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Antibody responses to borrelia IR$_6$ peptide variants and the C6 peptide in Swedish patients with erythema migrans.

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Abstract

AIM: The aim of this study was to evaluate the antibody responses to different VlsE protein IR₆ peptide variants and the synthetic C6 peptide in acute and convalescent (2-3 and 6 months) serum samples from Swedish patients with clinical erythema migrans (EM).

METHODS: Serum samples were prospectively collected from 148 patients with EM and compared to serum samples obtained from 200 healthy, blood donors. The IgG responses to three IR₆ peptide variants originating from *Borrelia burgdorferi* (*B. burgdorferi*) sensu stricto, *B. garinii*, and *B. afzelii* were measured by enzyme-linked immunosorbent assays (ELISA) and compared to a commercial C6 peptide ELISA.

RESULTS: Seropositivity rate in the IR₆ or C6 peptide ELISAs ranged from 32% to 58% at presentation, 30% to 52% at 2-3 months and 20% to 36% at 6 months. At presentation, positive antibodies in any of the four ELISA were found in 66%. In 7/52 (13%) C6 negative EM cases, serological reaction was found to the *B. burgdorferi* sensu stricto derived IR₆ peptide. In patients reporting previous LB compared to those without previous LB, significantly higher seropositivity rates were noted for all IR₆ peptides but not for the C6 peptide.

CONCLUSIONS: In the serology of EM in Europe, C6 ELISA does not seem to cover all cases. An ELISA using a mixture of *B. burgdorferi* sensu stricto IR₆ peptide and the C6 peptide could be of value in the serodiagnosis of LB in Europe. Further studies on combinations of variant IR₆ peptides and the C6 peptide in other manifestations of LB are needed to address this issue.

**Keywords:** Lyme borreliosis; Erythema migrans; Serology; ELISA; IR₆ peptide; C6
Introduction

In Europe, the causative agent of Lyme borreliosis (LB) is the tick-transmitted spirochete *Borrelia burgdorferi* (*B. burgdorferi*) sensu lato, consisting mainly of the three genospecies *B. burgdorferi* sensu stricto (s.s.), *B. garinii* and *B. afzelii*. The most common clinical manifestation of LB is the skin rash erythema migrans (EM), followed by other manifestations such as neuroborreliosis, arthritis, acrodermatitis, lymphocytoma and carditis (Berglund et al., 1995). The clinical manifestations of LB are classified into three different stages (I, II and III) according to localization and duration of infection. EM is an early clinical manifestation of LB and therefore regarded as stage I, whereas other manifestations indicate disseminated LB and belong to the later stages (II and III). The diagnosis of EM is generally based on patient history and typical skin rash. Laboratory confirmation, e.g. demonstration of anti-borrelial antibodies, is not usually recommended in the acute phase of EM due to low serosensitivity (20-50%). In stages II and III of LB, serosensitivity varies from 70% to nearly 100% (Wilske, 2005). Therefore, there is a greater potential for improvement in serosensitivity in cases of EM compared to stages II-III. Furthermore, when studying the early immune response in LB, serology in patients with EM is of particular interest. Clinically an improved serosensitivity in EM can be of use in cases of atypical rash where misdiagnosis may occur (Feder and Whitaker, 1995).

Recently, a commercial ELISA using a synthetic 26-mer peptide antigen called C6 has been evaluated and introduced in the serodiagnosis of LB (Cinco and Murgia, 2006; Smismans et al., 2006; Nyman et al., 2006; Tjernberg et al., 2007; Sillanpää et al., 2007). The C6 antigen is based on the sixth invariant region (IR₆) of the variable major protein-like sequence, expressed (VlsE) by *B. garinii* (Liang et al., 1999). Although strong antibody response has been shown against the C6 peptide regardless of infecting strain of borrelia (Liang et al., 2000), there is evidence of variation in the IR₆ amino acid sequences between *B. afzelii, B. garinii* and *B. burgdorferi* B31 (Goettner et al., 2004). The purpose of this study was to investigate patients with EM from an endemic area of Sweden and to compare early antibody responses
to the C6 peptide and three IR6 peptides representing the three main borreliol genospecies. In addition, we wanted to study the antibody responses over time and in relation to previously reported LB in order to determine differences in antibody response dynamics between different VlsE based antigens.
Material and methods

Patients with LB in Kalmar County in Sweden were prospectively included in a clinical study in 2003 (Tjernberg et al., 2007). Clinical data was recorded in a study protocol and serum samples were drawn at the first consultation (sample I), at 2-3 months (sample II) and 6 months (sample III), see Table 1 and Table 2.

