Other tick-borne diseases in the limelight

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Chapter 2

Serological and molecular evidence for Spotted Fever Group *Rickettsia* and *Borrelia burgdorferi* sensu lato co-infections in the Netherlands

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Abstract

Only a few reported cases indicate that *Rickettsia helvetica* and *Rickettsia monacensis* can cause disease in humans. Exposure to these two spotted fever group (SFG) rickettsiae occurs through bites of *Ixodes ricinus*, also the primary vector of Lyme borreliosis in Europe. To date, it is unclear how often exposure to these two microorganisms results in infection or disease.

We show that of all the *Borrelia burgdorferi s.l.*-positive ticks, 25% were co-infected with rickettsiae. Predominantly *R. helvetica* was detected while *R. monacensis* was only found in approximately 2% of the ticks. In addition, exposure to tick-borne pathogens was compared by serology in healthy blood donors, erythema migrans (EM)-patients, and patients suspected of Lyme neuroborreliosis (LNB). As could be expected, seroreactivity against *B. burgdorferi* sensu lato was lower in blood donors (6%) compared to EM patients (34%) and suspected LNB cases (64%). Interestingly, seroreactivity against SFG *Rickettsia* antigens was not detected in serum samples from blood donors (0%), but 6% of the EM patients and 21% of the LNB suspects showed anti-rickettsial antibodies. Finally, the presence of *B. burgdorferi* s.l. and *Rickettsia* spp. in cerebrospinal fluid samples of a large cohort of patients suspected of LNB (n=208) was investigated by PCR. DNA of *B. burgdorferi* s.l., *R. helvetica* and *R. monacensis* was detected in seventeen, four and one patient, respectively.

In conclusion, our data show that *B. burgdorferi* s.l. and SFG rickettsiae co-infection occurs in Dutch *I. ricinus* and that Lyme borreliosis patients, or patients suspected of Lyme borreliosis, are indeed exposed to both tick-borne pathogens. Whether SFG rickettsiae actually cause disease, and whether co-infections alter the clinical course of Lyme borreliosis, is not clear from our data, and warrants further investigation.

Introduction

Tick-borne rickettsioses are caused by the obligate intracellular bacteria from the spotted fever group (SFG) rickettsiae. Hard ticks (Ixodidae) have been identified as the vectors of SFG rickettsiae in humans [1, 2]. *Ixodes* ticks are also the main vector of *Borrelia burgdorferi* sensu lato (s.l.), the causative agent of Lyme borreliosis.

The clinical symptoms of SFG rickettsiosis usually begin four to ten days after a tick bite, and vary depending on the *Rickettsia* sp. involved. Typical signs include fever, headache, malaise, muscle pain, rash and local lymphadenopathy. For most SFG rickettsioses, a characteristic skin lesion at the site of tick bite, known as an eschar, may occur. Clinical diagnostics relies on the detection of antibodies against *Rickettsia* spp., which is the reference method. In general the sensitivity of IgG detection increases over time after onset of disease, starting at 46% from 5-9 days post-onset to approximately 100% after four weeks. There is a large extent of cross-reactivity in serology between the different members of the SFG rickettsiae [3, 4].

To date, at least 15 different *Rickettsia* spp. have been linked to SFG rickettsioses in humans. The three most prominent members are *R. rickettsii*, the causative agent of Rocky Mountain spotted fever, *R. conorii*, the causative agent of Mediterranean spotted fever and *R. africae*, the causative agent of African tick-bite fever. Some patients diagnosed with Mediterranean spotted fever, however, were actually infected with other SFG rickettsiae, such as *R. aeschlimannii, R. massiliae* and *R. monacensis* [5-7]. Two related spotted fevers, Tick-borne lymphadenopathy (TIBOLA) and Lymphangitis-associated rickettsiosis (LAR), have been attributed to other *Rickettsia* species, such as *R. slovaca, R. raoultii* and *R. sibirica mongolitimonae* [8].

For the two SFG rickettsiae frequently detected in *Ixodes ricinus*, *R. helvetica* and *R. monacensis*, the pathogenic potential has been gradually revealed and is still controversial. *R. helvetica* is distributed in *I. ricinus* ticks all around Europe, but only a few human cases have been reported. Two cases of sudden cardiac death with perimyocarditis were attributed to infection with *R. helvetica* [9]. *R. helvetica* infection was reported in three French and three Italian patients with fever without a skin rash [10, 11], and one Swedish patient with fever and rash [12]. Two patients with neurological infections (meningitis and meningoencephalitis) associated with *R. helvetica* infection were reported more recently [13, 14]. Finally, in several case reports, acute rickettsiosis with fever and rash caused by *R. monacensis* has also been described in Europe [7, 15].

In the Netherlands, *R. helvetica* infection in questing *I. ricinus* is widespread. It has been found in more than 28 different locations with infection rates varying between 6 and 66% [16-18], but no cases of autochthonous SFG rickettsiosis have been reported. Furthermore, a prospective tick bite study did not reveal overt symptoms after a bite of ticks infected with *R. helvetica* [19].

