Immunodiagnostics of Lyme neuroborreliosis

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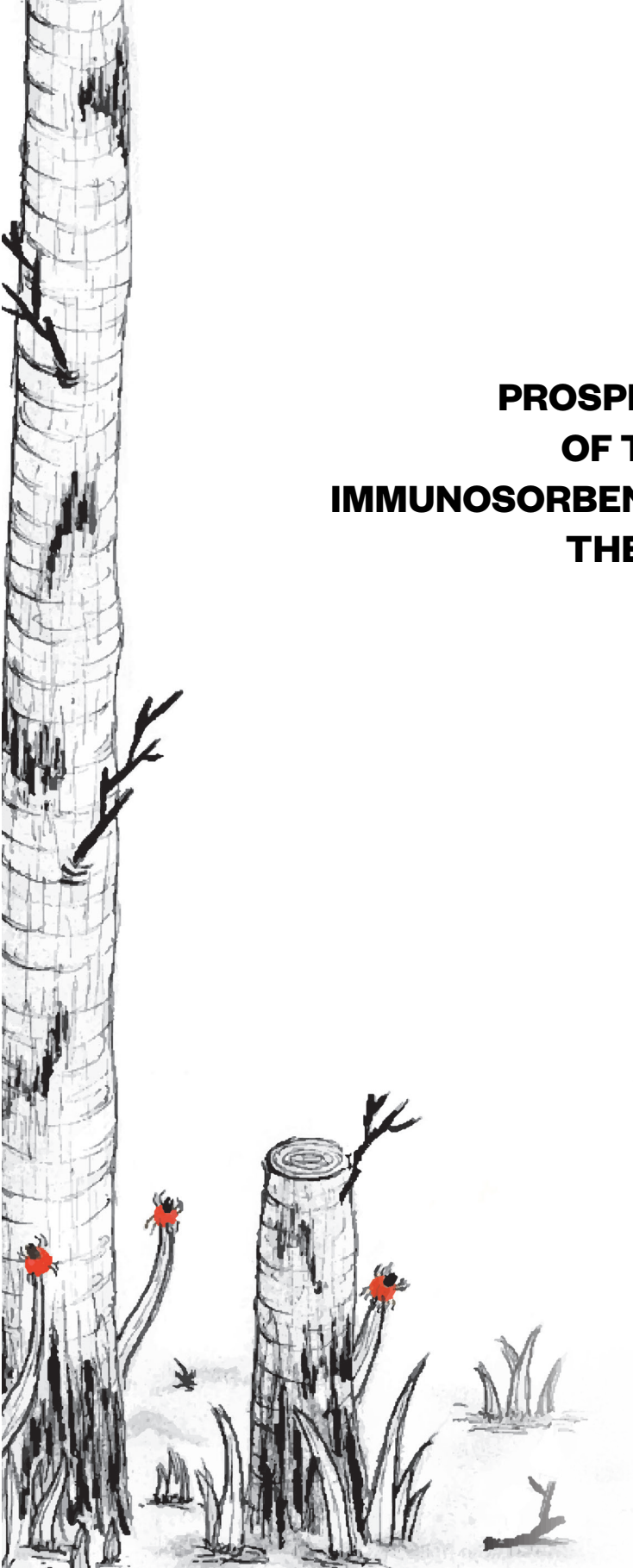

3 PROSPECTIVE COMPARISON OF TWO ENZYME-LINKED IMMUNOSORBENT SPOT ASSAYS FOR THE DIAGNOSIS OF LYME NEUROBORRELIOSIS

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ABSTRACT

Commercial cellular tests are used to diagnose Lyme borreliosis (LB), but studies on their clinical validation are lacking. This study evaluated the utility of an in-house and a commercial enzyme-linked immunosorbent spot (ELISpot) assay for the diagnosis of Lyme neuroborreliosis (LNB). Prospectively, peripheral blood mononuclear cells (PBMCs) were isolated from patients and controls and analysed using an in-house *Borrelia* ELISpot assay and the commercial LymeSpot assay. *B. burgdorferi* B31 whole-cell lysate and a mixture of outer surface proteins were used to stimulate the PBMCs and the numbers of interferon-gamma-secreting T cells were measured. Results were evaluated using receiver operating characteristic (ROC) curve analysis. Eighteen active and 12 treated LNB patients, 10 healthy individuals treated for an early (mostly cutaneous) manifestation of LB in the past and 47 untreated healthy individuals were included. Both assays showed a poor diagnostic performance with sensitivities, specificities, positive and negative predictive values ranging from 44.4-66.7%, 42.0-72.5%, 21.8-33.3% and 80.5-87.0%, respectively. The LymeSpot assay performed equally poorly when the calculation method of the manufacturer was used. Both the in-house and the LymeSpot assay are unable to diagnose active LNB or to monitor antibiotic treatment success.

KEYWORDS
Borrelia, Lyme neuroborreliosis, ELISpot, T cells, interferon-gamma

INTRODUCTION

Lyme borreliosis (LB) is a tick-borne disease caused by bacteria of the *Borrelia burgdorferi* sensu latu group. In Europe, the most prevalent species that cause LB are *B. afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto. The most common manifestation of LB is erythema migrans (EM); other manifestations include Lyme neuroborreliosis (LNB), Lyme arthritis (LA) and acrodermatitis chronica atrophicans (ACA). Most people, however, do not notice any symptoms and clear the infection unknowingly. In the Netherlands, surveys among general practitioners conducted in 1994 and 2017 showed a fourfold increase from an estimated 6,500 to an estimated 25,500 patients with EM [1, 2]. In addition, 1,500 cases of a disseminated manifestation of LB were reported in 2017 [1]. Thus, LB has an increasing impact on public health in the Netherlands [3].

The diagnosis of LB depends on clinical symptoms and can sometimes be difficult due to the lack of a 'gold standard' test, such as culture or polymerase chain reaction (PCR). Culture is only useful for skin manifestations such as EM or ACA, but is not recommended because of the varying sensitivity and long duration, and EM is mainly a clinical diagnosis [4-6]. PCR is particularly useful in skin manifestations and LA [5, 7]. For LNB, both culture and PCR show varying sensitivity and are mostly useful in the early phase of the disease [5, 7-10].

The most frequently used laboratory test for LB is based on the detection of *Borrelia*-specific antibodies. Unfortunately, the interpretation of serological tests can be difficult, as *Borrelia*-specific antibodies can persist lifelong and, hence, do not discriminate between an active LB and a cleared infection. Furthermore, the absence of *Borrelia*-specific antibodies in the early phase of the infection does not exclude LB [11]. Therefore, better diagnostic tools are needed that can establish an active LB, especially because early antibiotic therapy has proved to be effective [12].

In recent years, various cellular assays for the diagnosis of LB have been described. Some of these assays are based on the proliferation of T cells, such as the lymphocyte transformation test (LTT) described by von Baehr et al. [13] or the LTT-memory lymphocyte immunostimulation assay (MELISA) described by Valentine-Thon et al. [14]. Other assays detect cytokines which are secreted by T cells upon stimulation with *Borrelia* antigens, such as the Quantiferon test described by Callister et al. [15] or the enzyme-linked immunosorbent spot (ELISpot) assay (iSpot Lyme) described by Jin et al. [16]. Most studies on cellular assays have used poorly described study populations and lack clinical validations. Despite the lack of such validations, these assays are used in some laboratories for the diagnosis of LB [17-19], and when the test result is positive - thus when *Borrelia*-specific T cells are detected - (long-term) antibiotic treatment regimens are started for treatment of active LB [17], which is of major concern. Therefore, we recently validated an in-house *Borrelia* ELISpot assay for the detection of active LNB on a well-established study population of active LNB patients and various control groups [20]. We concluded that the T-cell activity measured in our in-house *Borrelia* ELISpot assay could not be used as a marker for active LNB. In the current study, we evaluated the diagnostic performance of a commercial LymeSpot assay that has not been validated previously, and compared this to the diagnostic performance of our in-house *Borrelia* ELISpot assay in patients suspected of LNB.

MATERIALS AND METHODS

STUDY POPULATION

Inclusion for this study started in March 2014 and ended in November 2017, and for a large part ran in parallel with two previously published studies [20, 21]. Therefore, most of the study participants in the current study also participated in the previous studies and, hence, the study groups of this study consisted of subgroups of the study groups of these previous studies.

All patients diagnosed with LNB in the Diakonessenhuis Hospital, Utrecht and the St Antonius Hospital, Nieuwegein, the Netherlands, were eligible for inclusion in the study if they fulfilled at least two criteria for LNB as defined by the European Federation of Neurological Societies (EFNS) [10]. These criteria are (i) the presence of neurological symptoms suggestive of LNB without other obvious explanations, (ii) cerebrospinal fluid (CSF) pleocytosis (≥ 5 leukocytes/µl) and (iii) *Borrelia*-specific intrathecal antibody production. If all three criteria were met, then a case was categorized as definite LNB; if two criteria were met, then a case was categorized as possible LNB. Patients were either recently diagnosed with active LNB or had been treated for LNB in the past. Clinical symptoms of LNB patients were classified as cranial or peripheral nerve infections – further divided into radiculopathy, cranial or peripheral neuropathy – or as central nervous system disease (which also included meningoencephalitis). Active LNB patients were recruited from March 2014 to November 2017 and were included if blood was drawn within 2 weeks after the start of antibiotic therapy. Treated LNB patients, who had been diagnosed between September 2006 and September 2014, were enrolled from February 2015 to March 2015 and were included at least 4 months after the completion of antibiotic therapy for LNB. The clinical outcome of both active and treated LNB patients was assessed by a neurologist after antibiotic treatment for active LNB was finished. The clinical outcome was interpreted as either a recovery of clinical symptoms or as no (or incomplete) recovery of clinical symptoms.

Healthy individuals were recruited during the period from March 2014 to December 2015 from personnel of the Diakonessenhuis Hospital, Utrecht, the St Antonius Hospital, Nieuwegein and the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands. Healthy individuals also included boy scout patrol leaders, owners of hunting dogs and recreational runners. All were invited to participate if they pursued recreational activities in high-risk areas for tick bites, such as gardens, forests, grasslands and dunes [22]. Thus, the healthy individuals in this study represented a subgroup of healthy individuals, with a high risk of tick exposure. The healthy individuals were further subdivided into two groups. The first group consisted of healthy individuals who had received antibiotic treatment for LB-related symptoms in the past, as they had reported in the Lyme-specific questionnaire, and were referred to as treated healthy individuals. The second group comprised all other healthy individuals and these were referred to as untreated healthy individuals.

All study participants were asked to complete a Lyme-specific questionnaire. This questionnaire included questions on tick bites, the presence of EM, antibiotic treatment for LB and self-reported complaints at the moment of inclusion and during possible earlier episodes of LB. Information regarding the clinical symptoms, pleocytosis and intrathecal antibody production during active disease of the LNB patients was extracted from the hospital information system. Healthy individuals were recruited only if they reported no complaints at the time of inclusion in the study.

ANTIBODY DETECTION IN SERUM AND SERUM–CSF PAIRS

For the detection of *Borrelia*-specific antibodies in serum, the C6 enzyme-linked immunosorbent assay (ELISA) (Immunetics, Boston, MA, USA) was used [23]. Equivocal and positive C6 ELISA results were confirmed using the *recom*Line immunoglobulin (Ig)M and IgG immunoblot test (Mikrogen GmbH, Neuried, Germany) [24]. Detection of intrathecally produced *Borrelia*-specific IgM and IgG antibodies was performed using the second-generation IDEIA LNB test (Oxoid, Hampshire, UK), which was adapted from the original publication by Hansen et al. [25]. Most importantly, the dilution of CSF was adjusted from 1:10 to 1:5, and various incubation times (of patient samples, conjugate and substrate) were shortened. The C6 ELISA and the IDEIA LNB tests were performed using a DS2-automated ELISA instrument (Dynex Technologies, Chantilly, VA, USA) and analyzed with the DS-Matrix™ software (Dynex Technologies). The immunoblot results

were recorded with an automated *recom*Scan system using the *recom*Scan software (Mikrogen GmbH). All assays were performed according to the instructions of the respective manufacturers and were interpreted as described previously [20].

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Isolation of peripheral blood mononuclear cells (PBMCs) was performed from whole blood specimens which were collected in lithium heparin tubes. If isolation of PBMCs started within 8 h after venipuncture, 3 ml of fresh, pre-warmed (37°C) Roswell Park Memorial Institute (RPMI) medium (Life Technologies, Invitrogen, Bleiswijk, the Netherlands) was added to 5 ml of blood and, after gently mixing, transferred into a Leucosep tube (Oxford Immunotec Ltd, Abingdon, UK). PBMCs were separated through density gradient centrifugation (Hettich Rotanta 460 RS; rotor 5624) at room temperature for 15 min at 1000 *g*. If isolation of PBMCs was performed between 8 and 32 h after venipuncture, then a T-Cell Xtend (Oxford Immunotec Ltd) step was performed prior to the addition of 3 ml of RPMI medium and density gradient centrifugation, as previously described [20, 26, 27]. After centrifugation, the PBMC fraction was collected and washed twice in 10 ml RPMI medium. The first wash step was performed at room temperature for 7 min at 600 *g*; the second wash step was also performed at room temperature for 7 min at 300 *g*. If necessary, excess erythrocytes were removed between the first and second wash step using human erythrocyte lysis buffer [0.010 M $KHCO_3$, 0.0001 M ethylenediamine tetraacetic acid (EDTA), 0.150 M NH_4Cl (pH 7.3 ± 0.1)]. After addition of 5 ml of lysis buffer, the solution was incubated for 5 min at 2°C and subsequently centrifuged using the first wash step centrifugation program. The final pellet was suspended in 1.1 ml of fresh, prewarmed (37°C) AIM-V medium (Life Technologies) and PBMCs were counted using the AC.T diff 2 analyser (Beckman Coulter, Woerden, the Netherlands), as described previously [20]. After isolation, the PBMCs were adjusted to a concentration of 2.5×10^6/ml using AIM-V medium, 100 µl of which (2.5×10^5 PBMCs) was tested in the in-house *Borrelia* ELISpot assay and the commercial LymeSpot assay [Autoimmun Diagnostika (AID) GmbH, Straßberg, Germany].

THE IN-HOUSE *BORRELIA* ELISPOT ASSAY

The in-house *Borrelia* ELISpot assay was performed as previously described [20]. In brief, a precoated polyvinylidene difluoride (PVDF) ELISpot^{PRO} 96-well plate (Mabtech, Nacka Strand, Sweden) was used, and four wells were tested for each study participant. These wells contained 50 µl of positive control [anti-human CD3 monoclonal antibody (mAb) CD3-2 (0.1 µg/ml); Mabtech], 50 µl of negative control (AIM-V medium), 50 µl of *B. burgdorferi* B31 whole-cell lysate (5 µg/ml; AID), hereafter referred to as *B. burgdorferi* B31, and 50 µl of outer surface protein (Osp)-mix (5 µg/ml; AID), respectively, which were used to stimulate the PBMCs. The Osp-mix consisted of a pool of 9-mer to 11-mer peptides of Osp-A (*B. burgdorferi*, *B. afzelii* and *B. garinii*), native Osp-C (*B. afzelii*) and recombinant p18. For the current study, this protocol was extended by the addition of two wells: the first additional well contained 100 µl of *B. burgdorferi* B31 (5 µg/ml) and the second additional well contained 100 µl of Osp-mix (5 µg/ml) to stimulate the PBMCs (Supporting information, Table S1).

The numbers of *Borrelia*-specific interferon (IFN)-γ-secreting T cells/2.5×10^5 PBMCs (displayed as black spots) were measured with an ELISpot reader (AID) and counted by two different people using the ELISpot software (AID), hereafter referred to as the numbers of spot-forming cells (SFCs). SFCs were counted without prior knowledge of the medical background of the study participants. The SFC size used was based on the expected SFC size of an IFN-γ-producing T cell, as determined by Feske et al. [28], and was set on –2.8 log (mm^2). Samples that had a discrepancy in the numbers of SFCs between the two counting persons were recounted by a third person, whose result was leading. For samples that were stimulated with 50 µl of *Borrelia* antigen, the conditions for recounting have been described previously [20]. For samples which were stimulated with 100 µl

of *B. burgdorferi* B31, a recount was performed for those samples which had a discrepancy in the numbers of SFCs in the critical area (between 0 and 10 SFCs), determined by receiver operating characteristic (ROC) curve analysis. When 100 µl of Osp-mix was used, those samples which had a discrepancy in the numbers of SFCs in the critical area (between 0 and 5 SFCs), determined by ROC curve analysis, were recounted. The results of the in-house *Borrelia* ELISpot assay were only interpreted when the assay was valid; i.e., when the numbers of SFCs upon stimulation in the positive control well were ≥ 20 and in the negative control well were ≤ 6 (the latter representing spontaneous SFCs) (Supporting information, Table S1). If the assay was valid, the final numbers of SFCs in the *Borrelia* antigen-stimulated wells were determined. For the wells containing 50 µl of *Borrelia* antigen, this was performed by subtraction of the numbers of SFCs in the negative control well from the numbers of SFCs in the *Borrelia* antigen-stimulated well. For the wells containing 100 µl of *Borrelia* antigen, the final numbers of SFCs were calculated by first multiplying the numbers of SFCs in the negative control well by 2 before subtracting them from the numbers of SFCs in the *Borrelia* antigen-stimulated well (Supporting information, Table S1). The final numbers of SFCs corresponded with the numbers of SFCs after stimulation with either *B. burgdorferi* B31 or Osp-mix. For some cases, the *Borrelia* antigens were tested several times and, for such cases, the median T-cell count was used to determine the final numbers of SFCs. Using the extended version of our in-house *Borrelia* ELISpot assay, we were able to compare our in-house *Borrelia* ELISpot assay with the LymeSpot assay on the basis of exactly the same (absolute) amount of *Borrelia* antigens (100 µl of a 5 µg/ml concentration per well), as prescribed in the LymeSpot assay protocol. In addition, we could also study the effect of various amounts of *Borrelia* antigen (50 versus 100 µl of a concentration of 5 µg/ml) on the numbers of SFCs for the in-house *Borrelia* ELISpot assay (Supporting information, Table S1).

