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Original article

Borrelia miyamotoi infection leads to cross-reactive antibodies to the C6 peptide in mice and men

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ABSTRACT

Objectives: *Borrelia miyamotoi* is a relapsing fever *Borrelia*, transmitted by hard (*Ixodes*) ticks, which are also the main vector for *Borrelia burgdorferi*. A widely used test for serodiagnosis of Lyme borreliosis is an enzyme immunoassay (EIA) based on the C6 peptide of the *B. burgdorferi* sl VlsE protein. We set out to study C6 reactivity upon infection with *B. miyamotoi* in a large well-characterized set of *B. miyamotoi* disease (BMD) patient sera and in experimental murine infection.
Methods: We performed *in silico* analyses, comparing the C6-peptide to immunodominant *B. miyamotoi* variable large proteins (Vlps). Next, we determined C6 reactivity in sera from mice infected with *B. miyamotoi* and in a unique longitudinal set of 191 sera from 46 BMD patients.
Results: *In silico* analyses revealed similarity of the C6 peptide to domains within *B. miyamotoi* Vlps. Cross-reactivity against the C6 peptide was confirmed in 21 out of 24 mice experimentally infected with *B. miyamotoi*. Moreover, 35 out of 46 BMD patients had a C6 EIA Lyme index higher than 1.1 (positive). Interestingly, 27 out of 37 patients with a C6 EIA Lyme index higher than 0.9 (equivocal) were negative when tested for specific *B. burgdorferi* sl antibodies using a commercially available immunoblot.
Conclusions: We show that infection with *B. miyamotoi* leads to cross-reactive antibodies to the C6 peptide. Since BMD and Lyme borreliosis are found in the same geographical locations, caution should be used when relying solely on C6 reactivity testing. We propose that a positive C6 EIA with negative immunoblot, especially in patients with fever several weeks after a tick bite, warrants further testing for *B. miyamotoi*. **J. Koetsveld, Clin Microbiol Infect 2020;26:513.e1–513.e6**

Introduction

Borrelia miyamotoi is a relapsing fever *Borrelia*, transmitted by *Ixodes* ticks, that may cause *B. miyamotoi* disease (BMD), characterized by an acute febrile illness in immunocompetent patients and chronic meningo-encephalitis in immunocompromised patients [1–7]. *Ixodes* ticks are also the vector for *Borrelia burgdorferi sensu lato* (sl), the causative agent of Lyme borreliosis. In *Ixodes* ticks, a correlation is found in infection rates of *B. miyamotoi* and *B. burgdorferi* sl [3].BMD is diagnosed by qPCR on blood during the acute phase of infection [1,4]. In patients with longer disease duration, outside of febrile episodes or for epidemiological purposes serology can be performed, relying on detection of antibodies against glycerophosphodiester phosphodiesterase (GlpQ) [4,8]. GlpQ is found in all relapsing fever *Borrelia*; however, the only relapsing fever *Borrelia* found in *Ixodes* ticks is *B. miyamotoi* [9].

A widely used serodiagnostic test for Lyme borreliosis is the C6 enzyme immunoassay (EIA). This test is based on the C6 peptide, a 25 amino acid peptide from the outer membrane protein VlsE that is highly conserved across *B. burgdorferi* sl species [10,11]. The C6

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EIA is considered to be highly specific and sensitive to diagnose disseminated Lyme borreliosis, while sensitivity in early-localized disease (erythema migrans) is limited (approximately 50–70%) [11].

