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

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SUMMARY

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 *Ixodes ricinus* tick-borne pathogen (TBP) composition, information about temporal TBP patterns remain scarce. These data are crucial for predicting seasonal/annual patterns which could improve understanding and prevent tick-borne diseases (TBD). We examined TBP dynamics in *Ixodes 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. We detected *Anaplasma phagocytophilum* (5.3%), *Rickettsia helvetica* (4.5%), *Borrelia burgdorferi* s.l. (3.7%), *Borrelia miyamotoi* (1.2%), *Babesia venatorum* (1.5%), *Babesia divergens* (0.1%), and the agent of the cat-flea typhus, *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. afzelii*/*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*), emphasising that regularly monitoring ticks and TBP is essential to appropriately assess acarological risk.

KEY WORDS

Tick-borne pathogens, dynamics, temporal patterns, pathogen co-occurrence

36 **INTRODUCTION**

37 Ticks are obligatory hematophagous arthropods and consequently, are one of the most
38 important pathogen vectors (Jongejan and Uilenberg, 2004; de la Fuente *et al.*, 2008; Dantas-Torres *et*
39 *al.*, 2012). Lyme borreliosis (LB) is the most commonly reported tick-borne disease (TBD) in the
40 northern hemisphere and is caused by bacteria belonging to the *Borrelia burgdorferi* s.l. complex. In
41 Western Europe, *Ixodes ricinus* is known to be involved in the transmission of these bacteria to both
42 humans and animals. This tick species has also been reported to be a vector for many other tick-borne
43 pathogens (TBP) with potentially significant consequences for human and animal health (*Anaplasma*,
44 *Rickettsia*, *Bartonella*, *Babesia*…) (Strle, 2004; Bonnet *et al.*, 2007a; Bonnet *et al.*, 2007b; Cotté *et al.*,
45 2008; Bonnet *et al.*, 2009; Sprong *et al.*, 2009).

46 While multiple different pathogens have been identified and confirmed in *I. ricinus* ticks, very
47 little is known about their seasonal and inter-annual variations. Time-series studies are thus crucial to
48 understanding natural variability in microbial communities over time. Over the last decade, only a
49 handful of surveys have assessed seasonal and monthly TBP variation patterns (Gassner *et al.*, 2010;
50 Reye *et al.*, 2010; Coipan *et al.*, 2013; Takken *et al.*, 2016; Chvostáč *et al.*, 2018). Although these results
51 have heightened our general understanding of TBP dynamics, several of these studies were performed
52 over short periods of less than two years, rendering it impossible to infer inter-annual discrepancies or
53 to detect bias due to a particularly exceptional year. Only Coipan *et al*. (2013) analysed several
54 pathogenic genera in ticks sampled over more than two years. These studies did demonstrate
55 relationships between seasons and TBP prevalence (*Borrelia*, *Rickettsia*, *Anaplasma*, *Neoehrlichia*, and
56 *Babesia*) in questing tick populations. These variations were mainly attributed to the varying availability
57 of reservoir hosts.

58 Tick density is also heavily influenced by the presence of suitable hosts, most notably wild
59 ungulates that sustain adults, thus enabling tick population renewal (Gilbert *et al.*, 2012; van Wieren
60 and Hofmeester, 2016). However, it's important to emphasise that immediate tick survival and questing
61 activities are highly dependent on suitable and specific environmental conditions (temperatures

comprised between 5 to 30°C; and up to 80% humidity). Simultaneously, several studies have investigated whether pathogen presence influences tick behaviour. Herrman and Gern (2010, 2012) suggested that *I. ricinus* infected with *B. burgdorferi* s.l. can tolerate increased levels of desiccation, and Neelakanta *et al*. (2010) 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 Candidatus *Neoehrlichia mikurensis*), *Rickettsia*, *Bartonella*, *Francisella*, *Coxiella*, *Theileria*, *Babesia*, and *Hepatozoon*.

86 **MATERIAL AND METHODS**

87 ***Tick collection***

88 *I. ricinus*, nymphs and adults, were monthly collected during three years, from April 2014 to May
89 2017, in the Sénart forest in the south of Paris. Samplings were performed by flagging (Vassallo *et al*.,
90 2000) on 10 transects of 10 square meters, localized on the parcel 96 (48°39'34.6"N 2°29'13.0"E, Figure
91 1). Flagging was always performed 3 consecutive times on each transect. After morphological
92 identification, ticks were stored at -80°C. In total 1167 *I. ricinus* (nymphs and adults) were collected.

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

95 Ticks were first washed once in ethanol 70% for 5 minutes and rinsed twice in sterile MilliQ water
96 for 5 minutes each time, before performing mechanical crushing. Ticks were individually crushed in
97 375µL of DMEM with decomplemented Foetal Calf Serum (10%) and six steel beads using the
98 homogenizer Precellys®24 Dual (Bertin, France) at 5500 rpm for 20 seconds.

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

103 Two controls were performed: (1) the crushing control, corresponding to a DMEM tube in which
104 crushing and DNA extraction were performed in the same conditions than on samples; and (2) the
105 extraction control, corresponding the DNA extraction step performed on water.

106

107 ***Tick-borne pathogens detection***

108 A high-throughput screening of the most common bacterial and parasitic species of tick-borne
109 pathogens, known to circulate in Europe was performed, allowing us to detect simultaneously the
110 presence of 31 pathogenic species, 7 genera and 1 phylum: the Borrelia genus and eight Borrelia
111 species (*B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. spielmanii*, *B. lusitaniae*, *B. bissettii* and

112 *B. miyamotoi*); the Anaplasma genus and five Anaplasma species (*A. marginale*, *A. phagocytophilum*,
113 *A. platys*, *A. centrale*, *A. bovis*); the Ehrlichia genus and *E. canis*; *Candidatus* Neoehrlichia mikurensis;
114 the Rickettsia genus and six Rickettsia species (*R. conorii, R. slovaca, R. massiliae, R. helvetica*,
115 *R. aeshlimanii* and *R. felis*); the Bartonella genus and *B. henselae*; *Francisella tularensis*;
116 *Coxiella burnettii*; the apicomplexa phylum and seven Babesia species (*B. divergens, B. microti,*
117 *B. caballi, B. canis, B. venatorum, B. bovis, B. ovis)*, but also the two parasitic genus Theileria and
118 Hepatozoon.

119 TBP DNA was detected using the BioMark™ real-time PCR system (Fluidigm, USA), that is a
120 microfluidic system allowing to perform 48 or 96 real-time PCR reactions on 48 or 96 different samples
121 as described in (Michelet *et al*., 2014; Moutailler *et al*., 2016). Briefly, each samples and primers/probe
122 set are deposited in individual wells. A pressure system allowed to load them on the chip, via
123 microchannels, in individual reaction chambers of 10nL, where each sample will meet individually each
124 primers/probe set.

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126 *Primers and probes*

127 Primers and probes used for this analysis are coming from Michelet *et al*. (2014) and Gondard *et*
128 *al*. (2019). They have been designed to specifically amplified pathogens (bacteria and parasites) which
129 are usually found in ticks in Europe. Their sequences, amplicon size, as well as targeted genes and
130 pathogens are registered in Table S1. It is important to note that, because of cross-reaction of
131 primers/probe combination (i.e. design) targeting *B. burgdorferi* s.s. and *B. spielmanii* with respectively
132 *B. garinii*/*B. valaisiana* and *B. afzelii* DNA, positive samples for the two formers were considered as
133 negative when associated to the latter. Therefore, potential associations between
134 *B. burgdorferi* s.s./*B. garinii*, *B. burgdorferi* s.s./*B. valaisiana* and *B. spielmanii*/*B. afzelii* cannot be
135 detected and the co-infection percentage may be under-estimated.

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138 *DNA pre-amplification*

139 Before high throughput real time PCR step, each sample was first pre-amplified using the TaqMan
140 PreAmp Master Mix (Applied Biosystems, France). Basically, the different primer pairs, used for the real
141 time PCR, were pooled combining equal volume of primers with a final concentration of 0.2µM. Due
142 to the high concentration of *Escherichia coli* (positive control) or tick DNA, the pre-amplification step
143 was not needed and the corresponding targeting primers were thus not added into the pool. The only
144 one exception was the primer pair named "Tick_spp_16S", targeting mitochondrial DNA, which was
145 added to the 0.2x pool.

