Ticks and Tick-borne Diseases 10 (2019) 166–169

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Immunoblot reactivity at follow-up in treated patients with Lyme neuroborreliosis and healthy controls

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ARTICLE INFO

Keywords:
Lyme disease
Lyme neuroborreliosis
Immunoblot
Follow-up
Comparison
Line immunoblot

ABSTRACT

About 5–20% of the general population in endemic areas have seroprevalence for anti-borrelial antibodies. Previous studies have shown a high rate of 25–97% of persisting anti-borrelial antibodies in patients with treated Lyme neuroborreliosis (LNB) at follow-up. These studies used immunoblots with antigens from whole-cell sonicates, which could be less specific than modern recombinant antigens. We assessed the seroprevalence of anti-borrelial antibodies in serum from patients with definite LNB and healthy controls with a line immunoblot using highly specific recombinant antigens.

We retrospectively identified patients with treated definite LNB who were treated at the Medical Center–University of Freiburg. Serum from LNB patients at a mean follow-up period of 4.9 years (SD: 3.3) and serum from healthy controls were assessed for anti-borrelial antibodies with a line immunoblot with recombinant antigens.

A total of 45 patients with definite LNB and 40 healthy controls were included. Ten LNB patients (22.7%) had persisting antibodies (IgG and/or IgM) in serum at follow-up. Serum samples from six healthy controls (15%) were positive for anti-borrelial antibodies (IgG and or IgM). Prevalence of positive IgM or IgG antibodies showed no statistically significant difference between LNB patients at follow-up and healthy controls (IgM $p = 0.32$, IgG $p = 0.54$). Immunoblot reactivity patterns at follow-up in LNB patients did not have statistically significant differences from healthy controls.

The discrepancy regarding earlier studies reporting higher amounts of LNB patients with persisting antibodies could be due to a higher specificity of the antigens used in recombinant immunoblots compared to other immunoblots (e.g., whole-cell sonicates). The results of our study should be replicated in a larger prospective multicenter study.

1. Introduction

Lyme borreliosis is a tick-borne infectious disease caused by the spirochete bacterium Borrelia burgdorferi sensu lato. About 3–15% of patients with Lyme borreliosis develop affections of the nervous system (Halperin, 2012; Huppertz et al., 1999; Wilking and Stark, 2014). Typical manifestations of Lyme neuroborreliosis (LNB) are polyradiculoneuritis, often with cranial nerve involvement (known as Bannwarth's syndrome) and meningitis (Pfister et al., 1987). Diagnosis of LNB is based on clinical presentation, serologic testing, and analysis of cerebrospinal fluid (CSF) (Mygland et al., 2010). International tiered case definitions exist regarding the likelihood of diagnosis depending on available diagnostic results (Kaiser, 1998; Mygland et al., 2010).

About 9.4% (95% CI 8.4–10%) of the general population in endemic areas show seroprevalence of anti-borrelial antibodies (Wilking et al., 2015). Estimates for Germany are 13% for males and 6% for females (Wilske et al., 2007). Seroprevalence rises with age. In certain populations (e.g., forest workers), seroprevalence can be as high as 34% (Oehme et al., 2002).

As high seroprevalence of anti-borrelial antibodies in serum leads to a low positive predictive value for diagnosing LNB in serologic testing alone, case definitions require CSF analysis for probable and definite diagnosis of LNB (Mygland et al., 2010). A meta-analysis of diagnostic test accuracy of serologic tests in Lyme borreliosis found a sensitivity of 0.81 (95% confidence interval [CI]: 0.57–0.94) and a specificity of 0.92 (95% CI: 0.88–0.95) for immunoblot testing in serum (Leeflang et al., 2016). Immunoblot reactivity seems to be linked to disease manifestation of Lyme borreliosis. In early manifestations, the spectrum of

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https://doi.org/10.1016/j.ttbdis.2018.09.011
Received 20 July 2018; Received in revised form 19 September 2018; Accepted 24 September 2018
Available online 26 September 2018
1877-959X/

detectable bands is narrow, usually containing IgM antibodies against OspC, Vlse, and flagellin (Goettner et al., 2005; Rauer et al., 1998; Wilske et al., 1993).

In late manifestations, a broader spectrum of bands is detectable, usually with additional IgG reactivity and positive bands for the antigens p83, p58, p43, p30, p21, DbpA, and p14 (Goettner et al., 2005; Rauer et al., 1998; Wilske et al., 1993). Different generations of antigens used in immunoblot assays exist. More recent immunoblots using specific recombinant antigens show higher diagnostic test accuracy compared to tests using earlier techniques of providing antigens, like whole-cell sonicates or purified antigens (Leeflang et al., 2016).