THE LYMESPOT ASSAY

The LymeSpot assay (AID) was run in parallel with the in-house *Borrelia* ELISpot assay. The LymeSpot assay uses a 96-well PVDF plate coated with anti-human IFN-γ antibodies. The assay was performed according to the manufacturer's protocol (Supporting information, Table S1), except for the isolation of the PBMCs and the amount of PBMCs/well, for which our standard protocol was used as described above and in Supporting information, Table S1. In a pilot study we investigated the influence of this deviation from the LymeSpot protocol, and showed that this had no impact on the diagnostic performance of the LymeSpot assay (see Supporting information, Data S4). Stimulation of the PBMCs in the LymeSpot assay was performed using a negative control (100 µl of AIM-V medium), a positive control (100 µl of Pokeweed; AID), 100 µl of *B. burgdorferi* B31 (5 µg/ml; AID) and 100 µl of Osp-mix (5 µg/ml; AID). Both the *B. burgdorferi* B31 and the Osp-mix antigens were identical to the *Borrelia* antigens used for the in-house *Borrelia* ELISpot assay described above. If the PBMC yield was sufficient, both controls and antigens were tested in duplicate (Supporting information, Table S1).

The final LymeSpot results were only calculated when the assay was valid. Following the manufacturer's instructions, the LymeSpot results were valid when the positive control well had ≥ 50 SFCs and the negative control well had ≤ 10 SFCs. The final LymeSpot results were calculated in two ways. First, the average numbers of SFCs were calculated, similarly as described above for the in-house *Borrelia* ELISpot assay, to allow an objective comparison of the results of the LymeSpot assay with those of the in-house *Borrelia* ELISpot assay (Supporting information, Table S1). Secondly, stimulation indices (SIs) were calculated following the protocol of the manufacturer (Supporting information, Fig. S2). For this, the numbers of SFCs of the negative control needed to be established first. If these numbers were between 3 and 10, SIs were calculated by dividing the numbers of *Borrelia*-specific SFCs by the numbers of SFCs of the negative control. If the numbers of SFCs of the negative control were between 0 and 2, SIs were calculated by dividing the final numbers of *Borrelia*-specific SFCs by 1. The final LymeSpot results were based on the

combination of the results of the SIs of both the *B. burgdorferi* B31 and the Osp-mix antigens, and a case could either be categorized as negative, positive (highly specific), or require diagnostic verification (Supporting information, Fig, S2).

DATA HANDLING AND STATISTICAL ANALYSIS

The results of the in-house *Borrelia* ELISpot assay using 50 µl and using 100 µl of *Borrelia* antigen and the results of the LymeSpot assay were compared with regard to their ability to detect active LNB patients and to distinguish them from the study participants in the other three groups. The 50-µl results were published previously, as part of a larger study population (n = 243) [20]. For both ELISpot assays, a comparison was performed based on the individual, as well as the combined results of the numbers of SFCs after stimulation with either 50 or 100 µl *B. burgdorferi* B31 and 50 or 100 µl Osp-mix. In addition, for the LymeSpot assay, the *B. burgdorferi* B31-specific SI, the Osp-mix-specific SI and the final results based on the combination of both SIs (Supporting information, Fig. S2) were compared between the four study groups. Dichotomous, unrelated data were analyzed using the $\chi 2$ or Fisher's exact test. Quantitative, unrelated data comparing more than two groups were analyzed using the Kruskal–Wallis $\chi 2$ test, and subsequent two-group comparisons were analyzed using the Dunn's test [29]. Quantitative, unrelated data comparing two groups were analyzed using the Wilcoxon rank sum test. Quantitative, related data comparing greater than or equal to two groups were analyzed using the Wilcoxon signed-rank test with continuity correction.

To assess the diagnostic performance of both ELISpot assays, various ROC curves were constructed and used to calculate the area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with 95% confidence intervals (CIs) [30]. The optimal threshold was calculated using the point on each ROC curve for which the distance to the upper left corner (where both sensitivity and specificity are 100%) was shortest, and was determined by the square root of $[(1\text{-sensitivity})^2 + (1\text{-specificity})^2]$. The sensitivity, specificity, PPV and NPV were calculated based on the optimal threshold. For both ELISpot assays, a ROC curve was constructed for each antigen separately by comparing the numbers of *Borrelia*-specific SFCs among active LNB patients with those among the other three groups, as well as for the results of both antigens together. To assess the diagnostic performance of each ELISpot assay using the results of both antigens together, a binomial logistic regression (BLR) model was built before the ROC curves were constructed. The numbers of *B. burgdorferi* B31-specific SFCs and the numbers of Osp-mix-specific SFCs, without and with their interaction term, were included as predictor variables in the model; the outcome variable was binary: sick (all active LNB patients) or not-sick (all other study participants). The performance of the BLR model was assessed by calculating the prediction error using cross-validation. For the LymeSpot assay, ROC curve analysis was also performed based on the SIs after stimulation with *B. burgdorferi* B31 and Osp-mix, as described in the Materials and Methods section covering the LymeSpot assay and in Supporting information, Fig. S2. The final results that needed diagnostic verification were classified as 'positive', and were combined with the positive results. Comparison of the ROC curves was performed using DeLong's test for two correlated ROC curves [30].

Raw P values < 0.05 were interpreted as statistically significant, which were subsequently followed by two-group comparisons where appropriate. To account for the multiple statistical analyses in this study, we applied the Benjamini-Hochberg (BH) procedure and controlled the false discovery rate at the level of 2.5%, i.e., no more than one false positive was allowed to be found in our list of rejections [31]. For all statistical analyses and construction of the figures, Rstudio version 1.1.383, 2009-2017 (Rstudio, Boston, MA, USA) was used.

RESULTS

STUDY POPULATION

Ninety-two study participants were eligible for inclusion in the study; however, five (5.4%) patients were excluded. Four study participants, one active LNB patient, one untreated and two treated healthy individuals, were excluded because of insufficient amounts of PBMCs to perform both ELISpot assays. One untreated healthy individual was excluded because the negative control well in the in-house *Borrelia* ELISpot assay was invalid (> 6 SFCs). Eighty-four (96.6%) of the 87 study participants comprised a subgroup of our recently published study [20]; 77 (88.5%) of the 87 study participants were also part of another published study [21] (Supporting information, Table S3).

CHARACTERISTICS OF THE ACTIVE LNB PATIENTS

Eighteen active LNB patients were included in the study, with a median of 6.0 days after the start of antibiotic treatment for their active disease; their median age was 54.7 years (Table 1). Based on the EFNS criteria [10], 12 of 18 (66.7%) active LNB patients were classified as definite LNB cases and the remaining six (33.3%) were classified as possible LNB cases because of the lack of intrathecally produced *Borrelia*-specific antibodies (Table 1). Clinical symptoms consisted of radiculopathy (n = 2), cranial neuropathy (n = 7) or central nervous system disease (n = 5). Four patients had a combination of different symptoms: one patient had radiculopathy and cranial neuropathy, one patient had radiculopathy, cranial and peripheral neuropathy, one patient had radiculopathy and central nervous system disease and the last patient had cranial neuropathy and central nervous system disease (data not shown). Most active LNB patients had *Borrelia*-specific antibodies in their blood [15 of 18 (83.3%)], which was greater compared to treated LNB patients [one of 12 (8.3%)] and untreated healthy individuals [seven of 47 (14.9%)] (adjusted P value ≤ 0.002) (Table 1). The majority [13 of 18 (72.2%)] of the active LNB patients showed complete recovery after the end of antibiotic therapy for active LNB, which was assessed by the neurologist with a median of 38.0 days after antibiotic treatment ended (Table 1).

CHARACTERISTICS OF THE TREATED LNB PATIENTS

Twelve treated LNB patients were included in the study, who were diagnosed with active LNB on average 5.4 years ago (Table 1). The median age of the treated LNB patients at inclusion was 56.3 years and the majority (91.7%) were classified as definite LNB cases at the time of diagnosis of active LNB in the past. One (8.3%) patient was classified as a possible LNB case because of the absence of pleocytosis (Table 1). Clinical symptoms included radiculopathy (n = 1), cranial neuropathy (n = 4) or central nervous system disease (n = 3). Four treated LNB patients had combined symptomology: one patient had radiculopathy, cranial neuropathy and central nervous system disease, one patient had radiculopathy and cranial neuropathy, one patient had radiculopathy and peripheral neuropathy and one patient had cranial and peripheral neuropathy (data not shown). Ten (83.3%) of the 12 treated LNB patients showed complete recovery after the end of antibiotic therapy for active LNB in the past, which was assessed by the neurologist with a median of 37.0 days after antibiotic treatment ended (Table 1). At inclusion in this study, however, eight (66.7%) of the 12 treated LNB patients reported complaints in the Lyme-specific questionnaire (Table 1). These self-reported symptoms included fatigue, neuropathic complaints, myalgias, arthralgias and cognitive complaints (data not shown).

Table 1. Demographic and clinical characteristics of the four study groups

Parameters	Active LNB patients (*n* = 18)	Treated LNB patients (*n* = 12)	Treated healthy individuals (*n* = 10)	Untreated healthy individuals (*n* = 47)	Statistics	
					BH[a] (overall)	BH[a] (2-group)
Gender (n of males; %)	10 (55.6)	7 (58.3)	8 (80.0)	23 (48.9)	0.680	ND[b]
Age (median years; IQR)	54.7 (45.8-63.8)	56.3 (51.2-68.0)	55.2 (41.6-59.5)	35.1 (23.2-44.9)	< 0.001	≤ 0.018[c]
Tick bite (yes; %)	8 (44.4)	8 (66.7)	9 (90.0)	37 (78.7)	0.129	> 0.025[d]
EM (yes; %)	1 (5.6)	3 (25.0)	8 (80.0)[e]	2 (4.3)	0.007	≤ 0.002[f]
Serology (no. of positives; %)	15 (83.3)	1 (8.3)	4 (40.0)	7 (14.9)	0.007	≤ 0.002[g]
Intrathecal *Borrelia*-specific antibody production (no. of positives; %)	12 (66.7)	12 (100)	NA	NA	0.225	NA
Pleocytosis (yes; %)	18 (100)	11 (91.7)	NA	NA	0.687	NA
EFNS criteria						
Definite LNB	12 (66.7)	11 (91.7)	NA	NA	0.462	NA
Possible LNB	6 (33.3)	1 (8.3)				
Time between end of AB and blood sampling (median years; IQR)	NA	5.4 (3.6-6.1)	5.0 (2.0-7.0)	NA	0.888	NA
Time between start of AB and blood sampling (median days; IQR)	6.0 (3.3-7.0)	NA	NA	NA	NA	NA
Recovery[h]						
Time between end of AB and visit at neurologist (median days; IQR)	38.0 (22.5-67.2)	37.0 (15.5-53.0)	NA	NA	0.883	NA
Complete recovery (yes; %)	13 (72.2)	10 (83.3)	NA	NA	0.875	NA
Symptoms at the start of the Study (yes; %)	18 (100)	8 (66.7)	0 (0)	0 (0)	0.007	≤ 0.017[i]

LNB, Lyme neuroborreliosis; *n*, number of study participants; BH, Benjamini–Hochberg; ND, not done; IQR, interquartile range; EM, erythema migrans; AB, antibiotic treatment for Lyme borreliosis (LB); EFNS, European Federation of Neurological Societies [10]; NA, not applicable.

a. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
b. As the initial comparison was not significantly different (raw *P* value > 0.050), two-group comparisons were not performed.
c. Untreated healthy individuals versus treated healthy individuals, treated Lyme neuroborreliosis (LNB) patients and active LNB patients (adjusted *P* values are 0.018, 0.002 and 0.001, respectively).
d. As the initial comparison was significantly different (raw *P* value < 0.050), two-group comparisons were also performed.
e. One treated healthy individual had an atypical skin rash, one had flu-like symptoms after the tick bite.
f. Treated healthy individuals versus untreated healthy individuals and active LNB patients (adjusted *P* values are < 0.001 and 0.002, respectively).
g. Active LNB patients versus treated LNB patients and untreated healthy individuals (adjusted *P* values are 0.002 and < 0.001, respectively).
h. The clinical outcome of both active and treated LNB patients was assessed by the neurologist after antibiotic treatment for active LNB was finished. The clinical outcome was interpreted as either a recovery of clinical symptoms or as no (or incomplete) recovery of clinical symptoms.
i. Treated healthy individuals versus treated and active LNB patients (adjusted *P* values are 0.017 and < 0.001, respectively), and untreated healthy individuals versus treated and active LNB patients (adjusted *P* values are < 0.001 for both).

CHARACTERISTICS OF THE HEALTHY INDIVIDUALS

A total of 57 healthy individuals were included. Ten (17.5%) reported having had antibiotic treatment for an early manifestation of LB in the past, which took place on average 5.0 years ago, and who were therefore classified as treated healthy individuals (Table 1). The median age of the treated healthy individuals was 55.2 years. Nine (90.0%) of the 10 treated healthy individuals reported having had a tick bite, and although this percentage was higher than among the other three groups, it was not statistically significant. Eight (80.0%) of the treated healthy individuals reported an EM, which was higher than among active LNB patients [one of 18 (5.6%)] and untreated healthy individuals [two of 47 (4.3%)] (adjusted *P* value ≤ 0.002) (Table 1). The other two either reported flu-like symptoms or an atypical skin rash after the tick bite.

The remaining 47 (82.5%) healthy individuals all reported never to have had antibiotic treatment for LB, and thus were classified as untreated healthy individuals. Their median age was 35.1 years, which was younger than the other three groups (adjusted *P* value ≤ 0.018) (Table 1).

INFLUENCE OF THE DIFFERENT AMOUNTS OF *BORRELIA* ANTIGEN USED ON THE MEDIAN NUMBERS OF SFCS IN THE IN-HOUSE *BORRELIA* ELISPOT ASSAY

PBMCs of all 87 study participants were stimulated with 50 µl [20] and 100 µl of *B. burgdorferi* B31 and Osp-mix, and subsequently tested in our in-house *Borrelia* ELISpot assay (Table 2). Overall, when 50 µl of *B. burgdorferi* B31 was used to stimulate the PBMCs, a lower median number of SFCs was obtained than when 100 µl of antigen was used (2.0 versus 4.0) (adjusted *P* value < 0.001) (Table 2). When the four study groups were analyzed separately, the association between the use of lower amounts of antigen as stimulant and the lower median number of SFCs remained for untreated healthy individuals (1.5 versus 2.0) (adjusted *P* value = 0.006) (Table 2). Stimulation of PBMCs with either 50 or 100 µl of Osp-mix did not result in a difference between the median numbers of SFCs in the in-house *Borrelia* ELISpot assay (1.0 versus 1.0) (adjusted *P* value = 0.786) (Table 2).

INFLUENCE OF THE DIFFERENT *BORRELIA* ANTIGENS USED FOR PBMC STIMULATION ON THE MEDIAN NUMBERS OF SFCS IN THE TWO ELISPOT ASSAYS

Analysis of the results of all 87 study participants showed that PBMC stimulation with 50 µl of *B. burgdorferi* B31 resulted in a higher median number of SFCs than stimulation with 50 µl of Osp-mix in the in-house *Borrelia* ELISpot assay (2.0 versus 1.0) (adjusted *P* value < 0.001) (Table 2). These results are similar to the results we have published previously using a study population of 243 study participants [20]. When the four study groups were analyzed separately, the median numbers of *B. burgdorferi* B31-specific SFC counts were higher compared to the median numbers of Osp-mix-specific SFC counts, although not significant (adjusted *P* values > 0.025) (Table 2). A higher median number of SFCs after PBMC stimulation with *B. burgdorferi* B31 compared to PBMC stimulation with Osp-mix was also seen when a volume of 100 µl of *Borrelia* antigen was used in the in-house *Borrelia* ELISpot assay (4.0 versus 1.0) (adjusted *P* value < 0.001) (Table 2). Comparisons within each of the four groups showed that this difference remained significant for active LNB patients (adjusted *P* value = 0.017) (Table 2).

In the LymeSpot assay, the higher yield of the *B. burgdorferi* B31 over the Osp-mix remained when the median numbers of SFCs were compared (5.0 versus 1.5) (adjusted *P* value < 0.001) (Table 2). When the four study groups were analysed separately, *B. burgdorferi* B31 remained superior in the LymeSpot assay for untreated healthy individuals and active LNB patients (adjusted *P* values ≤ 0.005) (Table 2).

COMPARISON OF THE MEDIAN NUMBERS OF SFCS IN THE TWO ELISPOT ASSAYS BETWEEN THE FOUR STUDY GROUPS AFTER STIMULATION OF THE PBMCS WITH *B. BURGDORFERI* B31

The PBMCs of treated healthy individuals were stimulated the most when either 50 µl of *B. burgdorferi* B31 was used in the in-house *Borrelia* ELISpot assay or 100 µl of *B. burgdorferi* B31 was used in the LymeSpot assay. The PBMCs of treated LNB patients were stimulated the most when 100 µl of *B. burgdorferi* B31 was used in the in-house *Borrelia* ELISpot assay (Table 2; Fig. 1a,c,e). In contrast, the PBMCs of untreated healthy individuals were stimulated the least, irrespective of the volume and the ELISpot assay used. An increased T-cell activation for patients and treated healthy individuals after PBMC stimulation with *B. burgdorferi* B31 was also seen in our previous study, which included more study participants (n = 243), and suggests that the ELISpot activity is related to exposure to the *Borrelia* bacterium [20].

