Ticks and Tick-borne Diseases 12 (2021) 101759

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Are other tick-borne infections overlooked in patients investigated for Lyme neuroborreliosis? A large retrospective study from South-eastern Sweden

Paula Gyllemark [a,b,*], Peter Wilhelmsson [b,c], Camilla Elm [c], Dieuwertje Hoornstra [d], Joppe W. Hovius [d,i], Marcus Johansson [b,e], Ivar Tjernberg [b,f], Per-Eric Lindgren [b,c,i], Anna J. Henningsson [b,c,g,i,1], Johanna Sjöwall [b,h,1]

[a] Department of Infectious Diseases, Region Jönköping County, Jönköping, Sweden
[b] Department of Biomedical and Clinical Sciences, Division of Inflammation and Infection, Linköping University, Linköping, Sweden
[c] Department of Clinical Microbiology, Region Jönköping County, Jönköping, Sweden
[d] Amsterdam, University Medical Centers location AMC, Center for Experimental and Molecular Medicine, Amsterdam, the Netherlands
[e] Department of Clinical Microbiology, Region Kalmar County, Kalmar, Sweden
[f] Department of Clinical Chemistry and Transfusion Medicine, Region Kalmar County, Kalmar, Sweden
[g] Department of Clinical Microbiology, Region Östergötland County, Linköping, Sweden
[h] Department of Infectious Diseases, Region Östergötland County, Linköping/Norrköping, Sweden
[i] ESCMID Study Group for Lyme Borreliosis – ESGBOR, European Society for Clinical Microbiology and Infectious Diseases, Switzerland

ARTICLE INFO

Keywords:
PCR
Co-infection
B. burgdorferi s.l

ABSTRACT

In Europe, the hard tick *Ixodes ricinus* is considered the most important vector of human zoonotic diseases. Human pathogenic agents spread by *I. ricinus* in Sweden include *Borrelia burgdorferi* sensu lato (s.l.), *Anaplasma phagocytophilum, Rickettsia helvetica,* the recently described *Neoehrlichia mikurensis, Borrelia miyamotoi*, tick-borne encephalitis virus (TBEV), and *Babesia* spp. (*Babesia microti, Babesia venatorum* and *Babesia divergens*). Since these pathogens share the same vector, co-infections with more than one tick-borne pathogen may occur and thus complicate the diagnosis and clinical management of the patient due to possibly altered symptomatology. *Borrelia burgdorferi* s.l., TBEV and *B. miyamotoi* are well-known to cause infections of the central nervous system (CNS), whereas the abilities of other tick-borne pathogens to invade the CNS are largely unknown. The aim of this study was to investigate the presence and clinical impact of tick-borne pathogens other than *B. burgdorferi* s.l. in the cerebrospinal fluid (CSF) and serum samples of patients who were under investigation for Lyme neuroborreliosis (LNB) in a tick-endemic region of South-eastern Sweden. CSF and serum samples from 600 patients, recruited from the Regions of Östergötland County, Jönköping County and Kalmar County in South-eastern Sweden and investigated for LNB during the period of 2009–2013, were retrospectively collected for analysis. The samples were analysed by real-time PCR for the presence of nucleic acid from *B. burgdorferi* s.l., *B. miyamotoi, A. phagocytophilum, Rickettsia* spp., *N. mikurensis,* TBEV and *Babesia* spp. Serological analyses were conducted in CSF and serum samples for all patients regarding *B. burgdorferi* s.l., and for the patients with CSF mononuclear pleocytosis, analyses of antibodies to *B. miyamotoi, A. phagocytophilum*, spotted fever group (SFG) rickettsiae, TBEV and *B. microti* in serum were performed. The medical charts of all the patients with CSF mononuclear pleocytosis and patients with positive PCR findings were reviewed. Of the 600 patients, 55 (9%) presented with CSF mononuclear pleocytosis, 13 (2%) of whom had *Borrelia*-specific antibodies in the CSF. One patient was PCR-positive for *N. mikurensis*, and another one was PCR-positive for *Borrelia* spp. in serum. No pathogens were detected by PCR in the CSF samples. Four patients had serum antibodies to *B. miyamotoi*, four patients to *A. phagocytophilum*, five patients to SFG rickettsiae, and six patients to TBEV. One patient, with antibodies to SFG

Abbreviations: SFG, spotted fever group; CNS, central nervous system; LNB, Lyme neuroborreliosis; TBEV, tick-borne encephalitis virus; HGA, human granulocytic anaplasmosis; CSF, cerebrospinal fluid; NA, nucleic acid; ELISA, enzyme-linked immunosorbent assay; WB, Western blot; WCL, whole cell lysates; GlpQ, glycerophosphodiester phosphodiesterase; BMD, *B. miyamotoi* disease; IFA, indirect immunofluorescence assay; EIA, enzyme immuno-assay; CT, computed tomography; MRI, magnetic resonance imaging; CMV, cytomegalovirus; AI, antibody index; MS, multiple sclerosis; DPNP, demyelinated polyneuropathy; LP, lumbar puncture; EM, erythema migrans.

* Corresponding author at: Department of Infectious Diseases, Ryhov County Hospital, SE-551 85, Jönköping, Sweden.
E-mail address: paula.gyllemark@rjl.se (P. Gyllemark).

[1] Authors contributed equally.

https://doi.org/10.1016/j.ttbdis.2021.101759
Received 5 April 2020; Received in revised form 12 May 2021; Accepted 19 May 2021
Available online 4 June 2021

rickettsiae, had both clinical and laboratory signs suggestive of a current infection. Nine patients had serum antibodies to more than one pathogen, although none of these was assessed as a current co-infection. We can conclude from this study that tick-borne co-infections are uncommon in patients who are being investigated for suspected LNB in South-eastern Sweden, an area endemic for borreliosis and TBE.

1. Introduction

Tick-borne infections are increasing in the Northern hemisphere, and new pathogens causing human disease are being identified (Socolovschi et al., 2009). In Europe, the hard tick *Ixodes ricinus* is the predominant tick species, and several bacterial species spread by *I. ricinus* are known to cause human diseases, including the *Borrelia burgdorferi* sensu lato (s.l.) group of spirochaetes, *Borrelia miyamotoi, Anaplasma phagocytophilum, Neoehrlichia mikurensis*, and several species of the spotted fever group (SFG) rickettsiae (Socolovschi et al., 2009; Wass et al., 2019; Welinder-Olsson et al., 2010). These microorganisms have all been reported with different prevalence in collected ticks in Sweden (Andersson et al., 2013; (Henningsson et al., 2015b); Lindblom et al., 2016; Wilhelmsson et al., 2013). The spread of *B. burgdorferi* s.l. to the central nervous system (CNS) causes Lyme neuroborreliosis (LNB). Meningitis due to *Rickettsia helvetica*, a SFG rickettsiae and the most frequently detected *Rickettsia* species in Swedish ticks, has been described in humans in Sweden (Lindblom et al., 2016; Nilsson et al., 2010; Nilsson et al., 2011). Although rare, infection by *A. phagocytophilum* may cause meningoencephalitis or affect the peripheral nervous system, e.g., brachial plexopathy or an isolated facial palsy (Dumler et al., 2007; Young and Klein, 2007). Since the CNS symptoms of these three infections are similar, they can be difficult to distinguish clinically.

The first case of human disease caused by *N. mikurensis* was reported in 2010 in Sweden (Welinder-Olsson et al., 2010). The organism mainly affects immuno-compromised patients, although immuno-competent individuals may also develop serious complications and experience symptoms of fever and myalgia as well as suffer from thrombo-embolic events (Grankvist et al., 2014; Welinder-Olsson et al., 2010; von Loewenich et al., 2010). The estimated prevalence of *N. mikurensis* in *I. ricinus* in Sweden is 6.0% (Andersson et al., 2013). For this pathogen, dissemination to the CNS has not yet been described in either humans or animals.

Borrelia miyamotoi, which belongs to the relapsing-fever group of *Borrelia*, is spread by different tick species belonging to the genus *Ixodes*, including *I. ricinus*, and it was first described to invade the CNS in humans in 2013 (Gugliotta et al., 2013; Hovius et al., 2013). Apparently, meningoencephalitis mostly affects immuno-compromised individuals, although CNS infection in an immuno-competent patient was recently described. The patients presented with LNB-associated symptoms, such as headache, neck pain and fever (Henningsson et al., 2019). The prevalence of *B. miyamotoi* in ticks in Southern Sweden is approximately 1% (Wilhelmsson et al., 2013).

In addition to bacteria, *I. ricinus* transmits tick-borne encephalitis virus (TBEV) in Europe. TBEV is a neurotrophic virus that may cause meningoencephalitis with serious and sometimes fatal neurological complications. The prevalence of TBEV in engorged ticks, which have bitten humans, in Southern Sweden, is estimated to be 0.2% (Lindblom et al., 2014).

Babesia microti, Babesia venatorum and *Babesia divergens* are tick-transmitted protozoans with a reported prevalence of approximately 3% (*B. microti*), 1% (*B. venatorum*) and 0.2% (*B. divergens*), respectively, in ticks in Southern Sweden (Karlsson and Andersson, 2016). All three species have been described to cause human disease. Babesiosis may be a serious illness, especially in immuno-compromised and splenectomised individuals (Bloch et al., 2019; Krause, 2019). While CNS involvement in cats and dogs has been described, the occurrence of CNS infection with *Babesia* spp. in humans is unknown (Bosman et al., 2013; Welzl et al., 2001).

Apart from *B. burgdorferi* s.l., *B. miyamotoi* and TBEV, the pathogenicity of other tick-borne microorganisms and their potentials to cause CNS infection in humans is poorly understood. Since knowledge of the clinical presentations of these infections is scarce amongst health-care providers in general, investigations of these pathogens are not commonly performed. Thus, these infections, and possible co-infections with multiple tick-borne pathogens, may be overlooked and symptoms may overlap, for which reason is why a broader diagnostic approach should be considered. Furthermore, as immuno-suppressive treatment is becoming more common in the population, co-infections, such as babesiosis, human granulocytic anaplasmosis (HGA) and infections caused by *B. miyamotoi* may pose a greater risk and the clinical picture may differ amongst patients (Sanchez et al., 2016).