Previous studies have stated that the majority of LNB patients remain seropositive for anti-borrelial antibodies after antibiotic treatment and at long-term follow-up. Using an immunoblot with antigens from whole-cell sonicates, one study found persisting anti-borrelial IgM antibodies in 97% of 46 patients with either LNB or Lyme arthritis 27 months after initial diagnosis (Hilton et al., 1997). Another study using an immunoblot with antigens from whole-cell sonicates found persisting anti-borrelial IgM antibodies in 18% and anti-borrelial IgG antibodies in 25% of 79 patients with early Lyme borreliosis (erythema migrans, early LNB, and other early disseminated infection) and after a follow-up period of 10–20 years (Kalish et al., 2001). Another study reported that 65% of LNB patients were seropositive 3.5–6 years after initial diagnosis using an ELISA to detect antibodies against purified flagellin from Borrelia burgdoferi (Hammers-Berggren et al., 1994).

In light of the high seroprevalence of anti-borrelial antibodies in the general population and the limited diagnostic test accuracy in the available studies, we assessed immunoblot reactivity using a test with highly specific recombinant antigens in LNB patients at follow-up after treatment as well as in healthy controls.

2. Material & methods

We retrospectively included adult patients with LNB treated from 2003 to 2014 at the Medical Center–University of Freiburg. Healthy controls were recruited in the same region as the LNB patients, where Lyme borreliosis is endemic. The study protocol was approved by the ethics committee of the Medical Center–University of Freiburg (EK-Fr. 269/13).

Patients had to fulfill the criteria for “definite” LNB as defined by Kaiser and Mygland et al. (Kaiser, 1998; Mygland et al., 2010). For definite LNB, patients were required to have compatible neurological symptoms, CSF pleocytosis, antibodies against *Borrelia burgdorferi s.l.* in serum and CSF, and evidence of a *Borrelia*-specific intrathecal synthesis of immunoglobulins (defined as an antibody index ≥2). Healthy controls were recruited through posted flyers at the Medical Center–-University of Freiburg. All patients and healthy controls gave their written informed consent to take part in the study.

Data on quality of life, fatigue, depression, and cognition of a portion of the included patients and healthy controls were reported elsewhere (Dersch et al., 2015). Surplus serum and CSF from the initial diagnostic workup from LNB patients were collected and stored at −80 °C. Serum was collected from LNB patients and healthy controls at the time of the follow-up examination. Immunoblot reactivity was assessed using a line assay for IgM and IgG antibodies with recombinant antigens from Ravo Diagnostika. Immunoblots with serum and CSF samples were performed according to the manufacturer’s instructions. Antigens detected by the used immunoblot are p83, p58, p14, p39, Vlse, OspA, OspC, DbpB, and DbpA.

According to the manufacturer’s instructions, immunoblot reactivity was assessed as positive for IgM when two or more of the antigens p39, OspC, 14 kDa, DbpA, or VlsE showed clearly visible reactivity or when an intense reactivity for the antigen OspC alone was detected. Immunoblot reactivity was regarded as positive for IgG when two or more of the antigens p83 (syn. p100), p58, p39, OspC, 14 kDa, DbpA, or VlsE showed clearly visible reactivity.

Table 1
demographic characteristics of LNB patients and healthy controls, and routine CSF parameters at time of initial diagnosis in LNB patients. Data is shown as mean, with standard deviation in brackets. CSF: cerebrospinal fluid; LNB: Lyme neuroborreliosis; n.a.: not available; WBC: white blood cell count.

	LNB (n = 44)	Healthy controls (n = 40)	p-value
Age [years]	54.34 (16.97)	43.55 (18.37)	0.0316
Sex [female]	21 (47.7%)	24 (54.5%)	0.2818
Follow-up after initial diagnosis [years]	4.9 (3.3)	n.a.	
CSF WBC [/µL]	209.9 (215.0)	n.a.	
Albumin quotient	24.82 (16.9)	n.a.	
CSF Lactate [mmol/l]	2.5 (0.9)	n.a.	

Immunoblot reactivity was independently assessed by three researchers. Disagreements were resolved by discussion among all researchers.

As we were primarily interested in immunoblot reactivity and reactivity patterns to single antigens we did not perform an ELISA for anti-borrelial antibodies.

Statistical comparisons for continuous data between two groups were performed using the Mann–Whitney U test; comparisons for dichotomous data were performed with the Fisher’s exact test. For estimating correlations, Spearman’s r test was used. Statistics were performed with Prism 4.0b for Macintosh GraphPad Software, San Diego, Calif.

