A three-years assessment of *Ixodes ricinus*-borne pathogens in a French peri-urban forest

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Abstract

Background: *Ixodes ricinus* is the predominant tick species in Europe and the primary pathogen vector for both humans and animals. These ticks are frequently involved in the transmission of *Borrelia burgdorferi* sensu lato, the causative agents of Lyme borreliosis. While much more is known about *I. ricinus* tick-borne pathogen composition, information about temporal tick-borne pathogen patterns remain scarce. These data are crucial for predicting seasonal/annual patterns which could improve understanding and prevent tick-borne diseases.

Methods: We examined tick-borne pathogen (TBPs) dynamics in *I. ricinus* collected monthly in a peri-urban forest over three consecutive years. In total, 998 nymphs were screened for 31 pathogenic species using high-throughput microfluidic real-time PCR.

Results: We detected DNA from *Anaplasma phagocytophilum* (5.3%), *Rickettsia helvetica* (4.5%), *Borrelia burgdorferi* s.l. (3.7%), *Borrelia miyamotoi* (1.2%), *Babesia venatorum* (1.5%) and *Rickettsia felis* (0.1%). Among all analysed ticks, 15.9% were infected by at least one of these microorganisms, and 1.3% were co-infected. Co-infections with *B. afzeli*/*B. garinii* and *B. garinii*/*B. spielmanii* were significantly over-represented. Moreover, significant variations in seasonal and/or inter-annual prevalence were observed for several pathogens (*R. helvetica, B. burgdorferi* s.l.*, B. miyamotoi*, and *A. phagocytophilum*).

Conclusions: Analysing TBPs prevalence in monthly sampled tick over three years allowed us to assess seasonal and inter-annual fluctuations of the prevalence of TBPs known to circulate in the sampled area, but also to punctually detect less common species. All these data emphasize that sporadic tick samplings are not sufficient to determine TBPs prevalence and that regular monitoring is necessary.

Key words: Tick-borne pathogens, dynamics, temporal patterns, pathogen co-occurrence

Background

Ticks are obligatory hematophagous arthropods and consequently, are one of the most important pathogen vectors [1–3]. Lyme borreliosis (LB) is the most commonly reported tick-

borne disease (TBD) in the northern hemisphere and is caused by bacteria belonging to the *Borrelia burgdorferi* s.l. complex. In Western Europe, *Ixodes ricinus* is known to be involved in the transmission of these bacteria to both humans and animals. This tick species has also been reported to be a vector for many other tick-borne pathogens (TBP) with potentially significant consequences for human and animal health (*Anaplasma*, *Rickettsia*, *Bartonella*, *Babesia*…) [4–9].

While multiple different pathogens have been identified and confirmed in *I. ricinus* ticks, very little is known about their seasonal and inter-annual variations. Time-series studies are thus crucial to understanding natural variability in microbial communities over time. Over the last decade, only a handful of surveys have assessed seasonal and monthly TBP variation patterns [10–14]. Although these results have heightened our general understanding of TBP dynamics, several of these studies were performed over short periods of less than two years, rendering it impossible to infer inter-annual discrepancies or to detect bias due to a particularly exceptional year. Only Coipan *et al*. [12] analysed several pathogenic genera in ticks sampled over more than two years. This study did demonstrate relationships between seasons and TBP prevalence (*Borrelia*, *Rickettsia*, *Anaplasma*, *Neoehrlichia*, and *Babesia*) in questing tick populations. These variations were mainly attributed to the varying availability of reservoir hosts.

Tick density is also heavily influenced by the presence of suitable hosts, most notably wild ungulates that sustain adults, thus enabling tick population renewal [15,16]. However, it's important to emphasise that immediate tick survival and questing activities are highly dependent on suitable and specific environmental conditions (temperatures between 8 to 24°C; and up to 80% humidity). Simultaneously, several studies have investigated whether pathogen presence influences tick behaviour. Herrmann and Gern [17,18] suggested that *I. ricinus* infected with *B. burgdorferi* s.l. can tolerate increased levels of desiccation, and Neelakanta *et al*. [19] demonstrated that *I. scapularis* infected with *Anaplasma phagocytophilum* are more resistant to cold. The presence of these TBP could therefore enhance survival or questing activities of the infected ticks under challenging abiotic conditions, suggesting the existence of a potential link between pathogen prevalence in questing ticks and seasons.

Tick density and TBP prevalence can thus be influenced by several variables, and can therefore potentially fluctuate both seasonally and annually. Studying these dynamics is essential to better understanding and anticipating TBP risk.

Peri-urban forests containing both TBP-reservoir hosts and ticks, and which are highly frequented by people and their pets, represent a particularly interesting area to study tick and TBP dynamics. The Sénart forest, located to the south of Paris, harbours many large ungulates and abundant and diverse populations of other TBP reservoir hosts (bank voles, wood mice, Siberian chipmunks, roe deer, hedgehogs, …), and accommodates more than three million visitors every year. This forest is therefore particularly adapted to studying ticks and tick-borne pathogen dynamics.

In this study, we assessed the seasonal and inter-annual variability of *I. ricinus*-borne pathogens in the Sénart forest over three consecutive years (from April 2014 to May 2017), and determined whether any significant associations existed between these pathogens. We investigated a total of 31 pathogenic species (bacteria and parasites), belonging to 11 genera: *Borrelia*, *Anaplasma*, *Ehrlichia*, *Neoehrlichia* (only *Neoehrlichia mikurensis*), *Rickettsia*, *Bartonella*, *Francisella*, *Coxiella*, *Theileria*, *Babesia*, and *Hepatozoon*.

Methods

Tick collection

I. ricinus, nymphs and adults, were monthly collected during three years, from April 2014 to May 2017, in the Sénart forest in the south of Paris. Ticks were collected between 10 am and noon. Samplings were performed by dragging (Vassallo *et al*., 2000) on 10 transects of 10 square meters, localized on the parcel 96 (48°39'34.6"N 2°29'13.0"E, **Figure 1**). Dragging was always performed 3 consecutive times on each transect by the same persons to limit sampling bias. The presence of *Dermacentor* spp. was occasionally reported but no investigation has been led further. The presence of *I. ricinus* larvae was also sometimes noticed. Their small size making their individual DNA extraction and analysis difficult, we chose to not collect them. After morphological identification, ticks were stored at -80°C. In total 1167 ticks were collected.

103 **Tick washing, crushing and DNA extraction**

104 Ticks were first washed once in ethanol 70% for 5 minutes and rinsed twice in sterile
105 MilliQ water for 5 minutes. Ticks were then individually crushed in 375µL of Dulbecco's
106 Modified Eagle Medium (DMEM) with decomplemented Foetal Calf Serum (10%) and six steel
107 beads using the homogenizer Precellys®24 Dual (Bertin, France) at 5500 rpm for 20 seconds.

108 DNA extractions were then performed on 100µL of tick crushing, using the DNA
109 extraction kit NucleoSpin® Tissue (Macherey-Nagel, Germany), and following the standard
110 protocol for human or animal tissue and cultured cells, from the step 2. DNA extracts were
111 eluted in 50µL of elution buffer and then stored at -20°C until further use.

112 Two controls were performed: (1) the crushing control, corresponding to a DMEM tube
113 in which crushing and DNA extraction were performed in the same conditions than on
114 samples; and (2) the extraction control, corresponding to the DNA extraction step without tick
115 samples.

116

117 **Tick-borne pathogens detection**

118 A high-throughput screening of the most common bacterial and parasitic species
119 known to circulate in ticks in Europe was performed. This allowed us to detect simultaneously
120 the presence of 31 pathogenic species, 7 genera and 1 phylum: the Borrelia genus and eight
121 Borrelia species (*B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. spielmanii*,
122 *B. lusitaniae*, *B. bissettii* and *B. miyamotoi*); the Anaplasma genus and five Anaplasma species
123 (*A. marginale*, *A. phagocytophilum*, *A. platys*, *A. centrale*, *A. bovis*); the Ehrlichia genus and
124 *E. canis*; *Neoehrlichia mikurensis*; the Rickettsia genus and six Rickettsia species (*R. conorii,*
125 *R. slovaca, R. massiliae, R. helvetica*, *R. aeshlimanii* and *R. felis*); the Bartonella genus and
126 *B. henselae*; *Francisella tularensis*; *Coxiella burnettii*; the apicomplexa phylum and seven
127 Babesia species (*B. divergens, B. microti, B. caballi, B. canis, B. venatorum, B. bovis, B. ovis)*,
128 but also the two parasitic genus Theileria and Hepatozoon.

129 TBP DNA was detected using the BioMark™ real-time PCR system (Fluidigm, USA), a
130 microfluidic system allowing to perform 48 or 96 real-time PCR reactions on 48 or 96 different
131 samples as described in Michelet *et al*. [20] and Moutailler *et al*. [21]. Briefly, each sample and

primers/probe set were included in individual wells. A pressure system allowed to load them on the chip, *via* microchannels, in individual reaction chambers of 10nL, where each sample will meet individually each primers/probe set.

Primers and probes

Primers and probes used for this analysis have been developed and validated by Michelet *et al*. [20] and Gondard *et al*. [22]. They have been designed to specifically amplify DNA from pathogens (bacteria and parasites) which are usually found in ticks in Europe. Their sequences, amplicon size, as well as targeted genes and pathogens are presented in the Additional **table1**. Please note that, due to potential cross-reactions between primer/probe combination (i.e. design) targeting *B. burgdorferi* s.s. and *B. spielmanii* with respectively *B. garinii*/*B. valaisiana* and *B. afzelii* DNA [described in 20], positive samples for the two formers were considered as negative when associated to the latter. Therefore, potential associations between *B. burgdorferi* s.s./*B. garinii*, *B. burgdorferi* s.s./*B. valaisiana* and *B. spielmanii*/*B. afzelii* cannot be detected and the co-infection percentage may be under-estimated.

DNA pre-amplification

DNA pre-amplifiations were performed using the TaqMan PreAmp Master Mix (Applied Biosystems, France). Basically, the different primer pairs, used for the real time PCR, were pooled combining equal volume of primers with a final concentration of 0.2µM. For each sample, 1.25µL of DNA extract was pre-amplified using the Perfecta PreAmp SuperMix reagent (1x) and the 0.2x pool (0.05µM), in a final reactive volume of 5µL. PCR cycle comprised a first cycle at 98°C for 2 minutes, followed by 14 cycles with 2 steps, the first one at 95°C for 10 seconds and the second one at 60°C for 3 minutes. Pre-amplified DNA were then diluted (1:10) by addition of 45µL of sterile deionised water before use.

160 **High throughput real time PCR**

161 For each pre-amplified sample, the BioMark™ real-time PCR system (Fluidigm, USA)
162 was used for high-throughput microfluidic real-time PCR amplification using the 48.48
163 microfluidic dynamic array (Fluidigm Corporation, USA). Amplifications were performed using
164 FAM- and black hole quencher (BHQ1)-labeled TaqMan probes with TaqMan Gene Expression
165 Master Mix in accordance with manufacturer's instructions (Applied Biosystems, France).
166 Thermal cycling conditions were used as follows: 95°C for 5 min, 45 cycles at 95°C for 10 s,
167 60°C for 15 s, and 40°C for 10s. Data were acquired on the BioMark Real-Time PCR system and
168 analysed using the Fluidigm Real-Time PCR Analysis software to obtain crossing point (CP)
169 values. Three tick species control (*I. ricinus*, *Dermacentor reticulatus*,
170 *Dermacentor marginatus*), one negative water control and one positive *Escherichia coli* control
171 were included in each chip.

