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Original article

Detection of *Borrelia burgdorferi* sensu lato DNA in cerebrospinal fluid samples following pre-enrichment culture

Trine Andreasen Leth [a,b], Anita Nymark [c,d,e,f,g], Fredrikke Christie Knudtzen [c,d,e], Sanne Løkkegaard Larsen [c,f,g], Marianne N. Skov [c,f,g], Thøger Gorm Jensen [c,f,g], Malene Bek-Thomsen [a], Henrik Boye Jensen [b,h], Joppe W. Hovius [i], Sigurdur Skarphédinsson [c,d,e], Jens Kjølseth Møller [a,b], Nanna Skaarup Andersen [a,c,f,g,*]

[a] *Department of Clinical Microbiology, Lillebaelt Hospital – University Hospital of Southern Denmark, Denmark*
[b] *Department of Regional Health Research, Faculty of Health Sciences, University of Southern Denmark, Denmark*
[c] *Clinical Centre for Emerging and Vector-borne Infections, Odense University Hospital, J.B. Winsløwsvej 21. 2., Odense DK-5000, Denmark*
[d] *Department of Infectious Diseases, Odense University Hospital, Odense, Denmark*
[e] *Research Unit of Infectious Diseases, Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark*
[f] *Department of Clinical Microbiology, Odense University Hospital, Odense, Denmark*
[g] *Research Unit for Clinical Microbiology, Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark*
[h] *Department of Neurology, Lillebaelt Hospital – University Hospital of Southern Denmark, Denmark*
[i] *Amsterdam UMC, location AMC, Center for Experimental and Molecular Medicine, Amsterdam Multidisciplinary Lyme Borreliosis Center, University of Amsterdam, Amsterdam, The Netherlands*

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ABSTRACT

Molecular methods for diagnosing Lyme neuroborreliosis (LNB) have shown suboptimal diagnostic sensitivities. The objective of this study was to improve the clinical sensitivity of PCR detection of *Borrelia burgdorferi* sensu lato spirochetes by inoculating cerebrospinal fluid (CSF) from patients suspected of LNB directly into culture medium at the time of lumbar puncture, with this pursuing enrichment of *Borrelia* spirochetes before PCR analysis. Adult patients with symptoms suggestive of LNB were prospectively enrolled at two hospitals in the Region of Southern Denmark. The CSF-culture samples were incubated for at least eight weeks. During this period, culture sample aliquots were analysed for the presence of *Borrelia* DNA by separate PCR protocols in two independent clinical laboratories. The included patients were diagnosed with definite (n=12) or possible (n=2) LNB, and non-LNB (n=171) based on clinical and paraclinical findings. Patients in the LNB and the non-LNB group had a median duration from symptom onset to lumbar puncture of 40 days (IQR [23–90] days) and 120 days (IQR [32–365] days), respectively. Pre-enrichment growth of *Borrelia* spirochetes was accomplished from three patients (21 %) in the LNB group. The positive culture samples were confirmed by both the digital droplet PCR and the real-time PCR methods employed. All CSF samples were PCR negative in the non-LNB group. The results of this study do not support the use of *Borrelia*-specific PCR as a general routine diagnostic tool in adults. Still, they suggest it may prove of additional value in selected patients with a limited time from symptom onset to sample collection.

1. Introduction

Lyme borreliosis (LB) caused by spirochetes of the *Borrelia burgdorferi* sensu lato (*Bb*) complex (Burgdorfer et al., 1982; Steere et al., 1983) is the most common tick-borne disease in Europe. The clinical manifestations vary and depend somewhat on *Bb* genospecies (Stanek et al., 2012; Stanek and Strle, 2018). *Bb* sensu stricto, *B. afzelii* and *B. garinii* usually cause infection of joints, skin, and nervous system, respectively, while *B. valaisiana* and *B. spielmanii* are less frequently described as a cause of human disease (Stanek and Reiter, 2011; Stanek and Strle, 2018). All these genospecies are described in ticks in Denmark (Skarphédinsson et al., 2007; Jensen et al., 2017; Petersen et al., 2019). Lyme neuroborreliosis (LNB), the most severe manifestation of LB, can affect both the peripheral and the central nervous system (CNS), typically with

* Corresponding author.
E-mail address: nanna.skaarup.andersen@rsyd.dk (N.S. Andersen).