3. Results

A total of 43 patients with definite LNB and 40 healthy controls were included. Healthy controls came from the same region as the LNB patients. Clinical and demographic data of the patients and healthy controls are shown in Table 1. LNB patients were statistically significantly older than the healthy controls. The mean follow-up period for the LNB patients was 4.9 years (standard deviation [SD]: 3.3) after the initial diagnosis.

Ten LNB patients (22.7%) had persisting antibodies in serum at follow-up (5 IgM, 3 IgG, and 2 IgM&IgG). Serum samples from LNB patients at follow-up (mean follow-up from initial diagnosis to follow-up investigation 4.9 years, SD: 3.3) were positive for IgM antibodies in seven patients (16.3%) and positive for IgG antibodies in five patients (11.6%). Serum samples from healthy controls were positive for IgM antibodies in three cases (7.5%) and positive for IgG antibodies in seven (17.5%) cases.

The mean total number for positive IgG antibodies against single antigens in LNB patients at follow-up was 3.4 (SD: 1.1) and in healthy controls was 2.7 (SD: 1.3). The mean total number of positive IgM antibodies against single antigens in LNB patients at follow-up was 3.7 (SD: 1.7) and in healthy controls was 3.4 (SD :2.5). There was not a statistically significant difference in the total number of positive bands for the LNB patients at follow-up and the healthy controls.

Overall six healthy controls (15%) showed positive anti-borrelial antibodies in immunoblot (1 IgM, 3 IgG, and 2 IgM&IgG). The prevalence of positive IgM or IgG antibodies at follow-up showed no statistically significant difference between the LNB patients and healthy controls at follow-up (IgM p = 0.32, IgG p = 0.54).

Whenever possible, immunoblot reactivity patterns from serum at follow-up was compared to reactivity patterns in serum from patients obtained at time of initial diagnosis. In most cases, initial immunoblot reactivity persisted in follow-up samples (especially reactivity against the OspC antigen), albeit the intensity of bands tended to be lower. In two samples, reactivity against p14 was lost in the follow-up samples.

IgG antibodies against the OspC antigen and IgM antibodies against the DbpA antigen tended to be more frequent in LNB patients, albeit

Table 2
IgG bands of single antigens in serum from LNB patients and healthy controls. P-value according to Fishers exact test.

antigen (IgG)	LNB patients (n = 44)	healthy controls (n = 40)	p-value
B.b.s.s. p83	1	3	0.3481
B. afz. P83	1	2	0.6029
p58 B.gar.	3	1	0.3481
B.b.s.s. 14 kDa	2	1	0.6029
B.afz. 14 kDa	1	1	1.0
B.gar. 14 kDa	2	3	0.6654
VlsE B.gar.	3	3	1.0
OspC B.sp.	1	1	1.0
OspC B.b.s.s.	2	1	0.6029
OspC B.afz.	1	0	1.0
OspC B.gar.	1	0	1.0
DbpA syn p18 B.b.s.s.	1	0	1.0
DbpA syn p18 B.afz.	5	1	0.2049

Table 3
IgM bands of single antigens in serum from LNB patients and healthy controls. P-value according to Fishers exact test.

antigen (IgM)	LNB patients (n = 44)	healthy controls (n = 40)	p-value
p58 B.gar.	1	0	1.0
B.b.s.s. 14 kDa	2	1	0.6029
B.gar. 14 kDa	3	4	0.7039
OspC B.sp.	6	2	0.2694
OspC B.b.s.s.	6	1	0.1122
OspC B.afz.	5	1	0.2049
OspC B.gar.	5	2	0.4367

this difference did not reach statistical significance (see Tables 2 and 3). Immunoblot reactivity patterns for single antigens in LNB patients at follow-up did not have a statistically significant difference from healthy controls (see Tables 2 and 3).

The prevalence of anti-borrelial antibodies at follow-up did not correlate with age in LNB patients ($p = 0.056$, Spearman's $r = 0.29$) or healthy controls ($p = 0.26$, Spearman's $r = 0.18$). Prevalence of anti-borrelial antibodies at follow-up did not correlate with time from initial diagnosis of LNB in LNB patients for IgG ($p = 0.59$, Spearman's $r = 0.09$) or IgM ($p = 0.7319$, Spearman's $r = 0.05$).

4. Discussion

We assessed serum and CSF samples from 44 patients with treated definite LNB at 4.9 years after initial diagnosis and serum samples from 40 healthy controls. A strength of our study is that all LNB patients had definite Lyme neuroborreliosis according to the strict case definition of definite LNB, with signs of acute infection in CSF analysis at time of initial diagnosis (Kaiser, 1998; Halperin et al., 2017; Mygland et al., 2010). All included patients had anti-borrelial antibodies in serum and CSF, pleocytosis, and intrathecal synthesis of anti-borrelial antibodies in CSF during acute disease.