172

173 **Nested PCR and sequencing**

174 Samples that were positive either only for species design but not for the genus design
175 or only for the genus design and not for the species design were all re-analysed by nested PCR.
176 We used primer pairs allowing to target another gene that the one tested into the fluidigm
177 experiment. Their sequences, amplicon size, as well as targeted genes and pathogen genus are
178 presented in the Additional **table 2**. Amplicons were then sequenced by the Eurofins company.
179 Sequences were then analysed using the Bioedit software and compared to the database NCBI
180 (National Center for Biotechnology Information) by sequence alignment using nucleotide
181 BLAST (Basic Local Alignment Search Tool).

182

183 **Statistical analysis**

184 ***TBP prevalences at the seasonal and multi-annual scale***

185 Differences in TBP prevalences were tested within and between years by using a
186 multivariable logistic regression model. We considered the calendar season level for the
187 within-year variability. Seasons were considered as following: Winter = December to February;
188 Spring = March to May; Summer = June to August and Autumn = September to November.

Logistic regression models were developed using the TBP status of each nymph as the outcome measure and season, year and the interaction between season and year as explanatory variables. We performed four specific models for the following group/species of TBP: (1) *B. burgdorferi* s.l. (considering *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. valaisiana* and *B. spielmanii*), (2) *B. miyamotoi*, (3) *A. phagocytophilum*, and (4) *R. helvetica*. The models were constructed from a generalized linear model [GLM, 23] using a binomial distribution (logit link). Model assessment was based on Akaike information criterion (AIC). Results were expressed as odds ratios (OR) and 95% confidence intervals. Statistical computations were performed in R 3.5.1. [24].

Statistical modelling of tick-borne pathogen associations

We tested the associations between the TBP species that belonged to the co-infection profiles of nymphs found in this study. We used the association screening approach [25]. For a given number of pathogen species tested (NP), the number of possible combination (NC) was calculated as $NC = 2^{NP}$. Assuming similar pathogen prevalence as those observed, a simulated dataset was built as an absence/presence matrix with hosts in lines and pathogen combinations in columns. With 5 000 simulations, we obtained the NC statistic distributions. We estimated a 95% confidence interval to obtain a profile that includes simultaneously all the combinations. From this profile, we inferred for each combination two quantiles, *Qinf* and *Qsup*. A global test was based on the 95% confidence envelope. When H0 was rejected, the local tests were based on the NC confidence intervals: [*Qinf* ; *Qsup*][25].

Results

Tick temporal dynamics

From April 2014 to May 2017, a total of 1167 *Ixodes ricinus* ticks were collected in the Sénart forest in the south of Paris (**Figure 1**). Please note that May and June 2016 were unfortunately not sampled due to logistic issues. Collected ticks were composed of 1098 nymphs, 35 females and 34 males. Adults were sporadically detected all over the three years Due to their low total abundance (more than 10 fold less compared with nymphs), we decided

to focus our temporal analysis on nymphs. The temporal dynamics of nymph densities over the three years is shown in **Figure 2**. Nymph densities followed similar patterns from one year to another, with a main peak of activity observed every year in spring, a strong decrease during summer and a second peak, smaller, observed in Autumn (**Figure 2**). In January and February, the average densities were less than 10 questing nymphs/100m^2. A clear rise was observed from March to May reaching an average peak of 95 nymphs/100m^2 in May. We then observed a decrease in summer up to a minimum average of 5 nymphs/100m^2 in September. The nymph densities increased slightly in October to reach an average of 13 nymphs/100m^2, before finally decreasing in November (2 nymphs/100m^2, sampled in 2015).

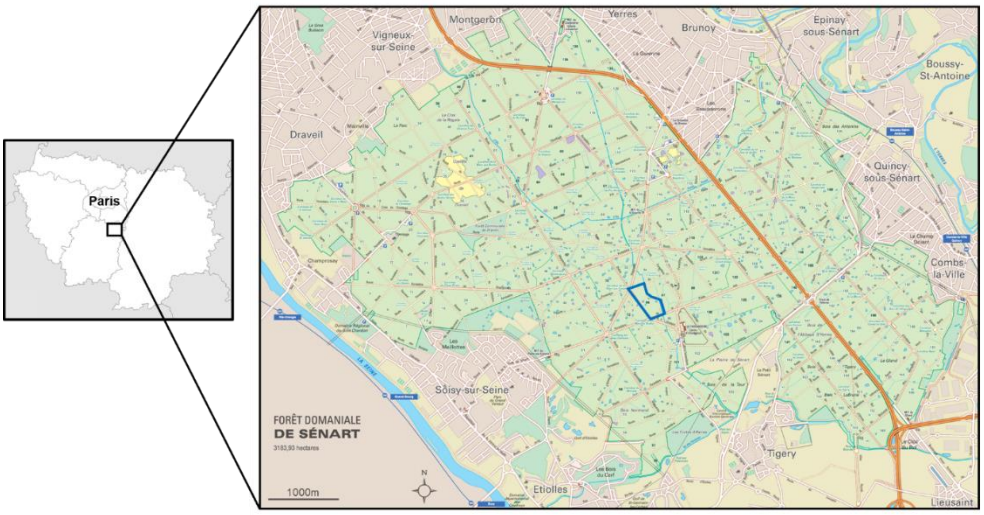


Figure 1. Sénart forest, location and parcel map. Sampling was made on the blue framed parcel.

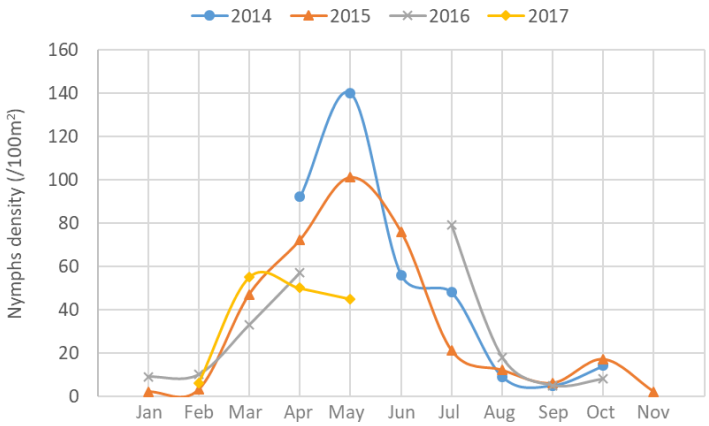


Figure 2. *Ixodes ricinus* nymphs monthly density (/100m^2) in 2014, 2015, 2016 and 2017. Ticks were sampled from April 2014 to May 2017. Please note that May and June 2016 were unfortunately not sampled.

233

234 **Detected pathogens and their prevalence in tick population**

235 Due to technical problems, DNA was extracted and analysed only from 1044 nymphs
236 among the 1098 previously mentioned. 46 were negative for at least one positive control and
237 thus have been removed from the analysis. From the 998 remaining DNA samples, 15.9%
238 [13.7%, 18.3%] were positive for at least one tested pathogen, which belong to three bacterial
239 and one protozoan genera: *Anaplasma*, *Borrelia*, *Rickettsia* and *Babesia* (**Table 1**).

240 Pathogens belonging to the *Anaplasma* genus were detected in 5.4% [4.1%, 7.0%] of
241 collected ticks. Most of them were positive for *Anaplasma phagocytophilum* (5.3% of all the
242 samples) and one DNA sample was only positive for the primers/probe combination specific
243 to *Anaplasma* spp.. This sample was confirmed by nested PCR and the amplicon was then
244 sequenced. The BLAST analysis on NCBI showed that this sequence matched at 99% of identity
245 with four different *Anaplasma* species (*A. phagocytophilum*, *A. marginale*, *A. ovis* and
246 *A. centrale*). Therefore, this sample was only considered as positive for *Anaplasma* spp..

247 Two species of *Rickettsia* were detected in questing *I. ricinus* nymphs.
248 *Rickettsia helvetica* was the most prevalent and was detected in 4.5% [3.3%, 6.0%] of nymphs.
249 *Rickettsia felis* was detected in only one nymph (0.1% [.003%, 0.6%]). The presence of *R. felis*
250 DNA was confirmed by nested PCR and sequencing of the *ompB* gene. The obtained sequence
251 (GenBank accession number MN267050) matched with the corresponding gene sequence of
252 *R. felis* (GenBank accession numbers GU182892.1) with 100% of identity and 100% of query
253 cover.

254 The genus *Borrelia* was represented by six different species detected in 4.9% [3.7%,
255 6.4%] of the surveyed nymphs. Five belonged to the LB group (3.7% [3.7%, 6.4%]), including
256 *B. burgdorferi* s.s. (1.5% [0.8%, 2.5%]), *B. garinii* (1.1% [0.6%, 2.0%]), *B. afzelii* (1.1% [0.6%,
257 2.0%]), *B. valaisiana* (0.6% [0.2%, 1.3%]) and *B. spielmanii* (0.4% [0.1%, 1.0%]). DNA of
258 *Borrelia miyamotoi*, belonging to the relapsing fever group, was detected in 1.2% [0.6%, 2.1%]
259 of the collected nymphs.

260 DNA from two species of *Babesia* were detected in questing nymph with the
261 microfluidic PCR: *Babesia venatorum* (1.5% [0.8%, 2.5%] of ticks) and *Babesia divergens* (0.1%
262 [0.003%, 0.6%]), detected in one tick). A deeper investigation of the *B. divergens* positive

sample, by nested PCR on the 18s rRNA gene and amplicon sequencing (GenBank accession number MN296295), allowed us to finally identify the DNA of *B. capreoli*, closely related species to *B. divergens*, circulating in wild ruminants and unable to infect Human and cattle erythrocytes [26].