Several European studies, including those from Sweden (Bjöersdorff et al., 1999; Lindblom et al., 2013; Lindblom et al., 2016; Wass et al., 2018), have reported serological evidence of co-infections with different tick-borne pathogens in humans (Dunaj et al., 2018; Jahfari et al., 2016; Koetsveld et al., 2016; Moniuszko et al., 2014; Ocias et al., 2018; Panczuk et al., 2016). However, few of these studies have used molecular detection methods and the primary focus has not been on CNS infection.

The aim of the present study was to investigate the presence of tick-borne pathogens other than *B. burgdorferi* s.l. in the cerebrospinal fluid (CSF), using both molecular and serological methods, in patients who have been investigated for LNB.

2. Patients and methods

2.1. Patients

Through laboratory databases, 600 patients were identified at the three accredited laboratories in Linköping, Jönköping and Kalmar, respectively, and they were consecutively chosen from analyses performed on patients under the investigation of LNB during the years 2009–2013. The selection criterion used was that for each patient at least 1 mL of the frozen sample volume of CSF and serum, respectively, was available. White blood cell counts in the CSF and serological analyses regarding *B. burgdorferi* s.l. were performed as part of the initial investigation at the time of sampling. All the patient samples had been stored at -20 °C. Real-time PCR was conducted on all 600 patient samples for the detection of *B. burgdorferi* s.l. (CSF and serum), *A. phagocytophilum* (CSF and serum), *Rickettsia* spp. (CSF and serum), *N. mikurensis* (CSF and serum), *B. miyamotoi* (CSF and serum), TBEV (CSF), and *Babesia* spp. (CSF and serum).

For the 55 patients with CSF mononuclear pleocytosis (>5 × 10^6 mononuclear cells per litre of CSF), the sera were analysed for antibodies to *B. miyamotoi, A. phagocytophilum*, SFG rickettsiae, TBEV, and *B. microti*. Three patient sera were not analysed for antibodies to *B. miyamotoi* due to lack of sera. We did not have access to serological assays for other *Babesia* spp. and a serological assay detecting antibodies to *N. mikurensis* has not yet been developed.

Medical charts were reviewed for all the patients with CSF mononuclear pleocytosis and for the patients in whom *N. mikurensis* and *Borrelia* spp. DNA was detected in the serum by real-time PCR.

2.2. Molecular detection methods

2.2.1. Total nucleic acid extraction and cDNA synthesis from CSF and serum samples

Total nucleic acids (NA) were extracted from 200 μL of CSF and 200

μL of serum using the MagNA Pure LC 2.0 Instrument (Life Science, Roche), and the MagNA Pure LC Total Nucleic Acid Isolation Kit (Life Science, Roche) using a 100-μL elution volume, according to the manufacturer's instructions. Each extraction batch of 32 samples consisted of 30 samples, one positive control, and one negative control. As a positive control, 20 μL of PBS solution (pH 7.2) that contained a known number of *B. afzelii* (strain Lu81) cells (ranging from 1 to 10^5 cells per 20 μl) were spiked into 180 μL CSF (obtained from routine diagnostic section at the Department of Clinical Microbiology, County Hospital, Jönköping, Sweden). The strain of *B. afzelii* used was kindly provided by Professor Sven Bergström, Umeå University, Umeå, Sweden, and cultivated at 35 °C for 8 days in Barbour-Stoenner-Kelly II medium with 6% rabbit serum, as described earlier, and counted using a phase-contrast microscope (Barbour, 1984). RNase-free water was used as a negative control. The NA were reverse-transcribed to cDNA using the illustra™ Ready-to-Go RT-PCR Beads kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Fifteen microlitres of NA and 10 μL pd (N)6 random hexamer primers (0.25 μg/μL) were incubated for 5 min at 97 °C and then mixed with one RT-PCR bead dissolved in 25 μL RNase-free water. The mixture was incubated for 30 min at 42 °C, followed by 5 min at 97 °C, producing 50 μL of cDNA.

All PCR analyses were performed at the Department of Clinical Microbiology, County Hospital, Jönköping, Sweden.

2.2.2. Detection of Borrelia spp. in CSF and serum samples

Detection of *Borrelia* spp. (including *B. miyamotoi*) was performed using a genus-specific TaqMan real-time PCR assay, as described in the supplementary. The primers *Borrelia*_F and *Borrelia*_R, and the probe *Borrelia*_P were designed to target the *Borrelia* spp. *16S* rRNA gene, to amplify a 116-bp long amplicon (Table 1). Positive samples were re-analysed using a *B. miyamotoi* species-specific TaqMan real-time PCR assay, as previously described (Hovius et al., 2013). The primers Bm_F and Bm_R, and the probe Bm_P are designed to target the *B. miyamotoi* flagellin B gene (*flaB*) to amplify a 156-bp long amplicon (Table 1). To determine other *B. burgdorferi* s.l. species of the samples positive in the real-time PCR assay, a nested, conventional PCR assay using primers targeting the intergenic spacer region between *5S* and *23S* rRNA genes, was applied as previously described (Postic et al., 1994; Wilhelmsson et al., 2010). Nucleotide sequencing of the PCR products amplified by the conventional PCR assay to determine species of *Borrelia* was performed by Eurofins GATC Biotech (Konstanz, Germany). All sequences were confirmed by sequencing both strands. The obtained chromatograms were initially edited and analysed using BioEdit Software v7.0 (Tom Hall, Ibis Therapeutics, Carlsbad, CA), and the sequences were examined using Basic Local Alignment Tool.

2.2.3. Detection of Anaplasma phagocytophilum in CSF and serum samples

Detection of *A. phagocytophilum* was performed using a TaqMan real-time PCR assay, as previously described ((Henningsson et al., 2015a). The primers ApF and ApR and the probe ApM are designed to target the *A. phagocytophilum* citrate synthase gene (gltA), to amplify a 64-bp long amplicon (Table 1). As a positive control, a synthetic plasmid containing the target sequence of the TaqMan real-time PCR assay was used. The plasmid contained the target sequence, spanning nucleotides 304–420 of the *A. phagocytophilum gltA* gene (GenBank: AF304137), synthesised and cloned into the pUC57 vector (GenScript Inc., Piscataway, NJ, USA).

2.2.4. Detection of Rickettsia spp. in CSF and serum samples

Detection of *Rickettsia* spp. was performed using a TaqMan real-time PCR assay, as previously described (Stenos et al., 2005). The primers CS-F and CS-R, and probe CS-P are designed to target the *Rickettsia* spp. citrate synthase gene (gltA), to amplify a 74-bp long amplicon (Table 1). As a PCR-positive control, a synthetic plasmid containing the target sequence of the TaqMan real-time PCR assay was used. The plasmid contained the target sequence, spanning nucleotides 1102–1231 of the *R. rickettsii gltA* gene (GenBank: 59,729), synthesised and cloned into the pUC57 vector (GenScript).

2.2.5. Detection of Neoehrlichia mikurensis in CSF and serum samples

Detection of *N. mikurensis* cDNA was performed using a SYBR Green

Table 1
Oligonucleotide primers and probes used for the molecular analysis of tick-borne pathogens.

Organism	Target gene	Primer/Probe name	Nucleotide sequence (5′→3′)	Amplicon length (bp)	Reference
Borrelia spp.	*16S* rRNA	Borrelia_F	GCT GAG TCA CGA AAG CGT AG	116	This study
		Borrelia_R	CAC TTA ACA CGT TAG CTT CGG TA		
		Borrelia_P	FAM-CGC TGT AAA CGA TGC ACA CTT GGT-MGB		
B. miyamotoi	*flaB*	BM_F	AGA AGG TGC TCA AGC AG	156	(Hovius et al., 2013)
		BM_R	TCG ATC TTT GAA AGT GAC ATA T		
		BM_P	FAM-AGC ACA GGA GGG AGT TCA AGC-BHQ1		
A. phagocytophilum	*gltA*	ApF	TTT TGG GCG CTG AAT ACG AT	64	(Henningsson et al., 2015a)
		ApR	TCT CGA GGG AAT GAT CTA ATA ACG T		
		ApM	FAM-TGC CTG AAC AAG TTA TG-BHQ1		
Rickettsia spp.	*gltA*	CS-F	TCG CAA ATG TTC ACG GTA CTT T	74	(Stenos et al., 2005)
		CS-R	TCG TGC ATT TCT TTC CAT TGT G		
		CS-P	FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ1		
N. mikurensis	*16S* rRNA	Neo_16S_F	GTA AAG GGC ATG TAG GCG GTT TAA	107	(Labbe Sandelin et al., 2015)
		Neo_16S_R	TCC ACT ATC CTC TCT CGA TCT CTA GTT TAA		
	groEL	Neo-F	CGG AAA TAA CAA AAG ATG GA	169	(Grankvist et al., 2015)
		Neo-R	ACC TCC TCG ATT ACT TTA G		
		Neo-probe	6FAM-TTG GTG ATG GAA CTA CA-MGB		
TBEV	11,054–11,121[a]	F-TBE 1	GGG CGG TTC TTG TTC TCC	68	(Schwaiger and Cassinotti, 2003)
		R-TBE 1	ACA CAT CAC CTC CTT GTC AGA CT		
		TBE-probe-WT	FAM-TGA GCC ACC ATC ACC CAG ACA CA-BHQ1		
	1329–1416[a]	TBEE-F6	GGC TTG TGA GGC AAA GAA	88	(Gäumann et al., 2010)
		TBEE-R2	TCC CGT GTG TGG TTC GAC TT		
		TBEE-P4	HEX-AAG CCA CAG GAC ATG TGT ACG CC-BHQ1		
Babesia spp.	*18S* rRNA	BJ1	GTC TTG TAA TTG GAA TGA TGG	411–452	(Wilhelmsson et al., 2020)
		BN2	TAG TTT ATG GTT AGG ACT ACG		

[a] Nucleotide fragment of the genome of TBEV strain Neudoerfl (U27495).
Abbreviations: FAM, 6-carboxy-fluorescine; HEX, 6-carboxy-hexachlorofluoriscine; BHQ, Black Hole Quencher; MGB, minor groove binder.

real-time PCR assay, as previously described (Labbe Sandelin et al., 2015). The primers Neo_*16S*_F and Neo_*16S*_R are designed to target the *N. mikurensis 16S* rRNA gene, to amplify a 107-bp long amplicon (Table 1). As a positive control, cDNA samples positive for *N. mikurensis*, as confirmed by sequencing in an earlier study (Labbe Sandelin et al., 2015), were used in each run. Confirmatory PCR (using the GroEL target gene) of one positive serum sample was conducted at the Department of Clinical Microbiology, Sahlgrenska University Hospital, Gothenburg, Sweden, as previously described (Grankvist et al., 2015).