The current study aimed to investigate whether patients with erythema migrans (EM) or patients suspected of Lyme neuroborreliosis (LNB) are co-exposed to *R. helvetica* or *R. monacensis* by assessing tick-infection rates, and by performing serology and molecular detection in humans. To this end, we determined *B. burgdorferi* s.l. and SFG *Rickettsia* co-infection rates in previously collected Dutch ticks and we used archived samples from patients (suspected) of different Lyme borreliosis manifestations, and healthy blood donors as controls.

Materials and methods

Tick data

Data on the co-infection rate of *B. burgdorferi* s.l. with SFG rickettsiae in ticks were collected from previous studies carried out in The Netherlands. Two studies [18, 20] collected questing *I. ricinus* nymphs and adults from the vegetation from various locations in the Netherlands. In addition, *I. ricinus* nymphs and adults that were feeding on humans were collected during an independent prospective tick bite study [19]. The presence of DNA from *Rickettsia* spp. and *B. burgdorferi* s.l. in tick lysates was detected and identified by PCR followed by reverse line blot analysis as described [18-20]. Data on infection with either one of the pathogens have been previously published [18-20], here we report co-infection rates of individual ticks (Supplemental Tables 1 and 2). Ticks with no information on the presence of both *Rickettsia* spp. and *B. burgdorferi* s.l. DNA were excluded from our analysis.

Patient sera and cerebrospinal fluid samples

A single serum sample from the following three groups was investigated for the presence of anti-*B. burgdorferi* s.l. and anti-SFG *Rickettsia* antibodies:

1 - Healthy blood donors from the Netherlands (n= 150). This control group was used to estimate the background level of antibodies in the Dutch general population and was expected to have a low seroprevalence for tick-borne diseases, including Lyme borreliosis and rickettsiosis.

2 - Patients with physician confirmed EM (n= 47). We considered EM patients as having a proven recent exposure to ticks of long enough duration to transmit pathogens. For this reason, EM patients were expected to have a higher prevalence of tick-borne diseases as compared to healthy blood donors.

3 - Sera from patients with neurological symptoms and a suspicion of LNB (n=33) were serologically examined for both anti-*B. burgdorferi* s.l. and anti-SFG *Rickettsia* antibodies. In addition to the investigation of serum samples, the presence of *B. burgdorferi* s.l. and *Rickettsia* spp. DNA was investigated in cerebrospinal fluid (CSF) from 208 samples from patients suspected of Lyme neuroborreliosis by means of specific PCRs. To this end we used samples that had been sent to the

Institute for Public Health and Environment (RIVM) for diagnostic purposes. Therefore only limited clinical information was available. However, for patients that proved to be *Rickettsia* positive in CSF by PCR, the responsible physicians at the hospitals at which patients were managed were asked to send their patients a short information document and an informed consent form for signed approval to access their medical history. Clinical information (relevant medical history, presenting symptoms, the physical examination, vital signs, laboratory findings, results of the lumbar puncture, prescribed treatment and the course of symptoms) was obtained retrospectively from all patients of whom a signed informed consent was received. Our methods were presented to the Institutional Review Board (IRB) at the Academic Medical Center (AMC) Amsterdam, who deemed our approach in compliance with the Dutch law on medical research in humans.

Serology

A commercially available Enzyme immunoassay (EIA) based on the C6-peptide of *B. burgdorferi* s.l. was used for the detection of *B. burgdorferi* s.l.-specific antibodies. The C6 peptide EIA was performed according to the manufacturer's instruction (Immunetics, Inc. Cambridge, MA). Results were scored as negative (Lyme index score <0.90, borderline (0.90 to 1.09), or positive (≥1.10)). The C6 peptide EIA does not distinguish between IgG and IgM antibodies and has a reported sensitivity of 23 to 90% in EM patients [21-23], and a sensitivity in LNB of 43 to 79%, with a high specificity (99-100%) [24].

For the detection of antibodies against SFG rickettsiae a commercially available IgG *R. conorii* IFA (Focus diagnostics, Cypress, CA) was used. The assay was performed according to the manufacturer's instruction. A positive serology was defined as an IgG titer of ≥1:64. Low IgG titers of 1:64 and 1:128 in a single serum sample were considered as evidence of a possible old infection or an early response in case of recent exposure to the pathogen. Single IgG end-point titers of ≥ 1:256 we considered to be serologically consistent with recent or current infection with *Rickettsia* spp. and defined as a probable case, as instructed by the manufacturer.