	Months	Analysed nymph number	*B. burgdorferi ss*	*B. garinii*	*B. afzelii*	*B. valaisiana*	*B. spielmanii*	*B. burgdorferi sl*	*B. miyamotoi*	*Borrelia spp*	*A. phagocytophilum*	*Anaplasma spp*	*R. helvetica*	*R. felis*	*Rickettsia spp*	*B. venatorum*	*Babesia spp*	Co-infections (2 TBP)	Co-infections (3 TBP)	Total
2014	Apr-14	89	0	0	0	0	0	0	0	**0**	10	**10**	7	0	**7**	0	**1**	0	0	**18**
	May-14	127	1	0	0	0	0	1	1	**2**	12	**12**	2	0	**2**	4	**4**	2	0	**18**
	Jun-14	54	0	1	1	1	0	1	0	**1**	4	**4**	9	1	**10**	2	**2**	1	1	**16**
	Jul-14	38	0	1	0	0	1	1	0	**1**	6	**6**	1	0	**1**	0	**0**	1	0	**8**
	Aug-14	9	0	0	0	0	0	0	0	**0**	0	**0**	1	0	**1**	0	**0**	0	0	**1**
	Sep-14	5	1	0	0	0	0	1	0	**1**	0	**0**	0	0	**0**	1	**1**	0	0	**2**
	Oct-14	13	0	0	0	1	0	1	0	**1**	1	**1**	0	0	**0**	0	**0**	0	0	**2**
2015	Jan-15	2	0	0	0	0	0	0	0	**0**	0	**0**	0	0	**0**	0	**0**	0	0	**0**
	Feb-15	3	0	0	1	0	0	1	0	**1**	0	**0**	0	0	**0**	0	**0**	0	0	**1**
	Mar-15	43	0	3	1	1	1	3	3	**6**	1	**1**	5	0	**5**	0	**0**	1	1	**12**
	Apr-15	69	2	0	0	0	0	2	0	**2**	3	**4**	4	0	**4**	0	**0**	0	0	**10**
	May-15	88	0	0	0	0	0	0	1	**1**	1	**1**	3	0	**3**	2	**2**	0	0	**7**
	Jun-15	78	1	0	0	0	1	2	2	**4**	2	**2**	1	0	**1**	2	**2**	1	0	**8**
	Jul-15	21	0	2	2	0	0	3	1	**4**	0	**0**	0	0	**0**	0	**0**	1	0	**4**
	Aug-15	6	0	0	0	0	0	0	0	**0**	0	**0**	0	0	**0**	0	**0**	0	0	**0**
	Sep-15	6	0	0	0	0	0	0	0	**0**	0	**0**	0	0	**0**	0	**0**	0	0	**0**
	Oct-15	17	4	0	0	0	0	4	0	**4**	0	**0**	0	0	**0**	0	**0**	0	0	**4**
	Nov-15	2	0	0	0	0	0	0	1	**1**	0	**0**	0	0	**0**	0	**0**	0	0	**1**
2016	Jan-16	9	0	0	1	0	0	1	0	**1**	0	**0**	1	0	**1**	0	**0**	0	0	**2**
	Feb-16	10	0	0	0	0	0	0	0	**0**	0	**0**	1	0	**1**	0	**0**	0	0	**1**
	Mar-16	26	2	1	2	0	0	4	0	**4**	1	**1**	0	0	**0**	0	**0**	1	0	**5**
	Apr-16	33	0	0	0	1	0	1	0	**1**	0	**0**	2	0	**2**	0	**0**	0	0	**3**
	Jul-16	78	2	2	2	0	1	5	0	**5**	2	**2**	4	0	**4**	1	**1**	1	1	**11**
	Aug-16	11	2	0	0	0	0	2	0	**2**	0	**0**	2	0	**2**	0	**0**	1	0	**3**
	Sep-16	5	0	0	0	0	0	0	0	**0**	0	**0**	1	0	**1**	0	**0**	0	0	**1**
	Oct-16	6	0	0	0	0	0	0	1	**1**	0	**0**	0	0	**0**	0	**0**	0	0	**1**
2017	Feb-17	6	0	0	0	0	0	0	1	**1**	0	**0**	0	0	**0**	0	**0**	0	0	**1**
	Mar-17	53	0	1	0	1	0	2	0	**2**	3	**3**	0	0	**0**	2	**2**	0	0	**7**
	Apr-17	50	0	0	1	1	0	2	0	**2**	3	**3**	0	0	**0**	1	**1**	0	0	**6**
	May-17	41	0	0	0	0	0	0	1	**1**	4	**4**	1	0	**1**	0	**0**	0	0	**6**
	Apr-14 to May-17	**998**	**15**	**11**	**11**	**6**	**4**	**37**	**12**	**49**	**53**	**54**	**45**	**1**	**46**	**15**	**16**	**10**	**3**	**159**
	Number of positive months	30	8	7	8	6	4	18	9	**23**	14	**14**	16	1	**16**	8	**9**	9	3	**27**

Table 1. Summary table of the TBP detection study results.

Temporal patterns of TBP in *Ixodes ricinus* nymphs

TBP prevalence at the monthly scale

In the following paragraph and corresponding figures, prevalences are those obtained for months with at least nine collected ticks.

Global infection rates fluctuated from 8% [3.3%, 15.7%] in May 2015 to 29.6% [18.0%, 43.6%] in June 2014 (**Figure 3**). At the genus level, variations in TBP prevalences and the number of months for which at least one tick was positive for each tested TBP are presented in **Figure 4** and **Table 1**.

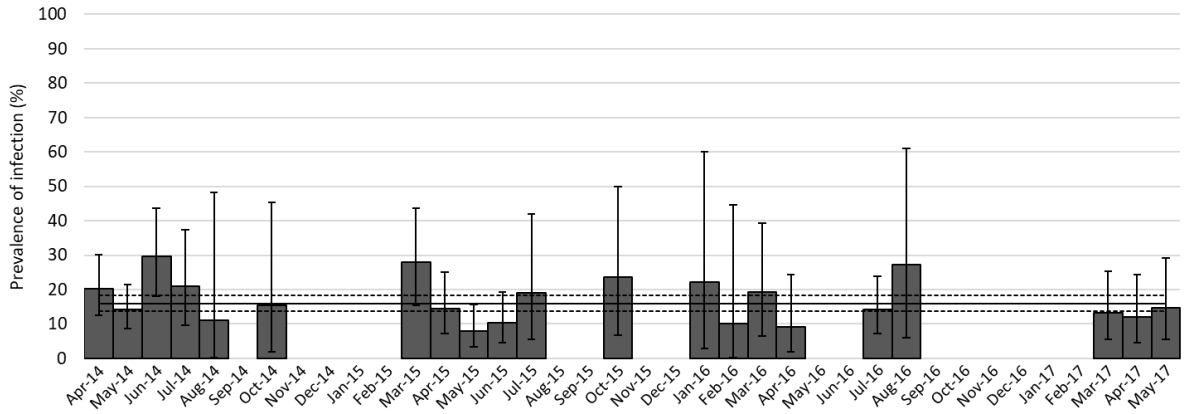
DNA from pathogens belonging to both genera *Rickettsia* and *Anaplasma* were detected respectively in 16 and 14 of the 30 sampled months respectively. When detected, prevalences fluctuated from 1.3% [0.03%, 6.9%] (June 2015) to 18.5% [9.3%, 31.4%] (June 2014) for *Rickettsia* and from 1.1% [0.03%, 6.2%] (May 2015) to 15.8% [6.0%, 31.3%] (July 2014) for *Anaplasma*. Both genera are mainly represented by one species: *R. helvetica* and *A. phagocytophilum* that are the most frequently detected species (16 and 14 /30 months respectively). These two species were found each year.

DNA from members of the *Borrelia* genus was detected in 23 of the 30 sampled months. This bacterial genus displayed the highest variability with prevalences fluctuating from 1.1% [0.03%, 6.2%] (May 2015) to 23.5% [6.8%, 49.9%] (October 2015). DNA from members of the LB group was detected in 18 of the 30 sampled months with prevalences ranging from 0.8% [0.03%, 6.2%] in May 2014 to 23.5% [6.8%, 49.9%] in October 2015. The most frequently identified species were *B. burgdorferi* s.s. (8 / 30 sampled months), *B. afzelii* (8 / 30) and *B. garinii* (7 / 30). DNA from these species was regularly detected over the three studied years. Conversely, *B. valaisiana* (6 / 30) and *B. spielmanii* (4 / 30) DNA was not detected during 11 (from April 2015 to March 2016) and 9 (from July 2015 to April 2016) consecutive months respectively. *Borrelia miyamotoi* (relapsing fever group) DNA was detected 9 times over the 30 sampled months with prevalences ranging from 0.8% [0.02%, 4.3%] in May 2014 to 7% [1.5%, 19.1%] in March 2015.

For parasites, DNA from the genus *Babesia* was detected in 9 months out of 30 sampled months. Prevalences presented the lowest variability ranging from 1.1% [0.03%, 6.1%] in April 2014 to 3.8% [0.5%, 13.0%] in March 2017 (**Figure 4**). The main detected species was

300 *B. venatorum* that was detected 9 times over 30 samplings and not detected during 9
301 consecutive sampled months, from June 2015 to April 2016.

302



303

304 **Figure 3. Nymph infection rate per month for at least one tested pathogen.** Months with less than
305 nine nymphs sampled have not been considered for percentage calculation. Error bars represent
306 confidence intervals of the percentage.

307

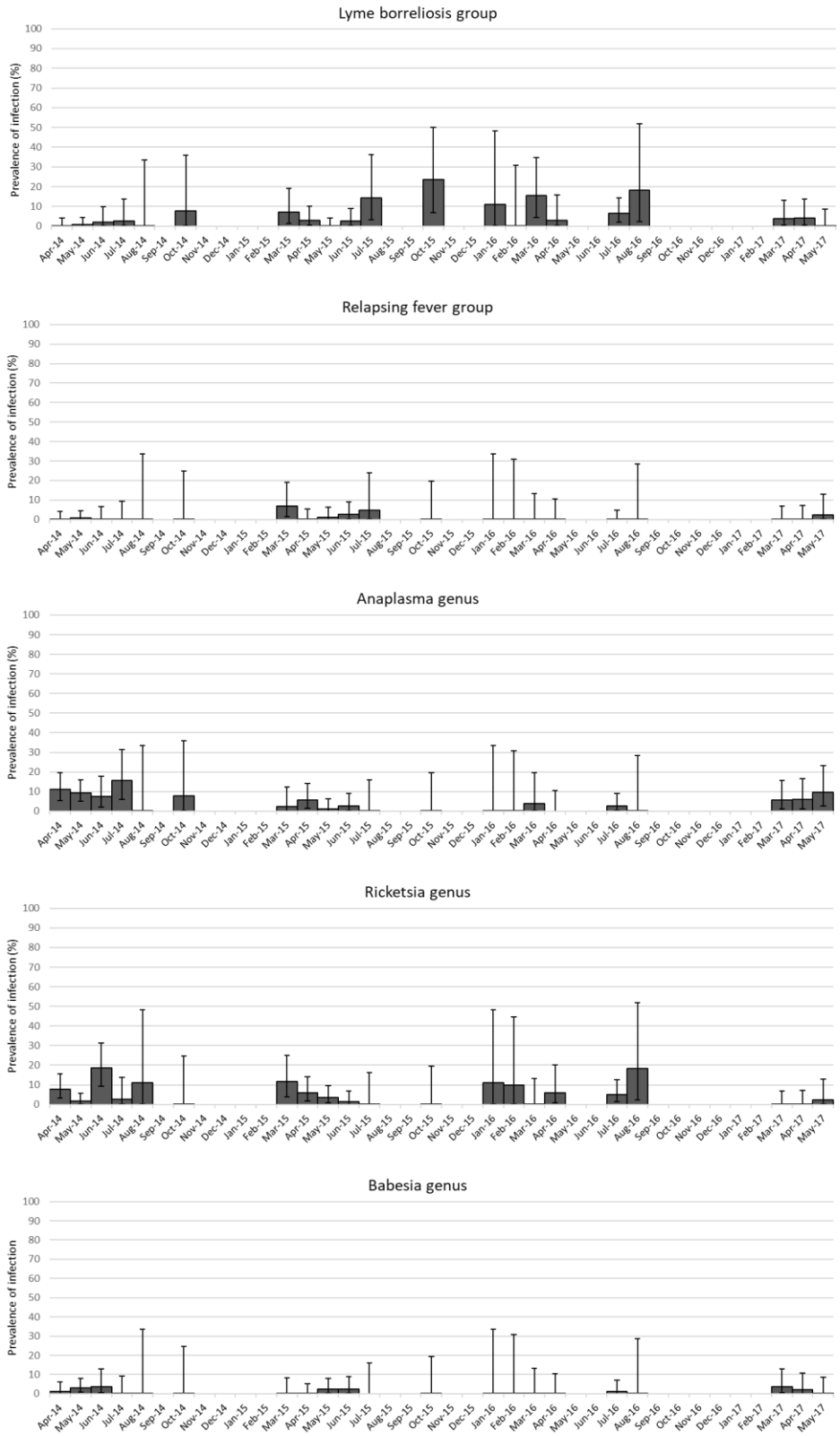


Figure 4. Nymph infection rate and confidence intervals per month for the different TBP. Months with less than nine nymphs sampled have not been considered. Error bars represent confidence intervals of the percentage.