Analysis of the different amounts of *B. burgdorferi* B31 showed that when 50 µl was used to stimulate the PBMCs in the in-house *Borrelia* ELISpot assay, the median number of SFCs of 1.5 for untreated healthy individuals was lower compared to the median number of SFCs of 9.3 for treated healthy individuals (adjusted P value = 0.015) (Table 2, Fig. 1a). When 100 µl of *B. burgdorferi* B31 was used to stimulate the PBMCs, no differences were found between the four study groups for the in-house *Borrelia* ELISpot assay (adjusted P values > 0.025) (Table 2, Fig. 1c). For the LymeSpot assay, the results were only significantly different between untreated (less ELISpot activity) and treated healthy individuals (more ELISpot activity) (adjusted P value = 0.014) (Table 2, Fig. 1e).

Overall, no difference was found between the median numbers of SFCs between both ELISpot assays when 100 µl of *B. burgdorferi* B31 was used to stimulate the PBMCs (adjusted P value = 0.360). Similarly, no differences were found when the four study groups were analyzed separately (adjusted P values > 0.025) (Table 2).

Table 2. Comparison of the ELISpot results expressed in the numbers of spot-forming cells for the in-house *Borrelia* ELISpot assay and the LymeSpot assay among the four study groups

			SFC count (median; IQR)			Statistics			
			In-house *Borrelia* ELISpot assay		LymeSpot assay	50 µl in-house *Borrelia* ELISpot *versus* 100 µl in-house *Borrelia* ELISpot		100 µl in-house *Borrelia* ELISpot *versus* 100 µl LymeSpot assay	
Study participants	***Borrelia* antigen**		**50 µl**	**100 µl**	**100 µl**	**BH[a] (overall)**	**BH[a] (within each group)**	**BH[a] (overall)**	**BH[a] (within each group)**
All study participants (*n* = 87)	*Bb* B31		2.0 (0.8-6.0)	4.0 (2.0-9.5)	5.0 (2.0-10.3)	<0.001	0.006[b]	0.360	>0.025
	Osp-mix		1.0 (0.0-2.0)	1.0 (0.0-3.0)	1.5 (0.5-3.3)	0.786	>0.025	0.685	>0.025
Active LNB patients (*n* = 18)	*Bb* B31		5.5 (1.3-7.8)	6.5 (1.5-11.5)	6.1 (2.6-10.2)				
	Osp-mix		0.0 (0.0-4.8)	1.0 (0.0-3.0)	1.3 (0.1-2.5)				
Treated LNB patients (*n* = 12)	*Bb* B31		5.5 (2.0-9.9)	9.5 (5.0-17.5)	8.4 (2.4-12.8)				
	Osp-mix		0.5 (0.0-4.5)	0.5 (0.0-2.5)	1.5 (0.9-3.3)				
Treated healthy individuals (*n* = 10)	*Bb* B31		9.3 (3.5-27.0)	6.5 (3.3-22.2)	26.1 (7.5-73.7)				
	Osp-mix		2.0 (1.1-6.0)	2.5 (0.5-7.8)	5.6 (3.3-12.0)				
Untreated healthy individuals (*n* = 47)	*Bb* B31		1.5 (0.0-3.0)	2.0 (1.0-5.5)	3.0 (1.4-6.3)				
	Osp-mix		1.0 (0.0-1.5)	1.0 (0.0-2.5)	1.0 (0.0-2.5)				
Statistics	Bb B31 *versus* Osp-mix	BH[a] (overall)	< 0.001	< 0.001	< 0.001				
		BH[a] (within each group)	> 0.025	0.017[c]	≤ 0.005[d]				
	Bb B31	BH[a] (overall)	0.018	0.132	0.075				
		BH[a] (two-group)	0.015[e]	> 0.025[f]	0.014[e,f]				
	Osp-mix	BH[a] (overall)	0.489	0.766	0.058				
		BH[a] (two-group)	ND[g]	ND[g]	0.005[e,f]				

SFC, spot-forming cell; IQR, interquartile range; ELISpot, enzyme-linked immunosorbent spot; BH, Benjamini–Hochberg; *n*, number of study participants; *Bb* B31, *Borrelia burgdorferi* B31; Osp, outer surface protein; ND, not done; LNB, Lyme neuroborreliosis.

a. To correct for multiple comparisons, the Benjamini-Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
b. The numbers of SFCs among untreated healthy individuals were significantly higher after stimulation with 100 µl of *B. burgdorferi* B31 compared to 50 µl of *B. burgdorferi* B31.
c. The numbers of SFCs after stimulation with 100 µl of *B. burgdorferi* B31 among active Lyme neuroborreliosis (LNB) patients were significantly higher compared to stimulation with 100 µl of Osp-mix.
d. The numbers of SFCs after stimulation with 100 µl of *B. burgdorferi* B31 among untreated healthy individuals and active LNB patients were significantly higher compared to stimulation with 100 µl of Osp-mix (adjusted *P* values are <0.001 and 0.005, respectively).
e. Untreated versus treated healthy individuals.
f. As the initial comparison was significantly different (raw *P* value <0.050), two-group comparisons were also performed.
g. As the initial comparison was not significantly different (raw *P* value >0.050), two-group comparisons were not performed.

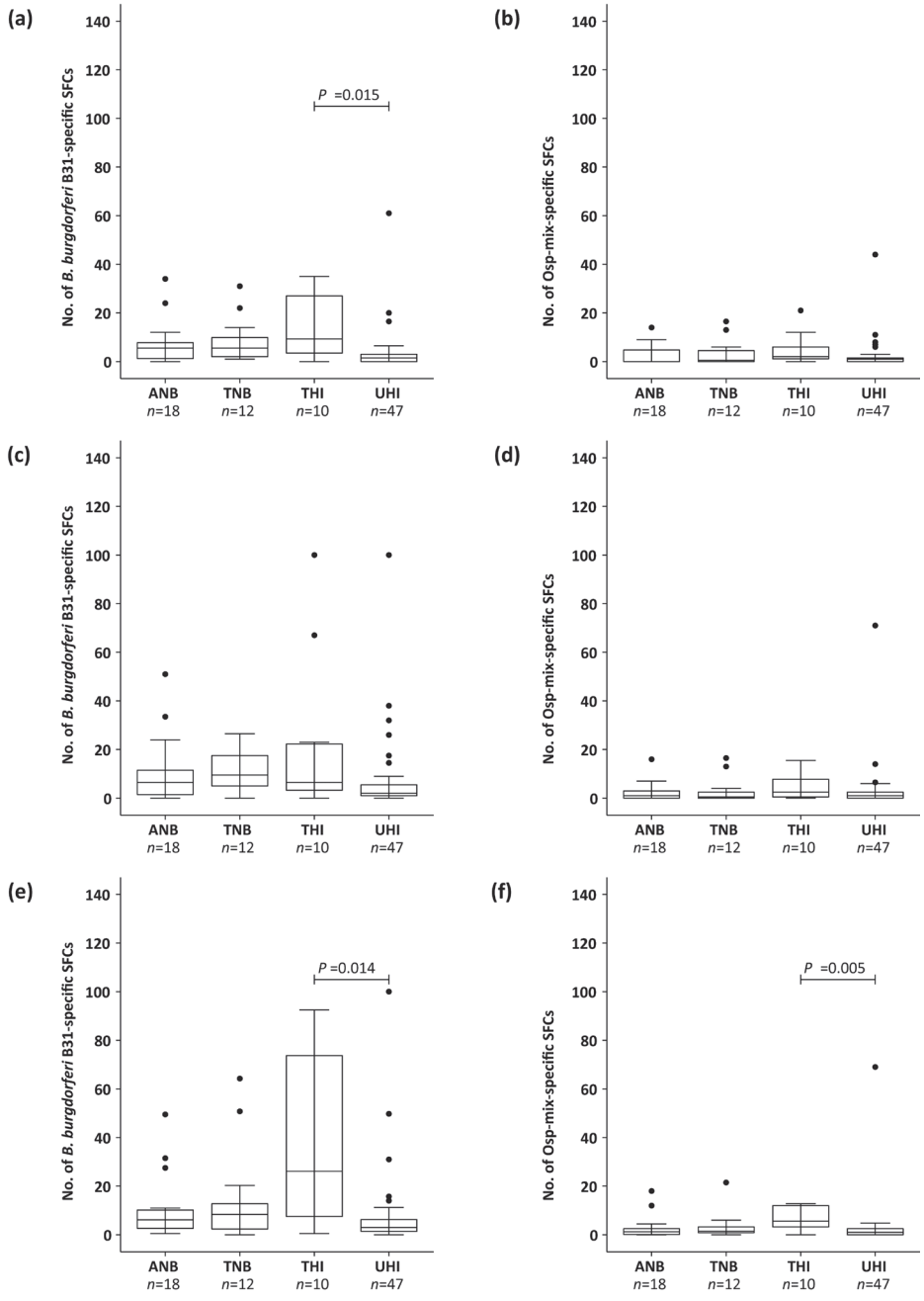

Fig. 1. Results of the in-house *Borrelia* enzym-linked immunosorbent spot (ELISpot) assay (a–d) and the LymeSpot assay (e–f) expressed in the numbers of spot-forming cells (SFCs). (a) (50 µl), (c) and (e) (both 100 µl) are the results after peripheral blood mononuclear cell (PBMC) stimulation with *Borrelia burgdorferi* B31, and (b) (50 µl), (d) and (f) (both 100 µl) are the results after PBMC stimulation with outer surface protein (Osp)-mix among active Lyme neuroborreliosis patients (ANB), treated Lyme neuroborreliosis patients (TNB), treated healthy individuals (THI) and untreated healthy individuals (UHI). The displayed *P* values are corrected and interpreted using the Benjamini–Hochberg procedure with a false discovery rate of 2.5% for multiple comparisons (only false discovery rates < 0.025 are displayed).

COMPARISON OF THE MEDIAN NUMBERS OF SFCS IN THE TWO ELISPOT ASSAYS BETWEEN THE FOUR STUDY GROUPS AFTER STIMULATION OF THE PBMCS WITH OSP-MIX

Similar to stimulation with *B. burgdorferi* B31, the PBMCs of treated healthy individuals were activated most upon stimulation with Osp-mix (Table 2, Fig. 1b,d,f). No significant differences between the four study groups were found for the in-house *Borrelia* ELISpot assay using 50 or 100 µl of Osp-mix (adjusted *P* values 0.489 and 0.766, respectively) (Table 2, Fig. 1b,d). For the LymeSpot assay, however, stimulation of the PBMCs with 100 µl of Osp-mix resulted in a significantly higher median number of SFCs of 5.6 for treated healthy individuals compared to the median number of SFCs of 1.0 for untreated healthy individuals (adjusted *P* value = 0.005) (Table 2, Fig. 1f).

Similar to the use of 100 µl of *B. burgdorferi* B31, no difference was seen between the median numbers of SFCs between both ELISpot assays upon stimulation of the PBMCs with 100 µl of Osp-mix (adjusted *P* value = 0.685). Subsequent comparisons within each group also did not show a difference (adjusted *P* values > 0.025) (Table 2).

THE DIAGNOSTIC PERFORMANCE OF THE TWO ELISPOT ASSAYS BASED ON THE NUMBERS OF SFCS

The diagnostic performance of the in-house *Borrelia* ELISpot assay and the LymeSpot assay were evaluated using ROC curve analysis, for which the numbers of SFCs were used. In order to enable a fair comparison between the two assays, the results obtained with PBMCs that were stimulated with 100 µl of *Borrelia* antigen were used for the in-house *Borrelia* ELISpot assay and compared with the results of the LymeSpot assay. The results obtained with 100 µl of *Borrelia* antigen were used, as this is the standard in the LymeSpot assay (Supporting information, Table S1). ROC curves were constructed based on the results obtained after PBMC stimulation with the *B. burgdorferi* B31 and the Osp-mix separately, as well as on the combined results of both *Borrelia* antigens. The calculated AUCs based on the individual *Borrelia* antigens were comparable to a random predictor, and ranged from 0.459 to 0.570 (Table 3, Fig. 2a,b). No difference was found between the AUC of the in-house *Borrelia* ELISpot assay and the AUC of the LymeSpot assay based on the numbers of *B. burgdorferi* B31-specific SFCs (AUC = 0.553 and 0.570, respectively) (adjusted *P* value = 0.974) (Table 3, Fig. 2a). Similarly, comparison of the AUCs from the two ELISpot assays based on the numbers of Osp-mix-specific SFCs also showed no difference (AUC = 0.479 for the in-house *Borrelia* ELISpot assay and AUC = 0.459 for the LymeSpot assay, respectively) (adjusted *P* value = 0.930) (Table 3, Fig. 2b).

Calculation of the optimal thresholds for the two assays using a single *Borrelia* antigen showed that the sensitivity and NPV was highest for the LymeSpot assay when *B. burgdorferi* B31 was used to stimulate the PBMCs (sensitivity = 66.7%, NPV = 87.0%) (Table 3). The specificity was highest for the in-house *Borrelia* ELISpot assay irrespective of whether *B. burgdorferi* B31 or Osp-mix was used to stimulate the PBMCs (66.7% each) (Table 3). The PPV was highest for the in-house *Borrelia* ELISpot assay when *B. burgdorferi* B31 was used to stimulate the PBMCs (30.6%) (Table 3).

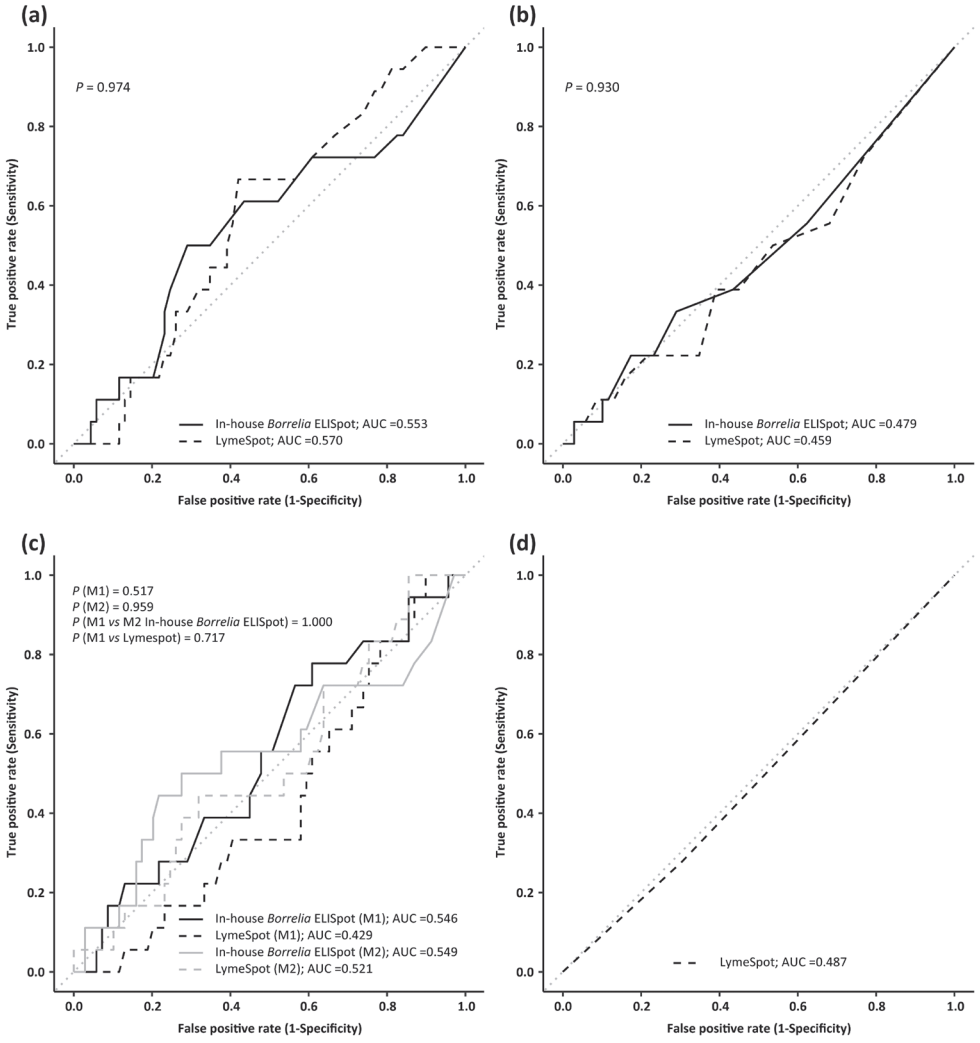

Fig. 2. The receiver operating characteristic (ROC) curves for both the in-house *Borrelia* enzyme-linked immunosorbent spot (ELISpot) assay (solid lines) and the LymeSpot assay (dashed lines) to discriminate active Lyme neuroborreliosis (LNB) patients from the other three groups. The dotted grey line represents the random predictor. (a) ROC curves based on the numbers of spot-forming cells after stimulation with 100 µl of *B. burgdorferi* B31. (b) ROC curves based on the numbers of spot-forming cells after stimulation with 100 µl of Osp-mix. (c) ROC curves based on the outcomes of the two binary logistic regression models (M) for which the combined results of both *Borrelia* antigens, which were based on the numbers of spot-forming cells, without (M1) and with (M2) their interaction term, were included as risk factors. *P* (M1) represents the adjusted *P* value for the comparison of both assays using the outcomes of model 1, *P* (M2) represents the adjusted *P*-value for the comparison of both assays using the outcomes of model 2, *P* (M1 versus M2 in-house *Borrelia* ELISpot) represents the adjusted *P* value for the comparison of the outcomes of models 1 and 2 for the in-house *Borrelia* ELISpot assay, *P* (M1 versus M2 LymeSpot) represents the adjusted *P* value for the comparison of the outcomes of models 1 and 2 for the LymeSpot assay. (d) ROC curve of the LymeSpot assay based on the final LymeSpot result (a combination of the stimulation indices of both antigens following the protocol of the manufacturer (Supporting information, Fig. S2)).