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symptoms of painful radiculitis, peripheral nerve palsy and lymphocytic meningitis (Hansen and Lebech, 1992; Mygland et al., 2010; Ogrinc et al., 2016; Knudtzen et al., 2017). The detection of intrathecal production of *Bb*-specific antibodies is a cornerstone in LNB diagnostics (Mygland et al., 2010). However, the *Bb* intrathecal antibody tests (*Bb*-CSF-AI) are suboptimal in the early stages of infection (< 6 weeks), as detectable antibodies have not yet formed (Ljøstad et al., 2007). Furthermore, it cannot distinguish between previous and current infection, rendering it possible for a patient to continue to have a positive test long after ended infection (Hansen and Lebech, 1991). Thus, physicians need supplemental laboratory diagnostics, especially early in the course of the disease. One approach to this is direct detection of *Bb* in CSF polymerase chain reaction (PCR) or culture. This approach is not recommended for routine diagnostics in suspected LNB patients due to a presently unknown diagnostic sensitivity ranging in published studies from 0-100% (Dessau et al., 2018). A limited number of spirochetes in the CSF is the best explanation for the low sensitivity (Krüger and Pulz, 1991; Lebech and Hansen, 1992; Lager et al., 2017; Barstad et al., 2018). However, one study suggests that the diagnostic sensitivity of *Bb*-specific PCR can be increased by increasing the volume of CSF used for analysis (Barstad et al., 2018). Another approach to increase the sensitivity of a *Bb*-specific PCR could be to increase the number of *Bb* spirochetes to a detectable level by pre-enrichment culture of CSF.

This prospective cross-sectional study aims to increase the sensitivity of PCR diagnostics by inoculation of CSF from patients suspected of LNB, directly into *Bb* specific culture medium at the time of lumbar puncture, hereby pursuing pre-enrichment of *Bb* spirochetes prior to *Bb*-specific PCR.

2. Materials

2.1. Study setting and population

This prospective cross-sectional study took place at two different hospitals in the Region of Southern Denmark:

I Lillebaelt Hospital, University Hospital of Southern Denmark (abbreviated SLB). The outpatient clinic at the Department of Neurology enrolled patients during the inclusion period from July 2019 to April 2021. The included patients were referred to the outpatient clinic by general practitioners and other hospital departments when LNB was suspected as a potential differential diagnosis.

II Clinical Centre for Emerging and Vector-borne Infections (CCEVI) at Odense University Hospital (OUH). The outpatient clinic at CCEVI enrolled patients from September 2019 throughout April 2021. The included patients were referred to CCEVI on suspicion of LNB or for second opinion examinations.

The patient inclusion criteria for this study were a) age (≥ 18 years), b) a lumbar puncture performed for *Bb*-CSF-AI analysis on clinical suspicion of LNB, and c) written consent after receiving both oral and written information about the study.

The clinicians enrolling the patients were introduced to how a typical Danish LNB patients presents based on the study by Knudtzen et al. (2017). However, it was the clinician's choice which patients to invite to participate in the study. At time of study inclusion results of CSF cell count, *Bb*-CSF-AI or CSF-CXCL13 were not available to the clinician and the LNB suspicion was therefore based only on symptom presentation and potentially a history of a tick bite or tick risk behaviour.

2.2. Culture protocol

At the time of the lumbar puncture, we inoculated one mL CSF directly into a 10 ml sample tube containing 7 ml in-house modified Kelly-Pettenkofer (MKP) medium acclimated to room temperature (Strle et al., 2006). This culture step will be called pre-enrichment culture throughout the article. The sample was designated P0. The P0 sample was transported at room temperature to the Departments of Clinical Microbiology at SLB or OUH, where it was incubated at 35°C under microaerophilic conditions (Anoxomat II system, Advanced Instruments, MA, USA).

All samples were cultured for at least eight weeks. At week 2, two times 1 mL aliquots were drawn from each culture for PCR examinations, and the culture tube was refilled with 2 mL fresh medium. At week 5, each culture was passaged by inoculating 1 mL sample into a new sample tube containing 7 mL fresh medium (designated P1). The original tube (P0) and the passaged tube (P1) were maintained for an additional three weeks before new aliquots were drawn from both P0 and P1 for new PCR examinations. All culture aliquots for PCR, regardless of the location of patient enrolment, were examined by *Bb*-specific PCR at both SLB and OUH. Furthermore, the SLB culture samples were evaluated visually by dark-field microscopy at week 2 and week 8 of incubation. An overview of the culture protocol is illustrated in Fig. 1.

Production of the in-house modified Kelly-Pettenkofer (MKP) medium and preparation of tubes for sample collection is presented in detail in the supplemental material and supplemental Tables S1 and S2.