TBP prevalences at the seasonal and multi-annual scale

In order to determine if the prevalence of TBP was different within and between years, a multivariable logistic regression model was performed. The spring season and the year 2014 have been considered as references for the seasonal and yearly effect respectively. Because some TBPs had too low prevalences in the nymph population (producing unreliable statistics), analyses were only performed on the most prevalent TBPs: *A. phagocytophilum*, *R. helvetica*, *B. burgdorferi* s.l., *B. miyamotoi* and *B. venatorum*.

Significant differences were observed at the seasonal scale (**Figure 5**, **Table 2**) for *R. helvetica* (higher in summer compared to spring), *B. burgdorferi* s.l. (higher in autumn compared to spring) and *B. miyamotoi* (higher in winter than in spring). Please note that the smallest number of sampled ticks (30 in total) was found in winter, and that the difference observed for *B. miyamotoi* in winter corresponded to only one infected tick collected in February 2017.

Significant differences were also observed between years for bacteria belonging to the complex *B. burgdorferi* s.l. with higher infection rates in 2015 and 2016 compared to 2014; for *A. phagocytophilum*, which was lower in 2015 and in 2016 compared to 2014 and for *R. helvetica*, which was lower in 2017 than in 2014. However, please note that samplings were only performed from January to May in 2017. No significant differences were observed according to season or year for *B. venatorum*.

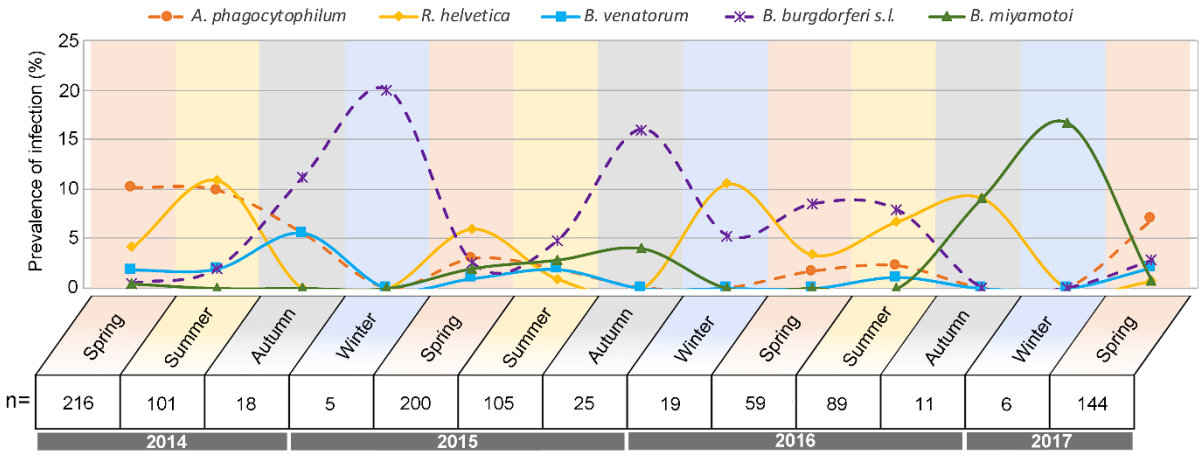


Figure 5. Percentage of positive nymphs per season for the most prevalent TBPs. Winter (pastel blue background) = January to February – Spring (pastel orange background) = March to May – Summer

(pastel yellow background) = June to August – Autumn (light grey background) = September to November – n = Number of analysed ticks.

Model	TBP	Variable	Odds Ratio	95% Confidence Interval		
				Low	High	
(1)	*B. burgdorferi sl*	Spring		REF		
		Autumn	**4.53**	**1.50**	**12.49**	*
		Summer	1.69	0.75	3.89	
		Winter	1.73	0.25	7.01	
		2014		REF		
		2015	**2.93**	**1.12**	**9.14**	*
		2016	**4.48**	**1.60**	**14.53**	*
		2017	2.45	0.57	9.95	
(2)	*B. miyamotoi*	Spring		REF		
		Autumn	0.00	NA	8.3275E+218	
		Summer	0.00	NA	2.26397E+88	
		Winter	**28.60**	**1.03**	**800.00**	*
(3)	*A. phagocytophilum*	2014		REF		
		2015	**0.20**	**0.08**	**0.42**	*
		2016	**0.16**	**0.04**	**0.45**	*
		2017	0.65	0.30	1.32	
(4)	*R. helvetica*	Spring		REF		
		Autumn	0.00	0.00	6.7759E+11	
		Summer	**3.10**	**1.27**	**7.85**	*
		Winter	0.00	NA	1.1447E+145	
		2014		REF		
		2015	1.34	0.54	3.39	
		2016	0.81	0.12	3.24	
		2017	**0.16**	**0.01**	**0.87**	*

Table 2. Multivariable logistic regression models assessing the seasonal and yearly TBP prevalence variations in nymphs. Odds ratios and their associated 95% confidence intervals obtained from the best model of TBP seasonal and yearly prevalence in questing nymphs. REF = Reference – NA = Not Applicable.

Pathogen associations

Among all the sampled ticks, 1% [0.5%, 1.8%] were co-infected with two pathogens and 0.3% [0.006%, 0.8%] were co-infected with three pathogens. Eight different co-infection profiles were found (**Table 3**). In most of cases (7/13), these co-infections concerned species belonging to the *Borrelia* genus: *B. garinii*/*B. afzelii*; *B. garinii*/*B. spielmanii*;

348 *B. garinii*/*B. afzelii*/*B. valaisiana* and *B. garinii*/*B. valaisiana*/*B. spielmanii*. Co-infections
349 profiles with species belonging to different genus were also observed:
350 *A. phagocytophilum*/*B. venatorum*; *A. phagocytophilum*/*R. helvetica*;
351 *B. burgdorferi* s.s./*R. helvetica* and *B. garinii*/*B. afzelii*/*R. helvetica*. All these associations
352 between pathogens were tested using the association screening approach (Vaumourin *et al*.,
353 2014). Compared to a random analysis, no associations were found to be under represented
354 while two were over represented: the first one between *B. garinii* and *B. afzelii* (observation =
355 3; min expected = 0; max expected = 2), and the second one between *B. garinii* and *B.*
356 *spielmanii* (observation = 2; min expected = 0; max expected = 1).

B. burgdorferi ss	*B. garinii*	*B. afzelii*	*B. valaisiana*	*B. spielmanii*	*A. phagocytophilum*	*R. helvetica*	*B. venatorum*	Co-occurrences number
	X	X						3
	X			X				2
	X	X	X					1
	X		X	X				1
					X		X	3
					X	X		1
X						X		1
	X	X				X		1

357

358 **Table 3. Summary table of the reported co-infection profiles.**

359

360 ## Discussion

361 ### *Ixodes ricinus* density and seasonal dynamics

362 This three-year survey demonstrated a clear seasonal pattern in *I. ricinus* density, with
363 a marked peak of questing nymphs in spring and a smaller peak in autumn. Low, but present
364 activity was detected in winter, as has been observed in Germany [27]. In addition to these
365 general patterns, some unexpected data were observed, the most striking being no peak
366 activity in spring 2017 (April and May) with tick densities very similar to those recorded in
367 March. Abiotic factors such as temperature, relative humidity, and rainfall, or fluctuating host
368 numbers in the sampling area are known to influence questing tick abundance and activity

patterns [28–33] and could explain these unusual observations. It's important to note that 2017 was distinguished by an abnormally wet March, with total rainfall much higher than that recorded in previous years in the same area (71.3, compared to 11.2, 33.6, and 61.7 mm rain/month in 2014, 2015, and 2016, respectively). Interestingly, the increased March rainfall was followed by an April drought (7.9 mm of rain/month in 2017, compared to 48.4, 27.2, and 66.2 mm rain/month in 2014, 2015, and 2016, respectively) (rainfall data estimated from the Orly station, Metéo-France data; https://donneespubliques.meteofrance.fr/?fond=produit&id_produit=90&id_rubrique=32). These unusual meteorological characteristics could explain the stable tick density from March to May 2017. Thereby, this finding clearly shows that the bimodal tick activity pattern usually observed during this study can punctually change with exceptional environmental conditions, reinforcing the importance of regular monitoring.

Ixodes ricinus-borne pathogen composition and prevalence over the three years

Most of the detected pathogen species corresponded to micro-organisms known to circulate in the Western Palearctic [34–41]. However, several species belonging to the *Bartonella* and *Francisella* genera, previously reported in the studied area [39,42], were not detected. The most prevalent pathogen species were *A. phagocytophilum* (5.4% of the examined nymphs), *R. helvetica* (4.5%), and *B. burgdorferi* s.l. (3.7%). Both high- and low-prevalence TBP were consistently detected in the sampling area for the duration of the study. Although prevalences varied between different TBPs, and some were not detected for long periods, they were all detected recurrently. Continued detection is consistent with the year-round presence of reservoir hosts in the sampling area (wood mice, bank voles, Siberian chipmunks, roe deer, common blackbird, European robin, song thrush…) [33,43,44]. The continued presence of reservoir hosts could facilitate the circulation of dominant species, and maintain, even at low rates, less prevalent pathogen species that may not be detected by a single sampling. This does support to regularly studying TBP temporal dynamics, to improve the assessment of their prevalence.

We also detected in a single tick, the DNA of the emergent human pathogen *R. felis*. Its detection is particularly scheming as this bacteria is known to be mainly transmitted from cat

399 to cat *via* fleas, with human contamination arising from cat or flea bites. As we only detected
400 DNA from *R. felis*, we cannot exclude that this detection could correspond to remnant DNA
401 from the previous blood meal. Nevertheless, several studies have already detected the
402 presence of *R. felis* or *R. felis*-like organisms in hematophagous arthropods [see in 45,46],
403 including in ticks collected from natural environments [47], and notably in two studies
404 performed on questing *I. ricinus*, including one based on RNA detection [48,49]. Rarely
405 investigated in studies dealing with TBP, the repeated detection of *R. felis* should encourage
406 increased surveillance for this spotted fever-causing pathogen in humans. Let's finally note
407 that all these findings suggest that a punctual sampling would certainly not facilitate the
408 detection of this pathogen, again highlighting the importance of collecting and analysing ticks
409 at a large temporal scale.

410 *B. divergens* and *B. capreoli* are two closely related species. During this study, we found
411 out that the design initially used to detect *B. divergens* was actually also able to detect the
412 DNA of *B. capreoli*. While *B. divergens* is responsible of babesiosis in human and cattle,
413 *B. capreoli* is only able to colonize erythrocytes from deer, therefore presenting no threat for
414 human or livestock [26]. These results emphases the importance of confirmation and careful
415 interpretations of microfluidic real time PCR [50].