Table 3. Diagnostic performance of the in-house *Borrelia* ELISpot assay and the LymeSpot assay based on the numbers of spot-forming cells obtained after peripheral blood mononuclear cell stimulation with *Borrelia burgdorferi* B31 and Osp-mix separately, and based on the combined numbers of spot-forming cells of both *Borrelia* antigens by using a binomial logistic regression model

Interpretation	ELISpot assay	Threshold[a] (95% CI)	AUC (95% CI)	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)	PPV (%; 95% CI)	NPV (%; 95% CI)	BH[b]	Prediction error BLR model (%)
SFC count after stimulation with *Bb* B31 (100 µl)	In-house *Borrelia* ELISpot assay	7.0 (2.5-8.5)	0.553 (0.382-0.714)	61.1 (38.9-77.8)	66.7 (42.0-81.2)	30.6 (20.5-44.8)	85.7 (79.1-92.2)	0.974	
	LymeSpot assay	5.3 (5.0-8.4)	0.570 (0.433-0.706)	66.7 (44.4-88.9)	59.4 (44.9-72.5)	29.7 (21.1-39.5)	87.0 (79.6-94.7)		
SFC count after stimulation with Osp-mix (100 µl)	In-house *Borrelia* ELISpot assay	2.5 (0.5-5.5)	0.479 (0.326-0.631)	44.4 (16.7-72.2)	66.7 (33.3-87.0)	23.3 (13.8-40.0)	80.9 (72.2-87.1)	0.930	
	LymeSpot assay	2.3 (0.3-4.3)	0.459 (0.305-0.614)	50.0 (22.2-77.8)	56.5 (24.6-84.1)	22.2 (14.6-36.4)	80.5 (72.3-87.5)		
SFC count of both antigens without interaction term in a BLR model[c,d]	In-house *Borrelia* ELISpot assay	0.21 (0.20-0.22)	0.546 (0.398-0.694)	66.7 (38.9-88.9)	49.3 (33.3-81.2)	26.5 (20.0-40.0)	85.7 (78.4-94.3)	0.517	21.8
	LymeSpot assay	0.21 (0.21-0.22)	0.429 (0.292-0.566)	61.1 (27.8-88.9)	42.0 (20.3-73.9)	21.8 (16.1-29.8)	80.8 (72.7-90.6)		21.8
SFC count of both antigens with interaction term in a BLR model[c,d]	In-house *Borrelia* ELISpot assay	0.21 (0.19-0.22)	0.549 (0.380-0.719)	55.6 (33.3-77.8)	72.5 (42.0-85.5)	33.3 (21.1-50.0)	85.7 (79.6-91.8)	0.959	20.7
	LymeSpot assay	0.21 (0.19-0.22)	0.521 (0.372-0.670)	50.0 (33.3-83.3)	68.1 (30.4-81.2)	27.6 (18.9-41.7)	83.9 (77.4-91.3)		20.7
Final result based on the stimulation indices of both antigens	LymeSpot assay	NA[e]	0.487 (0.367-0.606)	27.8 (11.1-50.0)	69.6 (58.0-79.7)	19.1 (7.7-32.0)	78.6 (73.7-84.0)	NA	

ELISpot, enzyme-linked immunosorbent spot; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; BH, Benjamini-Hochberg; BLR, binomial logistic regression; SFC, spot-forming cell; *Bb* B31, *B. burgdorferi* B31; Osp, outer surface protein; NA, not applicable.

a. The threshold is based on the numbers of spot-forming cells when the result of a single *Borrelia* antigen was analyzed, and on the linear predictors of the binary logistic regression model when the combined results of both *Borrelia* antigens were analyzed.
b. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
c. No significant difference (adjusted *P* value = 1.000) between the outcome of the BLR model without interaction term and the outcome of the BLR model with interaction term for the in-house *Borrelia* ELISpot assay.
d. No significant difference (adjusted *P* value = 0.717) between the outcome of the BLR model without interaction term and the outcome of the BLR model with interaction term for the LymeSpot assay.
e. See Supporting information, Fig. S2.

Combining the results of the two *Borrelia* antigens without or with their interaction term as risk factors in a BLR model also resulted in AUCs that were comparable to a random predictor (range = 0.429-0.549) (Table 3). Comparison of the AUCs for the in-house *Borrelia* ELISpot assay and the LymeSpot assay without their interaction term did not show a difference (AUC = 0.546 and 0.429, respectively) (adjusted *P* value = 0.517) (Table 3, Fig. 2c). Similarly, the AUCs for the in-house *Borrelia* ELISpot assay and the LymeSpot assay with their interaction term were also comparable (AUC = 0.549 and 0.521, respectively) (adjusted *P* value = 0.959) (Table 3, Fig. 2c). For both ELISpot assays, the prediction errors of the BLR models with the interaction term were only slightly better than the prediction errors of the BLR models without the interaction term (20.7% for both versus 21.8% for both) (Table 3). Thus, approximately one in five patients were wrongly diagnosed by using the BLR models.

Furthermore, no differences were found between the AUCs of both BLR models for the in-house *Borrelia* ELISpot assay (adjusted *P* value = 1.000) as well as for the LymeSpot assay (adjusted *P* value = 0.717) (Table 3, Fig. 2c). The highest sensitivity was found for the in-house *Borrelia* ELISpot assay when both antigens without their interaction term were included in the BLR model (66.7%); the highest specificity (72.5%) and PPV (33.3%) was found for the in-house *Borrelia* ELISpot assay when both antigens with their interaction term were included in the BLR model. The NPV was highest for the in-house *Borrelia* ELISpot assay, irrespective of whether or not the interaction term was included (85.7% each) (Table 3).

In conclusion, the two ELISpot assays showed a poor diagnostic performance for the diagnosis of active LNB when the numbers of SFCs were used in the ROC curve analyses, with sensitivities ranging from 44.4 to 66.7%, specificities from 42.0 to 72.5%, PPVs from 21.8 to 33.3% and NPVs from 80.5 to 87.0% (Table 3).

THE DIAGNOSTIC PERFORMANCE OF THE LYMESPOT ASSAY BASED ON THE SIS

ROC curve analysis based on a combination of the SIs of both antigens following the manufacturer's protocol (Supporting information, Fig. S2) resulted in an AUC of 0.487 (Table 3), which almost perfectly fitted the random predictor (Fig. 2d). Based on this analysis, the LymeSpot assay had a sensitivity of 27.8% to diagnose active LNB (Table 3). Thus, only five of the 18 active LNB patients were correctly identified using the LymeSpot assay (Table 4). Of these five active LNB patients, two had a positive LymeSpot result, and for three active LNB patients the LymeSpot results would still need diagnostic verification according to the manufacturer's instructions. The remaining 13 (72.2%) active LNB patients had a negative LymeSpot result (Table 4). When the results that needed diagnostic verification were excluded from the positive results, the sensitivity of the LymeSpot assay decreased to 11.1%. The specificity of the LymeSpot assay was 69.9% (Table 3), and 21 (30.4%) of the 69 controls either needed diagnostic verification [*n* = 11 (15.9%)] or had a positive LymeSpot result [*n* = 10 (14.5%)] (Table 4). Interestingly, the percentage of positive LymeSpot results was highest among treated healthy individuals [seven of 10 (70.0%)]; however, this was not significantly higher when compared to the other groups (adjusted *P* values > 0.025) (Table 4).

Table 4. Interpretation of the LymeSpot assay based on the stimulation indices according to the protocol of the manufacturer

			Statistics	
Study groups	**NEG (*n*; %)**	**POS[a] (*n*; %)**	**BH[b] (overall)**	**BH[b] (2-group)**
Active LNB patients (*n* = 18)	13 (72.2)	5[c] (27.8)	0.066	> 0.025[e]
Treated LNB patients (*n* = 12)	7 (58.3)	5[d] (41.7)		
Treated healthy individuals (*n* = 10)	3 (30.0)	7[f] (70.0)		
Untreated healthy individuals (*n* = 47)	38 (80.9)	9[g] (19.1)		
Control group[h] (*n* = 69)	48 (69.6)	21[i] (30.4)	NA	NA

NEG, negative; POS, positive; *n*, number of study participants; BH, Benjamini–Hochberg; LNB, Lyme neuroborreliosis;
NA, not applicable.
a. The positive results include those results that needed diagnostic verification.
b. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
c. Three (16.7%) of 18 active Lyme neuroborreliosis (LNB) patients required diagnostic verification.
d. Three (25.0%) of 12 treated LNB patients required diagnostic verification.
e. As the initial comparison was significantly different (raw *P* value < 0.050), two-group comparisons were also performed.
f. Two (20.0%) of 10 treated healthy individuals required diagnostic verification.
g. Six (12.8%) of 47 untreated healthy individuals required diagnostic verification.
h. The control group consists of all study participants except the active LNB patients.
i. 11 (15.9%) out of 69 controls required diagnostic verification.

ELISPOT RESULTS VERSUS CLINICAL SYMPTOMS, ANTIBIOTIC THERAPY AND RECOVERY STATUS

In total, 26 (29.9%) of the 87 study participants reported symptoms at inclusion in this study; all active LNB patients (*n* = 18) and eight (66.7%) of the 12 treated LNB patients. Overall, no association was found between the presence of symptoms and the results of the various ELISpot protocols (Table 5a). For treated LNB patients, the number of complaints reported at the start of the study was also not associated with the ELISpot results, irrespective of the ELISpot protocol used (Table 5a). As the treated healthy individuals were only included when they did not report any symptoms at the start of the study, elevated SFC counts in this group could not be linked to symptoms. Similarly, 16 (28.1%) of the 57 healthy individuals had a positive LymeSpot result, which could not be linked to symptoms.

Among active LNB patients, no association was found between the duration of symptoms prior to the blood sampling and the ELISpot results, irrespective of the ELISpot protocol used (Table 5b). For most active LNB patients, the antibiotic treatment had already started at the time of blood sampling; however, no association was found between the duration of antibiotic therapy prior to blood sampling and the ELISpot results using the various ELISpot protocols (Table 5c). Similarly, no association was found between the degree of recovery and the T-cell response of active LNB patients (Table 5d). For treated LNB patients, the degree of recovery was assessed at a median of 37.0 days after the end of antibiotic treatment for active disease in the past (approximately 5.4 years ago; Table 1), therefore, we did not compare the degree of recovery with the various ELISpot results obtained in the current study.

ELISPOT RESULTS VERSUS *BORRELIA*-SPECIFIC ANTIBODIES

In our previous study, elevated numbers of *B. burgdorferi* B31-specific T cells were significantly associated with the presence of *Borrelia*-specific serum antibodies [20]. In this study, which included a smaller number of study participants, comparison of the *B. burgdorferi* B31-specific SFC counts with the serology results showed a trend towards a combined B- and T-cell response when all study participants were analyzed together, irrespective of the ELISpot protocol used (Table 6a).

Within-group comparisons also showed a (non-significant) trend towards a combined B- and T-cell response, except for treated LNB patients, who showed elevated *B. burgdorferi* B31-specific SFC counts in the absence of *Borrelia*-specific antibodies. This was, again, in line with the results found in our previous study [20]. The presence of *Borrelia*-specific IgM or IgG also was not associated with elevated SFC counts (adjusted *P* values ranged from 0.199 to 1.000; data not shown).

Among active LNB patients, no significant association was found between the intrathecal production of *Borrelia*-specific antibodies and the T-cell response (Table 5b), which was similar to the results of our previous study in which 33 active LNB patients were included [20]. We also did not find a difference among the active LNB patients between negative and positive IgM AI results or between negative and positive IgG AI results when compared to the various SFC counts (adjusted *P* values ranged from 0.131 to 1.000; data not shown).

For treated LNB patients, the presence of intrathecally produced *Borrelia*-specific antibodies was determined at the time of active disease in the past, therefore, we did not compare these results with the ELISpot results using the various ELISpot protocols, as these were performed on average 5.4 years (Table 1), at the time the treated LNB patients were included in this study.

Table 5. Overview of the T-cell response, the presence and duration of clinical symptoms, and the degree of recovery (after antibiotic therapy) among the various study groups

				50 µl *B. burgdorferi* B31		100 µl *B. burgdorferi* B31			
				SFC count in-house *Borrelia* ELISpot assay		SFC count in-house *Borrelia* ELISpot assay		SFC count LymeSpot assay	
	Study groups	**Symptoms[a]**	***n* (%)**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**
(a)	All study participants (*n* = 87)	NO	61 (70.1)	2.0 (0.5-6.0)	0.439	3.0 (2.0-8.0)	0.486	4.5 (1.5-10.5)	0.916
		YES[c]	26 (29.9)	4.5 (1.3-7.4)		7.5 (1.5-13.9)		5.8 (2.1-9.6)	
	Treated LNB patients (*n* = 12)	NO	4 (33.3)	15.2 (7.9-24.2)	0.157	15.0 (5.0-25.4)	0.717	30.5 (9.9-54.1)	0.100
		YES[c]	8 (66.7)	2.0 (1.8-5.6)		9.5 (4.0-15.6)		3.3 (1.7-8.3)	
(b)	Symptom duration[d] (median; IQR)		*n* (%)	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]
	Active LNB patients	33.5 (15.8-59.5)	18 (100)	r_s = -0.200	0.703	r_s = -0.130	0.815	r_s = 0.170	0.746
(c)	Post AB-treatment time[e] (median; IQR)		*n* (%)	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]
	Active LNB patients	6.0 (3.3-7.0)	18 (100)	r_s = -0.160	0.770	r_s = -0.130	0.816	r_s = -0.370	0.374
(d)	Recovery status[f]		*n* (%)	Median (IQR)	BH[b]	Median (IQR)	BH[b]	Median (IQR)	BH[b]
	Active LNB	Incomplete	5 (27.8)	6.0 (2.0-9.5)	0.938	9.0 (5.0-14.5)	0.735	6.3 (1.0-10.0)	0.735
	patients (*n* = 18)	Complete	13 (72.2)	5.0 (1.0-7.0)		5.0 (1.0-10.0)		6.0 (3.0-11.0)	

Osp, outer surface protein; SFC, spot-forming cell; ELISpot, enzyme-linked immunosorbent spot; *n*, number of study participants; IQR, interquartile range; BH, Benjamini–Hochberg; NEG, negative; POS, positive; LNB, Lyme neuroborreliosis.

a. Symptoms are defined as the presence of symptoms at the start of the study. For (un)treated healthy individuals and treated Lyme neuroborreliosis (LNB) patients, the presence of symptoms was assessed by the completion of a Lyme-specific questionnaire; (un)treated healthy individuals were only included if they did not report any symptoms at the start of the study. For active LNB patients, the presence of symptoms was extracted from the hospital information system.
b. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
c. In total eight (66.7%) of the 12 treated LNB patients reported complaints at the start of the study. For all treated LNB patients, the presence of complaints was reported on average 5.4 years after the diagnosis of active disease in the past (Table 1).
d. Symptom duration is defined as the number of days the study participant experienced complaints prior to blood sampling.
e. Post AB-treatment time is defined as the number of days between the start of antibiotic (AB) treatment and blood sampling (median days; IQR).
f. The degree of recovery (recovery status) was assessed after a median of 38.0 days after the end of antibiotic therapy for active disease (Table 1).