Molecular detection of bacteria in cerebrospinal fluid

Bacterial DNA from CSF was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *B. burgdorferi* s.l. was detected using a duplex qPCR based on the OspA and flagellin genes and carried out on a LightCycler 480 (Roche Diagnostics Nederland B.V, Almere, the Netherlands) as described before [25]. This qPCR doesn't react with *Borrelia miyamotoi* DNA*.* , Reactions were done in a final volume of 20 µl with iQ multiplex Powermix, 3 µl of template DNA and 0.2 µM for all primers [20]. Positive controls (*B. burgdorferi* senso stricto strain B31) and negative water controls were used on every plate tested. SFG rickettsiae

were detected using a conventional PCR on the 16S rRNA gene followed by TAE agarose gel-electrophoresis [17]. Positive control for the *Rickettsia* spp. PCR was a *R. africae* positive patient sample [26]. PCR products were sequenced using an ABI PRISM BigDye Terminator Cycle sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems). All sequences were confirmed by sequencing both strands. Sequences were compared with sequences in Genbank using BLAST. To minimize cross contamination and false-positive results, negative controls were included in each batch tested by PCR. In addition, DNA extraction, PCR mix preparation, sample addition, and PCR analysis were performed in separated, dedicated labs.

Statistical analysis

Two-tailed Fisher's exact tests were performed to determine the significant differences between groups. Differences with p-value ≤ 0.05 were considered significant. Titers of IFAs were log-transformed and for all assays an average was calculated. The Fisher Exact test using a binominal distribution was used to calculate the 95% confidence interval.

Results

Tick data

We here report that 25% (22-28%) of 643 ticks positive for *B. burgdorferi* s.l. were co-infected with SFG-*Rickettsia* spp. (Supplemental table 1) [18-20]. Of the 1393 *Rickettsia*-positive tick lysates, 94% reacted with the *R. helvetica* probe in the reverse line blot. The remaining *Rickettsia* spp. positive samples mainly reacted with the generic ("catch all") probes, and could not be further specified using probes. Sequencing of these samples revealed that the majority of the "catch all" positives lysates were *R. monacensis*. Therefore, the infection rate of *R. monacensis* in Dutch *I. ricinus* was estimated to be less than 2%.

Serology

Nine of 150 blood donors (6%) had a *B. burgdorferi* s.l.-specific C6 EIA response, which is comparable to the background seroprevalence (4-8%) in the Dutch general population [27, 28]. None of the blood donors had detectable *R. conorii*-reactive IgG antibodies in their serum sample (Table 1, Figure 1). In 16 (34%) of 47 EM patients *B. burgdorferi* s.l.-specific antibodies were detected and three EM patients (6%) were IgG reactive in the *R. conorii*-IFA. Of the 33 suspected LNB cases 21 (64%) and seven (21%) patients reacted positive in the *B. burgdorferi* s.l. and *R. conorii* assay, respectively (Table 1, Figure 1). Seroprevalence of both *B. burgdorferi* s.l. and SFG *Rickettsia* IgG-antibodies was significantly higher in the EM (p=0.0129) and suspected LNB (p=0.0001) patients as compared to blood donors. Thus, seroreactivity against *B. burgdorferi* s.l. and SFG *Rickettsia* antigens both gradually increased from the blood donors, EM patients, to the LNB suspected patients (Table 1).

Table 1: Seroprevalence of *B. burgdorferi* s.l. and SFG *Rickettsia*

	BD		**EM**		**LNB**	
	n	**(%)**	**n**	**(%)**	**n**	**(%)**
Total	150		47		33	
***B. burgdorferi* s.l. positive**	9	(6%)	16	(34%)	21	(64%)
SFG *Rickettsia* positive	0	(0%)	3	(6%)	7	(21%)
Both positive	0	(0%)	1	(2%)	6	(18%)

Table 1: Seroprevalence of *B. burgdorferi* s.l. and SFG *Rickettsia*. Seroprevalence of IgG antibodies against B. burgdorferi s.l. and SFG rickettsiae in healthy blood donors (BD), patients with erythema migrans (EM) and patients suspected of Lyme neuroborreliosis (LNB). Occurrence of IgG antibodies against both B. burgdorferi s.l. and SFG rickettsiae are shown in the last row.

Figure 1: Serology results comparing the three study groups.