416

417 **Seasonal and inter-annual dynamics of *I. ricinus*-borne pathogens**

418 Improving the prevention of TBD requires a better understanding of their temporal—
419 and in particular—their seasonal dynamics. However, only a few studies have addressed these
420 issues during a minimum three-year period [12,13]. As ticks were collected monthly for over
421 three years in this study, we detected significant seasonal or annual infection rate fluctuations
422 for four TBP: *R. helvetica*, *B. burgdorferi* s.l., *B. miyamotoi*, and *A. phagocytophilum*. Note that
423 the statistically significant highest prevalence of *B. miyamotoi* in winter is only due to the
424 detection of one positive tick sampled during winter in 2017. In our opinion, this result alone
425 is insufficient to presume that *B. miyamotoi* have an increased winter prevalence. However,
426 we can observe that even if very few ticks are questing during these periods, they may carry
427 TBP.

While significant seasonal and annual differences were observed for *B. miyamotoi* and *A. phagocytophilum*, respectively, the presence of *R. helvetica* and *B. burgdorferi* s.l. varied significantly according to both seasons and years. None of these micro-organisms presented a similar pattern to any others. Comparing our results to the pluri-annual studies previously mentioned, we observe that only *R. helvetica* presented similar seasonal patterns [12]. This finding again emphasises how the season, the year or the sampling area can influence TBP presence and prevalence in questing tick populations.

The most common explanation for temporal variations in TBP prevalences is the variable availability of reservoir hosts during tick previous stage feeding. This hypothesis was already suggested by Coipan *et al.* [12] while they observed that several micro-organisms, assumed to share the same reservoir host, also presented similar seasonal patterns. Because the tick lifecycle is fundamentally linked to its host, any changes to the available host spectrum will undoubtedly influence TBP prevalence in the tick community [51]. However, because the entire tick life cycle is pluri-annual, it is difficult anyway to know if nymphs questing at a same time did perform their previous blood meal at the same period. The same generation of questing nymphs could come from larvae that would have fed at different moment and thus potentially upon different host species. An alternative hypothesis, based on both the presence of pathogens and the tick physiology, could also explain these patterns. Carrying certain TBP was shown to improve tick resistance to challenging abiotic conditions. Herrmann and Gern [17,18] demonstrated that ticks carrying *Borrelia* species exhibited higher survival rates in desiccating conditions and a lower tendency to move to favourable conditions for maintaining water balance than non-infected ticks. This was associated to a higher reserve of energy in Borrelia infected ticks [52] which would therefore exhibit higher resistance capacities to hydric stress notably. This hypothesis would explain the higher prevalence of *Borrelia*-infected questing ticks observed during or after the summer period in the present study and in those of Coipan and Takken [12,13]. Similarly, Neelakanta [19] demonstrated a higher expression of *iafgp* gene, coding for an antifreeze glycoprotein, in *A. phagocytophilum*-infected ticks. This thus conferred to ticks a stronger resistance to cold that could lead to higher prevalence of *A. phagocytophilum*-infected questing ticks during or just after winter. This hypothesis was not consistent with our data as *A. phagocytophilum* was not observed in greater prevalence during the cold seasons of our study.

Our results, in combination with those from the literature, support the hypothesis that TBP prevalence is influenced by both biotic and abiotic factors, and suggest one more time that sporadic samplings are insufficient to assess it.

Pathogen co-occurrence

Tick co-infections are being identified more and more frequently [21,41,49,53–57]. Clinical co-infections with several TBP are commonly reported [58–60] and are known to affect both disease symptoms and severity [61,62]. It is thus essential to investigate TBP associations in ticks, to better identify potential clinical co-infections and to improve epidemiological knowledge of TBD.

In this longitudinal three-year study, two TBP associations were significantly over-represented compared to a random distribution: the first one was between *B. garinii* and *B. afzelii*, as has been previously observed in studies using similar detection tools [21,41], or different methods [16s rRNA gene sequencing, 63]; the second one was between *B. garinii* and *B. spielmanii*. Interestingly, these findings contrast with published results on *Ixodes ricinus* TBP. While performing a meta-analysis on data published from 2010 to 2016, Strnad *et al* [64] observed a negative correlation between *B. garinii* and *B. afzelii*. Similarly, Herrmann *et al.* [65] also detected a negative co-occurrence between these two species following the analysis of 7400 nymphs collected over three years. These results are coherent considering the host specificity of these Borrelia species. Indeed, *B. garinii* doesn't share the same reservoir host (birds) than *B. afzelii* or *B. spielmanii* (wood mice and bank voles, or hazel and garden dormice) [66–71], and none of these species are known to be transmitted transovarially.

Even though the associations we identified were statistically "over-represented", in actual fact we only observed one more association than the fixed over-representation threshold (i.e. observed associations = 3 and 2; minimum expected = 0 and 0; maximum expected = 2 and 1; for *B. garinii*/*B. afzelii* and *B. garinii*/*B. spielmanii* associations, respectively). This indicates that caution should be applied when drawing conclusions about permanent associations between these different bacteria in ticks. Several different hypotheses could potentially explain these associations in the same nymph. Firstly, hosts are likely to carry several adjacent feeding ticks. This phenomenon, known as co-feeding, could promote

pathogen exchange between ticks even in the absence of systemic host infection [72]. Secondly, as discussed by van Duijvendijk *et al*. [73], when bloodmeals are disrupted due to host grooming, immune response or death, ticks may feed on more than one host to completely engorge, and consequently be exposed to several pathogens. Thirdly, despite these TBP species segregating between bird and rodent hosts, all of them have been detected in hedgehogs [74,75], and *B. afzelii* and *B. garinii* have been simultaneously detected in one Siberian chipmunk [44]. Both of these mammals were found to host a large number of tick larvae [44,76], and Siberian chipmunks have been reported to induce higher *B. burgdorferi* s.l. infection rates in nymphs, compared to bank voles and wood mice [44] in the Sénart forest. A last hypothesis might be that our analyses methods are unable to distinguish the rodent-circulating *B. garinii* OspA serotype 4 (corresponding to *B. bavariensis*) [77] from other *B. garinii* serotypes.

Associations between *B. garinii* and *B. valaisiana* are frequently reported, which isn't surprising as these species share the same reservoir host [78]. This association was the most common TBP association in a meta-analysis of literature published between 1984 and 2003 [79], and has been reported several times since in later studies [11,65,80]. While we observed this association twice, both times in association with a third *Borrelia* species, either *B. afzelii* or *B. spielmanii*, it was not significantly over-represented compared to a random distribution. Among the three previously mentioned studies, only Herrmann *et al.* [65] demonstrated that this association was over-represented when compared to a randomly sampled analysis. However, our study was performed on a much smaller dataset (998 versus 7400 analysed nymphs), with a halved co-infection percentage (1.3% versus 3%), indicating that our statistical analysis may be less powerful, which could explain why this association wasn't detected.

These contrasting tick pathogen association results highlight the complexity in clearly identifying pathogen associations in field-collected ticks. Several other parameters can also potentially influence pathogen association (host spectrum within the studied area, sample size influencing analytical statistical power, identification bias…). In this context, performing investigations under controlled conditions (suitable TBP growing and tick breeding systems…) will be a crucial future step to experimentally test these different associations and improve our knowledge on TBP co-occurrence.

Conclusions

This three-year study of *I. ricinus*-borne pathogens; (1) identified several TBP previously reported in the area, consistent with reservoir host availability; (2) allowed the surprising detection of *R. felis* DNA, a micro-organism rarely reported in questing ticks; (3) highlighted significant variations in seasonal and inter-annual pathogen prevalence; and finally (4) identified several unexpected co-occurrences between pathogens belonging to the *B. burgdorferi* s.l. complex. All these results represent another step towards understanding the TBPs ecology and emphase the need to perform longitudinal study. Especially since the main factors that are supposed to influence tick and TBP ecology may change in the next coming years with climate changes. Associated to other factors such as host information or meteorological measures, this kind of data is crucial to allow a better understanding of TBP ecology and TBD epidemiology.

Additional files

Additional file 1: Table S1. Targeted gene, amplicon size, primers and probe sequences used for TBP and Tick species detection.

Additional file 2: Table S2. Targeted gene and primers sequences used for results confirmation.

Abbreviation

TBP: tick-borne pathogens; TBD: tick-borne disease; LB: lyme borreliosis; *I. ricinus*: *Ixodes ricinus*; s.l.: sensu lato; s.s.: sensu stricto; *B. burgdorferi*: *Borrelia burgdorferi*; *B. afzelii*: *Borrelia afzelii*; *B. garinii*: *Borrelia garinii*; *B. valaisiana*: *Borrelia valaisiana*; *B. spielmanii*: *Borrelia spielmanii*; *B. lusitaniae*: *Borrelia lusitaniae*; *B. bissettii*: *Borrelia bissettii*; *B. miyamotoi*: *Borrelia miyamotoi*; *A. phagocytophilum*: *Anaplasma phagocytophilum*; *A. marginale*: *Anaplasma marginale*; *A. platys*: *Anaplasma platys*; *A. centrale*: *Anaplasma centrale*; *A. bovis*: *Anaplasma bovis*; *B. venatorum*: *Babesia venatorum*; *B. divergens*: *Babesia divergens*; *B. capreoli*: *Babesia capreoli*; *B. microti*: *Babesia microti*; *B. caballi*: *Babesia caballi*; *B. canis*: *Babesia canis*; *B. bovis*: *Babesia bovis*; *B. ovis*: *Babesia ovis*; *B. henselae*: *Bartonella*

548 *henselae*; *E. canis*: *Ehrlichia* canis; *R. helvetica*: *Rickettsia helvetica*; *R. felis*: *Rickettsia felis*;
549 *R. conorii*: *Rickettsia conorii*; *R. slovaca*: *Rickettsia slovaca*; *R. massiliae*: *Rickettsia massiliae*;
550 *R. aeshlimanii*: *Rickettsia aeshlimanii*; DMEM: Dulbecco's Modified Eagle Medium; NCBI:
551 National Center for Biotechnology Information; BLAST: Basic Local Alignment Search Tool;
552 GLM: Generalized Linear Model; AIC: Akaike Information Criterion; OR: Odds Ratios; NP:
553 Number of Pathogen species tested; NC: Number of possible Combination; iafgp: *Ixodes*
554 *scapularis* antifreeze glycoprotein

555

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568

569 **Authors’ contributions**

570 Conceived and designed the experiments: TP, MVT, JFC, KCM, EL. Performed the experiments:
571 EL. Analysed the data: EL, TP, SM, MM, KCM. Wrote the paper: EL, MM, KCM, JFC, SM, MVT, TP

572

573 ## References

574 1. Jongejan F, Uilenberg G. The global importance of ticks. Parasitology. 2004;129:S3–14.

575 2. de la Fuente J, Estrada-Pena A, Venzal JM, Kocan KM, Sonenshine DE. Overview: ticks as vectors of
576 pathogens that cause disease in humans and animals. Front Biosci. 2008;13:6938–6946.

577 3. Dantas-Torres F, Chomel BB, Otranto D. Ticks and tick-borne diseases: a One Health perspective.
578 Trends Parasitol. 2012;28:437–46.