146 For each sample, 1.25µL of DNA extract was pre-amplified using the Perfecta PreAmp SuperMix
147 reagent (1x) and the 0.2x pool (0.05µM), in a final reactive volume of 5µL. PCR cycle comprised a first
148 cycle at 98°C for 2 minutes, followed by 14 cycles with 2 steps, the first one at 95°C for 10 seconds and
149 the second one at 60°C for 3 minutes. Pre-amplified DNA were then diluted (1:10) by addition of 45µL
150 of sterile deionised water before use.

151

152 *High throughput real time PCR*

153 For each pre-amplified sample, the BioMark™ real-time PCR system (Fluidigm, USA) was used for
154 high-throughput microfluidic real-time PCR amplification using the 48.48 microfluidic dynamic array
155 (Fluidigm Corporation, USA). Amplifications were performed using FAM- and black hole quencher
156 (BHQ1)-labeled TaqMan probes with TaqMan Gene Expression Master Mix in accordance with
157 manufacturer's instructions (Applied Biosystems, France). Thermal cycling conditions were as follows:
158 95°C for 5 min, 45 cycles at 95°C for 10 s, 60°C for 15 s, and 40°C for 10s. Data were acquired on the
159 BioMark Real-Time PCR system and analysed using the Fluidigm Real-Time PCR Analysis software to
160 obtain crossing point (CP) values. Three tick species control (*I. ricinus*, *Dermacentor reticulatus*,
161 *Dermacentor marginatus*), one negative water control and one positive *E. coli* control were included
162 per chip.

163

164 *Nested PCR and sequencing*

165 Samples that were positive only for species-specific design but not for the genus design or only
166 genus design and not for species designs were all re-analysed by nested PCR using primer pairs allowing
167 to target another gene that the one tested into the fluidigm experiment and that is specific to the TBP
168 genus. Their sequences, amplicon size, as well as targeted genes and pathogen genus are registered in
169 Table S2. Amplicons were then sequenced by Eurofins company. Sequences obtained were assembled
170 using the Bioedit software and compared to the database NCBI (National Center for Biotechnology
171 Information) by sequence alignment using nucleotide BLAST (Basic Local Alignment Search Tool).

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173 *Statistical analysis*

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

175 We tested if the prevalence of TBP was different within and between years of sampling by using a
176 multivariable logistic regression model. We considered the calendar season level for the within-year
177 variability. Seasons were considered as following: Winter = January to February; Spring = March to May;
178 Summer = June to August and Autumn = September to November. A logistic regression model was
179 developed using the TBP status of each nymph as the outcome measure and season, year and the
180 interaction between season and year as explanatory variables. We performed four specific model for
181 the following group/species of TBP: (1) *B. burgdorferi* s.l. (considering *B. burgdorferi* s.s., *B. garinii*,
182 *B. afzelii*, *B. valaisiana* and *B. spielmanii*), (2) *B. miyamotoi*, (3) *A. phagocytophilum*, and (4)
183 *R. helvetica*. The models were constructed with a generalized linear model (GLM, McCullagh and
184 Nelder, 1989) using a binomial distribution (logit link). Model assessment was based on Akaike
185 information criterion (AIC). Results were expressed as odds ratios (OR) and 95% confidence intervals.
186 Statistical computations were performed in R 3.5.1. (R Core Team, 2018).

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188 *Statistical modelling of tick-borne pathogens associations*

189 We tested the associations between the TBP species that belonged to the co-infection profiles of
190 nymphs found in this study. To do this, we used the association screening approach (Vaumourin *et al*.,
191 2014), which is based on the statistic distribution of the occurrence count of each possible combination
192 of pathogens under the null hypothesis H0 that pathogens associations are random. For a given number
193 of pathogen species tested (NP), the number of possible combination (NC) was calculated as $NC = 2^{NP}$.
194 Assuming similar pathogen prevalence as those observed, a simulated dataset was built as an
195 absence/presence matrix with hosts in lines and pathogen combinations in columns. With 5 000
196 simulations, we obtained the NC statistic distributions. We estimated a 95% confidence envelope to
197 obtain a profile that includes simultaneously all the combinations. From this profile, we inferred for
198 each combination two quantiles, *Qinf* and *Qsup*. A global test was based on the 95% confidence
199 envelope. When H0 was rejected, the local tests were based on the NC confidence intervals: [*Qinf* ;
200 *Qsup*] (Vaumourin *et al*., 2014).
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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). Collected ticks were composed of 1098 nymphs, 35 females and 34 males. We focused our temporal analysis on nymphs due to the low number of adults collected over the three years. The temporal dynamics of nymph densities over the three years is shown in Figure 2A. Nymph densities follow similar patterns from one year to another, with a main peak of activity observed every year during spring months, a strong decrease in tick densities during summer and a second peak, smaller, observed in October (Figure 2A). These patterns are more intensively highlighted in the figure 2B which presents the average density of nymphs per month. In January and February, the average density is less than 10 questing nymphs, a clear rise is observed from March to May reaching a peak of 95.3 nymphs/100m^2 in May [84.6%, 107.0%]. Densities then decrease to a minimum of 5.3 nymphs/100m^2 in September [3.0%, 8.7%], rise slightly in October (13 nymphs/100m^2 [9.2%, 17.8%]) before decreasing again in November (2 nymphs/100m^2 [0.2%, 7.2%]).

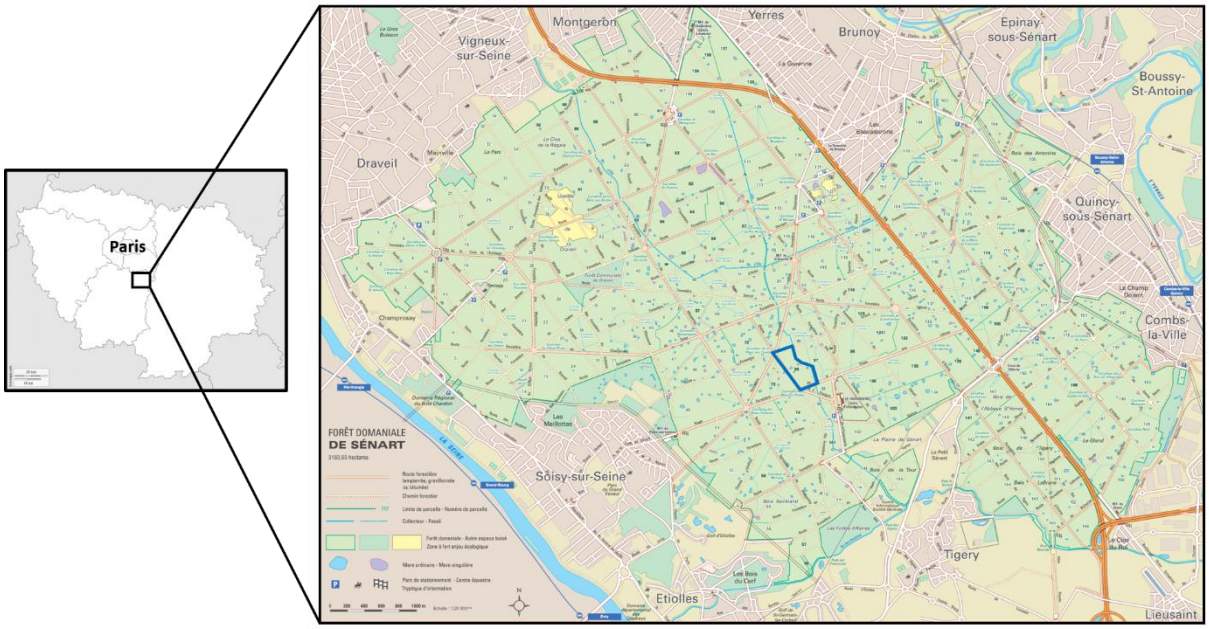

Figure 1. Senart forest, location and parcel map. Sampling was made on the blue framed parcel.