Interestingly, two studies describing the diagnostic accuracy of the C6 peptide, found limited cross-reactive antibodies in sera from patients with relapsing fever borreliosis [10,12]. A more recent study showed that individuals who were seropositive for *B. burgdorferi* were more likely to be GlpQ seropositive than those who were *B. burgdorferi* seronegative [13]. Also, two recent cases of BMD in the United States and in Europe were found to be reactive against the C6 peptide while *B. burgdorferi* immunoblots remained negative [14,15]. Importantly, another study recently showed that in a set of 43 sera from 24 patients with BMD from the United States the C6 ELISA was also positive in the vast majority (>90%) of convalescent phase serum samples [16]. Collectively, these findings suggest cross-reactivity of antibodies against *B. miyamotoi* proteins with the C6 peptide [8,17]. *B. miyamotoi* expresses Variable major proteins (Vmps) belonging either to the Variable large protein (Vlp) family, further divided into alpha, gamma and delta subfamilies, abbreviated as Vlp-α, Vlp-γ and Vlp-δ – homologous to *B. burgdorferi* sl VlsE – or to the Variable small protein (Vsp) family – homologous to *B. burgdorferi* sl outer surface protein C (OspC).

BMD and Lyme borreliosis are different disease entities that are caused by pathogens that are present in ticks in the same geographical locations. To date, little is known about the burden of BMD and little is known about the long-term effects of infection with *B. miyamotoi*. Although treatment regimens for both diseases and antimicrobial susceptibility of both pathogens are similar [18,19], it is important to be able to differentiate between these diseases. Based on the above, we aimed to determine C6 EIA cross-reactivity *in silico*, in experimentally *B. miyamotoi*-infected mice and in PCR-proven BMD patients from Russia.

Materials and methods

In silico analysis

All available *B. miyamotoi* Vmp amino acid sequences from each antigenic group (Vlp-α, Vlp-γ, Vlp-δ and Vsp) were downloaded from GenBank and/or Uniprot, with the exception of 'Reference Sequences' and duplicate sequences. As a next step we performed a NCBI Protein Blastp (protein–protein BLAST) search – restricted to *B. miyamotoi* (taxid: 47466) – using the C6 sequence (MKKDDQIAAAMVLRGMAKDGQFALK) as input. We subsequently selected all Vmp sequences with a query coverage >80% and identity with C6 peptide >50% (E-value >0.001). The Vmp amino acid sequences were aligned by Muscle and ClustalW, manually curated, and the similarity of the C6 peptide and Vmps sequences was assessed by MEGA 5.2 software (Pennsylvania State University, PA, USA).

As an independent and additional *in silico* analysis, we extracted all deposited protein sequences (open reading frames, ORFs) from eight *B. miyamotoi* strains (six Russian clinical isolates – Izh-4, Izh-5, Izh-14, Izh-16, Yekat-1 and Yekat-6 (BioProject PRJNA406856) – and two American tick-derived strains – CT13-2396 (BioProject PRJNA310783) and LB-2001 (BioProject PRJNA192621) – from Genbank by a custom Python script to a single fasta file. We next subjected the file containing all these amino acid sequences extracted from eight *B. miyamotoi* genomes to analysis by InterProScan software using default settings [20] and using the InterProScan match look-up service version 5.23-62.0, with a search against Pfam and SUPERFAMILY databases as an option. We subsequently retrieved all proteins matching PF00921 (Lipoprotein_2 family) of the Pfam database or SSF74748 (Variable surface antigen VlsE superfamily) of SUPERFAMILY databases from the fasta file by a custom Python script. Finally, alignments and phylogenetic analyses were performed using Muscle/ClustalW and MEGA 5.2 software [21].

Murine infection

Serum from previous *B. burgdorferi sensu stricto* (ss) N40 and *B. miyamotoi* LB-2001 (North American isolate) mouse infection experiments were used [8]. For these experiments 6- to 8-week-old female C3H/HeN mice (Charles River Laboratories) were infected by subcutaneous injection with 1×10^5 N40 spirochetes or by intraperitoneal (ip) injection with 1×10^7 LB-2001 spirochetes in phosphate-buffered saline (PBS). Sera were taken before infection and 20 days (N40) or 14 days (LB-2001) post infection (dpi) [8]. In addition, we infected 6- to 8-wk-old female Hsd:ICR (CD-1) mice by ip infection with 1×10^7 *B. miyamotoi* HT31 (Asian isolate) spirochetes in 200 μL of PBS, and sera were collected 15 or 22 dpi. Infection of mice was confirmed by dark-field microscopy (*B. miyamotoi* LB-2001 infected mice) or qPCR (*B. burgdorferi* ss N40 and *B. miyamotoi* HT31 infected mice).