2.2.6. Detection of TBEV in CSF samples

Detection of TBEV was performed using a duplex TaqMan real-time PCR assay, as previously described (Lindblom et al., 2014). The primers and probes are designed to target all three subtypes of TBEV, to amplify a 68-bp and an 88-bp long amplicon, respectively (Table 1).

2.2.7. Detection of Babesia spp. in CSF and serum samples

Detection of *Babesia* spp. was performed using a SYBR Green real-time PCR assay, as previously described (Wilhelmsson et al., 2020). Primers BJ1 and BN2 are designed to target the *Babesia* spp. *18S* rRNA gene, to amplify a 411–452 bp long amplicon depending on the species of *Babesia* (Table 1). As a PCR-positive control, a synthetic plasmid containing the target sequence of the SYBR Green real-time PCR assay was used. The plasmid contained the target sequence, spanning nucleotides 467–955 of the *B. divergens 18S* rRNA gene (GenBank: J439713), synthesized and cloned into the pUC57 vector (GenScript).

2.3. Serological detection methods

2.3.1. Detection of antibodies to Borrelia burgdorferi s.l. in CSF and serum

All 600 patients were previously analysed for *Borrelia*-specific antibodies in paired CSF and serum using the IDEIA Lyme Neuroborreliosis Kit (Oxoid, Hampshire, UK). The serological tests were performed according to the manufacturer's instructions at the Departments of Clinical Microbiology in Linköping, Jönköping, and Kalmar, respectively.

2.3.2. Detection of antibodies to Borrelia miyamotoi in serum

Serum samples from patients with CSF mononuclear pleocytosis (N=52) were analysed for the presence of *B. miyamotoi* IgM and IgG antibodies by previously described two-tier serology, using an enzyme-linked immunosorbent assay (ELISA) followed by confirmatory Western blot assays (WB), based on the recombinant protein Glycerophosphodiester phosphodiesterase (GlpQ) (Boyer et al., 2020; Hoornstra et al., 2018; Wagemakers et al., 2016). GlpQ is able to distinguish relapsing fever *Borrelia* from *B. burgdorferi* s.l. Additional whole cell lysate (WCL) WB analysis was performed using the *B. miyamotoi* strains NL-IR-1 and Izh-4 isolated from a Dutch tick (Kuleshov et al., 2019) and a patient with PCR-confirmed *B. miyamotoi* disease (BMD) in European Russia (Kuleshov et al., 2020), respectively. The amount of antigens used was 500 ng recombinant protein and 1 μg WCL. As IgM and IgG positive controls a pool of sera from 16 PCR-confirmed Russian BMD patients with high α-GlpQ antibody levels were used. The negative controls consisted of 10 serum samples obtained from healthy blood donors from a region in Norway non-endemic for ticks (Hoornstra et al., 2018). The cut-off for an ELISA positive sample was arbitrarily set at four standard deviations above the median read-out of the negative controls (Hoornstra et al., 2018; Krause et al., 2013). The interpretation of the confirmatory WB was assessed as published (Hoornstra et al., 2018; Koetsveld et al., 2018). The analyses were performed at the centre for Experimental and Molecular Medicine, Amsterdam, the Netherlands.

2.3.3. Detection of antibodies to Anaplasma phagocytophilum in serum

Serum samples from patients with CSF mononuclear pleocytosis (N=55) were analysed for the presence of *A. phagocytophilum* IgG antibodies using a commercially available indirect immunofluorescence assay (IFA) (Focus Diagnostics Inc., Cypress, CA, USA), and processed according to the manufacturer's instructions, except that dilutions of 1:80, 1:160, 1:320, etc. were used. The end-point titre was recorded as the reciprocal of the last serial dilution at which specific apple-green fluorescence of *Anaplasma* inclusion bodies focally located in the cytoplasm of the infected cells. IFA titre ≥1:160 was interpreted as positive. This cut-off value was based on the results obtained for local Swedish serum samples that were used when validating the method (Henningsson et al., 2015b). The analyses were performed at the Department of Clinical Microbiology, County Hospital, Jönköping, Sweden.

2.3.4. Detection of antibodies to SFG rickettsiae in serum

Serum samples from patients with CSF mononuclear pleocytosis (N=55) were analysed for the presence of IgM and IgG antibodies to *R. rickettsii*, a member of the SFG rickettsiae, using a commercially available IFA (Focus Diagnostics) and processed according to the manufacturer's instructions. IFA titre ≥1:64 for IgM and ≥1:512 for IgG were interpreted as positive; a higher titre than that set by the manufacturer, in order to achieve higher specificity and reduce the risk of cross-reactivity (Kantso et al., 2009). The analyses were performed at the Statens Serum Institut (SSI), Copenhagen, Denmark.

2.3.5. Detection of antibodies to TBEV in serum

Serum samples from the patients with CSF mononuclear pleocytosis (N=55) were analysed for the presence of IgM and IgG antibodies to TBEV using a commercially available enzyme immuno-assay (EIA) (Enzygnost anti-TBE IgG and IgM; Siemens Healthcare Diagnostics Products GmbH, Erlangen, Germany) according to the manufacturer's instructions. The analyses were performed at the Department of Clinical Microbiology, County Hospital, Jönköping, Sweden.

2.3.6. Detection of antibodies to Babesia microti in serum

Serum samples from patients with CSF mononuclear pleocytosis (N=55), were analysed for the presence of IgM and IgG antibodies to *B. microti* using a commercially available IFA (Focus Diagnostics), according to the manufacturer's instructions. IFA titre ≥1:64 was interpreted as positive. The analyses were performed at the SSI, Copenhagen, Denmark.

3. Results

Of the 600 patients, 341 (57%) were women and 259 (43%) were men, with a median age of 50 years (range, 4–85 years) for the women and 49 years (range, 3–87 years) for the men. Fifty-five (9%) of the patients had CSF mononuclear pleocytosis: 27 men and 28 women, with a median age of 35 years (range, 4–87 years) (Table 2).

Table 2
Characteristics of patients (N=55) with CSF mononuclear pleocytosis.

All patients N=55	
Pleocytosis, median × 10^6 (range × 10^6)[1]	30 (6–3150)
Men, N (%)	27 (49)
Women, N (%)	28 (51)
Median age, years (range)	35 (4–87)
Median duration of symptoms before LP, weeks (range)	1.4 (0.1–104)
Fatigue, N (%)	14 (25)
Fever, N (%)	13 (24)
Nausea, N (%)	14 (25)
Vertigo, N (%)	12 (22)
Concentration difficulties, N (%)	1 (1.8)
Head and/or neck pain, N (%)	33 (60)
Radiculitis, N (%)	10 (18)
Myalgia and/or arthralgia, N (%)	16 (29)
Numbness, N (%)	16 (29)
Cranial nerve palsy, N (%)	15 (27)

[1] Defined as >5 × 10^6 mononuclear cells per litre of CSF. LP, lumbar puncture.

3.1. PCR findings

One female patient (53 years of age) had detectable *N. mikurensis* in the serum by two PCR methods targeting different genes (*16S rRNA* and *groEL*). This patient was splenectomised during childhood due to spherocytosis and consulted health-care due to a couple of hours of headache, vertigo and vomiting. Information regarding tick bites was missing. Computed tomography (CT) and magnetic resonance imaging (MRI) of the brain did not reveal any pathology. The CSF analysis was devoid of pleocytosis. The intrathecal *Borrelia*-specific antibody index (AI) was not elevated. The patient recovered spontaneously without a conclusive diagnosis.

One male patient (45 years of age) had detectable *Borrelia* spp. in serum by real-time PCR targeting the *16S* rRNA gene (Cq-value >40). Based on the sequencing chromatograms of the intergenic spacer region between *5S* and *23S* rRNA genes, it was not possible to distinguish the *Borrelia* species, however the PCR targeting the *B. miyamotoi flaB* was negative. This patient had a peripheral facial palsy together with headache and neck pain for three days. The CSF was without CSF pleocytosis, the intrathecal *Borrelia*-specific AI was not elevated and the chemokine CXCL13 was below cut-off (<250 ng/L, ReaScan CXCL13, Reagena Ltd, Toivala, Finland). A CT of the brain revealed no pathology. He did not receive any antibiotic treatment and the facial palsy persisted to some extent for more than six months.

None of the other patients were PCR-positive concerning *B. burgdorferi* s.l. (CSF and serum), *A. phagocytophilum* (CSF and serum), *Rickettsia* spp. (CSF and serum), *B. miyamotoi* (CSF and serum), TBEV (CSF) or *Babesia* spp. (CSF and serum).

3.2. Serological findings

3.2.1. Patients with CSF mononuclear pleocytosis and elevated intrathecal Borrelia-specific antibody index

Thirteen patients (seven females and six males; median age, 10 years) had both mononuclear pleocytosis and an elevated intrathecal *Borrelia*-specific AI. Eleven patients had symptoms suggestive of LNB and were treated accordingly (Table 2). One patient received the diagnosis of suspected cytomegalovirus (CMV) myelitis with fever, symptoms of cauda equine syndrome and both IgM and IgG antibodies to CMV and one patient was without any conclusive diagnosis. These two patients were not treated with antibiotics and recovered within a few months.