50 µl Osp-mix		**100 µl Osp-mix**						
SFC count in-house *Borrelia* ELISpot assay		***SFC count in-house* *Borrelia* ELISpot assay**		**SFC count LymeSpot assay**		**LymeSpot assay**		
Median (IQR)	**BH[b]**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**	**NEG**	**POS**	**BH[b]**
1.0 (0.0-2.0)	0.994	1.0 (0.0-3.0)	0.811	1.5 (0.5-3.5)	0.720	42	19	1.000
0.0 (0.0-4.8)		1.0 (0.0-3.0)		1.3 (0.3-2.5)		19	7	
2.0 (0.0-6.3)	1.000	1.0 (0.0-2.5)	1.000	2.8 (1.4-8.4)	0.570	1	3	0.497
0.5 (0.0-3.0)		0.5 (0.0-4.0)		1.3 (0.4-2.3)		6	2	
Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	LymeSpot assay	Symptom duration[d] (median; IQR)	BH[b]
r_s = 0.180	0.721	r_s = 0.180	0.722	r_s = 0.440	0.250	NEG (*n*=13) POS (*n*=5)	32 (14.0-45.0) 55 (22.0-204.0)	0.677
Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	LymeSpot assay	Post AB-treatment time[e] (median; IQR)	BH[b]
r_s = -0.088	0.910	r_s = -0.098	0.889	r_s = -0.210	0.683	NEG (*n*=13) POS (*n*=5)	6.0 (5.0-8.0) 2.0 (2.0-6.0)	0.358
Median (IQR)	BH[b]	Median (IQR)	BH[b]	Median (IQR)	BH[b]	NEG	POS	BH[b]
0.0 (0.0-0.0)	0.460	1.0 (1.0-2.0)	0.992	0.5 (0.0-2.5)	0.784	4	1	1.000
1.0 (0.0-6.0)		0.0 (0.0-5.0)		1.5 (0.5-2.5)		9	4	

Table 6. Overview of the B- and T-cell response among the various study groups

				50 µl *Bb* B31		100 µl *Bb* B31	
				SFC count in-house *Borrelia* ELISpot assay		SFC count in-house *Borrelia* ELISpot assay	
	Study groups	**Serology result (IgM + IgG)**	***n* (%)**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**
(a)	All study participants combined (*n* = 87)	NEG	60 (69.0)	2.0 (0.4-5.3)	0.181	3.0 (1.8-6.0)	0.162
		POS	27 (31.0)	5.0 (1.5-8.8)		8.0 (3.0-14.5)	
	Active LNB patients (*n* = 18)	NEG	3 (16.7)	5.0 (2.5-19.5)	1.000	5.0 (2.5-28.0)	1.000
		POS	15 (83.3)	6.0 (1.5-7.5)		8.0 (2.0-11.0)	
	Treated LNB patients (*n* = 12)	NEG	11 (91.7)	6.0 (2.0-11.2)	0.783	7.0 (5.0-19.0)	1.000
		POS	1 (8.3)	2.0		12.0	
	Treated healthy individuals (*n* = 10)	NEG	6 (60.0)	4.5 (1.5-10.9)	0.359	3.5 (3.0-4.8)	0.130
		POS	4 (40.0)	24.0 (12.5-33.5)		45.0 (19.2-75.2)	
	Untreated healthy individuals (*n* = 47)	NEG	40 (85.1)	1.3 (0.0-3.0)	0.551	2.0 (1.0-4.3)	0.581
		POS	7 (14.9)	3.0 (0.8-5.5)		6.0 (2.0-11.2)	
		AI result (IgM + IgG)					
(b)	Active LNB patients	NEG	6 (33.3)	3.5 (2.3-19.0)	1.000	4.0 (1.5-26.4)	1.000
		POS	12 (66.7)	6.0 (0.8-7.3)		8.0 (2.3-10.5)	

Bb B31, *B. burgdorferi* B31; Osp, outer surface protein; SFC, spot-forming cell; ELISpot, enzyme-linked immunosorbent spot; AI, antibody index; *n*, number of study participants; IQR, interquartile range; BH, Benjamini–Hochberg; NEG, negative; POS, positive; LNB, Lyme neuroborreliosis; AB, antibiotic treatment for Lyme borreliosis.

a. The final LymeSpot result is based on a combination of the stimulation indices of both antigens following the protocol of the manufacturer (Supporting information, Fig. S2).

b. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).

		50 µl Osp-mix		100 µl Osp-mix						
SFC count LymeSpot assay		**SFC count in-house *Borrelia* ELISpot assay**		**SFC count in-house *Borrelia* ELISpot assay**		**SFC count LymeSpot assay**		**Final LymeSpot result[a]**		
Median (IQR)	**BH[b]**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**	**NEG**	**POS**	**BH[b]**
3.4 (1.4-8.6)	0.107	1.0 (0.0-2.0)	1.000	1.0 (0.0-3.0)	0.769	1.0 (0.4-3.0)	0.672	43	17	0.986
7.0 (3.8-19.4)		0.0 (0.0-4.5)		1.0 (0.0-4.0)		2.0 (0.5-4.0)		18	9	
11.0 (6.3-30.2)	0.685	1.0 (0.5-5.0)	0.630	5.0 (2.5-6.0)	0.652	4.5 (2.3-8.3)	0.691	1	2	0.430
6.0 (2.8-9.3)		0.0 (0.0-4.5)		1.0 (0.0-2.5)		1.0 (0.3-2.5)		12	3	
8.3 (2.3-15.2)	1.000	1.0 (0.0-5.0)	0.355	0.0 (0.0-3.0)	1.000	1.5 (0.8-3.5)	1.000	6	5	1.000
8.5		0.0		1.0		1.5		1	0	
8.5 (2.4-15.8)	0.111	1.8 (0.4-2.0)	0.724	2.0 (0.5-2.8)	0.418	3.8 (2.8-6.2)	0.309	3	3	0.471
78.8 (66.8-83.1)		9.5 (5.5-14.2)		11.0 (6.8-13.6)		12.2 (10.0-12.6)		0	4	
3.0 (1.2-5.6)	0.377	1.0 (0.0-1.3)	0.785	1.0 (0.0-2.0)	0.679	1.0 (0.0-2.1)	0.469	33	7	0.814
5.0 (3.5-10.0)		0.0 (0.0-1.0)		2.0 (0.5-4.0)		2.5 (1.0-3.8)		5	2	
4.3 (2.6-8.9)	0.847	2.0 (0.0-5.5)	0.845	0.5 (0.0-1.8)	0.890	0.3 (0.0-2.0)	0.625	4	2	1.000
6.6 (4.6-10.4)		0.0 (0.0-2.8)		1.0 (0.0-3.5)		1.5 (0.5-2.9)		9	3	

DISCUSSION

In the current study, the diagnostic performance of two ELISpot assays to diagnose active LNB were compared. The final study population consisted of 87 participants and comprised 18 active and 12 treated LNB patients, 10 healthy individuals who were treated for an early (mainly cutaneous) manifestation of LB in the past and 47 untreated healthy individuals. Both our in-house *Borrelia* ELISpot assay and the LymeSpot assay showed a poor diagnostic performance based on the numbers of SFCs with AUCs ranging from 0.429 to 0.570. The corresponding sensitivities, specificities, PPVs and NPVs ranged from 44.4 to 66.7%, 42.0 to 72.5%, 21.8 to 33.3% and 80.5 to 87.0%, respectively. The diagnostic performance of the LymeSpot assay, using so-called SIs following the manufacturer's protocol, resulted in a comparably low AUC of 0.487, with a corresponding sensitivity of 27.8%, a specificity of 69.6%, a PPV of 19.1% and a NPV of 78.6%. Our study showed that the two ELISpot assays, irrespective of the protocol used, cannot be used to diagnose LNB or to monitor antibiotic treatment success.

The results of the 87 study participants of the in-house *Borrelia* ELISpot assay after stimulation of the PBMCs with 50 µl of *B. burgdorferi* B31 in the current study represent a subset of the results of the 243 study participants published previously [20]. The SFC counts between the four study groups of the subgroup in this study were comparable with the SFC counts between the four study groups of the entire study population. Both studies showed significantly higher numbers of SFCs after stimulation with *B. burgdorferi* B31 for treated healthy individuals compared to untreated healthy individuals. Active LNB patients and treated LNB patients also showed higher numbers of SFCs after stimulation with *B. burgdorferi* B31 compared to untreated healthy individuals, although not significant in the current study. This is most probably explained by the lower number of study participants per group in the current study. The association between the B- and T-cell response that was found in our previous study was also seen in the current study, although it was not significant, most probably due to the smaller study population. The overall conclusion, that elevated numbers of SFCs are associated with a previous contact with the *Borrelia* bacterium [20]; however, was confirmed and could not be linked to symptomology nor to the degree of recovery or to antibiotic treatment. Elevated IFN-γ levels among asymptomatic individuals and previous LB patients have also been found by others [32-35].

Comparison of the in-house *Borrelia* ELISpot results after PBMC stimulation with either 50 or 100 µl of *B. burgdorferi* B31 showed similar results for three of the four study groups. However, among untreated healthy individuals, significantly higher numbers of SFCs were seen when 100 µl was used. This could be explained by the relatively higher number of untreated healthy individuals compared to the number of study participants in the other three groups.

Consistent with our previous study [20], we found that the use of Osp-mix as a T-cell stimulant resulted in very low numbers of SFCs, and cannot be used in its current composition to distinguish active LNB patients from the three control groups. Other studies also described a reduced performance of recombinant antigens compared to whole-cell lysates [13, 36]. This may, in part, be explained by the number of different antigens present: (a mixture of various) recombinant antigens contains far less antigens than a whole-cell lysate. Alternatively, recombinant antigens are more specific, therefore limiting the possibility of cross-reactivity. It is known that *Borrelia*-specific antibodies show cross-reactivity with other diseases [37] and that the bacterium shows high sequence homology with bacteria such as *Treponema* or *Leptospira* [38, 39]. Cross-reactivity could theoretically result in higher numbers of SFCs when a whole-cell lysate of *B. burgdorferi* B31 is used in patients with an active or previous infection caused by bacteria such as *Treponema* or *Leptospira*, or in healthy individuals who carry non-pathogenic *Treponema* or *Leptospira* species. Previously, we have tested two patients with active leptospirosis in our in-house *Borrelia* ELISpot assay, and one of them had high numbers of SFCs after stimulation with a whole-cell lysate of *B.*

burgdorferi B31 [20].

Overall, the numbers of SFCs after stimulation with *B. burgdorferi* B31 were also relatively low. In our experience, as well as described by others - for tuberculosis or cytomegalovirus infections - the numbers of IFN-γ-secreting T cells among exposed or infected individuals measured in an ELISpot assay using comparable amounts of PBMCs, ranging from 2.0×10^5 to 2.5×10^5, are generally much higher [40-42]. The lack of T-cell activity among the active LNB patients could be explained by the choice of *Borrelia* antigens. In the Netherlands, LNB is mainly caused by *B. garinii* and *B. bavariensis* [43] and less frequently by *B. burgdorferi* sensu stricto. As we have discussed previously [20], we do not believe that the use of *B. burgdorferi* B31 whole-cell lysate in the ELISpot assay resulted in the poor performance of both ELISpot assays, as *B. burgdorferi*, *B. garinii* and *B. bavariensis* are closely related and share many antigens. Von Baehr et al. [13] evaluated three *Borrelia* species and did not find any difference. Nordberg et al. [44] used *B. garinii* as a stimulating agent, CSF instead of blood and nitrocellulose-bottomed ELISpot plates instead of PVDF-bottomed plates, and also did not find higher numbers of activated T cells in their ELISpot assay. The Osp-mix we used contained antigens derived from an LNB-associated strain (*B. garinii*); however, the Osp-mix was inferior compared to the use of *B. burgdorferi* B31, as discussed in the previous paragraph. The lack of T-cell activity might also be explained by the inability of the human host to develop an adequate immune response against the *Borrelia* bacterium or the ability of the *Borrelia* bacterium to escape or suppress the immune system [45, 46]. It could also be due to the disease manifestation that was studied, as already debated previously [20], as LNB implies a local infection of the brain. Testing blood might thus be less suitable, as the immune cells could have migrated towards the central and/or peripheral nervous system [47, 48]. The testing of CSF, in combination with blood, may be more suitable [49]. Furthermore, IFN-γ may not the best marker to diagnose active LNB. It would be interesting to investigate whether other cytokines and/or chemokines could improve the ELISpot assays tested in this study. Recently, the LymeSpot assay has been adapted by the manufacturer by adding the detection of interleukin (IL)-2. However, no data are available yet with regard to the diagnostic performance of this modified LymeSpot assay.

For the LymeSpot assay, the PBMC isolation procedure used in this study deviated from the manufacturer's (AID) recommended protocol. These deviations from the LymeSpot protocol were made in order for the technician to be able to perform and process the ELISpot assays simultaneously, and to minimize the differences between the assays to allow for a more fair comparison. The PBMCs used in the LymeSpot assay were thus isolated according to the same protocol that was already in use in our laboratory for the in-house *Borrelia* ELISpot assay [20] and for the T-SPOT.TB test [20, 26, 50]. Consequently, the PBMC isolation differed at four points compared to the instruction manual of the LymeSpot assay.

First, the medium to dilute the blood prior to PBMC isolation differed, as RPMI medium was used instead of phosphate-buffered saline (PBS). Secondly, Leucosep tubes were used for the isolation of PBMCs, while the LymeSpot protocol advises to use standard tubes with a Ficoll gradient. As a consequence, the centrifugation steps of the isolation procedure were adjusted based on the instruction manual supplied with the Leucosep tubes. As the isolation of PBMCs is based on a gradient, we do not believe that the altered centrifugation time resulted in a different PBMC yield. An increased centrifugation speed could, potentially, result in a higher PBMC yield, but this should not influence the results of the LymeSpot assay, as the amount of PBMCs per well is standardized. This is confirmed by others [51, 52], who showed that PBMCs isolated by Leucosep tubes performed equally well in the ELISpot assay compared to PBMCs isolated using the Ficoll-gradient technique.

Thirdly, the centrifugation steps that were used to wash the PBMCs and the number of times the PBMCs were washed differed from the LymeSpot protocol. However, in the literature, various centrifugation speeds and times for washing the PBMCs are described, which range from 300 to

640 *g* for 7-10 min for the first wash step and from 300 to 470 *g* for 7-10 min for the second wash step [26, 41, 51, 53-55].

Finally, the amount of PBMCs used varied slightly, as we used 2.5×10^5 PBMCs/well, and according to the LymeSpot assay, 2.0×10^5 PBMCs/well should have been used. A higher number of PBMCs per well could result in increased numbers of SFCs, as the use of more PBMCs results in more antigen-presenting cells and more T cells that could become activated after stimulation with the *Borrelia* antigens.

The results of a comparative pilot experiment that we performed in which we assessed the influence of the deviations discussed above supported that these deviations from the recommended protocol are not critical as such (Supporting information, Data S4). Hence, the conclusion stands that both ELISpot assays cannot help to diagnose active LNB.

Probably some of the most critical steps that influence the performance of an ELISpot assay are the time between venipuncture and PBMC isolation, the time between PBMC isolation and incubation of the assay and the (overnight) incubation time of the assay [56, 57]. In this study, these times were all within the limits as described in the LymeSpot protocol, with the exception of the time between venipuncture and PBMC isolation, which was prolonged for various cases. A prolonged time between venipuncture and PBMC isolation is known to decrease the PBMC viability [56]. To compensate for this, for those cases for which the time between venipuncture and PBMC isolation was prolonged (8–32 h), we performed a T-Cell Xtend step prior to PBMC isolation. This T-Cell Xtend step has proved not to be detrimental to the PBMC yield and the ELISpot performance [26, 27, 58].

No data are provided in the instruction manual of the LymeSpot assay with regard to the diagnostic performance of this assay. To our knowledge, this is the first study that has investigated the diagnostic capacity of the LymeSpot assay for the diagnosis of active LNB. The diagnostic performance of the LymeSpot assay for other manifestations of LB has not yet been investigated thoroughly and remains unclear. Hopefully, more validation studies will be performed which will include other manifestations of LB, as well as follow-up studies to understand more clearly the diagnostic potential for treatment monitoring.

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DISCLOSURES

None.

REFERENCES

1. National Institute for Public Health and the Environment, the Netherlands. Lyme disease cases have quadrupled. Available at: https://www.rivm.nl/en/Documents_and_publications/Common_and_Present/Newsmessages/2018/Lyme_disease_cases_have_quadrupled. Accessed June 2018.
2. de Mik EL, van Pelt W, Docters-van Leeuwen BD, van der Veen A, Schellekens JF, Borgdorff MW. 1997. The geographical distribution of tick bites and erythema migrans in general practice in The Netherlands. Int J Epidemiol 26:451-7. doi: 10.1093/ije/26.2.451.
3. Hofhuis A, Harms M, Bennema S, van den Wijngaard CC, van Pelt W. 2015. Physician reported incidence of early and late Lyme borreliosis. Parasit Vectors 8:161. doi: 10.1186/s13071-015-0777-6.
4. Kwaliteitsinstituut voor de Gezondheidszorg (CBO). 2013. Dutch guidelines Lyme disease 2013. Available at [In Dutch]: https://www.rivm.nl/nieuws/cbo-richtlijn-lymeziekte-definitief.
5. Wilske B. 2003. Diagnosis of Lyme borreliosis in Europe. Vector Borne Zoonotic Dis 3:215-27. doi: 10.1089/153036603322662200.
6. Strle F. 1999. Principles of the diagnosis and antibiotic treatment of Lyme borreliosis. Wien Klin Wochenschr 111:911-5.
7. van Dam AP. 2011. Molecular diagnosis of *Borrelia* bacteria for the diagnosis of Lyme disease. Expert Opin Med Diagn 5:135-149. doi: 10.1517/17530059.2011.555396.
8. Cerar T, Ogrinc K, Cimperman J, Lotric-Furlan S, Strle F, Ruzic-Sabljic E. 2008. Validation of cultivation and PCR methods for diagnosis of Lyme neuroborreliosis. J Clin Microbiol 46:3375-9. doi: 10.1128/JCM.00410-08.
9. Karlsson M, Hovind-Hougen K, Svenungsson B, Stiernstedt G. 1990. Cultivation and characterization of spirochetes from cerebrospinal fluid of patients with Lyme borreliosis. J Clin Microbiol 28:473-9. doi: 10.1128/jcm.28.3.473-479.1990.
10. Mygland A, Ljostad U, Fingerle V, Rupprecht T, Schmutzhard E, Steiner I. 2010. EFNS guidelines on the diagnosis and management of European Lyme neuroborreliosis. Eur J Neurol 17:8-4. doi: 10.1111/j.1468-1331.2009.02862.x.
11. Engstrom SM, Shoop E, Johnson RC. 1995. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. J Clin Microbiol 33:419-27. doi: 10.1128/jcm.33.2.419-427.1995.
12. Wormser GP, Nadelman RB, Dattwyler RJ, Dennis DT, Shapiro ED, Steere AC, Rush TJ, Rahn DW, Coyle PK, Persing DH, Fish D, Luft BJ. 2000. Practice guidelines for the treatment of Lyme disease. The Infectious Diseases Society of America. Clin Infect Dis 31 Suppl 1:1-14. doi: 10.1086/314053.
13. von Baehr V, Doebis C, Volk HD, von Baehr R. 2012. The lymphocyte transformation test for *Borrelia* detects active Lyme borreliosis and verifies effective antibiotic treatment. Open Neurol J 6:104-12. doi: 10.2174/1874205X01206010104.
14. Valentine-Thon E, Ilsemann K, Sandkamp M. 2007. A novel lymphocyte transformation test (LTT-MELISA) for Lyme borreliosis. Diagn Microbiol Infect Dis 57:27-34. doi: 10.1016/j.diagmicrobio.2006.06.008.
15. Callister SM, Jobe DA, Stuparic-Stancic A, Miyamasu M, Boyle J, Dattwyler RJ, Arnaboldi PM. 2016. Detection of IFN-gamma secretion by T cells collected before and after successful treatment of early Lyme disease. Clin Infect Dis 62:1235-1241. doi: 10.1093/cid/ciw112.
16. Jin C, Roen DR, Lehmann PV, Kellermann GH. 2013. An enhanced ELISpot assay for sensitive detection of antigen-specific T cell responses to *Borrelia burgdorferi*. Cells 2:607-20. doi: 10.3390/cells2030607.
17. Alstrup K, Bech M. 2016. TV2_Documentar. [Snyd eller *borrelia*]. Available at: https://www.