2.3. Bb-specific PCR methods

Droplet digital PCR (ddPCR) performed at SLB: A culture aliquot was first centrifuged at 14,000 xg for 20 min, then 900 µL supernatant was removed by manual pipetting technique, and the remaining 100 µL was subjected to DNA extraction using the Maxwell® 16 Cell LEV DNA Purification Kit (Promega) together with the Maxwell® 16 Instrument (Promega) configured to an elution volume of 100 µL. All ddPCR analyses were performed using the QX100 Droplet Digital PCR System from Bio-Rad as described by Leth et al. (2022). Details of the primers and probes, gBlock controls, ddPCR program and a list of ddPCR reagents, analytical sensitivity, utensils and instruments are given in Supplementary, Tables S3-5. The *Bb*-specific ddPCR protocol was based on primer and probe sequences designed by Ornstein and Barbour (Ornstein and Barbour, 2006). Briefly described, reactions were assembled from the *Bb* and IPC primers (900 nM) and probes (300 nM), ddPCR enzyme supermix (Bio-Rad), and 12.5 µL purified sample DNA to a final volume of 50 µL. Subsequently, 22 µL was transferred to each duplicate well on a 96-well plate. The Automated Droplet Generator (Bio-Rad) produced droplets from 20 µL of the prepared reaction. PCR amplification was performed with a Verity PCR cycler (Table S4, Supplementary). Droplets were read on the QX100 Droplet Reader (Bio-Rad), and data were acquired using the QuantaSoft v.1.7.4.0917 software. Droplet counts <15,000 were not accepted, and the samples were re-run. Fluorescence amplitudes from positive and negative PCR controls were used to guide the manual threshold setting for classifying droplets as positives or negatives. Samples were run in replicates. Samples were defined as ddPCR positive if they contained ≥ two positive droplets in a pair of replicates. ddPCR was likewise performed on purified sample DNA directly from the CSF (1 mL) from each included SLB participant, without pre-enrichment culture. The analytical sensitivity was estimated to be approximately 10 *Borrelia* spirochetes per mL sample. Details are available in the supplemental material (Leth et al., 2022).

Real-time (rt)-PCR assay performed at OUH: DNA was extracted from 500 µL cultured aliquots on the MagNA Pure 96 system (Roche) using the "DNA and Viral NA Large Volume" kit (Roche) and eluted in 100 µL. Details of the primers and probes, amplicon sequences, PCR program, analytical sensitivity and a list of utensils and instruments are given in Supplementary, Tables S6-8. In brief, a PAN-*Borrelia* rt-PCR protocol modified from Tsao et al. (2004) was performed to detect any *Bb* species, including the species described in Danish ticks (*B. afzelii, Bb.* sensu stricto*, B. garinii, B. spielmanii, B. valaisiana* and *B. miyamotoi)*, positive samples were subsequently tested by a *Bb*-specific rt-PCR also modified