3.2.2. Patients with CSF mononuclear pleocytosis and serum antibodies to Borrelia miyamotoi

Six of the 52 patients with CSF pleocytosis showed IgM reactivity in their serum samples using the GlpQ ELISA. WB could not confirm these results. However, one patient showed IgM reactivity in the WCL WB. Five patients showed IgG reactivity in the GlpQ ELISA, of which none could be confirmed by WB. However, in the additional WCL WB four out of five patients showed reactivity. One of these four patients showed reactivity to both IgM and IgG. The four patients with positive GlpQ ELISA and WCL WB are included in Table 3. The clinical picture of two patients consisted of vertigo and myalgia respectively and they both showed a low-grade mononuclear pleocytosis in the CSF (7×10^6/L and 12×10^6/L). Concomitantly they tested positive for SFG rickettsiae IgM antibodies (titre 512) and TBEV IgG antibodies, respectively. None of these patients were treated with antibiotics and they were diagnosed with multiple sclerosis (MS) and demyelinating polyneuropathy (DPNP), respectively. The third patient underwent a headache investigation without a diagnosis and recovered within a month. The fourth patient, a child of eight years old, excibited a three-day lasting peripheral facial palsy and had an elevated intrathecal *Borrelia*-specific AI. None of the above four patients reported either fever, a low white blood cell count, thrombocytopenia, or elevated liver enzymes (measured in three of four patients).

Table 3
Patients with CSF mononuclear pleocytosis and intrathecal or serum antibodies (N= 32) detected to *B. burgdorferi* s.l., *B. miyamotoi, A. phagocytophilum*, SFG rickettsiae and TBEV, respectively.

–	Ab+ Bbsl. CSF* N=13	Ab+ B. m. serum N=4	Ab+ Anapl. serum N=4	Ab+ Rick. serum N=5	Ab+ TBEV serum N=6
Pleocytosis, median $\times 10^6$ (range $\times 10^6$)[1]	120 (7–470)	13 (7–120)	16 (7–80)	21 (12–44)	96 (7–200)
Men, N (%)	6 (46)	2 (50)	2 (50)	4 (80)	5 (83)
Women, N (%)	7 (54)	2 (50)	2 (50)	1 (20)	1 (17)
Median age, years (range)	10 (4–78)	36 (8–87)	58 (47–72)	49 (16–78)	57 (5–87)
Median duration symptoms before LP, weeks (range)	2 (0.1–78)	1.7 (0.4–4)	4.5 (1–78)	1.4 (0.1–6)	1.5 (0.3–4)
Fatigue, N (%)	6 (46)	1 (25)	1 (25)	2 (40)	3 (50)
Fever, N (%)	6 (46)	0 (0)	1 (25)	1 (20)	2 (33)
Nausea, N (%)	2 (15)	2 (50)	1 (25)	2 (40)	1 (17)
Vertigo, N (%)	0 (0)	1 (25)	2 (50)	2 (40)	1 (17)
Concentration difficulties, N (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Head -and/or neck pain, N (%)	8 (62)	1 (25)	2 (50)	1 (20)	5 (83)
Radiculitis, N (%)	6 (46)	0 (0)	1 (25)	1 (20)	3 (50)
Myalgia and/or arthralgia, N (%)	6 (46)	1 (25)	1 (25)	1 (20)	3 (50)
Numbness, N (%)	2 (15)	0 (0)	2 (50)	0 (0)	2 (33)
Cranial nerve palsy, N (%)	7 (54)	1 (25)	0 (0)	1 (20)	1 (17)

* Increased intrathecal *Borrelia*-specific antibody index. [1]Defined as $>5 \times 10^6$ mononuclear cells per litre of CSF. Ab, antibody; LP, lumbar puncture; Bbsl., *B. burgdorferi* s.l.; B. m., *B. miyamotoi*; Anapl., *A. phagocytophilum*; Rick, spotted fever group (SFG) rickettsiae; TBEV, tick-borne encephalitis virus

3.2.3. Patients with CSF mononuclear pleocytosis and serum antibodies to Anaplasma phagocytophilum

Four patients had a positive IgG titre (1:160) to *A. phagocytophilum* (Table 3). Of these, three patients had antibodies to several tick-borne pathogens (Table 4). One patient had low-grade mononuclear pleocytosis (8×10^6/L) and reported five weeks of nausea and vertigo. However, no explanation for the CSF mononuclear pleocytosis was given in the medical charts and the diagnosis remained unclear. Two patients received a diagnosis of LNB and TBE, respectively, and the third patient underwent a headache investigation but did not receive any conclusive diagnosis. No follow-up serological assays regarding *A. phagocytophilum* were performed.

3.2.4. Patients with CSF mononuclear pleocytosis and serum antibodies to SFG rickettsiae

Five patients had IgM or IgG antibodies to SFG rickettsiae (Table 3). They all had moderate CSF mononuclear pleocytosis (range $12–44 \times 10^6$/L). One patient had detectable IgG antibodies (titre 1:512) to SFG rickettsiae, an elevated intrathecal *Borrelia*-specific AI and was regarded as LNB (Table 4, case 4). The patient was treated with doxycycline and recovered within a couple of months. Two patients with vertigo, who had detectable IgM antibodies (titre 1:512) to SFG rickettsiae, subsequently received the diagnosis of MS and Miller Fisher syndrome, respectively. One patient with SFG rickettsiae IgG antibodies (titre 1:256) (Table 4, case 9) reported headache, neck pain and fatigue, and was later diagnosed with chronic subdural haemorrhage (SDH). One patient with IgG antibodies (titre 1:1024) had low-grade CSF mononuclear pleocytosis (24×10^6/L), seizures, and a low blood platelet count (52×10^6/L), but normal levels of liver enzymes. This patient was without any conclusive diagnosis and was treated with an intravenous cephalosporin with rapid recovery. No follow-up serological assays regarding SFG rickettsiae were performed.

Table 4
Patients with CSF mononuclear pleocytosis and possible co-infections.

Patient age gender	Organisms	AI (CSF) or titer (serum)	Symptoms at hospital admittance	Duration of symptoms before LP (weeks)	Treatment	Diagnosis	Duration of symptoms after treatment
Case 1 47, F	Bbsl. Anapl.	IgM and IgG (CSF) 1:160 IgG	Numbness legs	1	Doxycycline	LNB	<1 month
Case 2 58, M	Bbsl. Anapl.	IgG (CSF) I1:160 IgG	Headache	78	None	None	<4 months
Case 3 8, F	Bbsl. B. m.	IgG (CSF) IgG (serum)	Tiredness, facial palsy	0.4	Doxycycline	LNB	not recorded
Case 4 78, M	Bbsl. Rick.	IgM and IgG (CSF) 1:512 IgG (serum)	Tiredness, nausea, adiculitis, myalgia, arthralgia	6	Doxycycline	LNB	<3 months
Case 5 10, M	Bbsl. TBEV	IgM and IgG (CSF) IgG (weak) (serum)	Neckpain, radiculitis, myalgia, arthralgia	3	Doxycycline	LNB	<2 months
Case 6 30, F	B. m. Rick.	IgG (serum) 1:512 IgM (serum)	Nausea, vertigo	4	None	MS	not recorded
Case 7 87, M	B. m. TBEV	IgG (serum) IgG (serum)	Myalgia, arthralgia	1.6	None	dPNP	not recorded
Case 8 72, M	Anapl. TBEV	1:160 IgG (serum) IgM and IgG (serum)	Headache, tiredness, radiculitis, myalgia, arthralgia, vertigo, numbness	4	None	TBE	not recorded
Case 9 49, M	Rick. TBEV	1:256 IgG (serum) IgG (serum)	Head -and neckpain, tiredness	1.4	None	SDH	years

CSF, cerebrospinal fluid; AI, antibody index; LP, lumbar puncture; F, female; M, male; Bbsl., *B. burgdorferi* sensu lato; Anapl., *A. phagocytophilum*; LNB, Lyme neuroborreliosis; B. m., *B. miyamotoi*; Rick, spotted fever group rickettsiae; TBEV, tick-borne encephalitis virus; MS, multiple sclerosis; dPNP, demyelinating polyneuropathy; SDH, subdural haemorrhage.

3.2.5. Patients with CSF mononuclear pleocytosis and serum antibodies to TBEV

Six patients had antibodies to TBEV (Table 3). Two unvaccinated patients had both IgM- and IgG-antibodies to TBEV, presented with CSF mononuclear pleocytosis of 80 × 10^6 and 112 × 10^6 per litre of CSF, respectively, and had symptoms compatible with TBE. Two patients who were previously vaccinated against TBE had anti-TBE IgG antibodies and low-grade CSF mononuclear pleocytosis (7 × 10^6/L and 21 × 10^6/L, respectively). They later received the diagnoses of DPNP and SDH, respectively. Two patients were regarded as having LNB and were treated accordingly. Of these, one patient was vaccinated. For the other patient information regarding vaccination status was missing.

3.2.6. Patients with CSF mononuclear pleocytosis and serum antibodies to Babesia microti

None of the patients with CSF pleocytosis had detectable serum antibodies to *B. microti*.

3.2.7. Patients with serum antibodies to more than one pathogen

Nine patients had serum antibodies to more than one pathogen (Table 4). Cases 1, 3, 4 and 5 had symptoms compatible with LNB, had CSF mononuclear pleocytosis and an elevated AI to *Borrelia* (IgM and/or IgG), thereby fulfilling the diagnostic criteria for LNB. They received treatment for LNB and had concomitant IgG antibodies to *B. miyamotoi, A. phagocytophilum*, SFG rickettsiae and TBEV, respectively. They all had a short duration of disease prior to the lumbar puncture (LP) (range 0.4–6 weeks) and they recovered within three months following antibiotic (doxycycline) treatment. Case 2 had long-term headache, low-grade CSF mononuclear pleocytosis (7 × 10^6/L), an elevated *Borrelia*-specific AI (IgG) and antibodies to *A. phagocytophilum*. This patient recovered spontaneously within four months. Two patients (cases 6 and 7) had symptoms of vertigo and myalgia/arthralgia, respectively, and had low-grade CSF mononuclear pleocytosis (7 × 10^6/L and 12 × 10^6/L). They both had antibodies to *B. miyamotoi* and concomitant IgM antibodies to SFG rickettsiae (titre 1:512) and IgG antibodies to TBEV, respectively. These patients later received the diagnosis of MS and DPNP. Case 8 who had IgM and IgG antibodies to TBEV, as well as IgG antibodies to *A. phagocytophilum* had symptoms compatible with TBE. One patient who was previously vaccinated against TBE (Case 9) presented with head and neck pain and had IgG antibodies to TBEV, as well as IgG antibodies to SFG rickettsiae (titre 1:256). This patient was a forestry worker who later received the diagnosis of SDH.