Mouse ELISA

To detect IgM and IgG antibodies directed against GlpQ, high-binding half-surface plates (Greiner Bio-One) were coated overnight at 4°C with 4 μg/mL full length, *E. coli* expressed, his-tagged recombinant GlpQ derived from HT31 in PBS. Plates were washed in PBS–Tween 20 (0.05%) and incubated for 2 hr in blocking buffer (PBS plus 1% bovine serum albumin (BSA)) at room temperature (RT). To measure IgM and IgG directed against the C6 peptide, high-binding half-surface plates were coated overnight at 4°C with 4 μg/mL streptavidin (cat. no. 21122, Thermo-Fisher, The Netherlands) in 50 mM carbonate buffer (pH 9.2). Next, plates were washed and incubated for 2 hr with 5 μg/mL biotinylated C6 peptide (Genemed Synthesis, San Antonio, TX, USA) in blocking buffer at RT. Plates were subsequently washed and incubated for 1 hr at RT with 1:200 mouse sera in blocking buffer. Plates were washed and incubated for 1 hour with 1:2000 anti-mouse IgG-HRP (Cell Signaling Technology, Danvers, MA, USA) or 1:2000 anti-mouse IgM (SouthernBiotech, Birmingham, AL, USA), in blocking buffer. Finally, plates were washed and developed using TMB substrate, and absorbance was read at 450–655 nm (BioRad, Hercules, CA, USA). GlpQ ELISAs on mouse sera were performed as previously described [8]. GlpQ and C6 ELISAs were considered positive when values were greater than the mean plus three standard deviations of eight non-infected mice.

C6 EIA and B. burgdorferi Western Blot in PCR-proven BMD cases

Stored sera were used from 46 patients with PCR-confirmed *B. miyamotoi* infection from two locations, Izhevsk (European Russia) and Yekaterinburg (Asian Russia) [1,22,23]. A total of 191 sera drawn at different time points were available from these 46 patients were tested by C6 Lyme enzyme immunoassay (EIA) (Immunetics, Oxford, UK) according to the manufacturer's instructions. Number of available sera and sampling times are listed in Table S1. As per the manufacturer's recommendations, the cut-off (Lyme index) for positivity was 1.1, a Lyme index between 0.9 and 1.1 was designated as an equivocal result and a Lyme index below 0.9 was considered negative. Of all patients in which at least one serum sample was equivocal or positive – i.e. a C6 EIA Lyme-index ≥ 0.9 – the serum sample with the highest Lyme index was tested for IgM and IgG antibodies against *B. burgdorferi* antigens using a commercially available blot (Mikrogen diagnostic, Neuried,

Germany). Blots were also interpreted according to the manufacturer's instructions.

Ethics statement

The animal research protocols were approved by the Academic Medical Centre's Ethical Committee for Animal Research (protocol no. DIX103058), Yale University's Institutional Animal Care and Use Committee (protocol no. 2014-07941) and by the Wageningen University Ethical Committee for Animal Research (protocol no. 2016003). All Russian patients signed an informed consent form in accordance with, and approval from, the institutional review boards of the Republican Hospital of Infectious Diseases (Udmurt Republic, Russia), Medical association 'Novaya Bolnitsa' (Yekaterinburg, Russia) and the Council on Bioethics of the Izhevsk State Medical Academy (Minutes no. 17 of 24.12.2012).

Statistical analysis

Data analyses were performed using Graphpad Prism 7.0.2 software (Graphpad Prism Software Inc., La Jolla, CA, USA). Column statistics were performed to calculate medians and standard errors of the mean. Non-parametric Mann–Whitney tests were performed to calculate statistical differences between groups. A p values < 0.05 was considered statistically significant.