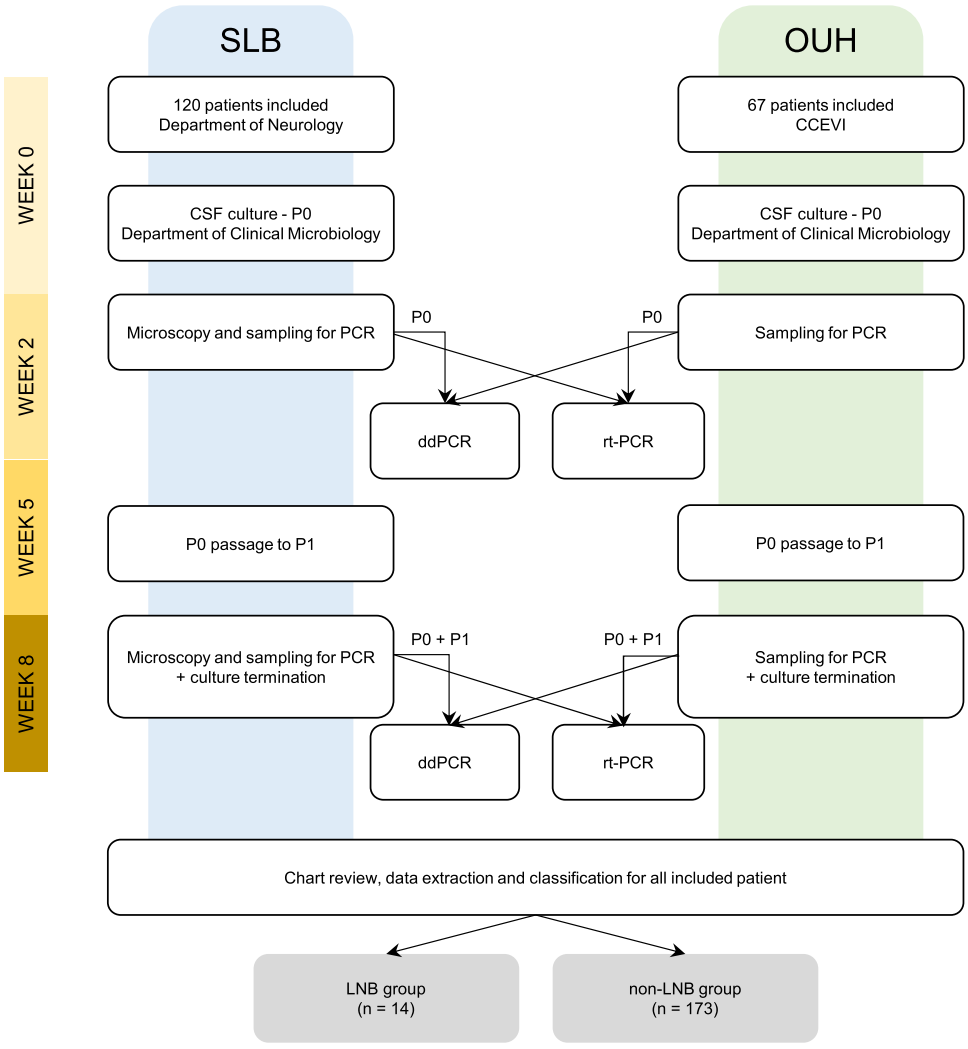

Fig. 1. Illustration showing the design of this study. In total, 187 patients were enrolled at the Department of Neurology at Hospital Lillebaelt (SLB) and Clinical Center for Emerging and Vector-borne Infections (CCEVI) at Odense University Hospital (OUH). Cerebrospinal fluid (CSF) samples were inoculated directly into sample tubes during lumbar puncture (P0). The samples were transported to the clinical microbiology laboratories at SLB and OUH, where they were incubated for at least 8 weeks. The cultivation timeline is shown as yellow blocks on the left side. After 2 weeks of incubation, aliquots were drawn from each culture and examined by droplet digital PCR (ddPCR), real-time PCR (rt-PCR) and dark-field microscopy. After 5 weeks of incubation, the P0 sample was passaged to P1, and both samples were cultured for an additional three weeks. At the end of the incubation period, aliquots were drawn from both P0 and P1 and subjected to droplet digital PCR (ddPCR), real-time PCR (rt-PCR) and dark-field microscopy. Included patients were classified and grouped into the LNB (Lyme neuroborreliosis) group or the non-LNB group based on clinical and paraclinical findings.

from Tsao et al., 2004 and a *B. miyamotoi*-specific rt-PCR modified from Graham et al. 2016. The rt-PCR was performed in a LightCycler® 480 Multiwell Plate 96-well plate format with a reaction volume of 20 µL using LightCycler® 480 Probes Master (Roche) and a primer and probe concentration of 1000 nM and 200 nM, respectively, and 6 µL template. PCR amplification was performed on the LightCycler® 480 Instrument System II (Roche) as previously described (Andersen et al., 2019). PAN-*Borrelia* PCR was likewise performed on an additional uncultured CSF specimen (1 mL) from each included OUH participant. The analytical sensitivity was estimated to be approximately 132 *Borrelia* spirochetes per mL sample. Details are available in the supplemental material.

2.4. Data collection

Laboratory results from CSF analyses included leukocyte cell counts, protein concentration, *Bb*-CSF-AI, CXCL13 concentration, and when available bacterial culture, species-specific PCR for viruses and bacteria, intrathecal production of herpes simplex virus 1 and 2 (HSV) and varicella-zoster virus antibodies. Laboratory results from serum analyses included *Bb* IgM and IgG and when available antibody measurements for other tick-borne pathogens (*Anaplasma phagocytophilum, Bartonella* species (spp.), tick-borne encephalitis virus (TBEV), *Rickettsia* spp.).

Through questionnaires and review of electronic patient charts, clinical data including age, sex, date of symptom onset, clinical symptoms, duration of symptoms, recollection of tick exposure and/or tick bite and/or erythema migrans, antimicrobial treatment before lumbar puncture, and diagnosis code at discharge or follow-up consultations were collected.

All data in this study were collected and managed using the data tools provided by the web-based software platform REDCap (Research Electronic Data Capture) hosted at OPEN, Open Patient data Explorative Network, Odense University Hospital, Region of Southern Denmark (Harris et al., 2009).

2.5. Classification

Following the European Federation of the Neurological Societies (EFNS) guidelines on the diagnosis of LNB (Mygland et al., 2010), patients were classified with definite LNB when there were:

I) Neurological symptoms suggestive of LNB
II) CSF pleocytosis ($\geq 5 \times 10^9$ leukocytes/L CSF)
III) Intrathecal production of *Bb* antibodies

Possible LNB was defined as patients with a clinical discharge diagnosis of LNB but only fulfilling two out of three EFNS criteria. The remaining patients were classified as non-LNB.

2.6. Statistics

Continuous variables describing patient characteristics were

summarised with medians and interquartile ranges (IQRs). Categorical variables were summarised using counts and percentages.

To test the significance between the LNB and the non-LNB groups, Fisher's exact test, Pearson Chi-square and Wilcoxon-Mann-Whitney were performed. A P-value <0.05 was considered statistically significant. All statistical analyses were performed using Stata version 17.0 (StataCorp LLC, USA).