4. Discussion

In this large retrospective study, both molecular and serological techniques were used to investigate the presence of *B. burgdorferi* s.l. and other tick-borne pathogens in 600 patients with clinically suspected LNB in a highly *Borrelia*- and TBEV-endemic region of South-eastern Sweden.

Thirteen patients fulfilled the diagnostic criteria for LNB, in accordance with the EFNS guidelines (Mygland et al., 2010). However, *B. burgdorferi* s.l. was not detected in the CSF samples in these patients by PCR. Although PCR is a sensitive detection method on itself, its sensitivity to detect *Borrelia* spp. in clinical CSF samples is low (Mygland et al., 2010). This can possibly be attributed to the spirochetal affinity to myelin and the likely low number of *Borrelia* spirochaetes circulating in the CSF (Cerar et al., 2008; Rupprecht et al., 2008). Additionally the relatively longer period of time between disease onset and CSF sampling may have been a contributing factor to the reduced PCR sensitivity in this study (Barstad et al., 2018). The patients with LNB had a median disease duration of two weeks prior to LP, and only two patients reported a symptom duration of less than one week.

Neoehrlichia mikurensis was detected by real-time PCR in the serum of one patient, who was previously splenectomised. This is in accordance with previous reports, describing an association of this pathogen to immune-suppressive conditions (Grankvist et al., 2014; Höper et al., 2020; Quarsten et al., 2020). In this particular case, however, no immuno-suppressive medication, history of autoimmune disease or haematological malignancy was reported. The finding is interesting, as it potentially implicates an under-reported pathogen. So far, around 65 cases of neoehrlichiosis have been diagnosed in Sweden (A. Grankvist, personal communication, March 2021), of whom 40 patients have been described in a recent study (Höper et al., 2020). *Neoehrlichia mikurensis* has been detected in human blood, plasma and serum samples but so far not in the CSF (Portillo et al., 2018). Our finding highlights the necessity to consider *N. mikurensis* in tick-exposed immuno-compromised individuals in Sweden, since the symptoms may be variable (Grankvist et al., 2014).

In this study, *Borrelia* spp. were detected in one patient by real-time PCR in the serum, but none in the CSF samples. This patient had no CSF

pleocytosis, but had symptoms compatible with LNB with peripheral facial palsy, headache and neck pain. Information regarding any erythema migrans (EM) were missing. The CSF was devoid of pleocytosis and negative regarding *Borrelia* AI, which could be in line with an infection caused by *B. afzelii*. This *Borrelia* spp. has been described to cause a CNS infection without CSF pleocytosis (Strle et al., 2006). Unfortunately, we were not able to sequence the PCR product in order to determine the *Borrelia* species in this particular patient, possible due to a low amount of *Borrelia* bacteria.

We were not able to detect *A. phagocytophilum* by PCR in either CSF or serum samples. In previous studies, *Anaplasma*-specific PCR has generally been performed on whole blood samples (Krause et al., 2002; Lee et al., 2018), and studies performed on serum have shown considerably lower sensitivity (Heo et al., 2002). The duration of symptoms may also be important to take into consideration, since Schotthoefer and co-workers reported a significantly higher sensitivity with PCR from patients with symptom duration of less than a few days (Schotthoefer et al., 2013). Nevertheless, the present study is, to our knowledge, the first to investigate the presence of *A. phagocytophilum* in CSF using molecular detection methods.

Rickettsia spp. were not detected by PCR in either CSF or serum samples. In a recent Danish study, in which CSF samples from patients with suspected LNB were investigated with molecular methods for *Rickettsia* spp. and serological methods for SFG rickettsiae, eight patients were found to be positive for *Rickettsia* spp. by PCR in the CSF, and most of them had symptoms and laboratory findings characteristic of LNB (Ocias et al., 2018). *Rickettsia* spp. DNA was also found in samples from patients without CSF mononuclear pleocytosis but in whom other non-infectious diagnoses were suspected. None of the PCR-positive patients in the Danish study had serum antibodies to SFG rickettsiae. All patients recovered spontaneously without any treatment. Similar findings have also been found in a Dutch study (Koetsveld et al., 2016).

In none of the 600 patients included in our study TBEV could be detected in the CSF by PCR. Only two patients were regarded as having a current TBEV infection according to their symptoms, CSF analysis and serology. We did not perform any PCR analysis of the sera, since the samples were drawn from patients with suspected neurological symptoms and TBEV is usually not detectable in the serum during the meningoencephalitic phase of the disease (Saksida et al., 2005). Interestingly, a few studies including a small number of patients iwith meningoencephalitis have detected TBEV with PCR in tissues other than the CSF, such as sera and urine (Nagy et al., 2018; Saksida et al., 2005; Veje et al., 2018).

It is not known whether *Babesia* spp. can infect the human CNS, and this study was not able to contribute to clarifying this issue, since *Babesia* spp. DNA was not detected in the CNS samples of any of the patients. *Babesia* spp. primarily infects red blood cells, causing mainly a systemic illness, which may be severe or lethal in immuno-suppressed and splenectomised patients (Krause, 2019).

In contrast to direct pathogen detection using molecular methods, the interpretation of serological results may be more challenging due to difficulties in differentiating between an on-going and a previous infection, especially in a tick-endemic region and without the availability of convalescent serum samples for evaluation of serodynamics.

In this study, four of the 52 patients with CSF pleocytosis had IgG, and one of these patients also had IgM antibodies to *B. miyamotoi* in serum, shown through both GlpQ ELISA and WCL WB reactivity. Although the conventional GlpQ WB was not reactive in any of these patients, the WCL WB was suggestive of previous exposure to *B. miyamotoi*. It is unclear why the GlpQ ELISA results could not be confirmed by WB, but it should be mentioned that all *B. miyamotoi* antibody assays are still experimental, including the GlpQ serology. It has been proposed that a GlpQ ELISA followed by GlpQ WB can be used for serodiagnosis of *B. miyamotoi* infection (Krause et al., 2013). A recent paper expressing concerns about the specificity of GlpQ (Reiter et al., 2020) is in stark contrast to many previously published papers (Boyer et al., 2020; Jahfari et al., 2017; Koetsveld et al., 2018; Schwan et al., 1996). Sample selection and technical issues could perhaps explain part of the observed and alledged discrepancy. For the current study we have followed another approach. Since the GlpQ ELISA results could not be confirmed by GlpQ WB, we sought for another second-tier test, i.e. a WCL WB, which actually is more rational since antibodies to other targets than GlpQ are analysed. In our manuscript, we only consider patients who are positive in the GlpQ ELISA as well as reactive in the WCL WB to have specific antibodies to *B. miyamotoi*. However, it should be noted that the use of WCL WB in addition to the GlpQ ELISA needs further evaluation and validation and thus the seroreactivity should be interpreted with caution. Only one of these patients received a diagnosis of tick-borne disease (LNB). Two patients either recovered spontaneously or had a quick recovery after treatment with antibiotics (doxycycline). The other two patients were found to have chronic neurological diseases, MS and DPNP, respectively. The detected IgG antibodies might reflect a previous exposure to *B. miyamotoi*, although an on-going infection at the time of sampling cannot be excluded, especially in the patient showing both IgG and IgM reactivity. This patient, however, did not have any typical symptoms or laboratory results supporting BMD (Hoornstra et al., 2018). It has previously been shown that *B. miyamotoi* can cause disease, including CNS infection, in both immunocompromised and immunocompetent individuals (Henningsson et al., 2019; Hovius et al., 2013; Platonov et al., 2011), and BMD has, as yet only rarely been described to resolve spontaneously without antibiotic treatment (Hoornstra et al., 2018; Sudhindra et al., 2016). In the Netherlands, it has been shown that 2% of healthy blood donors and 10% of forestry workers have antibodies to *B. miyamotoi* (i.e. GlpQ), suggesting that subclinical cases occur. Supporting the data in the Dutch study, it has been reported that 2% of ticks that have bitten humans in Sweden contain *B. miyamotoi* (Wilhelmsson et al., 2013), but so far, no studies of seroprevalence in the Swedish population have been performed.

Four of the patients with CSF pleocytosis had a positive, albeit a low IgG titre to *A. phagocytophilum*, probably reflecting a past exposure. Two patients with both CSF pleocytosis and an elevated *Borrelia* spp. AI were diagnosed with LNB and had serum antibodies to *A. phagocytophilum*. Neither of these patients had a severe disease course. Studies on mice that were simultaneously infected with *B. burgdorferi* s.l. and *A. phagocytophilum* have though showed the opposite (Thomas et al., 2001). One patient was serologically and from a clinical point of view regarded as having TBE and had no laboratory findings suggestive of HGA.

Five patients with CSF pleocytosis had either IgM or IgG antibodies to SFG rickettsiae in serum. While the measured antibody levels probably reflect a previous infection, one patient was considered to have a possible current SFG rickettsiae infection with thrombocytopenia and a high titre of IgG antibodies to SFG rickettsiae (1:1024). Knowledge regarding the kinetics of SFG rickettsiae antibodies is, however, scarce and follow-up serological analysis may be needed to determine whether it is a current or past infection. Interestingly, one patient with IgM antibodies to SFG rickettsiae (titre 1:512) was diagnosed with Miller-Fisher syndrome, a variant of Guillain-Barre syndrome that includes typical neurological symptoms, such as acute ophthalmoplegia, ataxia, and generalized areflexia, and has in fact been described in patients with acute HGA (Bakken et al., 1998). In this case, there was no medical information regarding the typical skin manifestations indicative of SFG rickettsiae infection.

Two patients had typical symptoms and IgM and IgG antibodies consistent with a current TBEV infection. Another four patients had IgG antibodies to TBEV but lacked symptoms compatible with TBE. At least three of them were previously vaccinated against TBE.

We found no serological evidence of current or past infections caused by *B. microti* amongst the study subjects with CSF mononuclear pleocytosis. In a recent study from Southern Sweden, serological responses to *B. microti* or *B. divergens* were seen in 10% of *Borrelia*-seropositive

patients, although this study used a different serological assay and may therefore not be fully comparable to our study (Svensson et al., 2019). In our study we could only analyse IgG antibodies to *B. microti*, an analysis reported to exhibit low cross-reactivity to *B. divergens* and *B. venatorum* (Lempereur et al., 2015).