Results

In silico analysis

A total of 57 *B. miyamotoi* Vmp sequences (23 Vlp-δ, 8 Vlp-γ, 5 Vlp-α and 21 Vsp) were obtained from GenBank and compared with the C6 peptide. The average identity of C6-like amino acid fragments of Vlp-δ and Vlp-γ with the C6 peptide was 0.58 (range 0.52–0.64, corresponding to 12 to 9 mismatches) and 0.51 (range 0.44–0.56, corresponding to 14 to 11 mismatches). Alignment of the C6 peptide with selected Vlps from these subfamilies is shown in Fig. 1. We identified ten highly conserved amino acid and several motifs that are present in the C6 and all Vlp-δ and Vlp-γ sequences. C6-like fragments of Vlp-δ and Vlp-γ were more homologous to each other than to the C6 peptide as shown in Fig. S1 using p-distance as a measure of homology. No significant similarities were found between the C6 peptide and Vlp-α (Fig. S2) or Vsp (data not shown) protein sequences. With an independent *in silico* analysis, described in more detail in Materials and methods, 222 Vlp protein sequences were identified in plasmids from eight *B. miyamotoi* strains. The file containing the complete sequences and their description is available in the supplementary material. The MEGA-file containing these aligned sequences is available in the supplementary material. The lengths of these complete Vlp protein sequences (or large fragments) varied from 314 to 365 amino acids. All could be successfully aligned to the C6 peptide. Four sequences represented Vlps that are expressed in Izh-4, Izh-5 and Izh-16 (Vlp-δ) and Izh-14 (Vlp-γ), while the other sequences represented 'archival' Vlps (Table 1 and Fig. S2). Thus, in line with the above-described *in silico* analysis, we show that Vlp-δ is most frequently represented and is most similar to the C6 peptide, followed by Vlp-γ, whereas Vlp-α has no fragments that are antigenically similar to the C6 peptide, and is the rarest annotated Vlp (Table S1).

Antibodies in Borrelia miyamotoi infected mice

None of the naive mice showed antibodies to GlpQ or the C6 peptide. GlpQ IgM and IgG antibodies were detected in seven out of eight mice infected with *B. miyamotoi* LB-2001 (Figs. 2A,B), but in none out of eight mice infected with *B. burgdorferi* (data not shown). In mice infected with *B. miyamotoi* HT31, GlpQ IgM was only detected in one out of eight mice 15 dpi and in none of the mice at 22 dpi. However, IgG antibodies against GlpQ were detected in all of these mice (Figs. 2A,B). IgM antibodies against the C6 peptide were detected in all *B. miyamotoi* LB-2001 infected mice 14 dpi (Fig. 2C) and six out of eight showed IgG antibodies against the C6 peptide (Fig. 2D). Both IgM and IgG antibodies against the C6 peptide were significantly higher in LB-2001-infected mice than in naive mice (Figs. 2C,D). Interestingly, the two C6 IgG seronegative mice also developed the lowest IgG response against GlpQ, one of which was GlpQ negative, and might actually not have been infected at all. In HT31 infected mice, four out of eight showed IgM antibodies and seven out of eight showed IgG antibodies against the C6 peptide 15 dpi. At 22 dpi one out of eight *B. miyamotoi* HT31 infected mice showed IgM antibodies to C6 and six out of eight mice had IgG antibodies reactive against the C6 peptide (Figs. 2C,D). As a reference, all *B. burgdorferi ss* strain N40 infected mice developed IgM and IgG antibodies against the C6 peptide (Fig. S3).