579 4. Strle F. Human granulocytic ehrlichiosis in Europe. Int J Med Microbiol Suppl. 2004;293:27–35.

580 5. Bonnet S, Jouglin M, L'Hostis M, Chauvin A. Babesia sp. EU1 from Roe Deer and Transmission within
581 Ixodes ricinus. Emerg Infect Dis. 2007;13:1208–10.

582 6. Bonnet S, Jouglin M, Malandrin L, Becker C, Agoulon A, L'hostis M, et al. Transstadial and transovarial
583 persistence of Babesia divergens DNA in Ixodes ricinus ticks fed on infected blood in a new skin-feeding
584 technique. Parasitology. 2007;134:197–207.

585 7. Cotté V, Bonnet S, Le Rhun D, Le Naour E, Chauvin A, Boulouis H-J, et al. Transmission of Bartonella
586 henselae by Ixodes ricinus. Emerg Infect Dis. 2008;14:1074–80.

587 8. Bonnet S, Brisseau N, Hermouet A, Jouglin M, Chauvin A. Experimental in vitro transmission of
588 Babesia sp. (EU1) by Ixodes ricinus. Vet Res. 2009;40:1–8.

589 9. Sprong H, Wielinga PR, Fonville M, Reusken C, Brandenburg AH, Borgsteede F, et al. Ixodes ricinus
590 ticks are reservoir hosts for Rickettsia helvetica and potentially carry flea-borne Rickettsia species.
591 Parasit Vectors. 2009;2:41.

592 10. Gassner F, van Vliet AJH, Burgers SLGE, Jacobs F, Verbaarschot P, Hovius EKE, et al. Geographic and
593 Temporal Variations in Population Dynamics of Ixodes ricinus and Associated Borrelia Infections in The
594 Netherlands. Vector-Borne Zoonotic Dis. 2010;11:523–32.

595 11. Reye AL, Hübschen JM, Sausy A, Muller CP. Prevalence and Seasonality of Tick-Borne Pathogens in
596 Questing Ixodes ricinus Ticks from Luxembourg. Appl Environ Microbiol. 2010;76:2923–31.

597 12. Coipan EC, Jahfari S, Fonville M, Maassen C, van der Giessen J, Takken W, et al. Spatiotemporal
598 dynamics of emerging pathogens in questing Ixodes ricinus. Front Cell Infect Microbiol [Internet]. 2013
599 [cited 2018 Jul 25];3. Available from:
600 https://www.frontiersin.org/articles/10.3389/fcimb.2013.00036/full

601 13. Takken W, van Vliet AJH, Verhulst NO, Jacobs FHH, Gassner F, Hartemink N, et al. Acarological Risk
602 of Borrelia burgdorferi Sensu Lato Infections Across Space and Time in The Netherlands. Vector-Borne
603 Zoonotic Dis. 2016;17:99–107.

604 14. Chvostáč M, Špitalská E, Václav R, Vaculová T, Minichová L, Derdáková M. Seasonal Patterns in the
605 Prevalence and Diversity of Tick-Borne Borrelia burgdorferi Sensu Lato, Anaplasma phagocytophilum
606 and Rickettsia spp. in an Urban Temperate Forest in South Western Slovakia. Int J Environ Res Public
607 Health. 2018;15:994.

608 15. Gilbert L, Maffey GL, Ramsay SL, Hester AJ. The effect of deer management on the abundance of
609 Ixodes ricinus in Scotland. Ecol Appl. 2012;22:658–67.

610 16. van Wieren SE, Hofmeester TR. 6. The role of large herbivores in Ixodes ricinus and Borrelia
611 burgdorferi s.l. dynamics. Ecol Prev Lyme Borreliosis [Internet]. Wageningen Academic Publishers; 2016
612 [cited 2018 Nov 19]. p. 75–89. Available from:
613 https://www.wageningenacademic.com/doi/abs/10.3920/978-90-8686-838-4_6

614 17. Herrmann C, Gern L. Survival of Ixodes ricinus (Acari: Ixodidae) Under Challenging Conditions of
615 Temperature and Humidity is Influenced by Borrelia burgdorferi sensu lato Infection. J Med Entomol.
616 2010;47:1196–204.

617 18. Herrmann C, Gern L. Do the level of energy reserves, hydration status and Borrelia infection
618 influence walking by Ixodes ricinus (Acari: Ixodidae) ticks? Parasitology. 2012;139:330–7.

619 19. Neelakanta G, Sultana H, Fish D, Anderson JF, Fikrig E. Anaplasma phagocytophilum induces Ixodes
620 scapularis ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold. J
621 Clin Invest. 2010;120:3179–90.

622 20. Michelet L, Delannoy S, Devillers E, Umhang G, Aspan A, Juremalm M, et al. High-throughput
623 screening of tick-borne pathogens in Europe. Front Cell Infect Microbiol. 2014;4:103.

624 21. Moutailler S, Moro CV, Vaumourin E, Michelet L, Tran FH, Devillers E, et al. Co-infection of Ticks:
625 The Rule Rather Than the Exception. PLoS Negl Trop Dis. 2016;10:e0004539.

626 22. Gondard M, Delannoy S, Pinarello V, Aprelon R, Devillers E, Galon C, et al. Upscaling surveillance of
627 tick-borne pathogens in the French Caribbean islands. bioRxiv. 2019;532457.

628 23. McCullagh P, Nelder JA. Generalized Linear Models. [Internet]. 2nd Edition. London: Chapman et
629 Hall; 1989 [cited 2019 Feb 28]. Available from: https://www.crcpress.com/Generalized-Linear-
630 Models/McCullagh-Nelder/p/book/9780412317606

631 24. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria:
632 R Foundation for Statistical Computing; 2018. Available from: https://www.R-project.org/

633 25. Vaumourin E, Vourc'h G, Telfer S, Lambin X, Salih D, Seitzer U, et al. To be or not to be associated:
634 power study of four statistical modeling approaches to identify parasite associations in cross-sectional
635 studies. Front Cell Infect Microbiol. 2014;4:62.

636 26. Malandrin L, Jouglin M, Sun Y, Brisseau N, Chauvin A. Redescription of Babesia capreoli (Enigk and
637 Friedhoff, 1962) from roe deer (Capreolus capreolus): Isolation, cultivation, host specificity, molecular
638 characterisation and differentiation from Babesia divergens. Int J Parasitol. 2010;40:277–84.

639 27. Dautel H, Dippel C, Kämmer D, Werkhausen A, Kahl O. Winter activity of Ixodes ricinus in a Berlin
640 forest. Int J Med Microbiol. 2008;298:50–4.

641 28. Perret J-L, Guigoz E, Rais O, Gern L. Influence of saturation deficit and temperature on Ixodes ricinus
642 tick questing activity in a Lyme borreliosis-endemic area (Switzerland). Parasitol Res. 2000;86:554–7.

643 29. Gilbert L. Altitudinal patterns of tick and host abundance: a potential role for climate change in
644 regulating tick-borne diseases? Oecologia. 2010;162:217–25.

645 30. Tagliapietra V, Rosà R, Arnoldi D, Cagnacci F, Capelli G, Montarsi F, et al. Saturation deficit and deer
646 density affect questing activity and local abundance of Ixodes ricinus (Acari, Ixodidae) in Italy. Vet
647 Parasitol. 2011;183:114–24.

648 31. Schulz M, Mahling Monia, Pfister Kurt. Abundance and seasonal activity of questing Ixodes ricinus
649 ticks in their natural habitats in southern Germany in 2011. J Vector Ecol. 2014;39:56–65.

650 32. Vourc'h G, Abrial D, Bord S, Jacquot M, Masséglia S, Poux V, et al. Mapping human risk of infection
651 with Borrelia burgdorferi sensu lato, the agent of Lyme borreliosis, in a periurban forest in France. Ticks
652 Tick-Borne Dis. 2016;7:644–52.

653 33. Marchant A, Coupanec AL, Joly C, Perthame E, Sertour N, Garnier M, et al. Infection of Ixodes ricinus
654 by Borrelia burgdorferi sensu lato in peri-urban forests of France. PLOS ONE. 2017;12:e0183543.

655 34. Capelli G, Ravagnan S, Montarsi F, Ciocchetta S, Cazzin S, Porcellato E, et al. Occurrence and
656 identification of risk areas of Ixodes ricinus-borne pathogens: a cost-effectiveness analysis in north-
657 eastern Italy. Parasit Vectors. 2012;5:61.

658 35. Overzier E, Pfister K, Herb I, Mahling M, Böck G, Silaghi C. Detection of tick-borne pathogens in roe
659 deer (Capreolus capreolus), in questing ticks (Ixodes ricinus), and in ticks infesting roe deer in southern
660 Germany. Ticks Tick-Borne Dis. 2013;4:320–8.

661 36. Pangrácová L, Derdáková M, Pekárik L, Hviščová I, Víchová B, Stanko M, et al. Ixodes ricinus
662 abundance and its infection with the tick-borne pathogens in urban and suburban areas of Eastern
663 Slovakia. Parasit Vectors. 2013;6:238.

664 37. Reye AL, Stegniy V, Mishaeva NP, Velhin S, Hübschen JM, Ignatyev G, et al. Prevalence of Tick-Borne
665 Pathogens in Ixodes ricinus and Dermacentor reticulatus Ticks from Different Geographical Locations
666 in Belarus. PLOS ONE. 2013;8:e54476.

667 38. Hansford KM, Fonville M, Jahfari S, Sprong H, Medlock JM. Borrelia miyamotoi in host-seeking
668 Ixodes ricinus ticks in England. Epidemiol Infect. 2015;143:1079–87.

669 39. Paul REL, Cote M, Le Naour E, Bonnet SI. Environmental factors influencing tick densities over seven
670 years in a French suburban forest. Parasit Vectors. 2016;9:309.

671 40. Sormunen JJ, Penttinen R, Klemola T, Hänninen J, Vuorinen I, Laaksonen M, et al. Tick-borne
672 bacterial pathogens in southwestern Finland. Parasit Vectors. 2016;9:168.

673 41. Raileanu C, Moutailler S, Pavel I, Porea D, Mihalca AD, Savuta G, et al. Borrelia Diversity and Co-
674 infection with Other Tick Borne Pathogens in Ticks. Front Cell Infect Microbiol [Internet]. 2017 [cited
675 2019 Jan 14];7. Available from: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00036/full

676 42. Reis C, Cote M, Paul REL, Bonnet S. Questing Ticks in Suburban Forest Are Infected by at Least Six
677 Tick-Borne Pathogens. Vector-Borne Zoonotic Dis. 2010;11:907–16.

678 43. Marsot M, Henry P-Y, Vourc'h G, Gasqui P, Ferquel E, Laignel J, et al. Which forest bird species are
679 the main hosts of the tick, Ixodes ricinus, the vector of Borrelia burgdorferi sensu lato, during the
680 breeding season? Int J Parasitol. 2012;42:781–8.

681 44. Marsot M, Chapuis J-L, Gasqui P, Dozières A, Masséglia S, Pisanu B, et al. Introduced Siberian
682 Chipmunks (Tamias sibiricus barberi) Contribute More to Lyme Borreliosis Risk than Native Reservoir
683 Rodents. PLOS ONE. 2013;8:e55377.

684 45. Reif KE, Macaluso KR. Ecology of Rickettsia felis: A Review. J Med Entomol. 2009;46:723–36.