2.7. Ethics statement

This study was approved by the Regional Committees on Health Research Ethics for Southern Denmark (116465-19) and is registered as public research of the Region of Southern Denmark (J.nr. 19/7404) according to the General Data Protection Regulation. The approval included additional sampling of cerebrospinal fluid specimens. All enrolled patients provided written informed consent to participate in the study.

3. Results

3.1. Patient characteristics

One-hundred and eighty-seven patients examined for LNB by *Bb*-CSF-AI consented to participate in this study during the inclusion periods at the two hospitals. There were 61% women included, and the median age was 51 years (IQR 36-63 years) (Table 1). The 187 patients were grouped into a LNB group (n = 14) containing twelve patients with definite LNB and two patients with possible LNB, and a non-LNB group (n = 173) (Fig. 1). Within the SLB cohort 5.8% (7/120) were classified with definite LNB, one patient (0.8%) was classified with possible LNB, while the majority 93.3% (112/120) were classified with non-LNB. From the OUH cohort, 7.5% (5/67) patients had definite LNB, one (1.5%) had possible LNB, while 91% of patients (61/67) were classified as non-LNB. The median duration from patient symptom onset to lumbar puncture was 40 days (IQR [23–90] days) in the LNB group, and 120 days (IQR [32–365] days) in the non-LNB group. CSF leukocyte cell counts, protein concentrations and CXCL13 levels were significantly higher in the LNB group compared to the non-LNB group (P< 0.01) (Table 1).

The minority of patients in our cohort had recollection of tick bite (36.4%) or erythema migrans (17.1 %) (Table 2). Within the LNB group, 21% (3/14) of patients had received antibiotic treatment during the 30 days prior to lumbar puncture. LNB group patients had a significantly higher proportion of radicular pain (92% vs. 58%; P = 0.01), peripheral facial nerve palsy (35% vs. 8%; P < 0.01) and neck stiffness (35% vs. 8%; P < 0.01) when compared to the non-LNB group and were the dominant symptoms in the LNB group. Most patients in both the LNB and the non-LNB groups had more than one symptom at the time of lumbar puncture, 85% (12/14) and 77% (142/187) respectively (P=0.30).

Table 1
Patient characteristics and cerebrospinal fluid laboratory results of the 187 included patients.

	LNB group (n = 14)	Non-LNB group (n = 173)	All patients (n = 187)	*P-value*
Sex, (F (%))	6 (42.9%)	108 (62.4%)	114 (61%)	0.149
Age in years, (median [IQR])	60 [50-67]	50 [36-62]	51 [36-63]	0.070
Duration from symptom onset to lumbar puncture in days, (median[IQR])	40 [23-90]	120 [32-365]	90 [30-361]	0.040
CSF - Leukocyte cell counts (x 10^9/L) (median [IQR])	83 [30-190]	3 [2-5]	4 [2-5]	< .001
CSF - Protein concentration (g/L) (median [IQR])	1.45 [0.57-1.74]	0.38 [0.32-0.49]	0.39 [0.32-0.53]	< .001
CSF - CXCL13 (ng/L) (median [IQR])	212 [54-561.4]	4.8 [2.4-10]	6.55 [2.6-10]	< .001

Sex is presented as F (%). The numeric values are described with medians and interquartile range (IQR) with 25-75th percentiles. Numbers in bold indicate a P-value <0.05 between the LNB and the non-LNB groups. LNB = Lyme neuroborreliosis.

Table 2
Anamnestic findings and clinical symptoms among the 187 patients included in this study.

	LNB (n = 14)	Non-LNB (n = 173)	All patients (n = 187)	*P-value*
Anamnestic				
Recollection of tick bite	1 (7.1%)	67 (38.7%)	68 (36.4 %)	0.02
Recollection of erythema migrans	3 (21.4%)	29 (16.8%)	32 (17.1%)	0.71
Antibiotic treatment in the 30 days prior to lumbar puncture	3 (21.4%)	13 (7.5%)	16 (8.6 %)	0.10
Immunocompromised	0	5 (2.9%)	5 (2.7%)	1.00
Symptoms				
Radicular pain	13 (92.9 %)	101 (58.4 %)	114 (61%)	0.01
Dysesthesia	5 (35.7 %)	30 (17.3 %)	35 (18.7 %)	0.14
Facial nerve palsy	5 (35.7 %)	19 (10.9 %)	24 (12.8 %)	0.02
Central facial nerve palsy	0	5 (2.9 %)	5 (2.7 %)	1.00
Peripheral facial nerve palsy	5 (35.7 %)	14 (8.1 %)	19 (10.2 %)	<0.01
Extremity paresis	5 (35.7 %)	36 (20.8 %)	41 (21.9 %)	0.19
Shoulders/Back	0	3 (1.7 %)	3 (1.6 %)	1.00
Arms/Hands	2 (14.3%)	18 (10.4 %)	20 (10.7 %)	0.65
Legs	3 (21.4%)	15 (8.7 %)	18 (9.6 %)	0.14
Fever	1 (7.1%)	11 (6.4 %)	12 (6.4 %)	1.00
Headache	3 (21.4%)	74 (42.8 %)	77 (41.2 %)	0.16
Neck stiffness	5 (35.7 %)	14 (8.1 %)	19 (10.2 %)	<0.01
Seizures	0	13 (7.5 %)	13 (7.0 %)	0.60
Confusion	1 (7.1%)	18 (10.4 %)	19 (10.2 %)	1.00
Other neuro-cognitive dysfunction	2 (14.3%)	16 (9.2 %)	18 (9.6 %)	1.00
Fatigue	9 (64.3%)	88 (50.9 %)	97 (51.9 %)	0.41
Nightly worsening of pain	4 (28.6%)	12 (6.9 %)	16 (8.6 %)	0.02
Nausea	1 (7.1%)	27 (15.6 %)	28 (15 %)	0.70
Dizziness	1 (7.1%)	61 (35.3 %)	62 (33.2 %)	0.04
Impaired hearing	1 (7.1%)	16 (9.2 %)	17 (9.1 %)	1.00
Impaired vision	2 (14.3%)	38 (22.0 %)	40 (21.4 %)	0.74