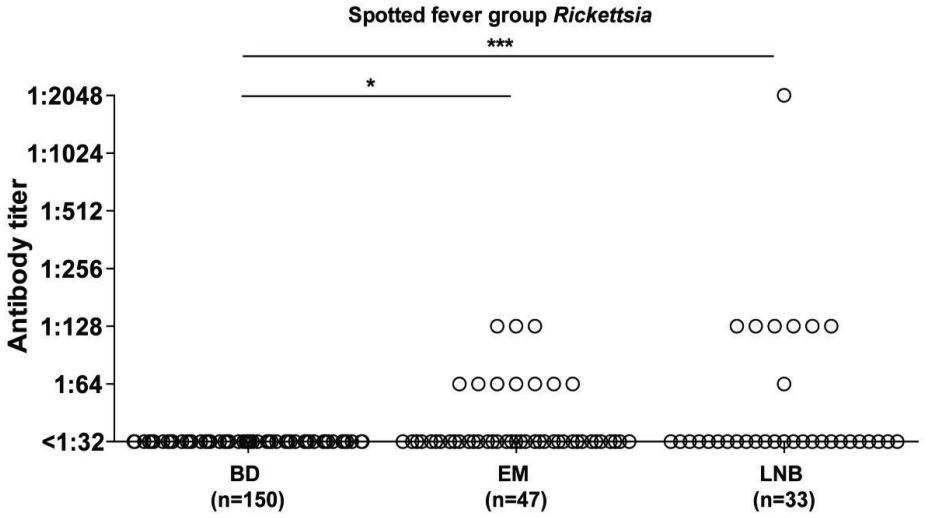

Figure 1: Serology results comparing the three study groups. IgG SFG *rickettsia* IFA titer values for the three study groups using a commercially available *R. conorii* IFA. The seroprevalence of antibodies against SFG rickettsiae was compared between healthy blood donors (BD), erythema migrans patients (EM) and patients suspected of Lyme neuroborreliosis (LNB)
* : P < 0.05, *** : P < 0.001

PCR

The presence of DNA from *B. burgdorferi* s.l. and SFG *Rickettsia* spp. was investigated in CSF from 208 suspected LNB cases by means of PCR. The DNA of *B. burgdorferi* s.l. was detected in 17 cases (8%) and DNA of *Rickettsia* spp. was amplified from five CSF samples (2%) (Table 2). Sequencing of a 360bp-fragment of the rickettsial 16S rRNA gene revealed that the sequences from the CSF of subject 2 and 3 were identical (100%) to a *R. helvetica* sequence deposited in Genbank (L36212. The sequences from the CSF of subject 1 and 4 were similar (>99%) to *R. helvetica* containing, respectively, only two- and one point mutations (Figure 2). The 16S rRNA sequence detected in the CSF sample of subject 5 was similar (>99%) to the *R. monacensis* sequence deposited in Genbank (DQ100164), and contained three point mutations (Figure 3).

Table 2: Detection of *B. burgdorferi* s.l. and SFG *Rickettsia* in CSF samples.

		***B. burgdorferi* s. l.**		**Total**
		Positive	**Negative**	
SFG *Rickettsia*	**Positive**	1	4	**5**
	Negative	16	187	**203**
Total		**17**	**191**	**208**

Table 2: Detection of *B. burgdorferi* s.l. and SFG *Rickettsia* in CSF samples. Detection of DNA from *B. burgdorferi* s.l. (in columns) and SFG *Rickettsia* (in rows) by PCR in CSF samples from patients suspected of Lyme neuroborreliosis.

Clinical data

We sent informed consent forms to all five subjects; subject 2 was lost to follow-up and the responsible physician of subject 4 declined to co-operate. The available laboratory and clinical information of all five subjects is summarized in table 3. From subject 1, 3, 4 and 5 serum was also available.

From subject 2 we did not have access to serum, nor did we have access to the clinical data (see above). Only subject 4, of which we had limited clinical data, had a positive *B. burgdorferi* s.l. PCR in CSF, however no detectable anti-*B. burgdorferi* s.l.- and anti-SFG rickettsial antibodies were found in serum of this subject.

In subject 1 and 3, DNA that was amplified from the CSF was identical or highly similar to *R. helvetica* (see above) and the *B. burgdorferi* s.l. PCR on CSF was negative. Anti-*B. burgdorferi* s.l. antibodies were found serum, with a Lyme-index (C6-EIA) of 1.8 and 9.9 respectively. Interestingly, neither of these subjects had detectable antibodies against *R. conorii* antigens (Table 3). Both subjects did not show clinical signs of infection based on their symptoms, physical examination or vital signs. Furthermore, both patients did not recall tick bites or skin lesions. Also, in both patients, no laboratory findings consistent with systemic infection were found and leukocytes, protein and glucose levels in the CSF were normal. Subject 5, in whom DNA from *R. monacensis* was amplified, showed symptoms of a radiculopathy. The patient did not recall a tick bite, nor did she notice skin lesions. No laboratory findings suggesting a systemic infection were found. Leukocytes, protein and glucose levels in the CSF were normal. In contrast to the *R. helvetica* positive patients, the *R. monacensis*-positive patient had a positive antibody response against *R. conorii* (Table 3). No *B. burgdorferi* s.l.- DNA was amplified in the CSF from this patient.