In the present study, nine patients had antibodies in serum or CSF to more than one of the analysed tick-borne pathogens. While four patients were diagnosed with LNB and one with TBE, none of them had symptoms or laboratory evidence of an on-going co-infection. This is consistent with other studies in Europe, in which patients with LNB or TBE have varying seroprevalence of other tick-borne pathogens but seldom exhibit symptoms compatible with a current co-infection (Koetsveld et al., 2016; Moniuszko et al., 2014; Ocias et al., 2018). Moniuszko et al (2014) investigated with both serological and molecular methods the presence of co-infections with *Borrelia* spp, *A. phagocytophilum* and *Babesia* spp. in patients with TBE and found one patient who was PCR-positive for *B. microti* in blood. PCR analysis was not performed in the CSF. None of the patients had symptoms compatible with a co-infection. Koetsveld et al. (2016) conducted PCR analysis of *R. helvetica* and *R. monacensis* DNA in CSF samples from patients investigated for suspected LNB and identified four patients positive for *R. helvetica* and one for *R. monacensis*, respectively. None had CSF mononuclear pleocytosis or symptoms compatible with an on-going co-infection, and only one of the five patients had antibodies to SFG rickettsiae. So far, co-infection with *B. miyamotoi* has only been described in one case with Lyme borreliosis with concomitant symptoms of high fever, myalgia, arthralgia, the presence of EM and elevated liver enzymes (Oda et al., 2017). Of the three patients with a possible co-infection with *B. miyamotoi*, one had typical symptoms of LNB and the other two were part of a neurological investigation and did not receive any diagnosis of tick-borne infection(s). None of the patients had any signs of an ongoing BMD and the *B. miyamotoi* serology might also reflect a past exposure.

In this study the strengths are the high number of patients included in three different *Borrelia*- and TBE-endemic regions and the use of corresponding paired CSF and serum samples. The retrospective design and lack of convalescent samples are limitations of the study, as is the use of serum rather than whole blood for the molecular detection of the obligatory intracellular pathogens such as *A. phagocytophilum, Rickettsia* spp. and *Babesia* spp. The assessment of whether the infections were previous or current may have been hampered by the retrospective study design, the limited clinical information in some cases, as well as the incomplete laboratory test results.

5. Conclusion

Despite the presence of various pathogens in ticks in South-eastern Sweden, the risk of contracting a tick-borne CNS infection, including co-infections with multiple tick-borne pathogens, appears to be low. Amongst the patients being investigated for LNB, there were few indications of current tick-borne co-infections. However, the results need to be confirmed in a prospective study conducted in a tick-endemic area with a higher number of patients with CSF pleocytosis.

Consent

The study was approved by the Regional Ethical Review Board in Linköping, Sweden (2016/136–31). The patients gave consent for their samples to be stored in a biobank and used for research purposes.

Funding sources

This study was supported by the Medical Research Council of South-eastern Sweden (FORSS), ALF Grants, Region Östergötland, and Futurum – the Academy for Health and Care, Department of Laboratory Medicine, Region Jönköping County, EU Interreg IVA project ScandTick Innovation, and NorthTick, an Interreg project supported by the North Sea Programme of the European Regional Development Fund of the European Union.

Authors contributions

JS, AH and PW planned the study. AH, JS and MJ performed collection of study samples. PW and CE developed and performed the PCR analyses. DH and JH performed serological analysis concerning *B. miyamotoi*. PG, JS, IT and MJ performed the collection of background data from medical charts. All the authors contributed to the manuscript original draft preparations. All the authors have read and approved the final manuscript.

Declaration of Competing Interest

Per-Eric Lindgren has been an external scientific expert to Valneva Austria GmbH, Vienna, Austria. The other authors declare no conflict of interest.

Acknowledgements

The authors thank Borbala Juhasz at the Department of Clinical Microbiology in Linköping for invaluable help with patient samples. We also wish to thank the following people for their excellent and invaluable help with the different laboratory analyses: Malin Lager and Anna Angel at the Department of Clinical Microbiology in Jönköping; Anna Grankvist at the Department of Clinical Microbiology in Gothenburg; Lukas Ocias, Karen Krogfelt and Charlotte Sværke Jørgensen and the laboratory staff at the Department of Virus and Microbiological Special Diagnostics at Statens Serum Institut, Copenhagen; and Henrik Nielsen, Lis Wassmann and Mehmet Kücükyildiz at the Department of Bacteria, Parasites and Fungi at Statens Serum Institut, Copenhagen. Professor Chris Anderson, for valuable help with language review.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2021.101759.

References

Andersson, M., Bartkova, S., Lindestad, O., Raberg, L., 2013. Co-infection with "*Candidatus* Neoehrlichia Mikurensis" and *Borrelia afzelii* in *Ixodes ricinus* ticks in Southern Sweden. Vector Borne Zoonotic Dis. 13, 438–442. https://doi.org/10.1089/vbz.2012.1118.

Bakken, J.S., Erlemeyer, S.A., Kanoff, R.J., Silvestrini, T.C., Goodwin, D.D., Dumler, J.S., 1998. Demyelinating polyneuropathy associated with human granulocytic ehrlichiosis. Clin. Infect. Dis. 27, 1323–1324.

Barbour, A.G., 1984. Isolation and cultivation of Lyme disease spirochaetes. Yale J. Biol. Med. 57, 521–525.

Barstad, B., Quarsten, H., Tveitnes, D., Noraas, S., Ask, I.S., Saeed, M., Bosse, F., Vigemyr, G., Huber, I., Oymar, K., 2018. Direct molecular detection and genotyping of *Borrelia burgdorferi* sensu lato in cerebrospinal fluid of children with Lyme neuroborreliosis. J. Clin. Microbiol. 56 https://doi.org/10.1128/jcm.01868-17.

Björsdorff, A., Brouqui, P., Eliasson, I., Massung, R.F., Wittesjö, B., Berglund, J., 1999. Serological evidence of *Ehrlichia* infection in Swedish Lyme borreliosis patients. Scand. J. Infect. Dis. 31, 51–55. https://doi.org/10.1080/00365549950161880.

Bloch, E.M., Kumar, S., Krause, P.J., 2019. Persistence of *Babesia microti* infection in humans. Pathogens 8, 102. https://doi.org/10.3390/pathogens8030102.

Bosman, A.M., Oosthuizen, M.C., Venter, E.H., Steyl, J.C., Gous, T.A., Penzhorn, B.L., 2013. *Babesia lengau* associated with cerebral and haemolytic babesiosis in two domestic cats. Parasit. Vectors. 6, 128. https://doi.org/10.1186/1756-3305-6-128.

Boyer, P.H., Koetsveld, J., Zilliox, L., Sprong, H., Talagrand-Reboul, É., Hansmann, Y., de Martino, S.J., Boulanger, N., Hovius, J.W., Jaulhac, B., 2020. Assessment of *Borrelia miyamotoi* in febrile patients and ticks in Alsace, an endemic area for Lyme borreliosis in France. Parasit. Vectors 13, 199. https://doi.org/10.1186/s13071-020-04071-9.

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, 3375–3379. https://doi.org/10.1128/jcm.00410-08.

Dumler, J.S., Madigan, J.E., Pusterla, N., Bakken, J.S., 2007. Ehrlichioses in humans: epidemiology, clinical presentation, diagnosis, and treatment. Clin. Infect. Dis. 45 https://doi.org/10.1086/518146. S45-S51, Suppl.

Dunaj, J., Moniuszko-Malinowska, A., Swiecicka, I., Andersson, M., Czupryna, P., Rutkowski, K., Zambrowski, G., Zajkowska, J., Grygorczuk, S., Kondrusik, M., Swierzbinska, R., Pancewicz, S., 2018. Tick-borne infections and co-infections in patients with non-specific symptoms in Poland. Adv. Med. Sci. 63, 167–172. https://doi.org/10.1016/j.advms.2017.09.004.

Gäumann, R., Muhlemann, K., Strasser, M., Beuret, C., 2010. High-throughput procedure for tick surveys of tick-borne encephalitis virus and its application in a national surveillance study in Switzerland. Appl. Environ. Microbiol. 76, 4241–4249. https://doi.org/10.1128/AEM.00391-10.

Grankvist, A., Andersson, P.O., Mattsson, M., Sender, M., Vaht, K., Höper, L., Sakiniene, E., Trysberg, E., Stenson, M., Fehr, J., Pekova, S., Bogdan, C., Bloemberg, G., Wennerås, C., 2014. Infections with the tick-borne bacterium "*Candidatus* Neoehrlichia mikurensis" mimic noninfectious conditions in patients with B cell malignancies or autoimmune diseases. Clin. Infect. Dis. 58, 1716–1722. https://doi.org/10.1093/cid/ciu189.

Grankvist, A., Sandelin, L.L., Andersson, J., Fryland, L., Wilhelmsson, P., Lindgren, P.E., Forsberg, P., Wennerås, C., 2015. Infections with *Candidatus* Neoehrlichia mikurensis and cytokine responses in 2 persons bitten by ticks. Sweden. Emerg. Infect. Dis. 21, 1462–1465. https://doi.org/10.3201/eid2108.150060.

Gugliotta, J.L., Goethert, H.K., Berardi, V.P., Telford 3rd, S.R., 2013. Meningoencephalitis from *Borrelia miyamotoi* in an immunocompromised patient. N. Engl. J. Med. 368, 240–245. https://doi.org/10.1056/NEJMoa1209039.

Henningsson, A.J., Asgeirsson, H., Hammas, B., Karlsson, E., Parke, A., Hoornstra, D., Wilhelmsson, P., Hovius, J.W., 2019. Two cases of *Borrelia miyamotoi* meningitis, Sweden, 2018. Emerg. Infect. Dis. 25, 1965–1968. https://doi.org/10.3201/eid2510.190416.

Henningsson, A.J., Hvidsten, D., Kristiansen, B.E., Matussek, A., Stuen, S., Jenkins, A., 2015a. Detection of Anaplasma phagocytophilum in Ixodes ricinus ticks from Norway using a realtime PCR assay targeting the Anaplasma citrate synthase gene gltA. BMC Microbiol. 15, 153. https://doi.org/10.1186/s12866-015-0486-5.