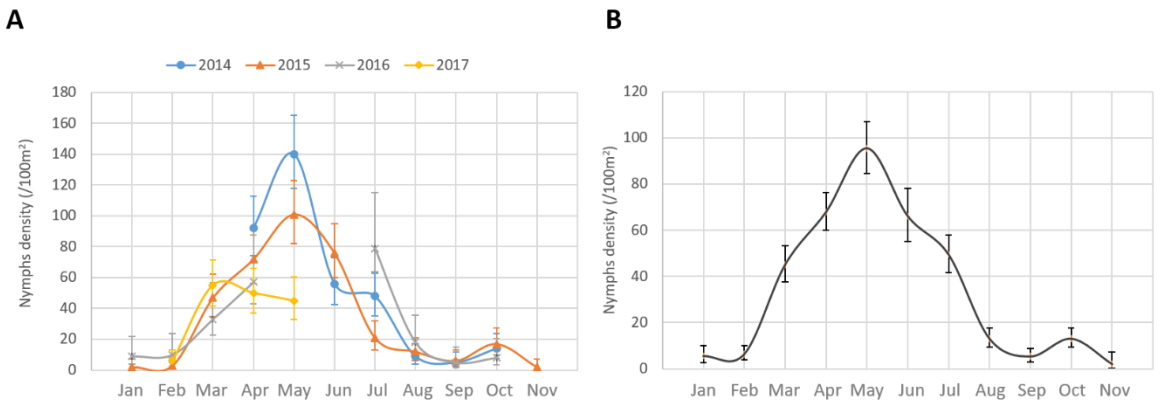

Figure 2. Ixodes ricinus nymph density. A: *Ixodes ricinus* nymphs monthly density (/100m^2) in 2014, 2015, 2016 and 2017. Ticks were sampled from April 2014 to May 2017. Please note that May and June 2016 were unfortunately not sampled. B: Average of nymph density per month (/100m^2). The calculation of the average nymph monthly density was made between samples corresponding to the same months through the three years of sampling. Error bars represent the Confidential Interval of the mean, calculated using the Poisson distribution.

Detected pathogens and their prevalence in tick population

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

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

Two species of *Rickettsia* were detected in questing *I. ricinus* nymphs. *Rickettsia helvetica* was the most prevalent and was detected in 4.5% [3.3%, 6.0%] of nymphs. *Rickettsia felis* was detected in only one nymph (0.1% [.003%, 0.6%]). The presence of *R. felis* DNA was confirmed by nested PCR and

247 sequencing as the obtained sequence of 248bp matched with the *R. felis* genome with 100% of identity
248 and 98% of query cover.

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

254 DNA from two species of protozoans belonging to the *Babesia* genus were detected in questing
255 nymph: *Babesia venatorum* (1.5% [0.8%, 2.5%] of ticks) and *Babesia divergens* (0.1% [.003%, 0.6%]),
256 detected in one tick).

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	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*	*B. divergens*	*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	**1**	0	0	**18**
	May-14	127	1	0	0	0	0	1	1	**2**	12	**12**	2	0	**2**	4	0	**4**	2	0	**18**
	Jun-14	54	0	1	1	1	0	1	0	**1**	4	**4**	9	1	**10**	2	0	**2**	1	1	**16**
	Jul-14	38	0	1	0	0	1	1	0	**1**	6	**6**	1	0	**1**	0	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	0	**1**
	Sep-14	5	1	0	0	0	0	1	0	**1**	0	**0**	0	0	**0**	1	0	**1**	0	0	**2**
	Oct-14	13	0	0	0	1	0	1	0	**1**	1	**1**	0	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	**0**
	Feb-15	3	0	0	1	0	0	1	0	**1**	0	**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	**0**	1	1	**12**
	Apr-15	69	2	0	0	0	0	2	0	**2**	3	**4**	4	0	**4**	0	0	**0**	0	0	**10**
	May-15	88	0	0	0	0	0	0	1	**1**	1	**1**	3	0	**3**	2	0	**2**	0	0	**7**
	Jun-15	78	1	0	0	0	1	2	2	**4**	2	**2**	1	0	**1**	2	0	**2**	1	0	**8**
	Jul-15	21	0	2	2	0	0	3	1	**4**	0	**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	**0**
	Sep-15	6	0	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	0	**4**
	Nov-15	2	0	0	0	0	0	0	1	**1**	0	**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	0	**2**
	Feb-16	10	0	0	0	0	0	0	0	**0**	0	**0**	1	0	**1**	0	0	**0**	0	0	**1**
	Mar-16	26	2	1	2	0	0	4	0	**4**	1	**1**	0	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	0	**3**
	Jul-16	78	2	2	2	0	1	5	0	**5**	2	**2**	4	0	**4**	1	0	**1**	1	1	**11**
	Aug-16	11	2	0	0	0	0	2	0	**2**	0	**0**	2	0	**2**	0	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	0	**1**
	Oct-16	6	0	0	0	0	0	0	1	**1**	0	**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	0	**1**
	Mar-17	53	0	1	0	1	0	2	0	**2**	3	**3**	0	0	**0**	2	0	**2**	0	0	**7**
	Apr-17	50	0	0	1	1	0	2	0	**2**	3	**3**	0	0	**0**	1	0	**1**	0	0	**6**
	May-17	41	0	0	0	0	0	0	1	**1**	4	**4**	1	0	**1**	0	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**	**1**	**16**	**10**	**3**	**159**
	Number of positive months	30	8	7	8	6	4	18	9	**23**	14	**14**	16	1	**16**	8	1	**9**	9	3	**27**

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

299 ***Temporal patterns of TBP in nymphal* I. ricinus**

300 *TBP prevalence at the monthly scale*

301 Global infection rates fluctuated over the three years from a minimum of 8% [3.3%, 15.7%] in May
302 2015 to a maximum of 29.6% [18.0%, 43.6%] in June 2014, yet monthly variation was not significant
303 probably due the low sample sizes ($p > 0.05$ according to Chi^2 test; Figure 3).

304 At the genus level, variations in TBP prevalences and the number of months for which at least one
305 tick was positive for each tested TBP are presented in Figure 4 and Table 1.

306 DNA from pathogens belonging to both genera *Rickettsia* and *Anaplasma* were detected
307 respectively in 16 and 14 of the 30 sampled months respectively. When detected, prevalence fluctuated
308 from 1.3% [0.03%, 6.9%] (June 2015) to 18.5% [9.3%, 31.4%] (June 2014) for *Rickettsia* and from
309 1.1% [0.03%, 6.2%] (May 2015) to 15.8% [6.0%, 31.3%] (July 2014) for *Anaplasma*. Both genera are
310 mainly represented by one species: *R. helvetica* and *A. phagocytophilum* that are the most frequently
311 detected species (16 and 14 /30 months respectively). These two species were found each sampled
312 year.

313 DNA from members of the *Borrelia* genus was detected in 23 of the 30 sampled months. This
314 bacterial genus displayed the highest variability with monthly prevalences fluctuating from
315 1.1% [0.03%, 6.2%] (May 2015) to 23.5% [6.8%, 49.9%] (October 2015). DNA from members of the LB
316 group was detected in 18 of the 30 sampled months with prevalences ranging from 0.8% [0.03%, 6.2%]
317 in May 2014 to 23.5% [6.8%, 49.9%] in October 2015. The most frequently identified species were
318 *B. burgdorferi* s.s. (8 / 30 sampled months), *B. afzelii* (8 / 30) and *B. garinii* (7 / 30). DNA from these
319 species was regularly detected over the three studied years. Conversely, *B. valaisiana* (6 / 30) and
320 *B. spielmanii* (4 / 30) DNA were not detected during 11 (from April 2015 to March 2016) and 9 (from
321 July 2015 to April 2016) consecutive sampled months respectively. *Borrelia miyamotoi* (relapsing fever
322 group) DNA was detected 9 times over the 30 sampled months with prevalences ranging between
323 0.8% [0.02%, 4.3%] in May 2014 and 7% [1.5%, 19.1%] in March 2015. Despite a prevalence and a
324 frequency of detection that is comparable to *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*, this species

DNA was not detected during two main periods: from June 2014 to March 2015 (7 samplings) and from January to September 2016 (7 samplings, but two sampling months were missing in this period). It was detected 5 times over 9 samplings from March to November 2015 and detected 3 times over 5 samplings from October 2016 to May 2017.