dailymotion.com/video/x4wmdyj. Accessed 11 February 2019.
18. Arminlabs. *Borrelia* ELISpot (T-Cell-Spot/IGRA: Interferon-Gamma-Release Assay). Available at: https://www.arminlabs.com/en/tests/elispot. Accessed November 2018.
19. BCA-clinic. Is the LymeSpot revised more effective than the traditional ELISpot. 2018. Available at: https://www.bca-clinic.de/en/is-the-lymespot-revised-more-effective-than-the-traditional-elispot/. Accessed February 2019.
20. van Gorkom T, Sankatsing SUC, Voet W, Ismail DM, Muilwijk RH, Salomons M, Vlaminckx BJM, Bossink AWJ, Notermans DW, Bouwman JJM, Kremer K, Thijsen SFT. 2018. An Enzyme-Linked Immunosorbent Spot Assay Measuring *Borrelia burgdorferi* B31-Specific Interferon Gamma-Secreting T Cells Cannot Discriminate Active Lyme Neuroborreliosis from Past Lyme Borreliosis: a Prospective Study in the Netherlands. J Clin Microbiol 56. doi: 10.1128/JCM.01695-17.
21. van Gorkom T, Kremer K, Voet W, Notermans DW, Vlaminckx BJM, Sankatsing SUC, Thijsen SFT. 2017. Disagreement between the results from three commercial tests for the detection of *Borrelia*-specific serum antibodies in the Netherlands associated with antibiotic treatment for Lyme borreliosis: a retrospective study. Eur J Clin Microbiol Infect Dis 36:2137-2146. doi: 10.1007/s10096-017-3037-1.
22. Sprong H, Hofhuis A, Gassner F, Takken W, Jacobs F, van Vliet AJ, van Ballegooijen M, van der Giessen J, Takumi K. 2012. Circumstantial evidence for an increase in the total number and activity of *Borrelia*-infected Ixodes ricinus in the Netherlands. Parasit Vectors 5:294. doi: 10.1186/1756-3305-5-294.
23. Liang FT, Steere AC, Marques AR, Johnson BJ, Miller JN, Philipp MT. 1999. Sensitive and specific serodiagnosis of Lyme disease by enzyme-linked immunosorbent assay with a peptide based on an immunodominant conserved region of *Borrelia burgdorferi* vlsE. J Clin Microbiol 37:3990-3996. doi: 10.1128/JCM.37.12.3990-3996.1999.
24. Wilske B, Fingerle V, Schulte-Spechtel U. 2007. Microbiological and serological diagnosis of Lyme borreliosis. FEMS Immunol Med Microbiol 49:13-21. doi: 10.1111/j.1574-695X.2006.00139.x.
25. Hansen K, Lebech AM. 1991. Lyme neuroborreliosis: a new sensitive diagnostic assay for intrathecal synthesis of *Borrelia burgdorferi*-specific immunoglobulin G, A, and M. Ann Neurol 30:197-205. doi: 10.1002/ana.410300212.
26. Bouwman JJM, Thijsen SFT, Bossink AW. 2012. Improving the timeframe between blood collection and interferon gamma release assay using T-Cell Xtend. J Infect 64:197-203. doi: 10.1016/j.jinf.2011.10.017.
27. Wang SH, Stew SS, Cyktor J, Carruthers B, Turner J, Restrepo BI. 2012. Validation of increased blood storage times with the T-SPOT.*TB* assay with T-Cell Xtend reagent in individuals with different tuberculosis risk factors. J Clin Microbiol 50:2469-71. doi: 10.1128/JCM.00674-12.
28. Feske ML, Medina M, Graviss EA, Lewis DE. 2012. IL-7 addition increases spot size and number as measured by T-SPOT.TB. Methods Mol Biol 792:229-41. doi: 10.1007/978-1-61779-325-7_18.
29. Dunn OJ. 1964. Multiple comparisons using rank sums. Technometrics 6:241-252.
30. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, Muller M. 2011. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 12:77. doi: 10.1186/1471-2105-12-77.
31. Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological) 57:289-300. doi: 10.2307/2346101.
32. Ekerfelt C, Forsberg P, Svenvik M, Roberg M, Bergstrom S, Ernerudh J. 1999. Asymptomatic *Borrelia*-seropositive individuals display the same incidence of *Borrelia*-specific interferon-gamma (IFN-gamma)-secreting cells in blood as patients with clinical *Borrelia* infection. Clin

Exp Immunol 115:498-502. doi: 10.1046/j.1365-2249.1999.00840.x.
33. Ekerfelt C, Masreliez C, Svenvik M, Ernerudh J, Roberg M, Forsberg P. 2001. Antibodies and T-cell reactivity to *Borrelia burgdorferi* in an asymptomatic population: a study of healthy blood donors in an inland town district in the south-east of Sweden. Scand J Infect Dis 33:806-8. doi: 10.1080/00365540110077376.
34. Jarefors S, Janefjord CK, Forsberg P, Jenmalm MC, Ekerfelt C. 2007. Decreased up-regulation of the interleukin-12Rbeta2-chain and interferon-gamma secretion and increased number of forkhead box P3-expressing cells in patients with a history of chronic Lyme borreliosis compared with asymptomatic *Borrelia*-exposed individuals. Clin Exp Immunol 147:18-27. doi: 10.1111/j.1365-2249.2006.03245.x.
35. Skogman BH, Hellberg S, Ekerfelt C, Jenmalm MC, Forsberg P, Ludvigsson J, Bergstrom S, Ernerudh J. 2012. Adaptive and innate immune responsiveness to *Borrelia burgdorferi* sensu lato in exposed asymptomatic children and children with previous clinical Lyme borreliosis. Clin Dev Immunol 2012:294587. doi: 10.1155/2012/294587.
36. Glickstein L, Moore B, Bledsoe T, Damle N, Sikand V, Steere AC. 2003. Inflammatory cytokine production predominates in early Lyme disease in patients with erythema migrans. Infect Immun 71:6051-3. doi: 10.1128/IAI.71.10.6051-6053.2003.
37. Fawcett PT, Gibney KM, Rose CD, Dubbs SB, Doughty RA. 1992. Frequency and specificity of antibodies that crossreact with *Borrelia burgdorferi* antigens. J Rheumatol 19:582-7.
38. Bruckbauer HR, Preac-Mursic V, Fuchs R, Wilske B. 1992. Cross-reactive proteins of *Borrelia burgdorferi*. Eur J Clin Microbiol Infect Dis 11:224-32. doi: 10.1007/BF02098084.
39. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. 2005. Diagnosis of Lyme borreliosis. Clin Microbiol Rev 18:484-509. doi: 10.1128/CMR.18.3.484-509.2005.
40. Jafari C, Ernst M, Kalsdorf B, Greinert U, Diel R, Kirsten D, Marienfeld K, Lalvani A, Lange C. 2006. Rapid diagnosis of smear-negative tuberculosis by bronchoalveolar lavage enzyme-linked immunospot. Am J Respir Crit Care Med 174:1048-54. doi: 10.1164/rccm.200604-465OC.
41. Barabas S, Spindler T, Kiener R, Tonar C, Lugner T, Batzilla J, Bendfeldt H, Rascle A, Asbach B, Wagner R, Deml L. 2017. An optimized IFN-gamma ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. BMC Immunol 18:14. doi: 10.1186/s12865-017-0195-y.
42. van Gemert JP, Thijsen SFT, Bossink AW. 2011. Tuberculosis-specific T-cell response after recent treatment and remote cure. Eur Respir J 38:1225-8. doi: 10.1183/09031936.00170010.
43. Jahfari S, Krawczyk A, Coipan EC, Fonville M, Hovius JW, Sprong H, Takumi K. 2017. Enzootic origins for clinical manifestations of Lyme borreliosis. Infect Genet Evol 49:48-54. doi: 10.1016/j.meegid.2016.12.030.
44. Nordberg M, Forsberg P, Nyman D, Skogman BH, Nyberg C, Ernerudh J, Eliasson I, Ekerfelt C. 2012. Can ELISpot be applied to a clinical setting as a diagnostic utility for neuroborreliosis? Cells 1:153-67. doi: 10.3390/cells1020153.
45. Liang FT, Yan J, Mbow ML, Sviat SL, Gilmore RD, Mamula M, Fikrig E. 2004. *Borrelia burgdorferi* changes its surface antigenic expression in response to host immune responses. Infect Immun 72:5759-67. doi: 10.1128/IAI.72.10.5759-5767.2004.
46. Verhaegh D, Joosten LAB, Oosting M. 2017. The role of host immune cells and *Borrelia burgdorferi* antigens in the etiology of Lyme disease. Eur Cytokine Netw 28:70-84. doi: 10.1684/ecn.2017.0396.
47. Ekerfelt C, Ernerudh J, Bunikis J, Vrethem M, Aagesen J, Roberg M, Bergstrom S, Forsberg P. 1997. Compartmentalization of antigen specific cytokine responses to the central nervous system in CNS borreliosis: secretion of IFN-gamma predominates over IL-4 secretion in response to outer surface proteins of Lyme disease *Borrelia* spirochetes. J Neuroimmunol 79:155-62. doi: 10.1016/s0165-5728(97)00118-5.

48. Grusell M, Widhe M, Ekerfelt C. 2002. Increased expression of the Th1-inducing cytokines interleukin-12 and interleukin-18 in cerebrospinal fluid but not in sera from patients with Lyme neuroborreliosis. J Neuroimmunol 131:173-8. doi: 10.1016/S0165-5728(02)00255-2.
49. Schub D. ST, Sester M. [Neues Verfahren zum verbesserten Nachweis Antigen-spezifischer Immunzellen in extrasanguinen Flüssigkeiten]. 2015. Available at: https://patents.google.com/patent/DE102014018047B3/de. Accessed July 2018.
50. Hofland RW, Thijsen SF, van Lindert AS, de Lange WC, van Gorkom T, van der Tweel I, Lammers JJ, Bossink AW. 2017. Positive predictive value of ELISpot in BAL and pleural fluid from patients with suspected pulmonary tuberculosis. Infect Dis (Lond) 49:347-355. doi: 10.1080/23744235.2016.1269190.
51. Nilsson C, Aboud S, Karlen K, Hejdeman B, Urassa W, Biberfeld G. 2008. Optimal blood mononuclear cell isolation procedures for gamma interferon enzyme-linked immunospot testing of healthy Swedish and Tanzanian subjects. Clin Vaccine Immunol 15:585-9. doi: 10.1128/CVI.00161-07.
52. Afonso G, Scotto M, Renand A, Arvastsson J, Vassilieff D, Cilio CM, Mallone R. 2010. Critical parameters in blood processing for T-cell assays: validation on ELISpot and tetramer platforms. J Immunol Methods 359:28-36. doi: 10.1016/j.jim.2010.05.005.
53. Sumonwiriya M, Paris DH, Sunyakumthorn P, Anantatat T, Jenjaroen K, Chumseng S, Im-Erbsin R, Tanganuchitcharnchai A, Jintaworn S, Blacksell SD, Chowdhury FR, Kronsteiner B, Teparrukkul P, Burke RL, Lombardini ED, Richards AL, Mason CJ, Jones JW, Day NPJ, Dunachie SJ. 2017. Strong interferon-gamma mediated cellular immunity to scrub typhus demonstrated using a novel whole cell antigen ELISpot assay in rhesus macaques and humans. PLoS Negl Trop Dis 11:e0005846. doi: 10.1371/journal.pntd.0005846.
54. Bathoorn E, Limburg A, Bouwman JJ, Bossink AW, Thijsen SF. 2011. Diagnostic potential of an enzyme-linked immunospot assay in tuberculous pericarditis. Clin Vaccine Immunol 18:874-7. doi: 10.1128/CVI.00031-11.
55. Wang SH, Powell DA, Nagaraja HN, Morris JD, Schlesinger LS, Turner J. 2010. Evaluation of a modified interferon-gamma release assay for the diagnosis of latent tuberculosis infection in adult and paediatric populations that enables delayed processing. Scand J Infect Dis 42:845-50. doi: 10.3109/00365548.2010.498021.
56. Bourguignon P, Clement F, Renaud F, Le Bras V, Koutsoukos M, Burny W, Moris P, Lorin C, Collard A, Leroux-Roels G, Roman F, Janssens M, Vandekerckhove L. 2014. Processing of blood samples influences PBMC viability and outcome of cell-mediated immune responses in antiretroviral therapy-naive HIV-1-infected patients. J Immunol Methods 414:1-10. doi: 10.1016/j.jim.2014.09.001.
57. Mallone R, Mannering SI, Brooks-Worrell BM, Durinovic-Bello I, Cilio CM, Wong FS, Schloot NC, T-Cell Workshop Committee IoDS. 2011. Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society. Clin Exp Immunol 163:33-49. doi: 10.1111/j.1365-2249.2010.04272.x.
58. Li J, Liu Y, Ma L, Gu L, Wang Q, Xu M, Ma R, Zhang Y, Yang Z, Deng J, Yi X. 2018. The performance of T-cell Xtend reagent in increasing blood storage times for interferon gamma release assays. J Clin Lab Anal 32. doi: 10.1002/jcla.22253.

SUPPLEMENTAL MATERIAL

Table S1. Comparison of the protocols used for the evaluation of the in-house *Borrelia* ELISPOT assay and the LymeSpot assay

Steps in the ELISPOT assay procedure	Protocol	
	In-house *Borrelia* ELISPOT assay	**LymeSpot assay**
ELISPOT plate	96-wells PVDF plate coated with anti-human IFN-γ antibodies (supplied by Mabtech)	96-wells PVDF plate coated with anti-human IFN-γ antibodies (supplied by AID)
PMBC isolation	In-house *Borrelia* ELISpot protocol as described in the "Materials and Methods" section of the published manuscript [1]	*Altered protocol*[a]: identical to the in-house *Borrelia* ELISPOT assay
PMBC concentration	100 µl of 2.5 x 10^6 PBMCs/ml	*Altered protocol*[a]: identical to the in-house *Borrelia* ELISPOT assay
Negative control	50 µl of AIM-V medium (supplied by Life technologies); tested in the singular	100 µl of AIM-V medium (supplied by Life technologies); tested in duplicate
Positive control	50 µl (0.1 µg/ml) of anti-human CD3 MAb CD3-2 (supplied by Mabtech); tested in the singular	100 µl of Pokeweed (supplied by AID); tested in duplicate
Borrelia antigens	Both 50 µl and 100 µl of *B. burgdorferi* B31 whole- cell lysate[b] (5 µg/ml) (supplied by AID); both tested in the singular	100 µl of *B. burgdorferi* B31 whole-cell lysate (5 µg/ml) (supplied by AID); tested in duplicate
	Both 50 µl and 100 µl of Osp-mix[c] (5 µg/ml) (supplied by AID); both tested in the singular	100 µl of Osp-mix[c] (5 µg/ml) (supplied by AID); tested in duplicate
Incubation time	20-24 hours at 37°C and 5% CO2	Identical to the in-house *Borrelia* ELISPOT assay
Wash step	Four times with an excess of PBS (pH 7.2 ± 0.1)	Six times 200 µl of washing buffer 'WP' (supplied by AID)
Conjugate	50 µl of 7-B6-1–alkaline phosphatase conjugated secondary antibody (supplied by Mabtech)	100 µl of alkaline phosphatase conjugated secondary antibody (supplied by AID)
Incubation time	One hour at 2°C	Two hours at room temperature in a humidified chamber
Wash step	Four times with an excess of PBS (pH 7.2 ± 0.1)	Six times with 200 µl of washing buffer 'WP' (supplied by AID)
Substrate	50 µl of BCIP/NBT plus substrate (supplied by Mabtech)	100 µl of BCIP/NBT substrate (supplied by AID)
Incubation time	7-10 minutes at room temperature or until SFCs become clearly visible	5-20 minutes at room temperature or until SFCs become clearly visible
Wash step	Four times with excess of tap water	Three times with excess of tap water
Dry plate	At least 90 minutes at 37°C	Identical to the in-house *Borrelia* ELISPOT assay
Calculation method of the final results of both ELISPOT assays	• Positive control should have ≥20 SFCs • Negative control should have ≤6 SFCs • 50 µl of *Borrelia* antigen: subtract no. of SFCs in the negative control well from those in the *Borrelia* antigen-stimulated well • 100 µl of *Borrelia* antigen: multiply no. of SFCs in the negative control well by two and subtract from no. of SFCs in the *Borrelia* antigen-stimulated well	Two calculation methods were performed: 1. A calculation based on the protocol of the in-house *Borrelia* ELISPOT assay, for which the no. of SFCs in the negative control were subtracted from the no. of SFCs in the *Borrelia* antigen-stimulated well (take average for all duplicates); 2. A calculation based on the protocol of the manufacturer (AID) as is shown in Fig. S2.