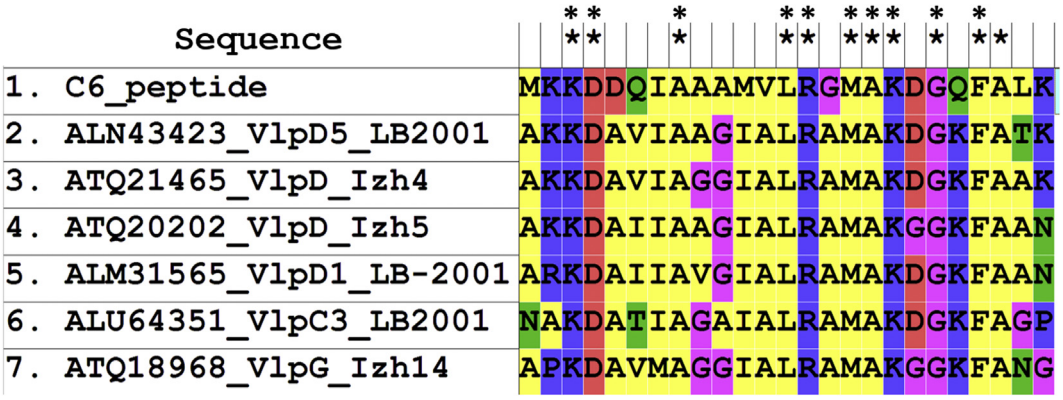

Fig. 1. Alignment of the C6-peptide with *B. miyamotoi* gamma and delta subfamily variable large proteins (Vlps). Depicted are exemplary amino acid, i.e. the most similar and least similar Vlp-δ sequences (no. 2 and no. 5, respectively) and Vlp-γ (no. 6 and no. 7, respectively), as well as three Vlps expressed in three Russian clinical isolates (no. 3, no. 4 and no. 7). Sequences are designated by their GenBank Accession Number_Type of Vlp_Name of the Source Strain. Conserved sites in the depicted sequences are marked by five-rayed stars and conserved sites in all retrieved Vlp-δ and Vlp-γ proteins (not shown) are marked by six-rayed stars. Amino acids are indicated by a letter and the background colors represent the group of amino acid types.

Table 1
Comparison of the C6 peptide with C6-like fragments of Vlps from 8 strains of *B. miyamotoi*

	B. miyamotoi strain								Total N	Identity with C6 peptide,%, mean ± SD	Identity with C6 peptide, %, range
	Izh-4 N	Izh-5 N	Izh-14 N	Izh-16 N	Yekat-1 N	Yekat-6 N	CT13-2396 N	LB-2001 N			
Vlp subfamily											
Vlp-δ	26	14	24	16	19	12	19	2	**132**	**59 ± 4**	**48 – 64**
Vlp-γ	9	6	15	7	8	6	4	0	**55**	**51 ± 5**	**40 – 60**
Vlp-α	5	4	10	7	3	4	1	1	**35**	**25 ± 2**	**24 – 28**
Total	40	24	49	30	30	22	24	3	**222**		

Table showing the total number (N) of genes encoding for Variable large proteins (Vlps) and the number of Vlps from three different Vlp subfamilies (Vlp-δ, Vlp-γ and Vlp-α) for 8 different strains of *B. miyamotoi* (Izh-4, Izh-5, Izh-14, Izh-16, Yekat-1, Yekat-6, CT13-2396 and LB-2001). The left two columns show the percentage of identity, including standard deviation (SD) and range, between these *B. miyamotoi* Vlps and the C6 peptide.

C6 and B. burgdorferi antibodies in patients with BMD

Of the 191 tested sera 15 sera were equivocal and 104 were positive for antibodies to the C peptide. Sera were (arbitrarily) divided into seven groups based on sampling time after onset of disease: 0–2, 3–5, 6–9, 10–19, 20–50, 51–126 and more than 136 days (Fig. 3, Table 2). C6 EIA Lyme index increased significantly over time: C6 EIA Lyme indexes were higher in sera from groups as early as 6–9 days after onset of disease, and in all groups sampled later, when compared with C6 EIA Lyme indexes in sera sampled between 0 and 2 days after onset disease (Fig. 3). In the earliest time window (0–2 days after onset of disease) eight out of 27 (29.6%, 95%CI 13.8–50.1) samples were positive and two showed equivocal results. The highest percentage of reactive sera occurred between 10 and 19 days after onset of disease: 18 out of 20 sera (90%, 95%CI 68.3–98.8) had a C6 EIA Lyme-index higher than 0.9 (Table 2). In total, of the 46 tested patients, 35 patients had a C6 Lyme index higher than 1.1 (positive) in at least one serum sample and four patients had a C6 Lyme index between 0.9 and 1.1 (equivocal) (Table S2). We previously assessed anti-Vmp antibody responses in the same patients by ELISA [11] and plane protein microarray [15]. Out of the 39 patients with C6 reactivity, 36 patients (92%) had IgM