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

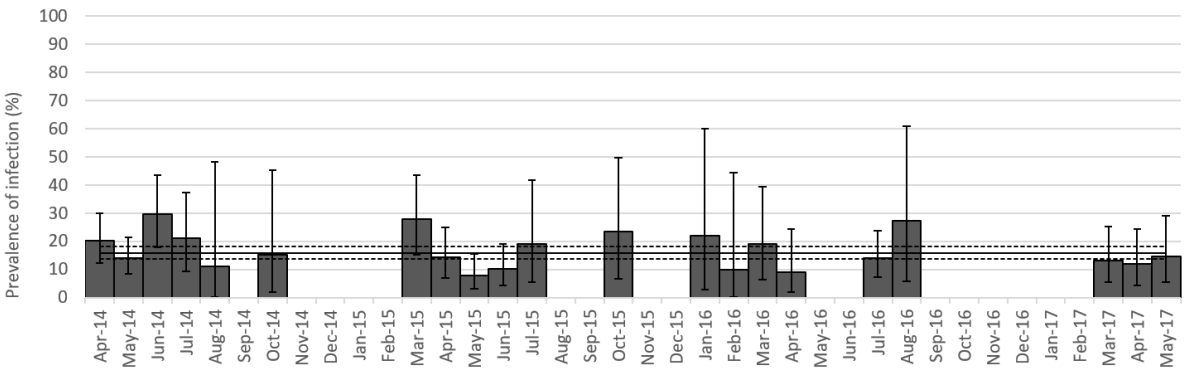

Figure 3. Nymph infection rate per month for at least one tested pathogen. Months with less than 9 nymphs sampled have not been considered for percentage calculation. Error bars represent confidence intervals of the percentage.

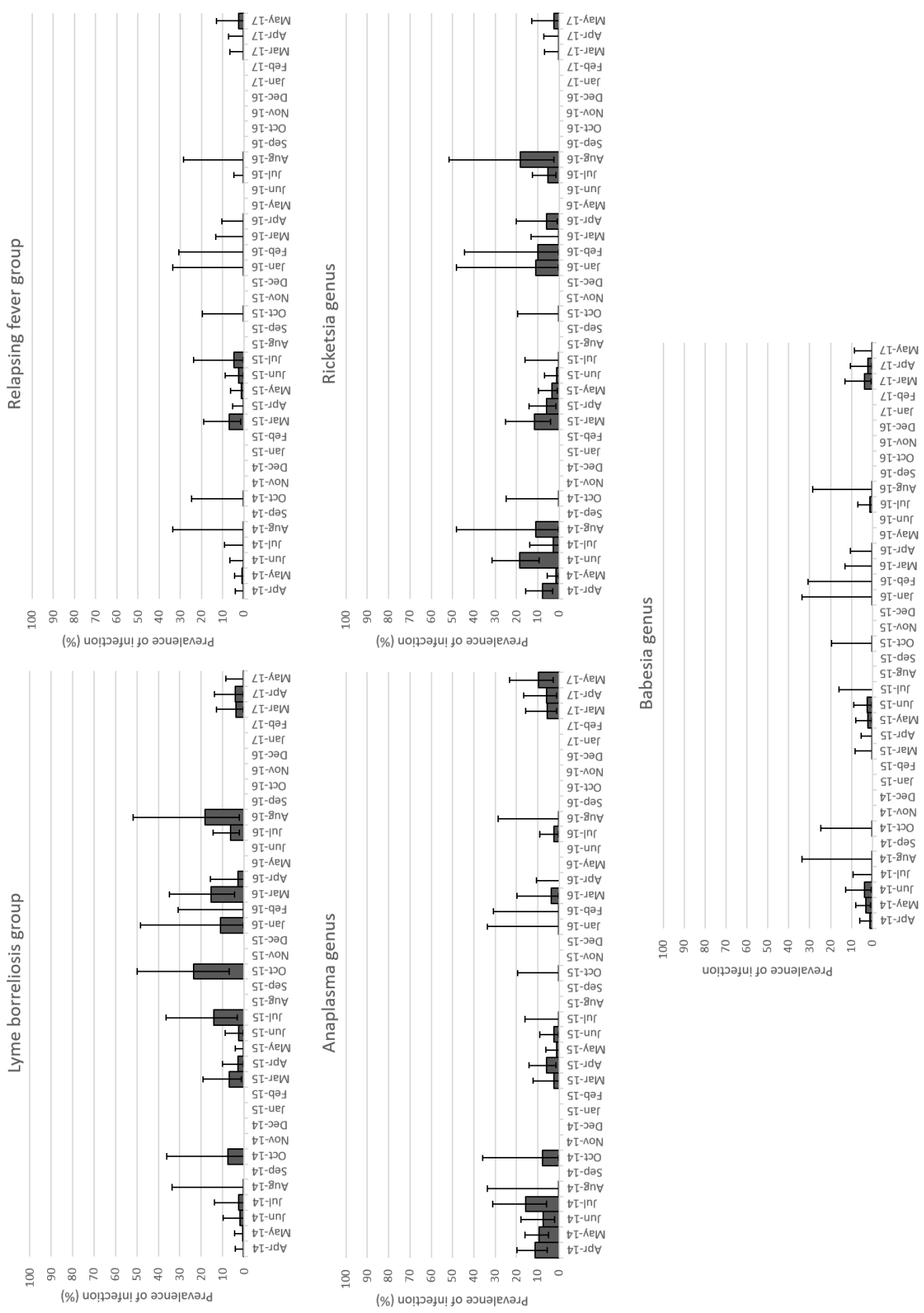

Figure 4. Nymph infection rate and confidence intervals per month for the different TBP. Months with less than 9 nymphs sampled have not been considered. Error bars represent confidence intervals of the percentage.

379 *TBP prevalence at the seasonal and multi-annual scale*

380 In order to determine if the prevalence of TBP was different within and between years of sampling,
381 a multivariable logistic regression model was performed. Because some samplings were missing and
382 because of the low number of nymphs collected in some months, the statistical analysis was performed
383 according to calendar seasons. Spring season and year 2014 have been considered as references for
384 the seasonal and yearly effect respectively. Because some TBP had too low prevalence in the nymph
385 population producing unreliable statistics, analyses were only performed on the most prevalent TBP:
386 *A. phagocytophilum*, *R. helvetica*, *B. burgdorferi* s.l., *B. miyamotoi* and *B. venatorum*.

387 Significant differences were observed at the seasonal scale (Table 2, Figure 5) for *R. helvetica*
388 (higher in summer compared to spring), *B. burgdorferi* s.l. (higher in autumn compared to spring) and
389 *B. miyamotoi* (higher in winter than in spring). It must be noticed that winter is the season with the
390 smallest number of sampled ticks (30 in total), and that the difference observed for *B. miyamotoi* in
391 winter correspond to only one tick infected sampled in February 2017.

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

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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.

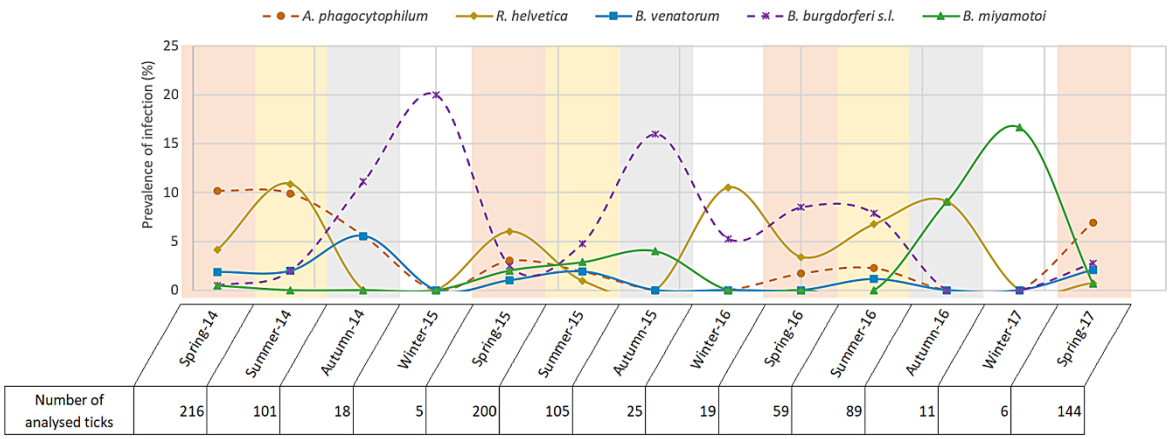

Figure 5. Percentage of positive nymphs per season for the most prevalent TBPs. Winter (white background)= January to February – Spring (orange background)= March to May – Summer (yellow background)= June to August – Autumn (grey background)= September to November.