Data are presented as No. (%). Numbers in bold indicate a P-value <0.05 between LNB and non-LNB groups. LNB = Lyme neuroborreliosis.

3.2. Bb pre-enrichment and PCR results

Three patients in the LNB group (Patient 1, Patient 3, and Patient 4) were pre-enrichment positive for *Bb* spirochetes, defined as the finding of DNA from *Bb* by PCR in the pre-enrichment culture samples, whereas these three patients were *Bb* PCR negative in CSF samples without pre-enrichment . One patient (Patient 2) in the LNB group was *Bb* PCR positive in the CSF sample only, without pre-enrichment culturing. This patient had received antibiotic treatment in the 30 days prior to lumbar puncture. Patient characteristics and paraclinical results of the *Bb* PCR positive patients are summarised in Table 3.

Table 3
Patient characteristics and laboratory results of the four PCR positive Lyme neuroborreliosis patients.

	Patient 1	Patient 2	Patient 3	Patient 4
Anamnestic				
Age (years)	50	57	71	69
Recollection of tick bite / EM	No / No	No / No	No / No	No / Yes
Duration of symptoms (days) prior to lumbar puncture	26	53	14	23
Month of lumbar puncture	July	June	August	September
Antibiotic treatment in the 30 days prior to lumbar puncture	No	Yes	No	No
Clinical characteristics				
Radicular pain	+	+	+	+
Dysesthesia	-	-	+	+
Facial nerve palsy	-	+	+	-
Peripheral nerve palsy	+	-	-	-
Neck stiffness	+	+	-	+
Fatigue	+	-	-	+
Radicular pain with nightly worsening	-	+	-	+
CSF laboratory characteristics				
CSF – Leukocyte cell count (x10^6/L)	160	30	44	240
CSF – Protein concentration (g/L)	1.9	0.6	0.6	4.4
CSF – CXCL13 (ng/L)	1198.8	14	53	148
Borrelia antibody index – IgM-value	0.4	5.9	12.1	4.5
Borrelia antibody index – IgG-value	1.0	0.1	234.9	76.1
ddPCR result after pre-enrichment culture				
P0 - week 2	5.8 copies / PCR	Negative	1.2 copies / PCR	2.4 copies / PCR
P0 - week 8	1.6 copies / PCR	Negative	Negative	1.8 copies / PCR
P1 - week 8	Negative	Negative	Negative	Negative
rt-PCR result[#] after pre-enrichment culture				
P0 - week 2	ct = 37.7	Negative	ct = 40	ct = 38.1
P0 - week 8	ct = 40.0	Negative	Negative	ct = 36.9
P1 - week 8	Negative	Negative	Negative	Negative
Lyme *Borrelia*	+	Negative	+	+
Borrelia culture result based on microscopy[*¤]	Negative	Negative	Negative	Negative
PCR result directly from CSF sample	Negative	Positive	Negative	Negative
Diagnosis	Definite LNB	Definite LNB	Definite LNB	Definite LNB

[#] Results from the PAN-Borrelia PCR analysis.
[*] The pre-enrichment culture samples were evaluated by dark-field microscopy at week 2 and week 8 of incubation.

EM = erythema migrans. CSF = cerebrospinal fluid. ddPCR = droplet digital PCR. rt-PCR = Real-time PCR. Ct. = cycle threshold. LNB = Lyme neuroborreliosis.