Figure 2: Alignment of the *R. helvetica* sequences derived from the CSF of patients suspected of Lyme neuroborreliosis

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R. helvetica   AACGCTATCGGTATGCTTAACACATGCAAGTCGAACGGACTAATTGGGGCTTGTTCCAGT
Subject 1      AACGCTATCGGTATGCTTAACACATGCAAGTCGAACGGACTAATTGGGGCTTGTTCCAGT
Subject 2      ----------GTATGCTTAACACATGCAAGTCGAACGGACTAATTGGGGCTTGTTCCAGT
Subject 3      AACGCTATCGGTATGCTTAACACATGCAAGTCGAACGGACTAATTGGGGCTTGTTCCAGT
Subject 4      ----------TATGCTTAACACATGCAAGTCGAACGGACTAATTGGGGCTTGCTCCAGT
R. conorii     AACGCTATCGGTATGCTTAACACATGCAAGTCGAACGGACTAATTGGGGCTTGCTCCAGT

R. helvetica   TAGTTAGTGGCAGACGGGTGAGTAACACGTGGGAATCTACCCATCAGTATGGAATAACTT
Subject 1      TAGTTAGTGGCAGACGGGTGAGTAACACGTGGGAATCTACCCATCAGTATGGAATAACTT
Subject 2      TAGTTAGTGGCAGACGGGTGAGTAACACGTGGGAATCTACCCATCAGTATGGAATAACTT
Subject 3      TAGTTAGTGGCAGACGGGTGAGTAACACGTGGGAATCTACCCATCAGTATGGAATAACTT
Subject 4      TAGTTAGTGGCAGACGGGTGAGTAACACGTGGGAATCTACCCATCAGTATGGAATAACTT
R. conorii     TAGTTAGTGGCAGACGGGTGAGTAACACGTGGGAATCTACCCATTAGTACGGAATAACTT

R. helvetica   TTAGAAATAAAAGCTAATACCATATATTCTCTATGGAGGAAAGATTTATCGCTGATGGAT
Subject 1      TTAGAAATAAAAGCTAATACCATATATTCTCTATGGAGGAAAGATTTATCGCTGATGGAT
Subject 2      TTAGAAATAAAAGCTAATACCATATATTCTCTATGGAGGAAAGATTTATCGCTGATGGAT
Subject 3      TTAGAAATAAAAGCTAATACCATATATTCTCTATGGAGGAAAGATTTATCGCTGATGGAT
Subject 4      TTAGAAATAAAAGCTAATACCATATATTCTCTATGGAGGAAAGATTTATCGCTGATGGAT
R. conorii     TTAGAAATAAAAGCTAATACCGTATATTCTCTGCGGAGGAAAGATTTATCGCTGATGGAT

R. helvetica   GAGCCCGCGTCAGATTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGACGATCTGTAG
Subject 1      GAGCCCGCGTCAGATTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGACGATCTGTAG
Subject 2      GAGCCCGCGTCAGATTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGACGATCTGTAG
Subject 3      GAGCCCGCGTCAGATTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGACGATCTGTAG
Subject 4      GAGCCCGCGTCAGATTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGACGATCTGTAG
R. conorii     GAGCCCGCGTCAGATTAGGTAGTTGGTGAGGTAATGGCTCACCAAGCCGACGATCTGTAG

R. helvetica   CTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
Subject 1      CTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGGCACGGCCCAGACTCCTACGGGA
Subject 2      CTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
Subject 3      CTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
Subject 4      CTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
R. conorii     CTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA

R. helvetica   GGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATA
Subject 1      GGCAGCAGTGGGGGATATTGGACAATGGGCGAAAGCCTGATCCAGCAATA
Subject 2      GGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATA
Subject 3      GGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATA
Subject 4      GGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATA
R. conorii     GGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATA
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Figure 2: Alignment of the *R. helvetica* sequences derived from the CSF of patients suspected of Lyme neuroborreliosis. Comparison of the alignment of the four *R. helvetica* 16S rDNA sequences found in the CSF of patients suspected of Lyme neuroborreliosis. *R. helvetica* (L36212) and *R. conorii* (NR_074480) sequences from Genbank are used for comparison. *R. helvetica* reference sequence is highlighted in green. Control sequence (*R. conorii*) is highlighted in blue. Differences in alignment, as compared to the reference sequence, are highlighted in yellow.

Figure 3: Alignment of the *R. monacensis* sequence derived from the CSF of a patient suspected of Lyme neuroborreliosis