685 46. Brown LD, Macaluso KR. Rickettsia felis, an Emerging Flea-Borne Rickettsiosis. Curr Trop Med Rep.
686 2016;3:27–39.

687 47. Oliveira K, S Oliveira L, C A Dias C, Silva A, R Almeida M, Almada G, et al. Molecular identification of
688 Rickettsia felis in ticks and fleas from an endemic area for Brazilian Spotted Fever. 2008.

689 48. Vayssier-Taussat M, Moutailler S, Michelet L, Devillers E, Bonnet S, Cheval J, et al. Next Generation
690 Sequencing Uncovers Unexpected Bacterial Pathogens in Ticks in Western Europe. PLOS ONE.
691 2013;8:e81439.

692 49. Lejal E, Moutailler S, Šimo L, Vayssier-Taussat M, Pollet T. Tick-borne pathogen detection in midgut
693 and salivary glands of adult Ixodes ricinus. Parasit Vectors. 2019;12:152.

694 50. Sprong H, Fonville M, Docters van Leeuwen A, Devillers E, Ibañez-Justicia A, Stroo A, et al. Detection
695 of pathogens in Dermacentor reticulatus in northwestern Europe: evaluation of a high-throughput
696 array. Heliyon. 2019;5:e01270.

697 51. Pfäffle M, Littwin N, Muders SV, Petney TN. The ecology of tick-borne diseases. Int J Parasitol.
698 2013;43:1059–77.

699 52. Herrmann C, Voordouw MJ, Gern L. Ixodes ricinus ticks infected with the causative agent of Lyme
700 disease, Borrelia burgdorferi sensu lato, have higher energy reserves. Int J Parasitol. 2013;43:477–83.

701 53. Halos L, Jamal T, Maillard R, Beugnet F, Menach AL, Boulouis H-J, et al. Evidence of Bartonella sp. in
702 questing adult and nymphal Ixodes ricinus ticks from France and co-infection with Borrelia burgdorferi
703 sensu lato and Babesia sp. Vet Res. 2005;36:79–87.

704 54. Schicht S, Junge S, Schnieder T, Strube C. Prevalence of Anaplasma phagocytophilum and
705 Coinfection with Borrelia burgdorferi Sensu Lato in the Hard Tick Ixodes ricinus in the City of Hanover
706 (Germany). Vector-Borne Zoonotic Dis. 2011;11:1595–7.

707 55. Andersson M, Bartkova S, Lindestad O, Råberg L. Co-Infection with 'Candidatus Neoehrlichia
708 mikurensis' and Borrelia afzelii in Ixodes ricinus Ticks in Southern Sweden. Vector-Borne Zoonotic Dis.
709 2013;13:438–42.

710 56. Cosson J-F, Michelet L, Chotte J, Le Naour E, Cote M, Devillers E, et al. Genetic characterization of
711 the human relapsing fever spirochete Borrelia miyamotoi in vectors and animal reservoirs of Lyme
712 disease spirochetes in France. Parasit Vectors. 2014;7:233.

713 57. Castro LR, Gabrielli S, Iori A, Cancrini G. Molecular detection of Rickettsia, Borrelia, and Babesia
714 species in Ixodes ricinus sampled in northeastern, central, and insular areas of Italy. Exp Appl Acarol.
715 2015;66:443–52.

716 58. Tijsse-Klasen E, Sprong H, Pandak N. Co-infection of Borrelia burgdorferi sensu lato and Rickettsia
717 species in ticks and in an erythema migrans patient. Parasit Vectors. 2013;6:347.

718 59. Moniuszko A, Dunaj J, Święcicka I, Zambrowski G, Chmielewska-Badora J, Żukiewicz-Sobczak W, et
719 al. Co-infections with Borrelia species, Anaplasma phagocytophilum and Babesia spp. in patients with
720 tick-borne encephalitis. Eur J Clin Microbiol Infect Dis. 2014;33:1835–41.

721 60. Hoversten K, Bartlett MA. Diagnosis of a tick-borne coinfection in a patient with persistent
722 symptoms following treatment for Lyme disease. Case Rep. 2018;2018:bcr-2018-225342.

723 61. Krause PJ, Telford SR, Spielman A, Sikand V, Ryan R, Christianson D, et al. Concurrent Lyme Disease
724 and Babesiosis: Evidence for Increased Severity and Duration of Illness. JAMA. 1996;275:1657–60.

725 62. Diuk-Wasser MA, Vannier E, Krause PJ. Coinfection by Ixodes Tick-Borne Pathogens: Ecological,
726 Epidemiological, and Clinical Consequences. Trends Parasitol. 2016;32:30–42.

727 63. Aivelo T, Norberg A, Tschirren B. Human pathogen co-occurrence in Ixodes ricinus ticks: effects of
728 landscape topography, climatic factors and microbiota interactions. bioRxiv. 2019;559245.

729 64. Strnad M, Hönig V, Růžek D, Grubhoffer L, Rego ROM. Europe-Wide Meta-Analysis of Borrelia
730 burgdorferi Sensu Lato Prevalence in Questing Ixodes ricinus Ticks. Stabb EV, editor. Appl Environ
731 Microbiol. 2017;83:e00609-17.

732 65. Herrmann C, Gern L, Voordouw MJ. Species co-occurrence patterns among Lyme borreliosis
733 pathogens in the tick vector Ixodes ricinus. Appl Environ Microbiol. 2013;AEM.02158-13.

734 66. Humair P-F, Postic D, Wallich R, Gern L. An Avian Reservoir (Turdus merula) of the Lyme Borreliosis
735 Spirochetes. Zentralblatt Für Bakteriol. 1998;287:521–38.

736 67. Kurtenbach K, Carey D, Hoodless AN, Nuttall PA, Randolph SE. Competence of Pheasants as
737 Reservoirs for Lyme Disease Spirochetes. J Med Entomol. 1998;35:77–81.

738 68. Huegli D, Hu CM, Humair P-F, Wilske B, Gern L. Apodemus Species Mice Are Reservoir Hosts of
739 Borrelia garinii OspA Serotype 4 in Switzerland. J Clin Microbiol. 2002;40:4735–7.

740 69. Richter D, Schlee DB, Allgower R, Matuschka F-R. Relationships of a Novel Lyme Disease Spirochete,
741 Borrelia spielmani sp. nov., with Its Hosts in Central Europe. Appl Environ Microbiol. 2004;70:6414–9.

742 70. Richter D, Schlee DB, Matuschka F-R. Reservoir Competence of Various Rodents for the Lyme
743 Disease Spirochete Borrelia spielmanii. Appl Environ Microbiol. 2011;77:3565–70.

744 71. Taragel'ova V, Koci J, Hanincova K, Kurtenbach K, Derdakova M, Ogden NH, et al. Blackbirds and
745 Song Thrushes Constitute a Key Reservoir of Borrelia garinii, the Causative Agent of Borreliosis in
746 Central Europe. Appl Environ Microbiol. 2008;74:1289–93.

747 72. Randolph SE, Gern L, Nuttall PA. Co-feeding ticks: Epidemiological significance for tick-borne
748 pathogen transmission. Parasitol Today. 1996;12:472–9.

749 73. van Duijvendijk G, Coipan C, Wagemakers A, Fonville M, Ersöz J, Oei A, et al. Larvae of Ixodes ricinus
750 transmit Borrelia afzelii and B. miyamotoi to vertebrate hosts. Parasit Vectors [Internet]. 2016 [cited
751 2019 Jan 14];9. Available from: http://www.parasitesandvectors.com/content/9/1/97

752 74. Skuballa J, Oehme R, Hartelt K, Petney T, Bücher T, Kimmig P, et al. European Hedgehogs as Hosts
753 for Borrelia spp., Germany. Emerg Infect Dis. 2007;13:952–3.

754 75. Skuballa J, Petney T, Pfäffle M, Oehme R, Hartelt K, Fingerle V, et al. Occurrence of different Borrelia
755 burgdorferi sensu lato genospecies including B. afzelii, B. bavariensis, and B. spielmanii in hedgehogs
756 (Erinaceus spp.) in Europe. Ticks Tick-Borne Dis. 2012;3:8–13.

757 76. Gern L, Rouvinez E, Toutoungi LN, Godfroid E. Transmission cycles of Borrelia burgdorferi sensu lato
758 involving Ixodes ricinus and/or I. hexagonus ticks and the European hedgehog, Erinaceus europaeus, in
759 suburban and urban areas in Switzerland. Folia Parasitol (Praha). 1997;44:309–14.

760 77. Margos G, Vollmer SA, Cornet M, Garnier M, Fingerle V, Wilske B, et al. A New Borrelia Species
761 Defined by Multilocus Sequence Analysis of Housekeeping Genes. Appl Environ Microbiol.
762 2009;75:5410–6.

763 78. Hanincova K, Taragelova V, Koci J, Schafer SM, Hails R, Ullmann AJ, et al. Association of Borrelia
764 garinii and B. valaisiana with Songbirds in Slovakia. Appl Environ Microbiol. 2003;69:2825–30.

765 79. Rauter C, Hartung T. Prevalence of Borrelia burgdorferi Sensu Lato Genospecies in Ixodes ricinus
766 Ticks in Europe: a Metaanalysis. Appl Environ Microbiol. 2005;71:7203–16.

767 80. Lommano E, Bertaiola L, Dupasquier C, Gern L. Infections and co-infections of questing Ixodes
768 ricinus ticks by emerging zoonotic pathogens in Western Switzerland. Appl Environ Microbiol.
769 2012;AEM.07961-11.