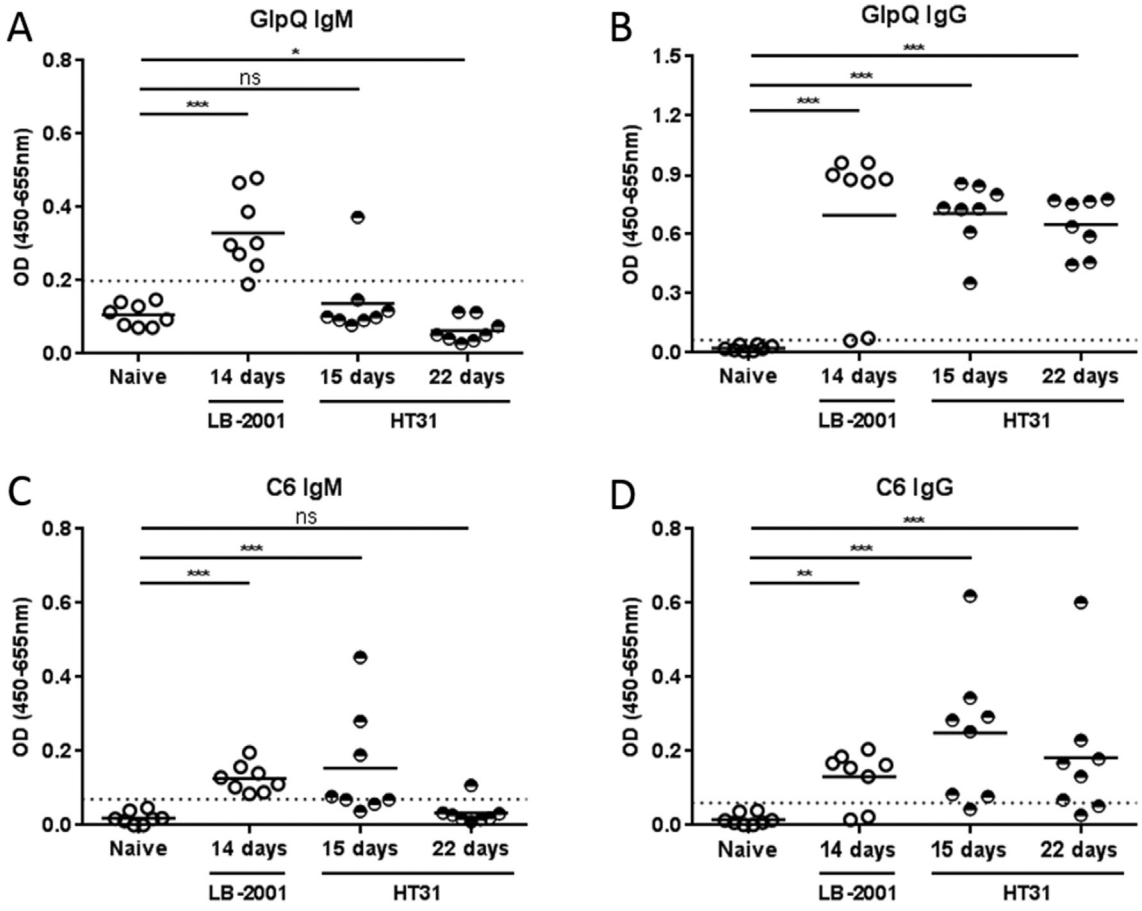

Fig. 2. IgM and IgG antibodies against GlpQ (glycerophosphodiester phosphodiesterase) and the C6 peptide in *B. miyamotoi* infected mice. Depicted are anti-GlpQ and anti-C6-peptide antibody levels in naive mice as well as in mice infected with *B. miyamotoi* LB-2001 and HT31. GlpQ IgM- and IgG antibodies are shown in A and B, respectively. Antibodies against the C6 peptide are shown in C (IgM) and D (IgG). On the *y*-axis the ELISA result is shown as optical density (OD). The dotted line in each panel indicates the cut-off, above which samples are considered positive. For IgM cut-offs were 0.2 for GlpQ and 0.07 for C6. For IgG cut-offs were 0.064 for GlpQ and 0.06 for C6. On the *x*-axis the different groups of mice are shown as indicated. Each circle shows one individual serum sample. Open circles show results from C3H/HEN mice, half-filled circles show results in CD-1 mice. Significance (compared to naive mice) is shown above each column. NS = not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

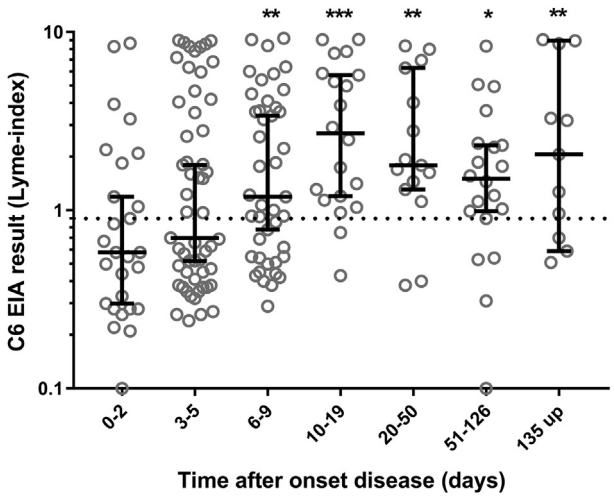

Fig. 3. C6 enzyme immunoassay (EIA) results in patients with *Borrelia miyamotoi* disease over time. C6 EIA results from patients with PCR-proven *Borrelia miyamotoi* disease (BMD) in sera sampled at different time windows after disease onset. On the *y*-axis the C6 EIA result is shown as the Lyme -index on a log10 scale. On the *x*-axis, time after onset of BMD is shown in days. The dotted line indicates the cut-off above which sera are considered equivocal or positive. Each circle shows one individual serum sample. Black line segments show the median C6 EIA result from serum samples within each time window. Error bars indicate the 95% CI from serum samples within each time window. Significant difference (compared to sera sampled between 0 and 2 days after onset of BMD) is shown above each column. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