Henningsson, A.J., Wilhelmsson, P., Gyllemark, P., Kozak, M., Matussek, A., Nyman, D., Ekerfelt, C., Lindgren, P.E., Forsberg, P., 2015b. Low risk of seroconversion or clinical disease in humans after a bite by an *Anaplasma phagocytophilum*-infected tick. Ticks Tick. Dis. 6, 787–792. https://doi.org/10.1016/j.ttbdis.2015.07.005.

Heo, E.J., Park, J.H., Koo, J.R., Park, M.S., Park, M.Y., Dumler, J.S., Chae, J.S., 2002. Serologic and molecular detection of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila* (human granulocytic ehrlichiosis agent) in Korean patients. J. Clin. Microbiol. 40, 3082–3085. https://doi.org/10.1128/jcm.40.8.3082-3085.2002.

Hoornstra, D., Koetsveld, J., Sprong, H., Platonov, A.E., Hovius, J.W., 2018. *Borrelia miyamotoi* disease in an immunocompetent patient, Western Europe. Emerg. Infect. Dis. 24, 1770–1772. https://doi.org/10.3201/eid2409.180806.

Hovius, J.W., de Wever, B., Sohne, M., Brouwer, M.C., Coumou, J., Wagemakers, A., Oei, A., Knol, H., Narasimhan, S., Hodiamont, C.J., Jahfari, S., Pals, S.T., Horlings, H. M., Fikrig, E., Sprong, H., van Oers, M.H., 2013. A case of meningoencephalitis by the relapsing fever spirochaete *Borrelia miyamotoi* in Europe. Lancet 382, 658. https://doi.org/10.1016/s0140-6736(13)61644-x.

Höper, L., Skoog, E., Stenson, M., Grankvist, A., Wass, L., Olsen, B., Nilsson, K., Martensson, A., Söderlind, J., Sakinis, A., Wennerås, C., 2020. Vasculitis due to *Candidatus* Neoehrlichia mikurensis: a cohort study of 40 Swedish patients. Clin. Infect. Dis. https://doi.org/10.1093/cid/ciaa1217.

Jahfari, S., Hofhuis, A., Fonville, M., van der Giessen, J., van Pelt, W., Sprong, H., 2016. Molecular detection of tick-borne pathogens in humans with tick bites and erythema migrans, in the Netherlands. PLoS Negl. Trop. Dis. 10, e0005042 https://doi.org/10.1371/journal.pntd.0005042.

Jahfari, S., Sarksyan, D.S., Kolyasnikova, N.M., Hovius, J.W., Sprong, H., Platonov, A.E., 2017. Evaluation of a serological test for the diagnosis of *Borrelia miyamotoi* disease in Europe. J. Microbiol. Methods 136, 11–16. https://doi.org/10.1016/j.mimet.2017.02.013.

Kantso, B., Svendsen, C.B., Jørgensen, C.S., Krogfelt, K.A., 2009. Evaluation of serological tests for the diagnosis of rickettsiosis in Denmark. J. Microbiol. Methods 76, 285–288. https://doi.org/10.1016/j.mimet.2008.12.012.

Karlsson, M.E., Andersson, M.O., 2016. *Babesia* species in questing *Ixodes ricinus*, Sweden. Ticks Tick. Dis. 7, 10–12. https://doi.org/10.1016/j.ttbdis.2015.07.016.

Koetsveld, J., Kolyasnikova, N.M., Wagemakers, A., Stukolova, O.A., Hoornstra, D., Sarksyan, D.S., Toporkova, M.G., Henningsson, A.J., Hvidsten, D., Ang, W., Dessau, R., Platonov, A.E., Hovius, J.W., 2018. Serodiagnosis of *Borrelia miyamotoi* disease by measuring antibodies against GlpQ and variable major proteins. Clin. Microbiol. Infect. 24, 1331–1338. https://doi.org/10.1016/j.cmi.2018.03.009.

Koetsveld, J., Tijsse-Klasen, E., Herremans, T., Hovius, J.W., Sprong, H., 2016. Serological and molecular evidence for spotted fever group *Rickettsia* and *Borrelia burgdorferi* sensu lato co-infections in the Netherlands. Ticks Tick. Dis. 7, 371–377. https://doi.org/10.1016/j.ttbdis.2015.12.010.

Krause, P.J., 2019. Human babesiosis. Int. J. Parasitol. 49, 165–174. https://doi.org/10.1016/j.ijpara.2018.11.007.

Krause, P.J., McKay, K., Thompson, C.A., Sikand, V.K., Lentz, R., Lepore, T., Closter, L., Christianson, D., Telford, S.R., Persing, D., Radolf, J.D., Spielman, A., 2002. Disease-specific diagnosis of coinfecting tickborne zoonoses: babesiosis, human granulocytic ehrlichiosis, and Lyme disease. Clin. Infect. Dis. 34, 1184–1191. https://doi.org/10.1086/339813.

Krause, P.J., Narasimhan, S., Wormser, G.P., Rollend, L., Fikrig, E., Lepore, T., Barbour, A., Fish, D., 2013. Human *Borrelia miyamotoi* infection in the United States. N. Engl. J. Med. 368, 291–293. https://doi.org/10.1056/NEJMc1215469.

Kuleshov, K.V., Hoornstra, D., Sprong, H., Platonov, A.E., Hovius, J.W., 2019. Draft whole-genome sequences of two Western European *Borrelia miyamotoi* isolates. Microbiol. Resour. Announc. 8, e01314-19 https://doi.org/10.1128/mra.01314-19 e01314-19.

Kuleshov, K.V., Margos, G., Fingerle, V., Koetsveld, J., Goptar, I.A., Markelov, M.L., Kolyasnikova, N.M., Sarksyan, D.S., Kirdyashkina, N.P., Shipulin, G.A., Hovius, J.W., Platonov, A.E., 2020. Whole genome sequencing of *Borrelia miyamotoi* isolate Izh-4: reference for a complex bacterial genome. BMC Genomics 21, 16. https://doi.org/10.1186/s12864-019-6388-4.

Labbe Sandelin, L., Tolf, C., Larsson, S., Wilhelmsson, P., Salaneck, E., Jaenson, T.G., Lindgren, P.E., Olsen, B., Waldenström, J., 2015. *Candidatus* Neoehrlichia mikurensis in ticks from migrating birds in Sweden. PLoS One 10, e0133250. https://doi.org/10.1371/journal.pone.0133250.

Lee, S.H., Park, S., Lee, Y.S., Lee, H.K., Hwang, S.D., 2018. Diagnosis and molecular characteristics of human infections caused by *Anaplasma phagocytophilum* in South Korea. J. Microbiol. 56, 847–853. https://doi.org/10.1007/s12275-018-8385-8.

Lempereur, L., Shiels, B., Heyman, P., Moreau, E., Saegerman, C., Losson, B., Malandrin, L., 2015. A retrospective serological survey on human babesiosis in Belgium. Clin. Microbiol. Infect. 21, 91–97. https://doi.org/10.1016/j.cmi.2014.07.004.

Lindblom, A., Wallmenius, K., Nordberg, M., Forsberg, P., Eliasson, I., Påhlson, C., Nilsson, K., 2013. Seroreactivity for spotted fever rickettsiae and co-infections with other tick-borne agents among habitants in Central and Southern Sweden. Eur. J. Clin. Microbiol. Infect. Dis. 32, 317–323. https://doi.org/10.1007/s10096-012-1742-3.

Lindblom, A., Wallmenius, K., Sjöwall, J., Fryland, L., Wilhelmsson, P., Lindgren, P.E., Forsberg, P., Nilsson, K., 2016. Prevalence of *Rickettsia* spp. in ticks and serological and clinical outcomes in tick-bitten individuals in Sweden and on the Åland Islands. PLoS One 11, e0166653. https://doi.org/10.1371/journal.pone.0166653.

Lindblom, P., Wilhelmsson, P., Fryland, L., Sjöwall, J., Haglund, M., Matussek, A., Ernerudh, J., Vene, S., Nyman, D., Andreassen, A., Forsberg, P., Lindgren, P.E., 2014. Tick-borne encephalitis virus in ticks detached from humans and follow-up of serological and clinical response. Ticks Tick. Dis. 5, 21–28. https://doi.org/10.1016/j.ttbdis.2013.07.009.

Moniuszko, A., Dunaj, J., Swiecicka, I., Zambrowski, G., Chmielewska-Badora, J., Zukiewicz-Sobczak, W., Zajkowska, J., Czupryna, P., Kondrusik, M., Grygorczuk, S., Swierzbinska, R., Pancewicz, S., 2014. Co-infections with *Borrelia* species, *Anaplasma phagocytophilum* and *Babesia* spp. in patients with tick-borne encephalitis. Eur. J. Clin. Microbiol. Infect. Dis. 33, 1835–1841. https://doi.org/10.1007/s10096-014-2134-7.

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, 8–16. https://doi.org/10.1111/j.1468-1331.2009.02862.x.

Nagy, A., Nagy, O., Tarcsai, K., Farkas, A., Takacs, M., 2018. First detection of tick-borne encephalitis virus RNA in clinical specimens of acutely ill patients in Hungary. Ticks Tick. Dis. 9, 485–489. https://doi.org/10.1016/j.ttbdis.2017.12.017.

Nilsson, K., Elfving, K., Påhlson, C., 2010. *Rickettsia helvetica* in patient with meningitis, Sweden, 2006. Emerg. Infect. Dis. 16, 490–492. https://doi.org/10.3201/eid1603.090184.

Nilsson, K., Wallmenius, K., Påhlson, C., 2011. Coinfection with *Rickettsia helvetica* and Herpes simplex virus 2 in a young woman with meningoencephalitis. Case Rep. Infect. Dis. 2011, 469194 https://doi.org/10.1155/2011/469194.

Ocias, L.F., Dessau, R.B., Lebech, A.M., Jørgensen, C.S., Petersen, R.F., Krogfelt, K.A., 2018. Evidence of rickettsiae in Danish patients tested for Lyme neuroborreliosis: a retrospective study of archival samples. BMC Infect. Dis. 18, 325. https://doi.org/10.1186/s12879-018-3210-x.

