQC6 test with both the VT ELISA and the Li CLIA.

For a serological test to be used as a "rule out" test, the sensitivity of the test should be high. A two-step procedure for the serodiagnosis of LB has been recommended both in the USA and in Europe [18, 19]. In the first step, a high-sensitivity ELISA test is recommended, which, if positive or equivocal, should be followed by an immunoblot for confirmation. Serosensitivity in later stages of borreliosis is generally high in different serodiagnostic *Borrelia* kits. In early localized borreliosis, however, serosensitivity has been generally poor. In order to evaluate new serodiagnostic kits for LB, cases of erythema migrans are therefore suitable to study. Even if erythema migrans is considered a clinical diagnosis, serosensitivity in erythema migrans, together with serosensitivity in other manifestations, is of importance in determining the performance of serodiagnostic kits for borreliosis.

In 20 sera with both evidence of intrathecally produced anti-*Borrelia* antibodies and pleocytosis in the CSF, the IQC6 test showed a serosensitivity of 95%, not significantly different from the combined IgM and IgG interpretation of the VT ELISA or the Li CLIA. In six sera with evidence of intrathecally produced anti-*Borrelia* antibodies without pleocytosis, the serosensitivity of the IQC6 test was 67%. Only a total of 26 sera was tested, and serosensitivity in sera from patients without pleocytosis in CSF as compared to serosensitivity in patients with pleocytosis was not significantly different ($p=0.12$). A positive test for intrathecally produced anti-*Borrelia* antibodies in the absence of pleocytosis suggests the possibility of a previous, non-ongoing case of neuroborreliosis. Even if the difference in serosensitivity was not significant, it indicates the need for further investigations based on larger numbers of specimens in order to determine the possibility of a higher serosensitivity of the IQC6 test in cases of ongoing neuroborreliosis, defined as both intrathecally produced anti-*Borrelia* antibodies and pleocytosis. The general serosensitivity in all 26 sera was 88% for the IQC6 test. This was not significantly different from any of the other serological tests. We consider 88% as too low a sensitivity for a "rule out" screen test in cases of neuroborreliosis. On the basis of the results of this study, we therefore still recommend detection of intrathecally produced anti-*Borrelia* antibodies in cases of suspected neuroborreliosis [19].

Cross-reactivity remains a problem using the IQC6 test. However, in sera positive for EBV infection, the number of false-positive results was significantly lower in comparison with the VT ELISA. Eighty percent of sera from patients with evidence of ongoing syphilis infection were positive in the IQC6 test, while 20% of sera from patients with treated syphilis were positive. Only a small number of sera was tested, and the difference in seropositivity was not significant. However, it suggests that, when evaluating serodiagnostic tests for borreliosis, sera with evidence of ongoing syphilis should be tested separately from sera from cases of treated syphilis to detect differences.

In Kalmar County, Sweden, LB is highly endemic. The seroprevalence in healthy adult blood donors was 8–24%, including equivocal tests. The seroprevalence of 8% in healthy blood donors measured with the IQC6 test was significantly lower than that of the combined IgM and IgG interpretation of the VT ELISA, 14%, and the Li CLIA, 12%. Assessment of seroprevalence is essential to understand the proportion of positive test results that may have originated from patients with non-ongoing LB. Seroprevalence is not to be confused with specificity, but the combination of test specificity and seroprevalence could be expressed in terms of "clinical specificity." The aim of a serodiagnostic kit for LB

must be to detect an ongoing infection with high sensitivity and to discriminate from other conditions not caused by LB or previous non-ongoing LB.

Of the 44 patients who did not complete the prospective study, 17 were children in whom symptoms may have resolved, resulting in a loss of motivation for completing the study with a follow-up sample.

In conclusion, this study showed that the IQC6 test seems to be more strongly correlated with ongoing LB than the other tests evaluated, primarily because of lower cross-reactivity in EBV-positive sera, and lower seroprevalence in our endemic area. No significant improvement in sensitivity in the different manifestations of borreliosis could be detected. Overall, our investigation suggests the IQC6 test is a suitable first-line screening test in cases of borreliosis, except those with erythema migrans and neuroborreliosis, provided that a positive or equivocal screening test result is followed by a confirmatory immunoblot.

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