429 ***Pathogen associations***

430 Among all the sampled ticks, 1% [0.5%, 1.8%] were co-infected with two pathogens and
431 0.3% [0.006%, 0.8%] were co-infected with three pathogens. Eight different co-infection profiles were
432 found (Table 3). In most of cases (7/13), these co-infections concerned species belonging to the *Borrelia*
433 genus: *B. garinii*/*B. afzelii*; *B. garinii*/*B. spielmanii*; *B. garinii*/*B. afzelii*/*B. valaisiana* and
434 *B. garinii*/*B. valaisiana*/*B. spielmanii*. Co-infections profiles with species belonging to different genus
435 were also observed: *A. phagocytophilum*/*B. venatorum*; *A. phagocytophilum*/*R. helvetica*;
436 *B. burgdorferi* s.s./*R. helvetica* and *B. garinii*/*B. afzelii*/*R. helvetica*. All these associations between
437 pathogens were tested using the association screening approach (Vaumourin *et al*., 2014). Compared
438 to a random analysis, no associations were found to be under represented while two were over
439 represented: the first one between *B. garinii* and *B. afzelii* (observation = 3; min expected = 0; max
440 expected = 2), and the second one between *B. garinii* and *B. spielmanii* (observation = 2; min expected
441 = 0; max expected = 1).

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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

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

450 **DISCUSSION**

451 ***Ixodes ricinus density and seasonal dynamics***

452 This three-year survey demonstrated a clear seasonal pattern in *I. ricinus* density, with a marked
453 peak of questing nymphs in spring and a smaller peak in autumn. Low, but present activity was detected
454 in winter, as has been observed in Germany (Dautel *et al.*, 2008). In addition to these general patterns,
455 some unexpected data were observed, the most striking being no peak activity in spring 2017 (April
456 and May) with tick densities very similar to those recorded in March. Abiotic factors such as
457 temperature, relative humidity, and rainfall, or fluctuating host numbers in the sampling area are
458 known to influence questing tick abundance and activity patterns (Perret *et al.*, 2000; Gilbert, 2010;
459 Tagliapietra *et al.*, 2011; Schulz *et al.*, 2014; Vourc'h *et al.*, 2016; Marchant *et al.*, 2017) and could
460 explain these unusual observations. It's important to note that 2017 was distinguished by an
461 abnormally wet March, with total rainfall much higher than that recorded in previous years in the same
462 area (71.3, compared to 11.2, 33.6, and 61.7 mm rain/month in 2014, 2015, and 2016, respectively.
463 Interestingly, the increased March rainfall was followed by an April drought (7.9 mm of rain/month in
464 2017, compared to 48.4, 27.2, and 66.2 mm rain/month in 2014, 2015, and 2016, respectively) (rainfall
465 data estimated from the Orly station, Metéo-France data;
466 https://donneespubliques.meteofrance.fr/?fond=produit&id_produit=90&id_rubrique=32). These unusual meteorological
467 characteristics could explain the stable tick density from March to May 2017. Thereby, this finding
468 clearly shows that the bimodal tick activity pattern usually observed during this study can punctually
469 change with exceptional environmental conditions, reinforcing the importance of regular monitoring.

470

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

472 Most of the detected pathogen species corresponded to micro-organisms known to circulate in the
473 Western Palearctic (Capelli *et al.*, 2012; Overzier *et al.*, 2013; Pangrácová *et al.*, 2013; Reye *et al.*, 2013;
474 Hansford *et al.*, 2015; Paul *et al.*, 2016; Sormunen *et al.*, 2016; Raileanu *et al.*, 2017). However, several
475 species belonging to the *Bartonella* and *Francisella* genera, previously reported in the studied area

476 (Reis *et al.*, 2010; Paul *et al.*, 2016), were not detected. The most prevalent pathogen species were
477 *A. phagocytophilum* (5.4% of the examined nymphs), *R. helvetica* (4.5%), and *B. burgdorferi* s.l. (3.7%).
478 Both high- and low-prevalence TBP were consistently detected in the sampling area for the duration of
479 the study. Although prevalence varied between different TBP, and some were not detected for long
480 periods, they were all detected recurrently. Continued detection is consistent with the year-round
481 presence of reservoir hosts in the sampling area (wood mice, bank voles, Siberian chipmunks, roe deer,
482 common blackbird, European robin, song thrush…) (Marsot *et al.*, 2012, 2013; Marchant *et al.*, 2017).
483 The continued presence of reservoir hosts could facilitate the circulation of dominant species, and
484 maintain, even at low rates, less prevalent pathogen species. These findings lend further support to
485 regularly studying TBP temporal dynamics to assess and monitor changing infection risks.

486 We also highlighted the infrequent presence of some TBP, such as *B. divergens*, which was only
487 detected in a single tick, as also reported by Paul *et al.* (2016). *I. ricinus* can host and transmit both
488 *B. divergens* and *B. venatorum* (Bonnet *et al.*, 2007a; Bonnet *et al.*, 2007b; Bonnet *et al.*, 2009), the
489 etiological agents of human babesiosis (Centeno-Lima *et al.*, 2003; Herwaldt *et al.*, 2003). Human
490 babesiosis is known to be a rare disease, but is classified as a potential emerging disease in Europe
491 (Hildebrandt *et al.*, 2013; Rizzoli *et al.*, 2014; Oechslin *et al.*, 2017). While *Babesia venatorum* is known
492 to circulate in roe deer (Bonnet *et al.*, 2007a) of which there are many in the Sénart forest, *Babesia*
493 *divergens* circulates in bovine species absent from the Sénart forest, which may explain its low
494 prevalence among tick populations. Similarly, we detected *R. felis* DNA in only one nymph. This bacteria
495 is also considered to be an emergent human pathogen, and has been associated with clinical
496 manifestations in patients from America, Asia, Africa, and Europe (see in Angelakis *et al.*, 2016). Its
497 detection is particularly interesting as this bacteria is known to be mainly transmitted from cat to cat
498 *via* fleas, with human contamination arising from cat or flea bites. Nevertheless, several studies have
499 detected the presence of *R. felis* or *R. felis*-like organisms in hematophagous arthropods (see in Reif
500 and Macaluso, 2009; Brown and Macaluso, 2016), including ticks collected from the natural
501 environment (Oliveira *et al.*, 2008), and notably in *Ixodes* species (Ishikura *et al.*, 2013). *R. felis* has been