Table 1. Baseline data for patients with erythema migrans (n=148). Patients were also divided into sub-groups based on self-reported previous Lyme borreliosis.

<table>
<thead>
<tr>
<th>Test</th>
<th>All EM (n=148)</th>
<th>Previous LB (n=46)</th>
<th>No previous LB (n=97)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age median years all (range)</td>
<td>58 (7-84)</td>
<td>61 (11-84)</td>
<td>54 (7-83)</td>
</tr>
<tr>
<td>Female</td>
<td>90</td>
<td>61</td>
<td>58</td>
</tr>
<tr>
<td>Age median years (range)</td>
<td>58 (7-84)</td>
<td>62 (44-84)</td>
<td>52 (7-83)</td>
</tr>
<tr>
<td>Male</td>
<td>58</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>Age median years (range)</td>
<td>56 (11-79)</td>
<td>56 (11-69)</td>
<td>56 (18-79)</td>
</tr>
<tr>
<td>Tickbite (yes/multiple/suspected/no)</td>
<td>64/19/48/14</td>
<td>20/7/14/5</td>
<td>40/11/34/9</td>
</tr>
<tr>
<td>Associated symptoms</td>
<td>46</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>Duration median days (range)</td>
<td>7 (1-45)</td>
<td>7 (1-30)</td>
<td>7 (1-45)</td>
</tr>
</tbody>
</table>

n = Numbers
EM = Erythema migrans
LB = Lyme borreliosis

Table 2. Proportion (%) of erythema migrans patients with antibodies to IR6 peptide variants P1, P2, or P4 (IgG) or C6 peptide (IgM/IgG) or EcoLine Western Blot (IgM/IgG) at presentation (I), at 2-3 months (II), or at 6 months (III). Patients were also divided into sub-groups based on self-reported previous Lyme borreliosis.

<table>
<thead>
<tr>
<th>Test</th>
<th>All patients (n=148)</th>
<th>Previous LB (n=46)</th>
<th>No previous LB (n=97)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>46</td>
<td>36</td>
<td>39</td>
</tr>
<tr>
<td>P2</td>
<td>32</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>P4</td>
<td>34</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>C6 IH</td>
<td>58</td>
<td>26</td>
<td>41</td>
</tr>
<tr>
<td>C6 Imm.</td>
<td>36</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td>Any IR6/C6 IH</td>
<td>66</td>
<td>74</td>
<td>63</td>
</tr>
<tr>
<td>WB</td>
<td>51</td>
<td>52</td>
<td>44</td>
</tr>
</tbody>
</table>

n = Numbers
EM = Erythema migrans
LB = Lyme borreliosis
C6 IH = C6 In house. Proportion positive samples based on mean optical density plus 3 SD of 171 blood donors.
C6 Imm. = Proportion positive samples based on manufacturer's cut off.
WB = EcoLine Western Blot (IgM/IgG)

Patients with haematological malignant disease, HIV infection, immunosuppressive treatment or ongoing antibiotic treatment were excluded from the study. Of 244 included patients 200
completed the study by fulfilling the study protocol and 158 (79%) presented with clinically
defined EM > 5 cm in diameter. The remaining 42 patients presented with other manifesta-
tions of LB. Of the 158 patients, a complete set of three serum samples (I, II and III) was
available for 148 patients. All EM patients were treated with antibiotics, in most cases with
phenoxy-methylpenicillin (Tjernberg et al., 2007). Baseline data for the 148 EM patients is
shown in Table 1. Patients were divided into two sub-groups according to presence or absence
of self-reported previous episodes of LB to investigate possible differences in serological re-
sponses. Clinical features such as fever, arthralgia, arthritis, pain, headache, vertigo, fatigue,
lymphocytoma, and multiple EM were regarded as associated symptoms. Serum samples
from 200 blood donors in the same area (Kalmar, Sweden) were collected as controls in April
2003, i.e. before the tick season (100 male, 100 female, mean age 45 years).