770 **Table S1.**

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
Borrelia burgdorferi	rpoB	Forward	GCTTACTCACAAAAGGCGTCTT		
		Reverse	GCACATCTCTTACTTCAAATCCT	83	[20]
		Probe	AATGCTCTTGGACCAGGAGGACTTTCA		
Borrelia garinii	rpoB	Forward	TGGCCGAACTTACCCACAAAA		
		Reverse	ACATCTCTTACTTCAAATCCTGC	88	[20]
		Probe	TCTATCTCTTGAAAGTCCCCCTGGTCC		
Borrelia afzelii	fla	Forward	GGAGCAAATCAAGATGAAGCAAT		
		Reverse	TGAGCACCCTCTTGAACAGG	116	[20]
		Probe	TGCAGCCTGAGCAGCTTGAGCTCC		
Borrelia valaisiana	ospA	Forward	ACTCACAAATGACAGATGCTGAA		
		Reverse	GCTTGCTTAAAGTAACAGTACCT	135	[20]
		Probe	TCCGCCTACAAGATTTCCTGGAAGCTT		
Borrelia lusitaniae	rpoB	Forward	CGAACTTACTCATAAAAGGCGTC		
		Reverse	TGGACGTCTCTTACTTCAAATCC	87	[20]
		Probe	TTAATGCTCTCGGGCCTGGGGGACT		
Borrelia spielmanii	fla	Forward	ATCTATTTTCTGGTGAGGGAGC		
		Reverse	TCCTTCTTGTTGAGCACCTTC	71	[20]
		Probe	TTGAACAGGCGCAGTCTGAGCAGCTT		
Borrelia bissettii	rpoB	Forward	GCAACCAGTCAGCTTTCACAG		
		Reverse	CAAATCCTGCCCTATCCCTTG	87	[20]
		Probe	AAAGTCCTCCCGGCCCAAGAGCATTAA		
Borrelia miyamotoi	glpQ	Forward	CACGACCCAGAAATTGACACA		
		Reverse	GTGTGAAGTCAGTGGCGTAAT	94	[20]
		Probe	TCGTCCGTTTTCTCTAGCTCGATTGGG		
Borrelia spp.	23S	Forward	GAGTCTTAAAAGGGCGATTTAGT		
		Reverse	CTTCAGCCTGGCCATAAATAG	73	[20]
		Probe	AGATGTGGTAGACCCGAAGCCGAGT		
Anaplasma marginale	msp1	Forward	CAGGCTTCAAGCGTACAGTG		
		Reverse	GATATCTGTGCCTGGCCTTC	85	[20]
		Probe	ATGAAAGCCTGGAGATGTTAGACCGAG		
Anaplasma platys	groEL	Forward	TTCTGCCGATCCTTGAAAACG		
		Reverse	CTTCTCCTTCTACATCCTCAG	75	[20]
		Probe	TTGCTAGATCCGGCAGGCCTCTGC		
Anaphasma phagocytophilum	msp2	Forward	GCTATGGAAGGCAGTGTTGG		
		Reverse	GTCTTGAAGCGCTCGTAACC	77	[20]
		Probe	AATCTCAAGCTCAACCCTGGCACCAC		
Anaplasma centrale	groEL	Forward	AGCTGCCCTGCTATACACG		
		Reverse	GATGTTGATGCCCAATTGCTC	79	[20]
		Probe	CTTGCATCTCTAGACGAGGTAAAGGGG		
Anaplasma bovis	groEL	Forward	GGGAGATAGTACACATCCTTG		
		Reverse	CTGATAGCTACAGTTAAGCCC	73	[22]
		Probe	AGGTGCTGTTGGATGTACTGCTGGACC		
Anaplasma spp.	16S	Forward	CTTAGGGTTGTAAAACTCTTTCAG		
		Reverse	CTTTAACTTACCAAACCGCCTAC	160	[22]
		Probe	ATGCCCTTTACGCCCAATAATTCCGAACA		
Ehrlichia spp.	16S	Forward	GCAACGCGAAAAACCTTACCA		
		Reverse	AGCCATGCAGCACCTGTGT	98	[22]
		Probe	AAGGTCCAGCCAAACTGACTCTTCCG		
Ehrlichia canis	gltA	Forward	GACCAAGCAGTTGATAAAGATGG		
		Reverse	CACTATAAGACAATCCATGATTAGG	136	[22]
		Probe	ATTAAAACATCCTAAGATAGCAGTGGCTAAGG		

(Continued)

771

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
Neoehrlichia mikurensis	groEL	Forward	AGAGACATCATTCGCATTTTGGA		
		Reverse	TTCCGGTGTACCATAAGGCTT	96	[20]
		Probe	AGATGCTGTTGGATGTACTGCTGGACC		
Rickettsia conorii	23S-5S ITS	Forward	CTCACAAAGTTATCAGGTTAAATAG		
		Reverse	CGATACTCAGCAAAATAATTCTCG	118	[20]
		Probe	CTGGATATCGTGGCAGGGCTACAGTAT		
Rickettsia slovaca	23S-5S ITS	Forward	GTATCTACTCACAAAGTTATCAGG		
		Reverse	CTTAACTTTTACTACAATACTCAGC	138	[20]
		Probe	TAATTTTCGCTGGATATCGTGGCAGGG		
Rickettsia massiliae	23S-5S ITS	Forward	GTTATTGCATCACTAATGTTATACTG		
		Reverse	GTTAATGTTGTTGCACGACTCAA	128	[20]
		Probe	TAGCCCCGCCACGATATCTAGCAAAAA		
Rickettsia helvetica	23S-5S ITS	Forward	AGAACCGTAGCGTACACTTAG		
		Reverse	GAAAACCCTACTTCTAGGGGT	79	[20]
		Probe	TACGTGAGGATTTGAGTACCGGATCGA		
Rickettsia aeschlimannii	ITS	Forward	CTCACAAAGTTATCAGGTTAAATAG		
		Reverse	CTTAACTTTTACTACGATACTTAGCA	134	[20]
		Probe	TAATTTTTGCTGGATATCGTGGCGGGG		
Rickettsia felis	orfB	Forward	ACCCTTTTCGTAACGCTTTGC		
		Reverse	TATACTTAATGCTGGGCTAAACC	163	[22]
		Probe	AGGGAAACCTGGACTCCATATTCAAAAGAG		
Rickettsia spp.	gltA	Forward	GTCGCAAATGTTCACGGTACTT		
		Reverse	TCTTCGTGCATTTCTTTCCATTG	78	[22]
		Probe	TGCAATAGCAAGAACCGTAGGCTGGATG		
Bartonella henselae	pap31	Forward	CCGCTGATCGCATTATGCCT		
		Reverse	AGCGATTTCTGCATCATCTGCT	107	[20]
		Probe	ATGTTGCTGGTGGTGTTTCCTATGCAC		
Bartonella spp.	ssrA	Forward	CGTTATCGGGCTAAATGAGTAG		
		Reverse	ACCCCGCTTAAACCTGCGA	118	[22]
		Probe	TTGCAAATGACAACTATGCGGAAGCACGTC		
Francisella tularensis	tul4	Forward	ACCCACAAGGAAGTGTAAGATTA		
		Reverse	GTAATTGGGAAGCTTGTATCATG	76	[20]
		Probe	AATGGCAGGCTCCAGAAGGTTCTAAGT		
	fopA	Forward	GGCAAATCTAGCAGGTCAAGC		
		Reverse	CAACACTTGCTTGAACATTTCTAG	91	[20]
		Probe	AACAGGTGCTTGGGATGTGGGTGGTG		
Coxiella burnettii	IS1111	Forward	TGGAGGAGCGAACCATTGGT		
		Reverse	CATACGGTTTGACGTGCTGC	86	[20]
		Probe	ATCGGACGTTTATGGGGATGGGTATCC		
	icd	Forward	AGGCCCGTCCGTTATTTTACG		
		Reverse	CGGAAAATCACCATATTCACCTT	74	[20]
		Probe	TTCAGGCGTTTTGACCGGGCTTGGC		
Apycomplexa	18S	Forward	TGAACGAGGAATGCCTAGTATG		
		Reverse	CACCGGATCACTCGATCGG	104	[22]
		Probe	TAGGAGCGACGGGCGGTGTGTAC		
Babesia microti	CCTeta	Forward	ACAATGGATTTTCCCCAGCAAAA		
		Reverse	GCGACATTTCGGCAACTTATATA	145	[20]
		Probe	TACTCTGGTGCAATGAGCGTATGGGTA		
Babesia canis	hsp70	Forward	TCACTGTGCCTGCGTACTTC		
		Reverse	TGATACGCATGACGTTGAGAC	87	[20]
		Probe	AACGACTCCCAGCGCCAGGCCAC		

(Continued)

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
Babesia ovis	18S	Forward	TCTGTGATGCCCTTAGATGTC		
		Reverse	GCTGGTTACCCGCGCCTT	92	[20]
		Probe	TCGGAGCGGGGTCAACTCGATGCAT		
Babesia bovis	CCTeta	Forward	GCCAAGTAGTGGTAGACTGTA		
		Reverse	GCTCCGTCATTGGTTATGGTA	100	[20]
		Probe	TAAAGACAACACTGGGTCCGCGTGG		
Babesia caballi	rap1	Forward	GTTGTTCGGCTGGGGCATC		
		Reverse	CAGGCGACTGACGCTGTGT	94	[20]
		Probe	TCTGTCCCGATGTCAAGGGGCAGGT		
Babesia venatorum (sp. EU1)	18S	Forward	GCGCGCTACACTGATGCATT		
		Reverse	CAAAAATCAATCCCCGTCACG	91	[20]
		Probe	CATCGAGTTTAATCCTGTCCCGAAAGG		
Babesia divergens	hsp70	Forward	GCGCGCTACACTGATGCATT		
		Reverse	CAAAAATCAATCCCCGTCACG	91	[20]
		Probe	CATCGAGTTTAATCCTGTCCCGAAAGG		
Theileria spp.	18S	Forward	GTCAGTTTTTACGACTCCTTCAG		
		Reverse	CCAAAGAATCAAGAAAGAGCTATC	213	[20]
		Probe	AATCTGTCAATCCTTCCTTTGTCTGGACC		
Hepatozoon spp.	18S	Forward	ATTGGCTTACCGTGGCAGTG		
		Reverse	AAAGCATTTTAACTGCCTTGTATTG	175	[22]
		Probe	ACGGTTAACGGGGGATTAGGGTTCGAT		
Ixodes ricinus	ITS2	Forward	CGAAACTCGATGGAGACCTG		
		Reverse	ATCTCCAACGCACCGACGT	77	[20]
		Probe	TTGTGGAAATCCCGTCGCACGTTGAAC		
Tick spp	16S	Forward	AAATACTCTAGGGATAACAGCGT		
		Reverse	TCTTCATCAAACAAGTATCCTAATC	99	[22]
		Probe	CAACATCGAGGTCGCAAACCATTTTGTCTA		
Dermacentor reticulatus	ITS2	Forward	AACCCTTTTCCGCTCCGTG		
		Reverse	TTTTGCTAGAGCTCGACGTAC	83	[20]
		Probe	TACGAAGGCAAACAACGCAAACTGCGA		
Dermacentor marginatus	ITS2	Forward	GCACGTTGCGTTGTTTGCC		
		Reverse	CCGCTCCGCGCAAGAATCT	139	[20]
		Probe	TTCGGAGTACGTCGAGCTCTAGCAGA		
Escherichia coli	eae	Forward	CATTGATCAGGATTTTTCTGGTGATA		
		Reverse	CTCATGCGGAAATAGCCGTTA	102	[20]
		Probe	ATAGTCTCGCCAGTATTCGCCACCAATACC		

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Targeted genus	Targeted gene	Name	Sequence	Reference
Babesia; Theileria; Hepatozoon	18s rRNA gene	BTH 18S 1st F BTH 18S 1st R BTH 18S 2nd F BTH 18S 2nd R	GTGAAACTGCGAATGGCTCATTAC AAGTGATAAGGTTCACAAAACTTCCC GGCTCATTACAACAGTTATAGTTTATTTG CGGTCCGAATAATTCACCGGAT	[81]
Anaplasma; Ehrlichia	16s rRNA gene	EHR1 EHR2 EHR3 EHR2	GAACGAACGCTGGCGGCAAGC AGTA(T/C)CG(A/G)ACCAGATAGCCGC TGCATAGGAATCTACCTAGTAG AGTA(T/C)CG(A/G)ACCAGATAGCCGC	[82]
Borrelia	flaB	FlaB280F FlaRL flaB_737F FlaLL	GCAGTTCARTCAGGTAACGG GCAATCATAGCCATTGCAGATTGT GCATCAACTGTRGTTGTAACATTAACAGG ACATATTCAGATGCAGACAGAGGT	[83]
Rickettsia	rompB	Rc.rompB.4362p Rc.rompB.4,836n Rc.rompB.4,496p Rc.rompB.4,762n	GTCAGCGTTACTTCTTCGATGC CCGTACTCCATCTTAGCATCAG CCAATGGCAGGACTTAGCTACT AGGCTGGCTGATACACGGAGTAA	[84]