502 reported in *I. ricinus* in two previous studies on questing ticks (Vayssier-Taussat *et al.*, 2013; Lejal *et al.*,
503 2018). The Vayssier-Taussat's one was based on RNA detection, demonstrating viability and active
504 replication of this bacteria in *I. ricinus*. Rarely investigated in studies dealing with TBP, the repeated
505 detection of *R. felis* should encourage increased surveillance for this spotted fever-causing pathogen in
506 humans. Let's finally note that all these findings suggest that a punctual sampling would certainly not
507 facilitate the detection of these pathogens, again highlighting the importance of collecting and
508 analysing ticks at a large temporal scale.
509
510
511 ***Seasonal and inter-annual dynamics of I. ricinus-borne pathogens***
512 Improving the prevention of TBD requires a better understanding of their temporal—and in
513 particular—their seasonal dynamics. However, only a few studies have addressed these issues during
514 a minimum three-year period (Coipan *et al.*, 2013; Takken *et al.*, 2016). As ticks were collected monthly
515 for over three years in this study, we detected significant seasonal or annual infection rate fluctuations
516 for four TBP: *R. helvetica*, *B. burgdorferi* s.l., *B. miyamotoi*, and *A. phagocytophilum*. Note that the
517 statistically significant highest prevalence of *B. miyamotoi* in winter is only due to the detection of one
518 positive tick sampled during winter in 2017. In our opinion, this result alone is insufficient to presume
519 that *B. miyamotoi* have an increased winter prevalence. However, we can observe that even if very few
520 ticks are questing during these periods, they may carry TBP.
521 While significant seasonal and annual differences were observed for *B. miyamotoi* and
522 *A. phagocytophilum*, respectively, the presence of *R. helvetica* and *B. burgdorferi* s.l. varied
523 significantly according to both seasons and years. None of these micro-organisms presented a similar
524 pattern to any others. Comparing our results to the pluri-annual studies previously mentioned, we
525 observe that only *R. helvetica* presented similar seasonal patterns (Coipan *et al.*, 2013). This finding
526 again emphasises how the season, the year or the sampling area can influence TBP presence and
527 prevalence in questing tick populations.

528 The most common explanation for temporal variations in TBP prevalence is the variable availability
529 of reservoir hosts during tick previous stage feeding. Because the tick lifecycle is fundamentally linked
530 to its host, any changes to the available host spectrum will undoubtedly influence TBP prevalence in
531 the tick community (see in Pfäffle *et al.*, 2013). Moreover, contact rates between ticks and their
532 reservoir hosts have been suggested as a major factor driving seasonal TBP prevalence (Estrada-Peña
533 and de la Fuente, 2016). Furthermore, Coipan *et al* (2013) observed that several micro-organisms
534 presumed to share the same reservoir host, also presented similar seasonal detection patterns, thereby
535 supporting the previous hypothesis. However, an alternate hypothesis could also explain these
536 patterns, as carrying certain TBP was shown to improve tick resistance to challenging abiotic conditions.
537 Herrmann and Gern (2010, 2012) demonstrated that ticks carrying *Borrelia* species exhibited higher
538 survival rates in desiccating conditions and a lower affinity for humid conditions than non-infected
539 ticks, suggesting a potentially higher prevalence of *Borrelia*-infected questing ticks during or after
540 summer. Similarly, Neelakanta (2010) demonstrated a higher expression of *iafgp* gene, coding for an
541 antifreeze glycoprotein, in *A. phagocytophilum*-infected ticks. This thus conferred to ticks a stronger
542 resistance to cold that could lead to higher prevalence of *A. phagocytophilum*-infected questing ticks
543 during or just after winter. In this context, TBP prevalence in questing tick populations could be also
544 influenced by abiotic meteorological conditions. Additionally, this hypothesis coincides with
545 observations from Coipan, Takken, and the current study concerning *B. burgdorferi* s.l. infected
546 questing ticks. Indeed, a higher prevalence was always observed during and/or after summer,
547 potentially implying an improved survival rate of infected ticks during hot and dry summer conditions.
548 In contrast, *A. phagocytophilum* was not observed in greater prevalence during the cold season.
549 Our results, in combination with those from the literature, support the hypothesis that TBP
550 prevalence is influenced by both biotic and abiotic factors, and suggest one more time that undertaking
551 regular TBP analysis is an optimal method by which to improve knowledge on TBP dynamics and to
552 better understand TBD epidemiology.
553

554 ***Pathogen co-occurrence***

555 Tick co-infections are being identified more and more frequently (Halos *et al.*, 2005; Schicht *et al.*,
556 2011; Andersson *et al.*, 2013; Cosson *et al.*, 2014; Castro *et al.*, 2015; Moutailler *et al.*, 2016; Raileanu
557 *et al.*, 2017; Lejal *et al.*, 2018). Clinical co-infections with several TBP are frequently reported (Tijsse-
558 Klasen *et al.*, 2013; Moniuszko *et al.*, 2014; Hoversten and Bartlett, 2018) and are known to affect both
559 disease symptoms and severity (Krause *et al.*, 1996; Diuk-Wasser *et al.*, 2016). It is thus essential to
560 investigate TBP associations in ticks, to better identify potential clinical co-infections and to improve
561 epidemiological knowledge of TBD.

562 In this longitudinal three-year study, two TBP associations were significantly over-represented
563 compared to a random distribution: the first was between *B. garinii* and *B. afzelii*, as has been
564 previously observed in studies using similar detection tools (Moutailler *et al.*, 2016; Raileanu *et al.*,
565 2017), or different methods (16s rRNA gene sequencing, Aivelo *et al.*, 2019); the second was between
566 *B. garinii* and *B. spielmanii*. Interestingly, these findings contrast with published results on
567 *Ixodes ricinus* TBP. While performing a meta-analysis on data published from 2010 to 2016, Strnad *et*
568 *al* (2017) observed a negative correlation between *B. garinii* and *B. afzelii*. Similarly, Herrmann *et al.*
569 (2013) also detected a negative co-occurrence between these two species following the analysis of
570 7400 nymphs collected over three years. These results are coherent considering the host specificity of
571 these Borrelia species. Indeed, *B. garinii* doesn't share the same reservoir host (birds) than *B. afzelii* or
572 *B. spielmanii* (wood mice and bank voles, or hazel and garden dormice) (Humair *et al.*, 1998;
573 Kurtenbach *et al.*, 1998; Huegli *et al.*, 2002; Richter *et al.*, 2004, 2011; Taragel'ova *et al.*, 2008), and
574 none of these species are known to be transmitted transovarially.

575 Even though the associations we identified were statistically "over-represented", in actual fact we
576 only observed one more association than the fixed over-representation threshold (i.e. observed
577 associations = 3 and 2; minimum expected = 0 and 0; maximum expected = 2 and 1; for
578 *B. garinii*/*B. afzelii* and *B. garinii*/*B. spielmanii* associations, respectively). This indicates that caution
579 should be applied when drawing conclusions about permanent associations between these different

580 bacteria in ticks. Several different hypotheses could potentially explain these associations in the same
581 nymph. Firstly, hosts are likely to carry several adjacent feeding ticks. This phenomenon, known as co-
582 feeding, could promote pathogen exchange between ticks even in the absence of systemic host
583 infection (Randolph *et al.*, 1996). Secondly, as discussed by van Duijvendijk *et al*. (2016), when
584 bloodmeals are disrupted due to host grooming, immune response or death, ticks may feed on more
585 than one host to completely engorge, and consequently be exposed to several pathogens. Thirdly,
586 despite these TBP species segregating between bird and rodent hosts, all of them have been detected
587 in hedgehogs (Skuballa *et al.*, 2007, 2012), and *B. afzelii* and *B. garinii* have been simultaneously
588 detected in one Siberian chipmunk (Marsot *et al.*, 2013). Both of these mammals were found to host a
589 large number of tick larvae (Gern *et al.*, 1997; Marsot *et al.*, 2013), and Siberian chipmunks have been
590 reported to induce higher *B. burgdorferi* s.l. infection rates in nymphs, compared to bank voles and
591 wood mice (Marsot *et al.*, 2013) in the Sénart forest. A last hypothesis might be that our analyses
592 methods are unable to distinguish the rodent-circulating *B. garinii* OspA serotype 4 (corresponding to
593 *B. bavariensis*) (Margos *et al.*, 2009) from other *B. garinii* serotypes.

594 Associations between *B. garinii* and *B. valaisiana* are frequently reported, which isn't surprising
595 as these species share the same reservoir host (Hanincova *et al.*, 2003). This association was the most
596 common TBP association in a meta-analysis of literature published between 1984 and 2003 (Rauter
597 and Hartung, 2005), and has been reported several times since in later studies (Reye *et al.*, 2010;
598 Lommano *et al.*, 2012; Herrmann *et al.*, 2013). While we observed this association twice, both times in
599 association with a third *Borrelia* species, either *B. afzelii* or *B. spielmanii*, it was not significantly over-
600 represented compared to a random distribution. Among the three previously mentioned studies, only
601 Herrmann *et al.* (2013) demonstrated that this association was over-represented when compared to a
602 randomly sampled analysis. However our study was performed on a much smaller dataset (998 versus
603 7400 analysed nymphs), with a halved co-infection percentage (1.3% versus 3%), indicating that our
604 statistical analysis may be less powerful, which could explain why this association wasn't detected.