and/or IgG antibodies to Vlp-δ and/or Vlp-γ. Out of the seven remaining C6-negative patients such antibodies were found only in three patients (43%): odds ratio 16.0 (95% CI 2.1–26.3, p 0.006). The proportion of patients producing antibodies to Vsp and/or Vlp-α in these two groups were similar: 15 of 39 (39%) and three of seven (43%), respectively.

In patients with a C6 Lyme index higher than 0.9 blot results showed that out of the 37 tested patients nine were positive by IgM immunoblot (and two indeterminate) and three were positive by IgG immunoblot (and three indeterminate). Thus, out of the 37 patients with either positive or equivocal C6 EIA results, 27 did not contain *B. burgdorferi*-specific IgM and/or IgG antibodies as determined by immunoblot (Table S2).

Discussion

Borrelia serology is the cornerstone of Lyme diagnostics [24,25]. A commonly used serological method is the C6 EIA, followed by an immunoblot confirmation, so-called two-tier testing [10,11]. We show that this C6 peptide aligns to several Vlps of *B. miyamotoi* (Table 1, Fig. 1, Figs. S1 and S2). We have shown that Vlps are immunodominant proteins in BMD and that antibodies against Vlps are produced in early BMD [8,17]. Therefore, cross-reactivity between anti-Vlp antibodies and the C6 peptide could be expected. Indeed, previous studies showed C6 reactivity in patients infected with *B. miyamotoi* [14–16]. However, these studies were merely observational and did not provide any further insight into the mechanism behind these findings. To more definitely determine whether *B. miyamotoi* induces cross-reactive antibodies to the C6 peptide we performed experimental murine studies. While the experimental ip inoculation of mice with 1×10^7 LB-2001 spirochetes does not reflect natural infection, we do show development of antibodies to the C6 peptide in these mice (Fig S1, Fig. 2). At the moment of inoculation LB-2001 spirochetes predominantly express Vsp-1 with a minority population of spirochetes expressing Vlp-γ and HT31 predominantly expresses Vlp-δ [8,17]. We postulate that reactive antibodies against these Vlps cause C6-cross-reactivity.