All three pre-enrichment positive patients had a shorter duration (≤ 26 days) from symptom onset to lumbar puncture compared to the other patients in the LNB group (median 40 days). The pre-enrichment positive samples were confirmed both by the *Bb*-ddPCR analysis, the PAN-*Borrelia* rt-PCR and the Lyme-*Borrelia* rt-PCR methods employed. The relatively small number of copies per ddPCR and relatively high cycle threshold values of the RT-PCR results indicate that the number of propagated *Bb* spirochetes were very low. Culture aliquots from pre-enrichment positive samples frozen at -80°C were re-inoculated in a new sample tube with MKP medium in an unsuccessful attempt to re-culture and propagate the *Bb* spirochetes for species identification. Therefore, no attempt was made to sequence the purified *Bb* DNA from the aliquots. In addition, simultaneously collected CSF samples from the three pre-enrichment positive patients were found negative by direct *Bb* PCR, demonstrating the enrichment of *Bb* target DNA by culture in the pre-enrichment positive samples.

None of the pre-enrichment positive patients had received antibiotic treatment before lumbar puncture, whereas 3 of the 11 pre-enrichment negative LNB group patients had received antecedent antibiotic therapy, including Patient 2 who was only *Bb* PCR positive directly in the CSF sample, without pre-enrichment culture.Discussion

In this prospective cross-sectional study, we attempted to detect *Bb* in CSF samples using a cohort of patients examined by *Bb*-CSF-AI on suspicionof LNB. Pre-enrichment of *Bb* spirochetes followed by PCR was successful in 21%) CSF samples from patients in the LNB group, counting only definite LNB cases this increased to 25%, using three independent PCR protocols in two independent clinical laboratories. In contrast, direct PCR on the same CSF samples without pre-enrichment culture were negative. As with other studies in this field, it is possible that, by chance, no *Bb* spirochetes were present in the 1 mL CSF sampled from each patient for the direct PCR, or that they were so few in number that the PCR assays, using only a limited volume of extracted DNA, were not sensitive enough to detect the low spirochete number, hence producing a false negative result. Our results indicate that pre-enrichment culture made it possible to increase the number of *Bb* spirochetes to a detectable level for three of 14 patients in the LNB group. However, in our pre-enrichment positive samples we demonstrated low copy numbers and high cycle threshold in the PCR's, and therefore the same risk of a false negative results, due to a very low spirochete presence could be true even for the remaining 11 pre-enrichment negative samples from patients in the LNB group.

The diagnostic sensitivity of our *Bb*-specific PCR assays combined with the pre-enrichment culturing of CSF samples (21%) is within the range of previous reports (15-50%) examining initiatives to increase the detection of *Bb* DNA in CSF (Lebech et al., 2000; Gooskens et al., 2006; Cerar et al., 2008; de Leeuw et al., 2014; Forselv et al., 2018).

In the LNB group, ten patients were PCR negative by both direct PCR on CSF samples and after pre-enrichment culture, two of these had received antibiotic therapy prior to lumbar puncture. One patient (Patient 2) was only PCR positive directly in the CSF sample, and not after pre-enrichment culture. This patient had also received antibiotics prior to lumbar puncture. We find that the antecedent antibiotic treatment of patients prior to lumbar puncture is a limitation in the pre-enrichment culture of patient samples in this study, as most antibiotics are effective agents against *Bb* growth both in vitro (Veinović et al., 2013) and in vivo (Borg et al., 2005; Mygland et al., 2010). This was shown in a recent study by Ogrinc and co-workers, who isolated *Bb* spirochetes in 5% (2/38) of CSF samples from patients who had received antibiotic treatment before CSF culture sampling, whereas they isolated *Bb* spirochetes from CSF in 12% (7/74) of patients without antibiotic therapy (Ogrinc et al., 2022).

Bb spirochetes are known to be very fastidious, slow-growing, and temperature-dependent and can therefore be difficult to isolate from human CSF specimens (Cerar et al., 2008; Ružić-Sabljić et al., 2014; Veinović et al., 2016). Consequently, we applied a culture protocol including the use of a nutrient-rich MKP culture medium and necessary temperature precautions to increase the possibility of *Bb* growth. *Bb* can be subjected to complement-mediated killing by serum components in the culture medium (Bhide et al., 2005; Ogrinc et al., 2022) however this was prevented using an in-house produced MKP culture medium with heat-inactivated rabbit serum. We furthermore inoculated the CSF specimens directly into sample tubes with pre-warmed (room temperature) medium before sampling and transported the samples at room temperature to avoid inhibition of *Bb* growth due to low temperatures, which had been previously shown to inhibit *Bb* growth (Veinović et al., 2016).