All sera were analysed by ELISA for antibodies to the C6 peptide (non-resolving assay for
IgM and IgG antibodies) and to three synthetic IR₆ peptides (IgG antibodies) produced at the
Core Facility of Protein Chemistry, Haartman Institute, University of Helsinki. The amino
acid sequence of peptide one (P1) corresponds to the IR₆ region of the VlsE gene sequence of
B. burgdorferi strain B31, peptide two (P2) corresponds to the IR₆ region of the VlsE gene
sequence of B. garinii strain IP90 and peptide four (P4) corresponds to the IR₆ region of the
VlsE recombination cassette four of B. afzelii ACAI (Sillanpää et al., 2007, Table 3). In order
to facilitate for the reader, the naming of the three IR₆ peptides has been kept the same in this
study. The commercial C6 peptide ELISA based on a B. garinii sequence (Liang et al., 1999)
was performed and interpreted according to the manufacturer’s instructions (Quick ELISA C6
Borrelia assay kit, Immunetics®, USA). The version of the Immunetics® C6 test available at
the time of this investigation used a conjugated antigen as a secondary measure to detect anti-
bodies specific for the C6 peptide (personal communication Immunetics®). Thus, it was not
selective for immunoglobulin classes. It was not fully comparable to the kit available on the
market at the time of submission, where conjugated goat-anti-human IgG/IgM was used in the
detection step. The IR₆ peptide antibody assays were performed as previously described
Table 3. IR₆ peptide sequences used in IgG ELISA tests.

<table>
<thead>
<tr>
<th>Code</th>
<th>Strain of origin</th>
<th>Amino acid sequence</th>
<th>MW</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td><em>B. burgdorferi</em> sensu stricto B31 VlsE gene</td>
<td><em>[H]MKKDDQIAAAIALRGMAKDGKFAVK</em>[OH]</td>
<td>2903</td>
<td>25 aa</td>
</tr>
<tr>
<td>P2</td>
<td><em>B. garinii</em> IP90 VlsE gene</td>
<td><em>[H]MKKDDQIAAAMVLRGMAKDGQFALKD</em>[OH]</td>
<td>2852</td>
<td>26 aa</td>
</tr>
<tr>
<td>P4</td>
<td><em>B. afzelii</em> ACAI vls recombination cassette 4</td>
<td><em>[H]MKKSDKIAAAIVLRGVAKDGKFAVA</em>[OH]</td>
<td>2588</td>
<td>25 aa</td>
</tr>
</tbody>
</table>

MW = Molecular weight
* = Site of biotin label.
aa = amino acids

(Heikkilä et al., 2003; Sillanpää et al., 2007). Sera from the 148 patients with EM and blood donor sera (n=200) were also analysed by an independent Western Blot kit (EcoLine, Viro-tech, Germany) in order to exclude seropositive samples when calculating cut-off levels for the different IR₆ peptides and an alternative comparable cut-off level for the C6 peptide.

Western Blot results were also used to confirm reactivity in the ELISA experiments. Although the manufacturer provided a cut-off level for the C6 test (C6 Imm; > 0.15), an in house cut-off level (C6 IH; ≥ 0.0689) was also calculated using the same method as for the IR₆ peptides in order to enable comparisons with all peptide tests on equal terms. In 29 blood donor samples with one or more IgG positive bands in the Western Blot, optical density (OD) values of the IR₆ peptide and C6 ELISAs were significantly higher, using non-parametric testing, compared to 171 blood donor samples with less than one positive IgG band regardless of IgM banding pattern (data not shown). These 29 blood donors with one or more positive IgG bands were therefore excluded from calculations of the cut-off levels. No significant differences in OD values were noted among subgroups of the remaining 171 blood donors with less than one positive IgG band (data not shown). These 171 blood donors could be separated into one group without bands (n=30), one group with borderline interpretation of one or more bands but no positive band (n=132) and finally one group with one positive IgM band and negative or borderline findings in IgG bands (n=9). The mean OD plus 3 standard deviations (SD) at 405 or 450 nm for the remaining 171 blood donors was used for the IR₆ peptides and C6 peptide results as cut-off levels, respectively. To compare the antibody responses to the variant IR₆ peptides and the C6 peptide between each other, the coefficient of determination ($R^2$) was
calculated for the OD values for each pair using all 444 EM samples collected from the three consecutive samples from each of the 148 patients.