PVDF, polyvinylidene difluoride; IFN-γ, interferon-gamma; AID, Autoimmun Diagnostika GmbH; PBMC, peripheral blood mononuclear cell; CD, cluster of differentiation; MAb, monoclonal antibody; PBS, phosphate-buffered saline; BCIP/NBT, 5-bromo-4-chloro-3'-indolylphosphate and nitroblue tetrazolium; SFCs, spot-forming cells (SFCs are based on the numbers of IFN-γ-secreting T cells/ 2.5 x 10^5 PBMCs).

a. The altered protocol showed various deviations from the LymeSpot protocol, which are described in the "Discussion" section of the published manuscript [1]. The influence of these deviations on the diagnostic performance of the LymeSpot assay are assessed in Data S4.

b. For some cases who were tested in the in-house *Borrelia* ELISPOT assay, more than one lot number of *B. burgdorferi* B31 whole-cell lysate (50 µl protocol) was used to stimulate the PBMCs. For these cases, the median number of SFCs was used for all subsequent analyses.

c. The outer surface protein (Osp)-mix consisted of a pool of 9-mer to 11-mer peptides of Osp-A (*B. burgdorferi, B. afzelii*, and *B. garinii*), native Osp-C (*B. afzelii*), and recombinant p18.

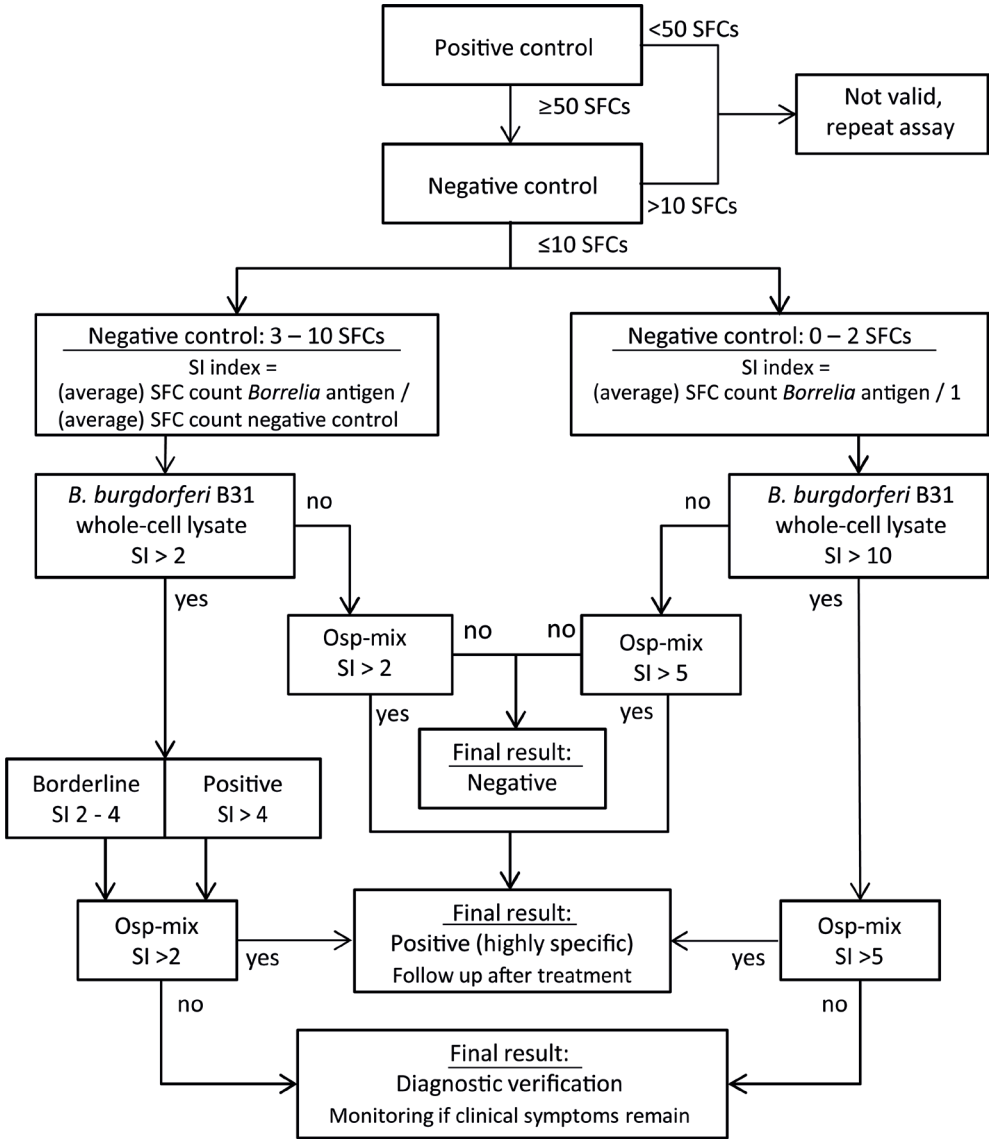

Fig. S2. Interpretation scheme of the LymeSpot assay according to the manufacturer. The spot-forming cell (SFC) count is based on the numbers of interferon-gamma-secreting T cells/2.5 x 10^5 PBMCs upon stimulation with Pokeweed (positive control), AIM-V medium (negative control), *B. burgdorferi B31* whole-cell lysate, or a mixture of various recombinant outer surface proteins (Osp). The Osp-mix consists of a pool of 9-mer to 11-mer peptides of Osp-A (*B. burgdorferi*, *B. afzelii*, and *B. garinii*), native Osp-C (*B. afzelii*), and recombinant p18. Depending on the numbers of SFCs in the negative control well, a stimulation index (SI) is calculated.

Table S3. Overview of the study populations used in the three studies

Studies		Time of inclusion of the study participants[a]																																					
		2010				2011				2012				2013				2014				2015				2016				2017				2018							
Reference	Study participants	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
	Total number of active Lyme NB patients (*n* = 40)					2		2	2			3		1	1	2	1	1	1	3	3	2	1	1	3	1		4	3			1	1	1					
2	*n* = 27					2		2	2			3		1	2	1	1	1	3	2		2	1	1	3														
3	*n* = 33					2		2	1			3		1	1	2	1	1	1	2	3		1	1	3	1		4	3										
1	*n* = 18																	1	1	1	2			1	2			4	3			1	1	1					
	Total number of treated Lyme NB patients (*n* = 39)					1								11				15				12																	
2	*n* = 36													10				15				11																	
3	n = 37					1								11				13				12																	
1	n = 12																					12																	
	Total number of treated healthy individuals (*n* = 28)													14				4				1	3		6														
2	*n* = 27													13				4				1	3		6														
3	*n* = 28													14				4				1	3		6														
1	*n* = 10																	2				3			5														
	Total number of untreated healthy individuals (*n* = 148)													69				64				10	2		3														
2	*n* = 147													69				63				10	2		3														
3	*n* = 145													67				64				9	2		3														
1	*n* = 47																	35				9	1		2														

Q1, first quarter; Q2, second quarter; Q3, third quarter; Q4, fourth quarter; LNB, Lyme neuroborreliosis

a. The number of study participants used in the three studies are in principle overlapping, unless for instance serum [2], or the number of peripheral blood mononuclear cells ([3], or current study [1]) were insufficient. The underlined numbers reflect the number of study participants (n=84) used in both ELISpot studies ([3] and current study [1]).

Data S4. A pilot study to investigate the influence of three different protocols for the isolation of peripheral blood mononuclear cells (PBMCs) on the LymeSpot results

INTRODUCTION OF DATA S4

PBMCs are immune cells that can be used to investigate the cellular immune response during an infection. To isolate PBMCs from whole blood, density gradient centrifugation can be applied followed by several wash steps to remove platelets, erythrocytes, granulocytes and residual Ficoll [4]. In the current manuscript of Van Gorkom et al. [1], we validated the LymeSpot assay. At the same time, the numbers of spot-forming cells (SFCs) obtained by using the LymeSpot assay were compared with those obtained by using our in-house *Borrelia* enzyme-linked immunosorbent spot (ELISpot) assay. To minimize the differences between both ELISpot assays and to facilitate the technician who performed both assays simultaneously, we used one single PBMC isolation protocol: the one that was already in use in our laboratory for the in-house *Borrelia* ELISpot assay. Consequently, the PBMC isolation protocol we used for the LymeSpot assay deviated from the protocol recommended by the manufacturer (Autoimmun Diagnostika (AID) GmbH, Straßberg, Germany). Our in-house PBMC isolation protocol differed from the LymeSpot protocol on four points: (i) the PBMC separation medium used, (ii) the various centrifugation steps, (ii) the medium used to wash the PBMCs, and (iv) the amount of PBMCs tested per well (Table S4.1). In 2018, after our study had finished, the manufacturer of the LymeSpot assay implemented a number of changes to their recommended PBMC isolation protocol, most importantly the speed of the various centrifugation steps. Interestingly, in this 'new' LymeSpot protocol, the centrifugation speeds were increased compared to the previous 'old' LymeSpot protocol, and now more closely resembled the centrifugation speeds of our in-house *Borrelia* ELISpot protocol. As variations in the PBMC isolation protocols could potentially influence the final LymeSpot results, we conducted a pilot study to assess the influence of the various PMBC isolation protocols on the diagnostic performance of the LymeSpot assay. In this experiment we compared the LymeSpot results obtained by using our in-house PBMC isolation protocol with the LymeSpot results obtained by using the old and the new LymeSpot protocol.

Table S4.1. Overview of the three PBMC isolation protocols used in this pilot study

	Old LymeSpot assay [a]	New LymeSpot assay[b]	In-house *Borrelia* ELISPOT assay[c]
PBMC separation medium	Ficoll Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)	Identical to old LymeSpot protocol	Leucosep tubes (OxFord Immunotec Ltd., Abingdon, UK)
Centrifugation step	30 min at 400 *g* without break	30 min at 1000 *g* without break	15 min at 1000 *g* without break
First wash step	Isolate the PBMC layer and add the layer to a tube containing 10 ml of PBS (pH 7.4) (Fisher Scientific, Landsmeer, The Netherlands)	Identical to old LymeSpot protocol	Isolate the PBMC layer and add to a tube containing 10 ml of RPMI medium (Life Technologies, Invitrogen, Bleiswijk, The Netherlands)
Centrifugation step	Centrifuge 10 min at 350 *g* with break	Centrifuge 10 min at 700 *g* with break	Centrifuge 7 min at 600 *g* with break
Second wash step	Discard PBS, resuspend PBMCs and fill with 10 ml of PBS	Identical to old LymeSpot protocol	Discard RPMI, resuspend PBMCs and fill with 10 ml of RPMI medium (Life Technologies)
Centrifugation step	Centrifuge 10 min at 350 *g* with break	Centrifuge 10 min at 700 *g* with break	Centrifuge 7min at 300 *g* with break
Third wash step	Discard PBS, resuspend PBMCs in 10 ml of RPMI medium	Discard PBS, resuspend PBMCs in 10ml of AIM-V medium (Life Technologies)	No third wash step
Centrifugation step	Centrifuge 10 min at 350 *g* with break	Centrifuge 10 min at 700 *g* with break	No third centrifugation step
Count PBMC suspension	Discard medium, resuspend pellet in 1.1 ml AIM-V medium (supplied by Life technologies) and count PBMCs as described in the 'Materials and Methods' section of the current manuscript of van Gorkom et al. [1]	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
Adjust PBMC suspension	Adjust PBMC suspension to 2.0 x 10^6 cells/ml with AIM-V medium	Identical to old LymeSpot protocol	Adjust PBMC suspension to 2.5 x 10^6 cells/ml with AIM-V medium
Volume of PBMC suspension/well	Add 100 µl of PBMC suspension in each well of the LymeSpot plate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
For each subject the following wells were tested:			
Negative control	• 100 µl of AIM-V medium; tested in duplicate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
Positive control	• 100 µl of Pokeweed (supplied by Autoimmun Diagnostika (AID) GmbH, Straßberg, Germany); tested in duplicate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
Borrelia antigens	• 100 µl of *B. burgdorferi* B31 whole- cell lysate (5 µg/ml) (supplied by AID); tested in duplicate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
	• 100 µl of Osp-mix[c] (5 µg/ml) (supplied by AID); tested in duplicate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol

PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; RPMI, Roswell Park Memorial Institute; Osp, outer surface protein
a. This protocol was valid from 2013 till 2018.
b. This protocol was made effective in 2018.
c. This protocol has been used in the current manuscript of Van Gorkom et al. [1].

MATERIALS AND METHODS OF DATA S4

SELECTION OF THE STUDY PARTICIPANTS

For this pilot study we recruited study participants, who represent a biased selection of the study participants described in the current manuscript of Van Gorkom et al. [1] and in the manuscript of Van Gorkom et al. published in 2018 [3]. These newly recruited study participants were a convenience sample of the healthy individuals. They were chosen because they were either working at the Diakonessenhuis Hospital or at the National Institute for Public Health and the Environment (RIVM) and, thus, were easily accessible, and because they previously had had positive ELISpot results, they had a good chance of yielding positive results again. Table S4.2 shows the characteristics of the seven participants included in this pilot study. Three study participants had previously been classified as untreated healthy individuals, and four had previously been classified as treated healthy individuals [1]. All study participants had previously been tested in the in-house *Borrelia* ELISpot assay and had shown elevated SFC counts and four of them had also been subjected to the LymeSpot assay and had tested positive. The remaining three study participants had not been tested in the LymeSpot assay previously, as they were initially recruited before we started the validation of the LymeSpot assay.

Table S4.2 Overview of the selected study participants for this pilot study and the results of previous ELISPOT assays

Study participant	Group	Year of first ELISPOT result	Median SFC count in-house *Borrelia* ELISPOT assay[a] using *B. burgdorferi* B31 whole-cell lysate (50µl)	LymeSpot results[a,b]
1	UHI	2013	16.3	ND
2	"	2013	8.5	ND
3	"	2015	20	POS
4	THI	2013	14	ND
5	"	2015	12.5	POS
6	"	2015	35	POS
7	"	2015	31	POS

UHI, untreated healthy individuals; THI, treated healthy individuals; SFC, spot forming cell; ND, not done; POS, positive

a. A total number of 2.5 x 10^5 peripheral blood mononuclear cells/well was used in the ELISPOT assay.
b. The LymeSpot result was based on a combination of both stimulation indices as described by the manufacturer (Fig. S2) of the current manuscript of Van Gorkom et al. [1].

PBMC ISOLATION AND LYMESPOT PROCEDURE

Table S4.1 summarizes the three protocols used for the isolation of the PBMCs in this pilot study. For each study participant, three lithium heparin tubes were collected. Separation of PBMCs was done by using density gradient centrifugation and for the old and new LymeSpot protocol we used Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). If isolation of PBMCs started within 8 hours after venipuncture, then PBMCs were isolated directly following the protocols described in Table S4.1. If isolation of PMBCs was done between 8 and 32 hours after venipuncture, then a T-Cell Xtend (OxFord Immunotec Ltd., Abingdon, United Kingdom) procedure was performed first, according to the protocol described in the 'Materials and Methods' of the current manuscript of Van Gorkom et al. [1]. The final LymeSpot result, as described in the instruction manual of the LymeSpot assay, was based on a combination of the stimulation indices of both antigens and was only calculated when the assay was valid (Fig. S2, and the 'Materials and Methods' section of the current manuscript of Van Gorkom et al. [1], respectively). To assess the differences between the SFC counts for the various isolation protocols, untreated and treated healthy individuals were analyzed separately, similar to the study population in the current manuscript of Van Gorkom et al. [1]. To determine the possible impact of the use of a

modified PBMC protocol for assessing the diagnostic performance of the LymeSpot assay, we classified final LymeSpot results that needed diagnostic verification as 'positive'. Subsequently, we combined those results with the positive results, as was done for the assessment of the diagnostic performance of the LymeSpot assay in the current manuscript of Van Gorkom et al. [1]

DATA HANDLING AND STATISTICAL ANALYSIS

The non-parametric Cochran's Q test for more than two related samples was used for comparison of the final LymeSpot results between the three protocols. Quantitative, related data comparing more than two groups were analyzed by using the Wilcoxon signed rank test with continuity correction. As all raw *P* values were >0.05, no correction was applied to account for the multiple statistical analyses in this pilot study.

RESULTS OF DATA S4

THE SFC COUNTS OBTAINED BY USING THE THREE PBMC ISOLATION PROTOCOLS

For five (71.4%) of the seven study participants, PBMCs were isolated within three hours of venipuncture, and for one untreated healthy individual (participant 1), and one treated healthy individual (participant 5), the venipuncture was performed on the previous day and therefore a pre incubation step with T-Cell Xtend was needed. Overall, all study participants that were included showed elevated SFC counts in at least one of the three protocols conducted, for at least one of the two *Borrelia* antigens tested (Table S4.3).

Comparison of the mean SFC counts among untreated healthy individuals and among treated healthy individuals did not show a difference for any of the protocols in case *B. burgdorferi* B31 whole-cell lysate was used to stimulate the PBMCs (raw *P* values 0.125 to 0.625) (Table S4.3). Similarly, no differences were found between the mean SFC counts for the three protocols when Osp-mix was used to stimulate the PBMCs (raw *P* values 0.125 to 1.000) (Table S4.3).

For both untreated and treated healthy individuals, more variation was seen between the mean SFC counts obtained by using the in-house *Borrelia* ELISpot protocol than between the mean SFC counts obtained by using the old and the new LymeSpot protocol (Table S4.3). The observed variation seemed to decrease when the mean SFC counts decreased. Comparison of the SFC counts between duplicate measurements also showed variation, which was largest for the in-house *Borrelia* ELISpot protocol when compared to the two LymeSpot protocols. The difference between the two duplicate measurements after stimulation of the PBMCs with *B. burgdorferi* B31 whole-cell lysate varied between 5 and 49 SFCs for the in-house *Borrelia* ELISpot protocol, between 1 and 27 SFCs for the new LymeSpot protocol, and between 0 and 8 SFCs for the old LymeSpot protocol (Table S4.3). The difference between the two duplicate measurements after stimulation of the PBMCs with Osp-mix varied between 0 and 11 SFCs for the in-house *Borrelia* ELISpot protocol, between 0 and 6 SFCs for the new LymeSpot protocol, and between 0 and 5 SFCs for the old LymeSpot protocol (Table S4.3).