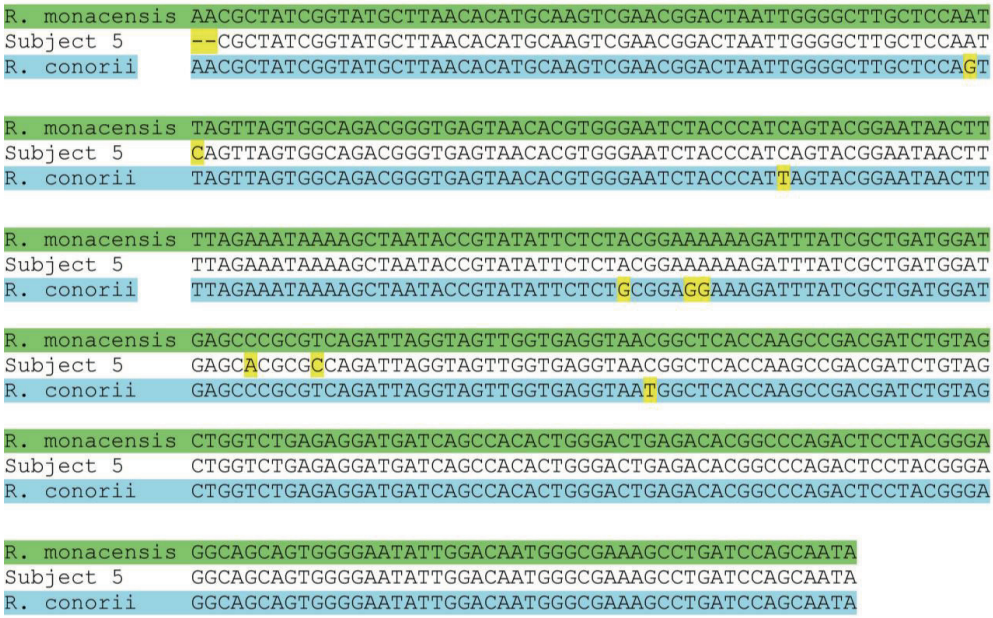

Figure 3: Alignment of the *R. monacensis* sequence derived from the CSF of a patient suspected of Lyme neuroborreliosis. Comparison of the alignment of the *R. monacensis* 16S rDNA sequences found in the CSF of a patient suspected of Lyme neuroborreliosis. *R. monacensis* (DQ100164) and *R. conorii* (NR_074480) sequences from Genbank are used for comparison. *R. monacensis* reference sequence is highlighted in green. Control sequence (*R. conorii*) is highlighted in blue. Differences in alignment, as compared to the reference sequence, are highlighted in yellow.

Table 3: Combined data of the patients with a SFG *Rickettsia* positive PCR in CSF samples

		Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Clinical data						
M/F		F	F	F	F	F
Age		61	77	39	38	53
Clinical data obtained		**Yes**	No	**Yes**	No	**Yes**
Reported tick bite		No	-	No	-	No
Fever		No	-	No	-	No
Skin symptoms (EM/rash)		No	-	No	-	No
Immunocompromised		No	-	No	-	No
Symptoms		- Forgetfulness - Afasia - Incontinency	-	- Headache - Forgetfulness - Stiff neck - Fatigue - Palpitations	-	- Pain left leg - Loss of sensation left leg
Clinical diagnosis		Fronto-temporal dementia	-	None	-	Radiculopathy
Laboratory data	**(ref values)**					
SFG *Rickettsia* IFA titer	(>1:64)	<1:32	-	<1:32	<1:32	**1:128**
Lyme-index (C6 EIA)	(<1.2)	**1,8**	-	**9,9**	0,3	**8,5**
B. burgdorferi s.l. IgG-blot		NEG	-	**POS**	-	**POS**
B. burgdorferi s.l. IgM-blot		NEG	-	NEG	-	NEG
Hb (mmol/l)	(7.5-10)	7.8	-	9	-	8.1
Leukocytes (x10^9/l)	(4-10)	6.7	-	6	-	7.3
Trombocytes (x10^9/l)	(150-400)	290	-	207	-	**494**
Sedimentation rate (mm/hr)	(<20)	**30**	-	2	-	5
CRP (mg/l)	(<10)	2	-	1	-	1
ASAT (U/l)	(<45)	-	-	14	-	23
ALAT (U/l)	(<40)	24	-	20	-	21
CSF data	**(ref values)**					
Sequencing result 16s DNA		***R. helvetica***	***R. helvetica***	***R. helvetica***	***R. helvetica***	***R. monacensis***
B. burgdorferi s.l. PCR		NEG	NEG	NEG	**POS**	NEG
B. burgdorferi s.l. IgG-blot		NEG	-	NEG	-	NEG
B. burgdorferi s.l. IgM-blot		NEG	-	NEG	-	NEG
CSF leukocytes (/3µl)	(<15)	10	-	0	-	3
CSF glucose (mmol/l)	(2.5-3.7)	**4.1**	-	3.5	-	3.3
CSF protein (mg/l)	(0.18-0.58)	0.26	-	0.35	-	0.47

Clinical data, laboratory results and CSF data collected from the patients in which SFG rickettsiae were detected in CSF samples. Whether there was a reported tick bite, fever, skin symptoms (such as EM or rash) or other (presenting) symptoms, whether or not the patient was immunocompromised, and the (final) clinical diagnosis, were all based on the available medical history and the correspondence from the treating physicians.

Positive results and abnormal findings are in boldface. M: male. F: female. Clinical data obtained: indicates whether we were able to obtain the clinical data from that subject. Age: age at the time of lumbar puncture. EM: Erythema migrans. NEG: Negative. POS: Positive.