Statistical analyses were performed using Fisher’s exact two-tailed test for proportions and non-parametric tests (Mann-Whitney’s U-test) when comparing OD values, age and symptom duration between groups (Statistica 7.1). A p < 0.05 was considered statistically significant. Receiver operating characteristic curve (ROC) analysis was performed using MedCalc® software version 9.4.2.0. The study was approved by the regional ethical board of Linköping University, Sweden.
Results

Clinical features

Of 148 EM patients, 46 (31%) reported a previous episode of LB (Table 1). Patients with self-reported, previous LB were older than those without previous LB (median 61 versus 54 years, \( p=0.01 \)). Regarding associated symptoms or duration of EM at presentation, no significant differences between the two groups were found. Among patients reporting previous LB, women were significantly older than men (median 62 versus 56 years, \( p=0.01 \)). No woman below 44 years of age reported previous LB.

Antibodies to C6 and IR\(_6\) peptides

The proportion of positive serum samples in the IR\(_6\) or C6 peptide ELISAs ranged from 32\% to 58\% at presentation, from 30\% to 52\% at 2-3 months, and from 20\% to 36\% at 6 months (Table 2: P1, P2, P4, C6 IH and C6 Imm.). Of the 148 patients at presentation, 97 (66\%) were positive for any IR\(_6\) or C6 IH peptide, and 11 (7\%) were positive for one or more IR\(_6\) peptides but not to C6 IH at presentation. At presentation, 76 (51\%) patients were positive in P1 or C6 Imm., and 95 (64\%) were positive in P1 or C6 IH. A total of 51 (34\%) were all negative, and 36 (24\%) were all positive in tested ELISAs (calculated data, not shown in Table). Thus, full concordance was achieved in 51 negative and 36 positive samples at presentation (59\%). No significant differences were found between women and men in seropositivity to IR\(_6\) peptides or C6 IH/Imm (data not shown).

The proportion of positive samples for the three IR\(_6\) peptides did not change significantly from sample I to sample II (P1: \( p=0.3523 \), P2: \( p=1.0 \), P4: \( p=0.3368 \)), but decreased significantly to sample III regarding P1 and P4 (P1: \( p=0.0070 \), P4: \( p=0.0093 \), Table 2). For the C6 peptide, however, the seropositivity rate tended to decrease from sample I to sample II with a further decrease to sample III, changes being significant for C6 IH between all samples (Sample I-II: \( p=0.0074 \); Sample II-III: \( p=0.0069 \)) and between samples II and III for C6 Imm. (Sample I-II: \( p=0.3873 \); Sample II-III: \( p=0.0436 \)). Of the 148 patients, 76 (51\%), 79 (53\%),
or 54 (36%) were positive for either C6 Imm or P1 or both at presentation, at 2-3 months, and at 6 months, respectively (calculated data, not shown in Table).

In patients with previous LB compared to those without previous LB, the seropositivity rate at presentation was significantly higher for all IR₆ peptides (p<0.02, data not shown) but not for C6 IH or C6 Imm (p=0.36 or p=0.10, respectively, data not shown).

The coefficient of determination (R²) considering all 444 EM samples varied from 0.77 to 0.81 between IR₆ peptides and from 0.59 (P4) to 0.72 (P2) between the C6 peptide and IR₆ peptides. The highest correlation between the C6 peptide and the three IR₆ peptides was with the IR₆ P2 peptide originating from B. garinii. The IR₆ peptides generally correlated better with each other than with the C6 peptide.