605 These contrasting tick pathogen association results highlight the complexity in clearly identifying
606 pathogen associations in field-collected ticks. Several other parameters can also potentially influence
607 pathogen association (host spectrum within the studied area, sample size influencing analytical
608 statistical power, identification bias…). In this context, performing investigations under controlled
609 conditions (suitable TBP growing and tick breeding systems…) will be a crucial future step to
610 experimentally test these different associations and improve our knowledge on TBP co-occurrence.
611

612 **CONCLUSIONS**

613 This three-year study of *I. ricinus*-borne pathogens; (1) identified several TBP previously reported
614 in the area, consistent with reservoir host availability; (2) highlighted the surprising presence of micro-
615 organisms not normally reported in questing ticks such as *R. felis*; (3) highlighted significant variations
616 in seasonal and inter-annual pathogen prevalence; and finally (4) identified several unexpected co-
617 occurrences between pathogens belonging to the *B. burgdorferi* s.l. complex. All these data underline
618 the importance of regularly performing tick collections and TBP analysis to improve our knowledge on
619 TBP presence and dynamics, and to better understand the ecology and epidemiology of TBD.

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AUTHOR CONTRIBUTIONS

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

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859 **SUPPORTING INFORMATION**

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
Borrelia burgdorferi	rpoB	Forward	GCTTACTCACAAAAGGCGTCTT		
		Reverse	GCACATCTCTTACTTCAAATCCT	83	Michelet *et al*., 2014
		Probe	AATGCTCTTGGACCAGGAGGACTTTCA		
Borrelia garinii	rpoB	Forward	TGGCCGAACTTACCCACAAAA		
		Reverse	ACATCTCTTACTTCAAATCCTGC	88	Michelet *et al*., 2014
		Probe	TCTATCTCTTGAAAGTCCCCCTGGTCC		
Borrelia afzelii	fla	Forward	GGAGCAAATCAAGATGAAGCAAT		
		Reverse	TGAGCACCCTCTTGAACAGG	116	Michelet *et al*., 2014
		Probe	TGCAGCCTGAGCAGCTTGAGCTCC		
Borrelia valaisiana	ospA	Forward	ACTCACAAATGACAGATGCTGAA		
		Reverse	GCTTGCTTAAAGTAACAGTACCT	135	Michelet *et al*., 2014
		Probe	TCCGCCTACAAGATTTCCTGGAAGCTT		
Borrelia lusitaniae	rpoB	Forward	CGAACTTACTCATAAAAGGCGTC		
		Reverse	TGGACGTCTCTTACTTCAAATCC	87	Michelet *et al*., 2014
		Probe	TTAATGCTCTCGGGCCTGGGGGACT		
Borrelia spielmanii	fla	Forward	ATCTATTTTCTGGTGAGGGAGC		
		Reverse	TCCTTCTTGTTGAGCACCTTC	71	Michelet *et al*., 2014
		Probe	TTGAACAGGCGCAGTCTGAGCAGCTT		
Borrelia bissettii	rpoB	Forward	GCAACCAGTCAGCTTTCACAG		
		Reverse	CAAATCCTGCCCTATCCCTTG	87	Michelet *et al*., 2014
		Probe	AAAGTCCTCCCGGCCCAAGAGCATTAA		
Borrelia miyamotoi	glpQ	Forward	CACGACCCAGAAATTGACACA		
		Reverse	GTGTGAAGTCAGTGGCGTAAT	94	Michelet *et al*., 2014
		Probe	TCGTCCGTTTTCTCTAGCTCGATTGGG		
Borrelia spp.	23S	Forward	GAGTCTTAAAAGGGCGATTTAGT		
		Reverse	CTTCAGCCTGGCCATAAATAG	73	Michelet *et al*., 2014
		Probe	AGATGTGGTAGACCCGAAGCCGAGT		
Anaplasma marginale	msp1	Forward	CAGGCTTCAAGCGTACAGTG		
		Reverse	GATATCTGTGCCTGGCCTTC	85	Michelet *et al*., 2014
		Probe	ATGAAAGCCTGGAGATGTTAGACCGAG		
Anaplasma platys	groEL	Forward	TTCTGCCGATCCTTGAAAACG		
		Reverse	CTTCTCCTTCTACATCCTCAG	75	Michelet *et al*., 2014
		Probe	TTGCTAGATCCGGCAGGCCTCTGC		
Anaphasma phagocytophilum	msp2	Forward	GCTATGGAAGGCAGTGTTGG		
		Reverse	GTCTTGAAGCGCTCGTAACC	77	Michelet *et al*., 2014
		Probe	AATCTCAAGCTCAACCCTGGCACCAC		
Anaplasma centrale	groEL	Forward	AGCTGCCCTGCTATACACG		
		Reverse	GATGTTGATGCCCAATTGCTC	79	Michelet *et al*., 2014
		Probe	CTTGCATCTCTAGACGAGGTAAAGGGG		
Anaplasma bovis	groEL	Forward	GGGAGATAGTACACATCCTTG		
		Reverse	CTGATAGCTACAGTTAAGCCC	73	Gondard *et al*., 2019
		Probe	AGGTGCTGTTGGATGTACTGCTGGACC		
Anaplasma spp.	16S	Forward	CTTAGGGTTGTAAAACTCTTTCAG		
		Reverse	CTTTAACTTACCAAACCGCCTAC	160	Gondard *et al*., 2019
		Probe	ATGCCCTTTACGCCCAATAATTCCGAACA		
Ehrlichia spp.	16S	Forward	GCAACGCGAAAAACCTTACCA		
		Reverse	AGCCATGCAGCACCTGTGT	98	Gondard *et al*., 2019
		Probe	AAGGTCCAGCCAAACTGACTCTTCCG		
Ehrlichia canis	gltA	Forward	GACCAAGCAGTTGATAAAGATGG		
		Reverse	CACTATAAGACAATCCATGATTAGG	136	Gondard *et al*., 2019
		Probe	ATTAAAACATCCTAAGATAGCAGTGGCTAAGG		