Discussion

Previous studies estimated that more than one million tick bites occur annually in The Netherlands, from which 183,000 were by ticks infected with *B. burgdorferi* s.l. [29, 30]. With an infection rate of 28% in questing ticks (Supplemental Table 1), we estimate that approximately 280,000 of the tick bites in The Netherlands will be with ticks infected with *Rickettsia* spp., mostly *R. helvetica* and to a lesser extent *R. monacensis*. We now show that with 25% of *B. burgdorferi* s.l. infected ticks being co-infected with *Rickettsia* spp. (Supplemental table 1), approximately 46,000 tick bites would be from these co-infected ticks. This substantial human exposure raises the question whether *R. helvetica* and other SFG rickettsiae can lead to infection, and consequently even to disease.

In our study, we detected a significant increase in the seroprevalence of antibodies against SFG rickettsiae in Lyme borreliosis patients compared to blood donors. These results suggest transmission of SFG rickettsiae to humans in the Netherlands. In the EM group we found 6% SFG rickettsiae seropositivity compared to 34% *B. burgdorferi* s.l. seropositivity, and in the LNB group 21%. Whether this can be attributed to a simultaneous or previous infection with *Rickettsia* spp. remains to be elucidated. Our data is in accordance with earlier studies in France and Sweden, in which an increase in SFG *Rickettsia* antibodies was found in areas where *R. helvetica* was found to be prevalent [10, 31, 32].

No commercial serological test for the specific detection of *R. helvetica* and *R. monacensis* antibodies is currently available. Due to the high degree of homology of *Rickettsia* spp. within the spotted fever group, the well-known high level of cross reactivity for different SFG rickettsiae [3, 4], and the fact that *R. conorii*, as well as other well-known pathogenic SFG rickettsiae are not encountered in the Netherlands, we used a commercial *R. conorii* IFA for our screening purposes. Case studies of patients infected with either *R. helvetica* or *R. monacensis* did also show antibodies against *R. conorii.* However, antibody responses to *R. conorii* were lower than the response to the causative agent of the disease in these patients (*R. helvetica* or *R. monacensis*) [7, 10, 15]. With *R. helvetica* and *R. monacensis* being by far the most prevalent SFG rickettsiae in the Netherlands, a positive IFA test to *R. conorii* is most probably caused by the presence of antibodies to these SFG rickettsiae. However, we realize that some of the positive reactions might have been due to exposure to *R. conorii* or SFG rickettsiae other than *R. helvetica* and *R. monacensis* in the past. On the other hand, the use of a different member of the SFG rickettsiae as an antigen may also have resulted in a lower sensitivity to detect antibodies to *R. helvetica* and *R. monacensis*. In addition, the reported low sensitivity of serology in SFG *Rickettsia* patients of approximately 50% in the first weeks after onset [2], could also have led to further underestimation.

In contrast to the indirect evidence of a current or past infection by positive serology, the detection of the microorganism itself or one of its components, for example its DNA, is more direct evidence of an ongoing infection. For this reason, we investigated the CSF of another panel of patients suspected of LNB by PCR for *Rickettsia* spp. and *B. burgdorferi* s.l.. We detected both *B. burgdorferi* s.l. and SFG *Rickettsia* DNA in the CSF from suspected neuroborreliosis patients (n=208). It is known that the sensitivity of PCR for detecting *B. burgdorferi* s.l. DNA in CSF in Lyme patients is low, ranging from 9 to 50% [33, 34]. The sensitivity for detecting *R. helvetica* in CSF is unknown, however, *R. helvetica* can be detected by PCR in the CSF [13, 14]. We detected *Rickettsia* spp. DNA in CSF of five patients suspected of Lyme neuroborreliosis, suggesting transmission of *R. helvetica* and *R. monacensis* from the tick to the host and dissemination to the central nervous system. We realize that isolation and culture directly from patient material would have been stronger evidence for *R. helvetica* or *R. monacensis* infection. However, we were limited to molecular methods to detect *R. helvetica* and *R. monacensis*, since serum and CSF from these LNB patients were used for routine diagnostics and subsequently frozen, making them ill-suited for isolation and culture. In addition, attempts to confirm our molecular results with a PCR on the gltA locus failed due to the limited availability of CSF from these patients (data not shown). However, sequencing revealed and confirmed that we indeed amplified rickettsial DNA. It should be emphasized that we did not use *R. helvetica* or *R. monacensis* DNA as a positive control and that all negative controls tested negative in the PCR runs.