To compare analytical performance at different sensitivity and specificity levels, ROC analysis was performed using the OD values of the IR₆ peptides and the C6 peptide from the 148 EM patients at presentation and the 171 selected blood donors (Figure 1). Area under curve (AUC) was calculated for all tests and compared pairwise. AUC in between IR₆ peptides did not differ significantly, however AUC for all IR₆ peptides were significantly higher (p≤0.021) than for the C6 peptide.

Of the selected 171 blood donor samples, two (1.0 %) were positive for P1, two, (1.0 %) for P2, and three (1.5 %) for P4 ELISA. Five of the 171 (2.5 %) blood donors were positive for C6 IH and none (0%) for C6 Imm (data not shown). Using the same cut-offs considering all 200 blood donors, seroprevalence would be 21 (10.5%) in P1, 20 (10%) in P2, 20 (10.0%) in P4, 23 (11.5%) in C6 IH and 15 (7.5%) in C6 Imm. Using a combination of C6 Imm and P1, a total of 21 (10.5%) blood donors would be positive.
Figure 1. Receiver operating characteristic curve of IR<sub>6</sub> peptides and C6 optical density results based on 148 patients with erythema migrans at presentation and 171 healthy blood donors.

- P1 – *B. burgdorferi* sensu stricto
- P2 – *B. garinii*
- P4 – *B. afzelii*
- C6 – Immunetics® Quick ELISA™ C6 Borrelia assay kit

In 52 patients, C6 IH was negative for all three samples. In nine of these patients, antibody responses to one or more of the IR<sub>6</sub> peptides were found, most frequently antibodies to P1, 7/52 (13%), see Table 4. Correspondingly, 46 patients were negative in all IR<sub>6</sub> peptide tests in all three samples. In 15 of these patients, a positive antibody response was detected using the C6 IH cut-off, but none were regarded positive by the higher C6 Imm. cut-off (data not shown).
Table 4. Positive IR$_6$ peptide (IgG) responses (% positive) in 52 C6 negative erythema migrans patients at presentation (I), at 2-3 months (II), or at 6 months (III).

<table>
<thead>
<tr>
<th>Peptide antigen</th>
<th>n</th>
<th>WB pos %</th>
<th>WB VlsE IgG pos %</th>
<th>n</th>
<th>WB pos %</th>
<th>WB VlsE IgG pos %</th>
<th>n</th>
<th>WB pos %</th>
<th>WB VlsE IgG pos %</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>7</td>
<td>71</td>
<td>100</td>
<td>12</td>
<td>67</td>
<td>58</td>
<td>6</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>5</td>
<td>80</td>
<td>80</td>
<td>4</td>
<td>75</td>
<td>75</td>
<td>2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>4</td>
<td>75</td>
<td>75</td>
<td>7</td>
<td>43</td>
<td>57</td>
<td>2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Any IR$_6$</td>
<td>9</td>
<td>67</td>
<td>89</td>
<td>15</td>
<td>53</td>
<td>47</td>
<td>7</td>
<td>71</td>
<td>57</td>
</tr>
</tbody>
</table>

n = Numbers
WB = EcoLine Western Blot (IgM/IgG)
VlsE = Variable major protein-like sequence, expressed

Western blot analysis of patients with EM

Western Blot results in the 148 EM patients at presentation and during follow-up are shown in Table 5. Already at presentation, IgG antibodies to the VlsE protein were found in 42% of the cases, and IgM antibodies against outer surface protein C in 39%.

Table 5. Banding pattern in EcoLine Western Blot results (% positive) from 148 erythema migrans patients at presentation, at 2-3 months or at 6 months.