Table S4.3. Results of the LymeSpot assay by using the three PBMC isolation protocols used in this pilot study

		Old LymeSpot protocol (protocol 1)			New LymeSpot protocol (protocol 2)			In-house *Borrelia* ELISPOT protocol (protocol 3)			Comparison of the three protocols based on			
		Mean SFC count (duplicate measurements)		Final LymeSpot result[a]	Mean SFC count (duplicate measurements)		Final LymeSpot result[a]	Mean SFC count (duplicate measurements)		Final LymeSpot result[a]	Mean SFC count			Final LymeSpot result[a]
Study participant	Group	*Bb* B31	Osp-mix		*Bb* B31	Osp-mix		*Bb* B31	Osp-mix		Protocols	Raw *P* values: *Bb* B31	Raw *P* values: Osp-mix	Raw *P* value
1[b]	Untreated healthy individuals	15 .0 (8.0-22.0)	ND[c]	DV/POS[c]	1.0 (0.0-2.0)	0.0 (0.0-0.0)	NEG	7.0 (4.0-10.0)	2.5 (1.0-4.0)	NEG	1 vs 2	0.250	1.000	0.223
2		11.5 (7.0-16.0)	2.0 (2.0-2.0)	DV	5.5 (4.0-7.0)	2.0 (0.0-4.0)	NEG	54.5 (43.0-66.0)	31.5 (31.0-32.0)	POS	1 vs 3	0.500	1.000	
3		28.5 (28.0-29.0)	9.0 (6.0-12.0)	POS	12.0 (12.0-12.0)	7.5 (5.0-10.0)	POS	41.5 (17.0-66.0)	6.5 (5.0-8.0)	DV	2 vs 3	0.250	0.500	
4	Treated healthy individuals	40.5 (27.0-54.0)	16.5 (15.0-18.0)	POS	11.5 (11.0-12.0)	6.0 (5.0-7.0)	POS	48.0 (42.0-54.0)	23.5 (18.0-29.0)	POS	1 vs 2	0.625	0.750	0.368
5[b]		6.0 (5.0-7.0)	4.5 (4.0-5.0)	NEG	12.5 (9.0-16.0)	4.0 (2.0-6.0)	DV	26.0 (23.0-29.0)	7.5 (5.0-10.0)	POS	1 vs 3	0.125	0.250	
6		25.5 (25.0-26.0)	ND[c]	DV/POS[c]	17.5 (16.0-19.0)	5.0 (3.0-7.0)	DV	54.5 (52.0-57.0)	10.0 (10.0-10.0)	POS	2 vs 3	0.125	0.125	
7		11.5 (11.0-12.0)	6.0[d]	POS	14.0 (10.0-18)	12.0 (11.0-13.0)	POS	53.0 (42.0-64.0)	27.5 (22.0-33.0)	POS				
Total no. of negative results for the LymeSpot assay				1 out of 7			2 out of 7			1 out of 7				
(no. out of total number of study participants (%))				14.3			28.6			14.3				

SFC, spot forming cell; *Bb* B31, *B. burgdorferi* B31 whole-cell lysate; Osp, outer surface protein, ND, not done; DV, diagnostic verification; POS, positive; NEG, negative Green boxes represent concordant results by at least two protocols. For this comparison, the final LymeSpot results that needed diagnostic verification were classified as 'positive', and were subsequently combined with the positive results, as was done for the assessment of the diagnostic performance of the LymeSpot assay in the current manuscript by Van Gorkom et al. [1].

a. The final result of the LymeSpot assay, as described in the instruction manual of the LymeSpot assay, was based on a combination of the stimulation indices of both antigens and was only calculated when the assay was valid (Fig. S2 and the 'Materials and Methods' of the current manuscript of Van Gorkom et al., respectively [1]).
b. For this study participant, a T-cell Xtend step was performed prior to the PBMC isolation as the venipuncture was performed the previous day.
c. The Osp-mix using the old LymeSpot protocol was not tested due to insufficient numbers of PBMCs and this result was therefore missing. Interpretation of the mean SFC count after stimulation with *B. burgdorferi* B31 whole-cell lysate resulted in a positive outcome, and thus the final result is either diagnostic verification (if the mean SFC count in the Osp-mix stimulated wells would result in a negative outcome), or positive (if the mean SFC count in the Osp-mix stimulated wells would result in a positive outcome).
d. The Osp-mix was tested in the singular due to insufficient amounts of PBMCs.

THE FINAL LYMESPOT RESULTS OBTAINED BY USING THE THREE PBMC ISOLATION PROTOCOLS

The majority (varying from 71.4% to 85.3% for the three protocols) of the study participants had a LymeSpot result that either needed diagnostic verification or was positive (Table S4.3). None of the study participants had a negative LymeSpot result for all three protocols.

Comparison of the final LymeSpot results among untreated healthy individuals did not show a difference between the three protocols in case *B. burgdorferi* B31 whole-cell lysate was used to stimulate the PBMCs (raw *P* value = 0.223) (Table S4.3). Similarly, no differences were found between final LymeSpot results for the three protocols among the treated healthy individuals (raw *P* value = 0.368) (Table S4.3).

The final results of the old LymeSpot assay which was active during the study period described in the current manuscript of Van Gorkom et al. [1], and of the in-house *Borrelia* ELISpot protocol were concordant for five cases; two untreated healthy individuals (participants 2 and 3), and three treated healthy individuals (participants 4, 6, and 7) (Table S4.3). For two cases, one untreated healthy individual (participant 1) and one treated healthy individual (participant 5), the old LymeSpot protocol and the in-house *Borrelia* ELISpot protocol yielded conflicting results (Table S4.3).

Comparison of the final results obtained with the new LymeSpot protocol, which is currently active, and the in-house *Borrelia* ELISpot protocol showed that both protocols were concordant for six cases (Table S4.3). For one untreated healthy individual (participant 2), the results obtained by using the new LymeSpot protocol and the in-house *Borrelia* ELISpot protocol were discordant (Table S4.3).

Interestingly, most discordant results were seen between the final results obtained with the old and the new LymeSpot protocol. For four cases, one untreated healthy individual (participant 3), and three treated healthy individuals (participants 4, 6, and 7), the results were concordant (Table S4.3). For three cases, two untreated healthy individuals (participants 1 and 2), and one treated healthy individual (participant 5), the final LymeSpot results by using the old and new LymeSpot protocol were discordant (Table S4.3).

To summarize, the final LymeSpot results obtained with the in-house *Borrelia* ELISPOT protocol always matched the results obtained with at least one of the two protocols recommended by the manufacturer, and most variation was found between the old and new LymeSpot protocol.

DISCUSSION DATA S4

Variations in any protocol can influence the results of the assay concerned. In this pilot study, we investigated the influence of various PBMC isolation protocols on the performance of the LymeSpot assay, and found that the largest number of discordant LymeSpot results was found between the old and the new LymeSpot protocol. However, no significant differences were found between the SFC counts, nor between the final LymeSpot results, for any of the three evaluated PBMC isolation protocols.

One of the differences between our in-house *Borrelia* ELISpot protocol and the two LymeSpot protocols was the method to separate the PBMCs. The Leucosep tubes we used in the in-house *Borrelia* ELISpot protocol also contain Ficoll-paque PLUS (identical to the separation medium used in the old and new LymeSpot protocols using standard tubes), and in addition, a porous barrier

consisting of polyethylene which prevents the mixing of blood with Ficoll. The results of our experiment showed some differences between our in-house *Borrelia* ELISpot protocol and the two LymeSpot protocols; however, no protocol was superior and most differences were found between the results obtained with the old and the new LymeSpot protocol. Other studies also showed that PBMCs isolated by Leucosep tubes performed equally well using an ELISpot assay compared to PBMCs isolated by using standard tubes using a Ficoll-gradient [4, 5].

Variations in centrifugation time and speed as well as variations in the various washing steps of the PBMCs could also have an effect on the performance of the LymeSpot assay. Interestingly, the manufacturer of the LymeSpot assay implemented a number of changes to their recommended PBMC isolation protocol in 2018, most importantly the speed of the various centrifugation steps. In a personal communication, the manufacturer informed us that the PBMC isolation protocol was adjusted upon request by several laboratories who mentioned low PBMC yields by using the old LymeSpot protocol. Comparison of the PBMC yields in our experiment confirmed that the PBMC yield indeed was lower for the old LymeSpot protocol (mean of 2.9 x 10^6 ± 1.4 PBMCs/ml) than for the new LymeSpot protocol (mean of 4.8 x 10^6 ± 1.7 PBMCs/ml). The PBMC yield using our in-house *Borrelia* ELISpot protocol was comparable with the PBMC yield of the new LymeSpot protocol (mean of 5.0 x 10^6 ± 1.1 PBMCs/ml). Although the PBMC yields were different between the old and new LymeSpot protocol, the amount of PBMCs tested per well in the LymeSpot assays was standardized, and, thus, equal for both protocols. Nevertheless, most differences in the final results of the LymeSpot assay were found between the old and new LymeSpot protocol. Since differences consisted of a negative old LymeSpot result versus a positive new LymeSpot result, and vice versa, we do not believe these differences are caused by the differences in speed of the various centrifugation steps and raises questions with regard to the robustness of the assay. A number of studies [4, 6-10] used centrifugation speeds ranging from 300 to 640 *g* for 7 to 10 minutes for the first wash step, and from 300 to 470 *g* for 7 to 10 minutes for the second wash step underlining that a broad range of centrifugation speeds can be used to wash the PBMCs.

Another factor that could have influenced the diagnostic performance of the LymeSpot assay in the current manuscript of van Gorkom et al. [1] was the amount of PBMCs tested per well. For the in-house *Borrelia* ELISpot protocol we tested 2.5 x 10^5 PBMCs/well. In contrast, for the old and new LymeSpot protocols we tested the recommended 2.0 x 10^5 PBMCs/well, each. The amount of PBMCs and the PBMC isolation procedure as described in our in-house *Borrelia* ELISpot protocol (Table S4.3) is similar to the one used in our laboratory for the T-SPOT.TB test (T-SPOT.TB, Oxford Immunotec Ltd., Abingdon, UK). Our laboratory has extensive experience using the T-SPOT.TB test [6, 8, 11], and annual (external) quality controls for the T-SPOT.TB test are always met (data not shown). As expected, we found higher mean SFC counts when using our in-house *Borrelia* ELISpot protocol, although this was not significant and did not result in a higher percentage of positive LymeSpot results. Interestingly, a higher mean SFC count coincided with an increased variation between the mean SFC counts, and was largest for the in-house *Borrelia* ELISpot protocol. The observed association between a higher variation for higher SFC counts has also been described by Smith et al. [12] who evaluated different ELISpot protocols used for the diagnosis of tuberculosis and concluded that the consequence of this variation is limited when cut-offs are established at low SFC counts.

Another critical factor known to influence the ELISpot performance is the time between the venipuncture and the processing of the blood samples, as the numbers of SFCs can decrease over time [12]. For two study participants in this experiment, blood was drawn one day prior to PBMC isolation. For these blood samples, we used a pre-treatment step with T-cell Xtend. Comparison of the final SFC counts for the three protocols showed that increased numbers of SFCs were found for both study participants for at least one *Borrelia* antigen in at least one of the protocols.

Therefore, we conclude that other factors are more critical and that the use T-Cell Xtend is not detrimental for the performance of the LymeSpot assay, which has also been shown by others using much larger study populations [6, 13, 14].

To conclude, although the current experiment is limited by the number of study participants, the results of this pilot study show that the four deviations from the LymeSpot protocol (the PBMC separation medium used, the various centrifugation steps, the medium used to wash the PBMCs, and the amount of PBMCs) are not the most critical steps in the assessment of the diagnostic performance of the LymeSpot assay. When using three different work-up protocols of the LymeSpot assay to test the blood of seven patients, no statistically significant differences were found in the resulting SFC counts and the final LymeSpot results. Based on these observations, we are confident that the conclusion of our study that the LymeSpot assay cannot be used to diagnose Lyme neuroborreliosis is not influenced by the different PBMC isolation protocols we used.

REFERENCES OF SUPPLEMENTAL MATERIAL

1. van Gorkom T, Voet W, Sankatsing SUC, Nijhuis CDM, Ter Haak E, Kremer K, Thijsen SFT. 2020. Prospective comparison of two enzyme-linked immunosorbent spot assays for the diagnosis of Lyme neuroborreliosis. Clin Exp Immunol 199:337-356. doi: 10.1111/cei.13393.
2. van Gorkom T, Kremer K, Voet W, Notermans DW, Vlaminckx BJM, Sankatsing SUC, Thijsen SFT. 2017. Disagreement between the results from three commercial tests for the detection of *Borrelia*-specific serum antibodies in the Netherlands associated with antibiotic treatment for Lyme borreliosis: a retrospective study. Eur J Clin Microbiol Infect Dis 36:2137-2146. doi: 10.1007/s10096-017-3037-1.
3. van Gorkom T, Sankatsing SUC, Voet W, Ismail DM, Muilwijk RH, Salomons M, Vlaminckx BJM, Bossink AWJ, Notermans DW, Bouwman JJM, Kremer K, Thijsen SFT. 2018. An Enzyme-Linked Immunosorbent Spot Assay Measuring *Borrelia burgdorferi* B31-Specific Interferon Gamma-Secreting T Cells Cannot Discriminate Active Lyme Neuroborreliosis from Past Lyme Borreliosis: a Prospective Study in the Netherlands. J Clin Microbiol 56. doi: 10.1128/JCM.01695-17.
4. Nilsson C, Aboud S, Karlen K, Hejdeman B, Urassa W, Biberfeld G. 2008. Optimal blood mononuclear cell isolation procedures for gamma interferon enzyme-linked immunospot testing of healthy Swedish and Tanzanian subjects. Clin Vaccine Immunol 15:585-9. doi: 10.1128/CVI.00161-07.
5. Afonso G, Scotto M, Renand A, Arvastsson J, Vassilieff D, Cilio CM, Mallone R. 2010. Critical parameters in blood processing for T-cell assays: validation on ELISpot and tetramer platforms. J Immunol Methods 359:28-36. doi: 10.1016/j.jim.2010.05.005.
6. Bouwman JJM, Thijsen SFT, Bossink AW. 2012. Improving the timeframe between blood collection and interferon gamma release assay using T-Cell Xtend. J Infect 64:197-203. doi: 10.1016/j.jinf.2011.10.017.
7. Barabas S, Spindler T, Kiener R, Tonar C, Lugner T, Batzilla J, Bendfeldt H, Rascle A, Asbach B, Wagner R, Deml L. 2017. An optimized IFN-gamma ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. BMC Immunol 18:14. doi: 10.1186/s12865-017-0195-y.
8. Bathoorn E, Limburg A, Bouwman JJ, Bossink AW, Thijsen SF. 2011. Diagnostic potential of an enzyme-linked immunospot assay in tuberculous pericarditis. Clin Vaccine Immunol 18:874-7. doi: 10.1128/CVI.00031-11.
9. Sumonwiriya M, Paris DH, Sunyakumthorn P, Anantatat T, Jenjaroen K, Chumseng S, Im-Erbsin R, Tanganuchitcharnchai A, Jintaworn S, Blacksell SD, Chowdhury FR, Kronsteiner B, Teparrukkul P, Burke RL, Lombardini ED, Richards AL, Mason CJ, Jones JW, Day NPJ, Dunachie SJ. 2017. Strong interferon-gamma mediated cellular immunity to scrub typhus demonstrated using a novel whole cell antigen ELISpot assay in rhesus macaques and humans. PLoS Negl Trop Dis 11:e0005846. doi: 10.1371/journal.pntd.0005846.
10. Wang WZ, Fredrikson S, Sun JB, Link H. 1995. Lyme neuroborreliosis: evidence for persistent up-regulation of *Borrelia burgdorferi*-reactive cells secreting interferon-gamma. Scand J Immunol 42:694-700. doi: 10.1111/j.1365-3083.1995.tb03713.x.
11. Hofland RW, Thijsen SF, van Lindert AS, de Lange WC, van Gorkom T, van der Tweel I, Lammers JJ, Bossink AW. 2017. Positive predictive value of ELISpot in BAL and pleural fluid from patients with suspected pulmonary tuberculosis. Infect Dis (Lond) 49:347-355. doi: 10.1080/23744235.2016.1269190.
12. Smith SG, Joosten SA, Verscheure V, Pathan AA, McShane H, Ottenhoff TH, Dockrell HM, Mascart F. 2009. Identification of major factors influencing ELISpot-based monitoring of cellular responses to antigens from Mycobacterium tuberculosis. PloS one 4:e7972. doi: 10.1371/journal.pone.0007972.

13. Li J, Liu Y, Ma L, Gu L, Wang Q, Xu M, Ma R, Zhang Y, Yang Z, Deng J, Yi X. 2018. The performance of T-cell Xtend reagent in increasing blood storage times for interferon gamma release assays. J Clin Lab Anal 32. doi: 10.1002/jcla.22253.
14. Wang SH, Stew SS, Cyktor J, Carruthers B, Turner J, Restrepo BI. 2012. Validation of increased blood storage times with the T-SPOT.*TB* assay with T-Cell Xtend reagent in individuals with different tuberculosis risk factors. J Clin Microbiol 50:2469-71. doi: 10.1128/JCM.00674-12.