860 *(Continued)*

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
Candidatus Neoehrlichia mikurensis	groEL	Forward	AGAGACATCATTCGCATTTTGGA		
		Reverse	TTCCGGTGTACCATAAGGCTT	96	Michelet *et al*., 2014
		Probe	AGATGCTGTTGGATGTACTGCTGGACC		
Rickettsia conorii	23S-5S ITS	Forward	CTCACAAAGTTATCAGGTTAAATAG		
		Reverse	CGATACTCAGCAAAATAATTCTCG	118	Michelet *et al*., 2014
		Probe	CTGGATATCGTGGCAGGGCTACAGTAT		
Rickettsia slovaca	23S-5S ITS	Forward	GTATCTACTCACAAAGTTATCAGG		
		Reverse	CTTAACTTTTACTACAATACTCAGC	138	Michelet *et al*., 2014
		Probe	TAATTTTCGCTGGATATCGTGGCAGGG		
Rickettsia massiliae	23S-5S ITS	Forward	GTTATTGCATCACTAATGTTATACTG		
		Reverse	GTTAATGTTGTTGCACGACTCAA	128	Michelet *et al*., 2014
		Probe	TAGCCCCGCCACGATATCTAGCAAAAA		
Rickettsia helvetica	23S-5S ITS	Forward	AGAACCGTAGCGTACACTTAG		
		Reverse	GAAAACCCTACTTCTAGGGGT	79	Michelet *et al*., 2014
		Probe	TACGTGAGGATTTGAGTACCGGATCGA		
Rickettsia aeschlimannii	ITS	Forward	CTCACAAAGTTATCAGGTTAAATAG		
		Reverse	CTTAACTTTTACTACGATACTTAGCA	134	Michelet *et al*., 2014
		Probe	TAATTTTTGCTGGATATCGTGGCGGGG		
Rickettsia felis	orfB	Forward	ACCCTTTTCGTAACGCTTTGC		
		Reverse	TATACTTAATGCTGGGCTAAACC	163	Gondard *et al*., 2019
		Probe	AGGGAAACCTGGACTCCATATTCAAAAGAG		
Rickettsia spp.	gltA	Forward	GTCGCAAATGTTCACGGTACTT		
		Reverse	TCTTCGTGCATTTCTTTCCATTG	78	Gondard *et al*., 2019
		Probe	TGCAATAGCAAGAACCGTAGGCTGGATG		
Bartonella henselae	pap31	Forward	CCGCTGATCGCATTATGCCT		
		Reverse	AGCGATTTCTGCATCATCTGCT	107	Michelet *et al*., 2014
		Probe	ATGTTGCTGGTGGTGTTTCCTATGCAC		
Bartonella spp.	ssrA	Forward	CGTTATCGGGCTAAATGAGTAG		
		Reverse	ACCCCGCTTAAACCTGCGA	118	Gondard *et al*., 2019
		Probe	TTGCAAATGACAACTATGCGGAAGCACGTC		
Francisella tularensis	tul4	Forward	ACCCACAAGGAAGTGTAAGATTA		
		Reverse	GTAATTGGGAAGCTTGTATCATG	76	Michelet *et al*., 2014
		Probe	AATGGCAGGCTCCAGAAGGTTCTAAGT		
	fopA	Forward	GGCAAATCTAGCAGGTCAAGC		
		Reverse	CAACACTTGCTTGAACATTTCTAG	91	Michelet *et al*., 2014
		Probe	AACAGGTGCTTGGGATGTGGGTGGTG		
Coxiella burnettii	IS1111	Forward	TGGAGGAGCGAACCATTGGT		
		Reverse	CATACGGTTTGACGTGCTGC	86	Michelet *et al*., 2014
		Probe	ATCGGACGTTTATGGGGATGGGTATCC		
	icd	Forward	AGGCCCGTCCGTTATTTTACG		
		Reverse	CGGAAAATCACCATATTCACCTT	74	Michelet *et al*., 2014
		Probe	TTCAGGCGTTTTGACCGGGCTTGGC		
Apycomplexa	18S	Forward	TGAACGAGGAATGCCTAGTATG		
		Reverse	CACCGGATCACTCGATCGG	104	Gondard *et al*., 2019
		Probe	TAGGAGCGACGGGCGGTGTGTAC		
Babesia microti	CCTeta	Forward	ACAATGGATTTTCCCCAGCAAAA		
		Reverse	GCGACATTTCGGCAACTTATATA	145	Michelet *et al*., 2014
		Probe	TACTCTGGTGCAATGAGCGTATGGGTA		
Babesia canis	hsp70	Forward	TCACTGTGCCTGCGTACTTC		
		Reverse	TGATACGCATGACGTTGAGAC	87	Michelet *et al*., 2014
		Probe	AACGACTCCCAGCGCCAGGCCAC		

(Continued)

Targeted TBP	Targeted gene	Identity	Sequence	Amplicon size (bp)	Reference
Babesia ovis	18S	Forward	TCTGTGATGCCCTTAGATGTC		
		Reverse	GCTGGTTACCCGCGCCTT	92	Michelet *et al*., 2014
		Probe	TCGGAGCGGGGTCAACTCGATGCAT		
Babesia bovis	CCTeta	Forward	GCCAAGTAGTGGTAGACTGTA		
		Reverse	GCTCCGTCATTGGTTATGGTA	100	Michelet *et al*., 2014
		Probe	TAAAGACAACACTGGGTCCGCGTGG		
Babesia caballi	rap1	Forward	GTTGTTCGGCTGGGGCATC		
		Reverse	CAGGCGACTGACGCTGTGT	94	Michelet *et al*., 2014
		Probe	TCTGTCCCGATGTCAAGGGGCAGGT		
Babesia venatorum (sp. EU1)	18S	Forward	GCGCGCTACACTGATGCATT		
		Reverse	CAAAAATCAATCCCCGTCACG	91	Michelet *et al*., 2014
		Probe	CATCGAGTTTAATCCTGTCCCGAAAGG		
Babesia divergens	hsp70	Forward	GCGCGCTACACTGATGCATT		
		Reverse	CAAAAATCAATCCCCGTCACG	91	Michelet *et al*., 2014
		Probe	CATCGAGTTTAATCCTGTCCCGAAAGG		
Theileria spp.	18S	Forward	GTCAGTTTTTACGACTCCTTCAG		
		Reverse	CCAAAGAATCAAGAAAGAGCTATC	213	Michelet *et al*., 2014
		Probe	AATCTGTCAATCCTTCCTTTGTCTGGACC		
Hepatozoon spp.	18S	Forward	ATTGGCTTACCGTGGCAGTG		
		Reverse	AAAGCATTTTAACTGCCTTGTATTG	175	Gondard *et al*., 2019
		Probe	ACGGTTAACGGGGGATTAGGGTTCGAT		
Ixodes ricinus	ITS2	Forward	CGAAACTCGATGGAGACCTG		
		Reverse	ATCTCCAACGCACCGACGT	77	Michelet *et al*., 2014
		Probe	TTGTGGAAATCCCGTCGCACGTTGAAC		
Tick spp	16S	Forward	AAATACTCTAGGGATAACAGCGT		
		Reverse	TCTTCATCAAACAAGTATCCTAATC	99	Gondard *et al*., 2019
		Probe	CAACATCGAGGTCGCAAACCATTTTGTCTA		
Dermacentor reticulatus	ITS2	Forward	AACCCTTTTCCGCTCCGTG		
		Reverse	TTTTGCTAGAGCTCGACGTAC	83	Michelet *et al*., 2014
		Probe	TACGAAGGCAAACAACGCAAACTGCGA		
Dermacentor marginatus	ITS2	Forward	GCACGTTGCGTTGTTTGCC		
		Reverse	CCGCTCCGCGCAAGAATCT	139	Michelet *et al*., 2014
		Probe	TTCGGAGTACGTCGAGCTCTAGCAGA		
Escherichia coli	eae	Forward	CATTGATCAGGATTTTTCTGGTGATA		
		Reverse	CTCATGCGGAAATAGCCGTTA	102	Michelet *et al*., 2014
		Probe	ATAGTCTCGCCAGTATTCGCCACCAATACC		

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

Targeted genus	Targeted gene	Name	Sequence	Reference
Babesia; Theileria; Hepatozoon	18s rRNA gene	BTH 18S 1st F	GTGAAACTGCGAATGGCTCATTAC	Masatani *et al*., 2017
		BTH 18S 1st R	AAGTGATAAGGTTCACAAAACTTCCC	
		BTH 18S 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	
		BTH 18S 2nd R	CGGTCCGAATAATTCACCGGAT	
Anaplasma; Ehrlichia	16s rRNA gene	EHR1	GAACGAACGCTGGCGGCAAGC	Rar *et al*., 2005
		EHR2	AGTA(T/C)CG(A/G)ACCAGATAGCCGC	
		EHR3	TGCATAGGAATCTACCTAGTAG	
		EHR2	AGTA(T/C)CG(A/G)ACCAGATAGCCGC	
Borrelia	flaB	FlaB280F	GCAGTTCARTCAGGTAACGG	Loh *et al*., 2016
		FlaRL	GCAATCATAGCCATTGCAGATTGT	
		flaB_737F	GCATCAACTGTRGTTGTAACATTAACAGG	
		FlaLL	ACATATTCAGATGCAGACAGAGGT	
Rickettsia	rompB	Rc.rompB.4362p	GTCAGCGTTACTTCTTCGATGC	Choi *et al*., 2005.
		Rc.rompB.4,836n	CCGTACTCCATCTTAGCATCAG	
		Rc.rompB.4,496p	CCAATGGCAGGACTTAGCTACT	
		Rc.rompB.4,762n	AGGCTGGCTGATACACGGAGTAA	

Table S2. Targeted gene, amplicon size and primers sequences used for results confirmation.