<table>
<thead>
<tr>
<th>Sample time</th>
<th>IgM</th>
<th>OspC</th>
<th>VlsE</th>
<th>p39</th>
<th>EBV</th>
<th>VlsE</th>
<th>p39</th>
<th>p83</th>
<th>BBA36</th>
<th>BBO323</th>
<th>Crasp3</th>
<th>pG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presentation</td>
<td>39</td>
<td>10</td>
<td>16</td>
<td>0</td>
<td>42</td>
<td>11</td>
<td>12</td>
<td>3</td>
<td>11</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2-3 months</td>
<td>48</td>
<td>8</td>
<td>13</td>
<td>0</td>
<td>46</td>
<td>15</td>
<td>12</td>
<td>5</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>6 months</td>
<td>43</td>
<td>5</td>
<td>11</td>
<td>0</td>
<td>38</td>
<td>16</td>
<td>12</td>
<td>5</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

EM = Erythema migrans
OspC = Outer surface-protein C
VlsE = Variable major protein-like sequence, expressed
EBV = Epstein-Barr Virus
Crasp3 = Complement regulator-acquiring surface protein 3-
BBA36, BBO323 and pG are in-vivo expressed B. burgdorferi antigens.
Discussion

This study demonstrated that while the majority of patients with EM showed concordant serological responses to the C6 peptide and IR6 peptides at presentation, 41% of the patients revealed variable responses between the peptides originating from the three genospecies of *B. burgdorferi* sensu lato. Interestingly, in 7/148 (5%) of the EM patients a seroresponse to the IR6 peptide originating from *B. burgdorferi* s.s. was detected at presentation, while a negative response was noted in the C6 test using the even more stringent in house cut-off level. In all of these cases a positive VlsE IgG band was detected by Western Blot. Taken together, these results suggest there may be cases of early Borrelia infection in Europe that the C6 test may not detect.

Generally, seropositivity for the different IR6 peptides corresponded well to recent findings in Finnish patients with EM (Sillanpää et al., 2007). However, seropositivity for the C6 peptide was 36% at presentation in this study compared to 55% and 65% in Finnish and Slovenian patients with EM at diagnosis and to 91% in early localised LB in Dutch patients (Sillanpää et al., 2007; Smismans et al., 2006). The variation in the C6 seropositivity rates between different studies may be related to differences in the patient material or timing of sampling. In addition, the relative distribution of different *Borrelia* genospecies in different geographical locations may explain the somewhat discrepant serological responses. Such differences have previously been documented: In a Finnish study, the majority of culture or PCR verified EM patients were positive for *B. garinii* (Oksi et al., 2001) while a recent Swedish study from Blekinge County showed a predominance of *B. afzelii* in a comparable patient material (Bennet et al., 2006).

Taking into account the heterogeneity of the IR6 region of the immunodominant surface protein VlsE among the three borrelial genospecies (Göttner et al., 2004, Sillanpää et al., 2007), a weaker or absent seroresponse to C6 could be predicted in subjects infected by *B. burgdorferi* s.s. or *B. afzelii*, at least in the early stage of the infection.
As expected, an early antibody immune response of IgM class in the EM patients was noted to outer surface protein C (OspC) as shown by a seropositivity rate of 39% at presentation in Western Blot. Regarding IgG Western Blot response, the VlsE antigen clearly dominated the investigated IgG bands with 42% seropositivity already at presentation. Changes over time in Western Blot results were minor.

The most interesting IR₆ peptide results from this study was with the P1 peptide derived from *B. burgdorferi* s.s. This is somewhat surprising, as this genospecies is believed to be unusual in this particular geographic area. In another study of EM in southern Sweden, skin biopsies from 118 patients with EM lesions were PCR positive for *Borrelia* of which 74% were infected by *B. afzelii* and 26% were infected by *B. garinii*. No cases infected with *B. burgdorferi* s.s. were reported (Bennet et al., 2006). However, the findings of the present study with a predominance of IR₆ antibody response to *B. burgdorferi* s.s. might be explained by cross reactivity between IR₆ antigens. Similar data has been reported where IR₆ antibodies to *B. burgdorferi* s.s. were detected in European patients with disseminated LB and conversely antibodies to IR₆ from *B. afzelii* seemed to be detectable equally efficiently in US patients supposedly infected by *B. burgdorferi* s.s. (Sillanpää et al., 2007).