The main result of our study is that seroprevalence of anti-borrelial antibodies in serum of treated LNB patients at five-year follow-up did not have a statistically significant difference from those of healthy controls from the same area with endemic Lyme borreliosis. At a mean follow-up period of 4.9 years (SD: 3.3), 10 LNB patients (22.7%) had persisting antibodies in serum (5 IgM, 3 IgG, and 2 IgM&IgG). Overall, six healthy controls (15%) showed positive anti-borrelial antibodies in immunoblot (1 IgM, 3 IgG, and 2 IgM&IgG). The prevalence of IgM or IgG antibodies at follow-up showed no statistically significant difference between LNB patients and healthy controls (IgM $p = 0.32$, IgG $p = 0.54$).

The seroprevalence in healthy controls in our study is in line with studies reporting similar seroprevalences of anti-borrelial antibodies in regions with endemic Lyme borreliosis (Wilking et al., 2015; Wilske et al., 2007). Despite minor differences in the immunoblot reactivity pattern between LNB patients and healthy controls, no clear pattern or single antigen indicative of a former definite LNB could be identified. Immunoblot reactivity seems to vanish in most LNB patients (77.3%) at follow-up. Treated LNB patients at follow-up and healthy controls show the same rate of seroprevalence for anti-borrelial antibodies in serum.

Our results are in contrast to earlier studies, which reported persisting anti-borrelial antibodies at follow-up in about 97% of patients with late Lyme borreliosis (Hilton et al., 1997) and in 25% of patients with early Lyme borreliosis (Kalish et al., 2001). These earlier studies used immunoblots with antigens from whole-cell sonicates. A meta-analysis of diagnostic test accuracy in serologic testing for Lyme borreliosis showed that sensitivity and specificity increased when newer generation tests with recombinant antigens were applied compared to tests with older generation antigens (whole-cell lysate or purified antigens) (Leeflang et al., 2016). The high seroprevalence of persisting antibodies in earlier studies, therefore, could be due to more unspecific reactivity in older generation immunoblots. We used a modern line immunoblot with highly specific recombinant antigens in our study. The results from earlier studies reporting high prevalence of persisting anti-borrelial antibodies in LNB patients could be due to a higher specificity of immunoblots using recombinant antigens.

Besides the use of immunoblots with antigens from whole-cell sonicates, the mentioned earlier studies also used ELISA for serologic testing. The application of ELISA testing alone without confirmation by a more specific diagnostic method could contribute to the higher rates of 'persistent seropositivity' in these earlier studies as compared to our study.

Another issue with these earlier studies is the lack of an epidemiologically matched control group to assess seroprevalence in the general population. Therefore, the seroprevalence in healthy controls regarding the respective immunoblot used in these studies is unknown. Regarding the lower specificity of immunoblots using whole-cell lysates, the earlier studies could have shown a seroprevalence in the general population similar to treated LNB patients at follow-up. However, such an issue cannot be tested retrospectively.

Several methodological issues regarding our study have to be discussed.

LNB patients were statistically significantly older than healthy controls in our study. As age is correlated with higher seroprevalence (Wilking et al., 2015), this difference could interfere with the results if we would have found a difference in seroprevalence between LNB patients and healthy controls. The fact that we did not find a difference between both groups despite the age difference therefore supports the reliability of our results.

A standardized follow-up period was not employed because of a retrospective study design. However, in our study regarding treated LNB patients at follow-up, time from initial diagnosis to follow-up investigation had no impact on immunoblot reactivity.

We did not perform CSF analysis at follow-up, which would have resulted in interesting data regarding prevalence of anti-borrelial antibodies in CSF at follow-up. However, additional CSF analysis was deemed unethical because LNB patients had no signs of ongoing disease and, therefore, CSF analysis would have been for scientific reasons only.

The results of our study should be replicated in a prospective, multicentric study. A prospective follow-up design investigating immunoblot reactivity over the course of the disease in LNB patients would lead to a better understanding of the role of serologic testing in Lyme borreliosis.

Conflicts of interest

RD, AS, MM, and OM have nothing to declare. SR reports receiving consulting and lecture fees, and grant and research support from Bayer Vital GmbH, Biogen Idec, Merck Serono, Novartis, Sanofi-Aventis,

Baxter, RG, and Teva. Furthermore, SR indicates that he is a founding executive board member of Ravo Diagnostika GmbH. OS reports receiving consulting and lecture fees, and grant and research support from Baxter, Bayer Vital GmbH, Biogen Idec, Genzyme, Merck Serono, Novartis, RG, Sanofi-Aventis and Teva.

Funding

Immunoblot tests were supplied by RAVO Diagnostika GmbH.

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