Oda, R., Kutsuna, S., Sekikawa, Y., Hongo, I., Sato, K., Ohnishi, M., Kawabata, H., 2017. The first case of imported *Borrelia miyamotoi* disease concurrent with Lyme disease. J. Infect. Chemother. 23, 333–335. https://doi.org/10.1016/j.jiac.2016.12.015.

Panczuk, A., Tokarska-Rodak, M., Koziol-Montewka, M., Plewik, D., 2016. The incidence of *Borrelia burgdorferi, Anaplasma phagocytophilum* and *Babesia microti* coinfections among foresters and farmers in Eastern Poland. J. Vector Borne Dis. 53, 348–354.

Platonov, A.E., Karan, L.S., Kolyasnikova, N.M., Makhneva, N.A., Toporkova, M.G., Maleev, V.V., Fish, D., Krause, P.J., 2011. Humans infected with relapsing fever spirochaete *Borrelia miyamotoi*. Russia. Emerg. Infect. Dis. 17, 1816–1823. https://doi.org/10.3201/eid1710.101474.

Portillo, A., Santibanez, P., Palomar, A.M., Santibanez, S., Oteo, J.A., 2018. "*Candidatus* Neoehrlichia mikurensis" in Europe. New Microbes New Infect. 22, 30–36. https://doi.org/10.1016/j.nmni.2017.12.011.

Postic, D., Assous, M.V., Grimont, P.A., Baranton, G., 1994. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of rrf (5S)-rrl (23S) intergenic spacer amplicons. Int. J. Syst. Bacteriol. 44, 743–752. https://doi.org/10.1099/00207713-44-4-743.

Quarsten, H., Salte, T., Lorentzen, A.R., Hansen, I.J.W., Hamre, R., Forselv, K.J.N., Øines, Ø., Wennerås, C., Noraas, S., 2020. Tick-borne pathogens detected in the blood of immunosuppressed norwegian patients living in a tick-endemic area. Clin. Infect. Dis. https://doi.org/10.1093/cid/ciaa971.

Reiter, M., Stelzer, T., Schötta, A.M., Markowicz, M., Leschnik, M., Harsch, A., Reiß, E., Kneusel, R.E., Stockinger, H., Stanek, G., 2020. Glycerophosphodiester phosphodiesterase identified as non-reliable serological marker for *Borrelia miyamotoi* disease. Microorganisms 8, 1846. https://doi.org/10.3390/microorganisms8121846.

Rupprecht, T.A., Koedel, U., Fingerle, V., Pfister, H.W., 2008. The pathogenesis of Lyme neuroborreliosis: from infection to inflammation. Mol. Med. 14, 205–212. https://doi.org/10.2119/2007-00091.

Saksida, A., Duh, D., Lotric-Furlan, S., Strle, F., Petrovec, M., Avsic-Zupanc, T., 2005. The importance of tick-borne encephalitis virus RNA detection for early differential diagnosis of tick-borne encephalitis. J. Clin. Virol. 33, 331–335. https://doi.org/10.1016/j.jcv.2004.07.014.

Sanchez, E., Vannier, E., Wormser, G.P., Hu, L.T., 2016. Diagnosis, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: a review. Jama 315, 1767–1777. https://doi.org/10.1001/jama.2016.2884.

Schotthoefer, A.M., Meece, J.K., Ivacic, L.C., Bertz, P.D., Zhang, K., Weiler, T., Uphoff, T. S., Fritsche, T.R., 2013. Comparison of a real-time PCR method with serology and blood smear analysis for diagnosis of human anaplasmosis: importance of infection time course for optimal test utilization. J. Clin. Microbiol. 51, 2147–2153. https://doi.org/10.1128/jcm.00347-13.

Schwaiger, M., Cassinotti, P., 2003. Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick borne encephalitis virus (TBEV) RNA. J. Clin. Virol. 27, 136–145. https://doi.org/10.1016/s1386-6532(02)00168-3.

Schwan, T.G., Schrumpf, M.E., Hinnebusch, B.J., Anderson Jr., D.E., Konkel, M.E., 1996. GlpQ: an antigen for serological discrimination between relapsing fever and Lyme borreliosis. J. Clin. Microbiol. 34, 2483–2492. https://doi.org/10.1128/jcm.34.10.2483-2492.1996.

Socolovschi, C., Mediannikov, O., Raoult, D., Parola, P., 2009. Update on tick-borne bacterial diseases in Europe. Parasite 16, 259–273. http://www.ncbi.nlm.nih.gov/pubmed/20092057.

Stenos, J., Graves, S.R., Unsworth, N.B., 2005. A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group *Rickettsiae*. Am. J. Trop. Med. Hyg. 73, 1083–1085.

Strle, F., Ruzić-Sabljić, E., Cimperman, J., Lotric-Furlan, S., Maraspin, V., 2006. Comparison of findings for patients with *Borrelia garinii* and *Borrelia afzelii* isolated from cerebrospinal fluid. Clin. Infect. Dis. 43, 704–710. https://doi.org/10.1086/506936.

Sudhindra, P., Wang, G., Schriefer, M.E., McKenna, D., Zhuge, J., Krause, P.J., Marques, A.R., Wormser, G.P., 2016. Insights into *Borrelia miyamotoi* infection from an untreated case demonstrating relapsing fever, monocytosis and a positive C6 Lyme serology. Diagn. Microbiol. Infect. Dis. 86, 93–96. https://doi.org/10.1016/j.diagmicrobio.2016.06.015.

Svensson, J., Hunfeld, K.P., Persson, K.E.M., 2019. High seroprevalence of *Babesia* antibodies among *Borrelia burgdorferi*-infected humans in Sweden. Ticks Tick. Dis. 10, 186–190. https://doi.org/10.1016/j.ttbdis.2018.10.007.

Thomas, V., Anguita, J., Barthold, S.W., Fikrig, E., 2001. Coinfection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis alters murine immune responses, pathogen burden, and severity of Lyme arthritis. Infect. Immun. 69, 3359–3371. https://doi.org/10.1128/iai.69.5.3359-3371.2001.

Wagemakers, A., Koetsveld, J., Narasimhan, S., Wickel, M., Deponte, K., Bleijlevens, B., Jahfari, S., Sprong, H., Karan, L.S., Sarksyan, D.S., van der Poll, T., Bockenstedt, L.K., Bins, A.D., Platonov, A.E., Fikrig, E., Hovius, J.W., 2016. Variable major proteins as targets for specific antibodies against *Borrelia miyamotoi*. J. Immunol. 196, 4185–4195. https://doi.org/10.4049/jimmunol.1600014.

Wass, L., Grankvist, A., Bell-Sakyi, L., Bergström, M., Ulfhammer, E., Lingblom, C., Wennerås, C., 2019. Cultivation of the causative agent of human neoehrlichiosis from clinical isolates identifies vascular endothelium as a target of infection. Emerg. Microbes Infect. 8, 413–425. https://doi.org/10.1080/22221751.2019.1584017.

Wass, L., Grankvist, A., Mattsson, M., Gustafsson, H., Krogfelt, K., Olsen, B., Nilsson, K., Mårtensson, A., Quarsten, H., Henningsson, A.J., Wennerås, C., 2018. Serological reactivity to *Anaplasma phagocytophilum* in neoehrlichiosis patients. Eur. J. Clin. Microbiol. Infect. Dis. 37, 1673–1678. https://doi.org/10.1007/s10096-018-3298-3.

Veje, M., Studahl, M., Johansson, M., Johansson, P., Nolskog, P., Bergström, T., 2018. Diagnosing tick-borne encephalitis: a re-evaluation of notified cases. Eur. J. Clin. Microbiol. Infect. Dis. 37, 339–344. https://doi.org/10.1007/s10096-017-3139-9.

Welinder-Olsson, C., Kjellin, E., Vaht, K., Jacobsson, S., Wennerås, C., 2010. First case of human "*Candidatus* Neoehrlichia mikurensis" infection in a febrile patient with chronic lymphocytic leukemia. J. Clin. Microbiol. 48, 1956–1959. https://doi.org/10.1128/jcm.02423-09.

Welzl, C., Leisewitz, A.L., Jacobson, L.S., Vaughan-Scott, T., Myburgh, E., 2001. Systemic inflammatory response syndrome and multiple-organ damage dysfunction in complicated canine babesiosis. J. S. Afr. Vet. Assoc. 72, 158–162.

Wilhelmsson, P., Fryland, L., Börjesson, S., Nordgren, J., Bergström, S., Ernerudh, J., Forsberg, P., Lindgren, P.E., 2010. Prevalence and diversity of *Borrelia* species in ticks that have bitten humans in Sweden. J. Clin. Microbiol. 48, 4169–4176. https://doi.org/10.1128/jcm.01061-10.

Wilhelmsson, P., Lindblom, P., Fryland, L., Ernerudh, J., Forsberg, P., Lindgren, P.E., 2013. Prevalence, diversity, and load of *Borrelia* species in ticks that have fed on humans in regions of Sweden and Åland Islands, Finland with different Lyme borreliosis incidences. PLoS One 8, e81433. https://doi.org/10.1371/journal.pone.0081433.

Wilhelmsson, P., Lövmar, M., Krogfelt, K.A., Nielsen, H.V., Forsberg, P., Lindgren, P.E., 2020. Clinical and serological outcome in humans bitten by *Babesia* species positive *Ixodes ricinus* ticks in Sweden and on the Åland Islands. Ticks Tick. Dis. 11, 101455 https://doi.org/10.1016/j.ttbdis.2020.101455.

von Loewenich, F.D., Geissdorfer, W., Disque, C., Matten, J., Schett, G., Sakka, S.G., Bogdan, C., 2010. Detection of "*Candidatus* Neoehrlichia mikurensis" in two patients with severe febrile illnesses: evidence for a European sequence variant. J. Clin. Microbiol. 48, 2630–2635. https://doi.org/10.1128/jcm.00588-10.

Young, N.P., Klein, C.J., 2007. Encephalopathy with seizures having PCR-positive *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*. Eur. J. Neurol. 14, 3–4. https://doi.org/10.1111/j.1468-1331.2006.01582.x.