The higher seropositivity rate at presentation against the C6 peptide compared to the delayed seroresponse to the various IR₆ peptides (IgG) in our investigation could possibly be explained by the fact that the commercial diagnostic kit used was not selective for antibody classes. However, due to insufficient specificity we chose not to include IgM ELISAs for the respective IR₆ peptides. Also, detection of IgM antibodies to the various IR₆ peptides did not improve detection of EM cases as compared to IgG antibodies (data not shown). As suggested earlier, there may also be other methodological differences affecting the results (Sillanpää et al., 2007). It could be of methodological interest to compare the IgG IR₆ peptide results in this study with an altered C6 test detecting only IgG antibodies. If doing so, a lower sensitivity
would be expected in a C6 IgG analysis compared to the commercial IgM/IgG kit. Although comparing partly different antibody classes, the highest correlation of serological responses was found between the C6 peptide test and the IR$_6$ peptide from *B. garinii*. This could be expected as the C6 peptide is based on an amino acid sequence from that particular genospecies (Liang et al., 1999). The main conclusion of the present study, that the C6 test may not cover all cases of erythema migrans, is in line with Sillanpää et al. (2007). However, the results from the present study showed the best correlation between the C6 ELISA and the *B. garinii* IR$_6$ peptide in contrast to the results by Sillanpää et al. (2007) where C6 correlated better with the *B. burgdorferi* sensu stricto IR$_6$ peptide. Moreover, in this study we have shown a number of C6 negative sera to be reactive with the *B. burgdorferi* sensu stricto IR$_6$ peptide. This is a new finding not observed in the study by Sillanpää et al. (2007).

In a highly endemic area for LB like Kalmar County in Sweden with a reported incidence of 160/100,000 inhabitants and year (Berglund et al., 1995), an increasing part of the population will have experienced one or more episodes of LB. Reinfection in our endemic area is common, and was reported by 31% of the patients in this study. Although LB and multiple episodes of LB are primarily related to a certain part of the population with behavioural habits generating a higher risk of tick bites, the population which previously had LB will expand and may cause further difficulties that must be taken into account when interpreting borrelia serology. A known problem of borrelia serology in our area is a high seroprevalence (8-14%) in the healthy adult population most probably generated by a previous exposure to *Borrelia* with a persisting detectable antibody response for many years not related to clinical symptoms or signs (Tjernberg et al. 2007). In this sense, seropositivity rates for all IR$_6$ peptides were indeed significantly higher in patients with self-reported previous LB as compared to those who did not report previous LB. However, regarding the C6 peptide this difference was not statistically significant suggesting that the C6 ELISA could be more suitable as a screening test for LB in individuals with a previous episode of LB. Another interesting finding was older age in women reporting previous LB in this study than in corresponding men. These results are con-
The ROC analysis showed a steep drop of specificity in the C6 peptide ELISA when reaching a sensitivity of around 65% which was not seen in the IR₆ peptide ELISAs. This is probably due to the analytical design of the C6 peptide ELISA. Apparently, the OD values of the C6 peptide ELISA in the blood donors group are very close to the OD values in the EM patients when reaching that sensitivity. This limits the possibilities to sensitize the C6 assay by in house modifications.

Although this study showed that in the majority of Swedish patients with EM the seroresponse to the tested IR₆ peptides and the C6 peptide are concordant at presentation, there are clinical cases of EM with negative C6 peptide results that may be detected mainly by seroresponse to a *B. burgdorferi* s.s. derived IR₆ peptide. Also, there were differences in the dynamics of the serological response to the C6 peptide as compared to the tested IR₆ peptides. These results may depend on differences in test formulations and differences in antigenic properties between the peptides. In Europe, differences in the local geographical distribution of borrelial genospecies may cause variation in serological response depending on the specific antigen used. Perhaps, an ELISA using a mixture of the C6 peptide and a *B. burgdorferi* s.s. derived IR₆ peptide could be of value in the serodiagnosis of LB in Europe. Further studies of combinations of antibody responses to IR₆ peptides and the C6 peptide in other LB manifestations with or without previous LB and control cases are needed to address this issue.
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