Whereas, the results above collectively suggest that Dutch LB patients are exposed to SFG rickettsiae, these data do not allow any speculations on the pathogenic potential of these microorganisms. Moreover, the clinical data obtained from the five patients, in which DNA of SFG rickettsiae was found in the CSF, was limited. However, sporadic cases have been reported in literature showing that *R. helvetica* and *R. monacensis* can be detected by PCR in various body fluids and are capable of infecting humans and cause disease [14, 35]. In our study, despite the fact that we were able to amplify DNA of *R. helvetica* in four subjects, serology results did not show evidence of (past) infection in these patients. There could be multiple explanations for this finding; 1) low sensitivity of our commercial *R. conorii* IFA to detect anti-*R. helvetica* antibodies, 2) laboratory contamination, although unlikely for reasons described above, and finally 3) the absence of specific antibodies actually allowed for dissemination of *R. helvetica*. The latter is supported a recent study from Tunisia were, using a qPCR on both blood samples and skin biopsies from patients suspected of rickettsial disease, high rates of positivity was found in patients with negative serology [36]. In contrast, in the patient where DNA of *R. monacensis* was amplified, serology did show evidence for exposure to SFG rickettsiae, confirming our findings in the CSF. Regardless, due to the non-specific nature of the

complaints and the lack of inflammation in the CSF and blood it is unlikely that the clinical picture in any of these five patients was caused by SFG rickettsiae.

To conclude, based on the simultaneous presence in Dutch *I. ricinus* ticks, as well as serological and molecular data, our results indicate that co-infection of *R. helvetica* or *R. monacensis* and *B. burgdorferi* s.l. occurs in the Netherlands. The implications of our findings for clinical practice are not yet clear and, based on our data, it cannot be concluded that *R. helvetica* and *R. monacensis* are capable of inducing disease. Nevertheless, the results presented here warrant future research. Larger cohorts of well-defined patients, of which extensive clinical information is available, should be studied to determine whether *R. helvetica* or *R. monacensis* are capable of causing disease by themselves and whether co-infection with *B. burgdorferi* s.l. could alter the susceptibility to, and the clinical manifestations of, Lyme borreliosis.

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Supplemental table 1: Infection rate of questing *Ixodes ricinus* nymphs and adults with *Borrelia burgdorferi* s.l. and SFG *Rickettsia*

Study (year)	Tick collection method	*I. ricinus*	*Borrelia* pos.		*Rickettsia* pos.		Co-infection		Co-infection in *Borrelia* pos.	
		No.	No.	(%)	No.	(%)	No.	(%)	%	(C.I.)
Tijsse-Klasen (2010)	Flagging and collected from lizards	713	106	(15%)	103	(14%)	15	(2%)	14%	(8-22%)
Tijsse-Klasen (2011)	Collected from humans	213	34	(16%)	40	(19%)	6	(3%)	18%	(7-35%)
Coipan (2013)	Flagging	3979	503	(13%)	1250	(31%)	139	(3%)	28%	(24-32%)
Total		**4905**	**643**	**(13%)**	**1393**	**(28%)**	**160**	**(3%)**	**25%**	**(22-28%)**

Supplemental table 1: Infection rate of questing *Ixodes ricinus* nymphs and adults with *Borrelia burgdorferi* s.l. and SFG *Rickettsia*. Data on *B. burgdorferi* s.l. and SFG *Rickettsia* positive (pos.) I. ricinus ticks as found in three previous studies performed in the Netherlands. Co-infection rates in ticks collected during these studies and the SFG rickettsiae infection rate in B. burgdorferi s.l. positive ticks are also shown.

Supplemental table 2: Information on the origin of Ixodes ricinus nymphs and adults used in Supplemental table 1.

Collection sites	Ticks collected	Time of collection	Collection method	Reference
Heumensoord	137	May - October in the years 2007 till 2009	Blanket dragging, removed from lizards	Tijsse-Klasen (2010)
Bergherbos	142	May - October in the years 2007 till 2009	Blanket dragging, removed from lizards	Tijsse-Klasen (2010)
Leusderheide	201	May - October in the years 2007 till 2009	Blanket dragging, removed from lizards	Tijsse-Klasen (2010)
Hullenberg	233	May - October in the years 2007 till 2009	Blanket dragging, removed from lizards	Tijsse-Klasen (2010)
Ballum (Ameland)	297	January 2004 to December 2008	Ticks removed from humans	Tijsse-Klasen (2011)
Apeldoorn	38	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Appelscha	79	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Bellingwedde	6	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Bijlmerweide	330	April – September in the years 2000 till 2010	Blanket dragging	Coipan (2013)
Bilthoven	40	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Duin& Kruidberg	1640	April – September in the years 2000 till 2010	Blanket dragging	Coipan (2013)
Ede	354	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Eijsden	232	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Gieten	136	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Haaksbergen	105	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Hoge Veluwe	8	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Hoog Baarlo	311	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Hoogeveen	163	April – September in the years 2000 till 2010	Blanket dragging	Coipan (2013)
Houtvest, Bos	510	April – September in the years 2000 till 2010	Blanket dragging	Coipan (2013)
Houtvest, Heide	88	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Kwade Hoek	162	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Montferland	1470	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Nijverdal	127	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Ruinen	94	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Twiske	292	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Veldhoven	242	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)
Wassenaar	204	January - December in the years 2006 till 2010	Blanket dragging	Coipan (2013)

Approximately 2% of all ticks collected/tested were adults, all others were nymphs. Please note that in some cases more ticks were collected than analyzed on both pathogens.