We also tested the C6 antibody response in a longitudinal set of BMD sera and showed that 39 out of 46 patients produced C6-reactive antibodies. As expected from *in-silico* analysis, a clear correlation between C6 positivity and the production of antibodies to Vlp-δ and/or Vlp-γ was observed. Interestingly, only ten of these patients were reactive in an immunoblot test commonly used in two-tier testing for Lyme disease (Table S1). These ten patients did not have erythema migrans or other Lyme borreliosis symptoms, but had a clinical presentation consistent with BMD with fever, thrombocytopenia and lymphopenia [1,22,23]. Since up to 40% of *Ixodes* ticks are infected with *B. burgdorferi* sl in the geographical location were these sera were collected, (asymptomatic) concurrent or previous *B. burgdorferi* sl could explain these positive blot results. An alternative explanation could be cross-reactive antibodies induced by *B. miyamotoi* to other *B. burgdorferi* sl antigens by immunoblot.

Our results demonstrate that cross-reactive antibodies against the C6 peptide regularly occur in patients with BMD, with as much as 90% (95% CI 68.3–98.8) of patients being C6-reactive in samples taken 10 to 19 days after onset of disease. In addition, we have shown that C6 reactivity can be detected as early as 6–days after onset of BMD. Since BMD and Lyme borreliosis are found in the same geographical locations, it is important to note that the C6 peptide by itself does not allow for serological distinction between these two hard tick-borne diseases. Therefore, two-tier testing, together with a critical evaluation of the clinical symptoms, is necessary to discriminate between Lyme borreliosis and BMD. In patients with clinical symptoms compatible with BMD, a positive C6 EIA and negative *B. burgdorferi* immunoblot, further PCR-based diagnostics and/or serological testing (e.g. the detection of anti-GlpQ antibodies) for *B. miyamotoi* might be warranted.

Transparency declaration

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Table 2
Median C6 Lyme index and percentage of C6 positive sera in samples drawn at different time windows after onset of disease

Time after onset disease	Number of samples	Median C6 Lyme index (95% CI)	*p* value	% C6 > 0.9 (95% CI)	% C6 > 1.1 (95% CI)
0–2	27	0.58 (0.3–1.19)	–	37.0 (19.4–57.6)	29.6 (13.8–50.1)
3–5	55	0.7 (0.52–1.8)	0.1	49.1 (35.4–62.9)	45.5 (32.0–59.5)
6–9	43	1.19 (0.78–3.39)	0.0069	62.8 (46.7–77.0)	51.2 (35.5–66.7)
10–19	20	2.71 (1.2–5.73)	0.0003	90 (68.3–98.8)	80 (56.4–94.3)
20–50	15	1.79 (1.31–6.3)	0.0052	86.7 (59.5–98.3)	86.7 (59.5–98.3)
51–126	20	1.51 (0.99–2.32)	0.0225	80 (56.3–94.3)	65 (40.8–84.6)
135 up	11	2.06 (0.59–8.95)	0.0083	72.7 (39.0–94.0)	63.6 (30.8–89.1)

Median C6 Lyme index for different time windows after onset of *B. miyamotoi* disease is shown with 95% confidence interval (CI). Significance (compared to sera sampled between 0–2 days after onset disease) is indicated as *p* values. The last and second to last columns show the percentage of sera (including 95% CI) with a Lyme index higher than 0.9 or higher than 1.1 for each group. $p<0.05$ was considered significant.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2019.07.026.

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