The inability to detect spirochetes in the *Bb* PCR pre-enrichment positive samples by dark-field microscopy could be explained by

interfering CSF immune response components inhibiting *Bb* growth or insufficient adaptation of the *Bb* spirochetes to the artificial MKP culture medium, especially in CSF samples with minimal numbers of spirochetes present. Our PCR results support these theories, by demonstrating low copy numbers and high cycle threshold values in PCR positive culture samples. Furthermore, only culture aliquots drawn after 2 weeks of incubation were PCR positive. We are aware that PCR can detect DNA not only from living but also from dead *Bb* spirochetes and that our results may be an expression of the latter since a decrease in the number of *Bb* targets were observed from culture week 2 to 8 and the fact that we were unable to re-culture the PCR positive cultures (Table 3). It is a limitation that the positive *Bb* PCR findings could not be verified by sequencing. However, given that three separate PCR protocols in two independent clinical laboratories confirmed each positive PCR result, combined with the clinical and paraclinical findings, we are confident that our *Bb* PCR findings represent the actual presence of *Bb* in the three definite LNB cases.

The detection rate of *Bb* may be improved by including a more optimal study population, comprising of children and adults in the very early and acute phase of the disease (Barstad et al., 2018; Ogrinc et al., 2022; Skogman et al., 2021). Our patient cohort only included adult patients, some of which referred for second opinion, and thus with a relatively long period from symptom onset to lumbar puncture. The longer duration of illness, the more disadvantageous it is when attempting to isolate *Bb* due to low levels of spirochetes caused by the immune response fighting the infection (Skogman et al., 2021; Strle et al., 2006; Veinović et al., 2016;). Indeed, a common feature of the PCR positive LNB cases was the shorter duration from symptom onset to lumbar puncture compared to the other enrolled LNB patients. This makes the above mentioned problems of potentially false negative PCR results even more apparent, especially if the time from symptom onset to lumbar puncture is several weeks. A study by Knudtzen et al. in 2017 showed that children with LNB have a significantly shorter period from onset of symptoms to lumbar puncture (10.5 days) compared to adults (21 days) (Knudtzen et al., 2017). Therefore, we believe that a study population including children would be an ideal target population for the detection of *Bb* spirochetes by culture and PCR.

The patients in this cohort study were included on a clinical suspicion of LNB, where *Bb*-CSF-AI were performed, but prior to the results of CSF cell count, CSF-CXCL-13 and *Bb*-CSF-AI. Less than 8 % of the included patient were diagnosed with LNB, most probably due to the relative broad inclusion criterion. In future studies it should be considered to exclude patients where a LNB diagnosis is unlikely based on the EFNS guidelines (Mygland et al., 2010). This way, some of the laborious and expensive laboratory work could be limited.

3.3. Perspectives

This study may serve as a pilot study for future research projects attempting to culture *Bb* spirochetes from CSF in patients suspected of having LNB. Isolation of *Bb* from CSF might prove successful in clinical selected cases, with emphasis on short duration from symptom onset to sample collection. Based on the results of this study, we suggest examining *Bb* pre-enrichment culture samples by PCR after 7 and 14 days of incubation for the detection of *Bb* DNA.

Techniques are now available to detect and characterise uncultured *Bb* spirochetes in ticks using metagenomic next-generation sequencing analysis (Lambert et al., 2019; Dunaj et al., 2020). However, without the isolation of *Bb* from clinical specimens, we will never truly know their biology and pathogenicity to humans. Thus, there is a motivation for further attempts to culture and characterise *Bb* spirochetes from humans.

Conclusion

Our results show that pre-enrichment by culture of *Bb* spirochetes from CSF specimens can improve the detection of *Bb* DNA in a cohort of well-classified LNB patients. The results of this study do not support the use of *Borrelia*-specific PCR as a general routine diagnostic tool in adults, but it may prove of additional value in selected patients with a limited time from symptom onset to sample collection, as often seen in children.

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Data availability

The dataset generated during this study is not publicly available due to the Danish Data Protection Law in accordance with approval by the Danish Data Protection Agency (J.nr. 19/7404). It is available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Trine Andreasen Leth: Conceptualization, Funding acquisition, Data curation, Investigation, Writing – original draft. **Anita Nymark:** Data curation, Writing – original draft. **Fredrikke Christie Knudtzen:** Data curation, Investigation, Writing – original draft. **Sanne Løkkegaard Larsen:** Data curation, Writing – original draft. **Marianne N. Skov:** Conceptualization, Investigation, Writing – original draft. **Thøger Gorm Jensen:** Funding acquisition, Investigation, Writing – original draft. **Malene Bek-Thomsen:** Conceptualization, Funding acquisition, Writing – original draft. **Henrik Boye Jensen:** Data curation, Writing – original draft. **Joppe W. Hovius:** Conceptualization. **Sigurdur Skarphédinsson:** Conceptualization, Funding acquisition, Data curation, Investigation, Writing – original draft, Writing – original draft. **Jens Kjølseth Møller:** Conceptualization, Funding acquisition, Data curation, Investigation, Writing – original draft. **Nanna Skaarup Andersen:** Conceptualization, Funding acquisition, Data curation, Investigation, Writing – original draft.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Supplementary materials

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