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1 **Co-infection of bacteria and protozoan parasites in *Ixodes ricinus* nymphs collected in**
2 **the Alsace region, France**

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23

24 **Keywords:** *Ixodes ricinus*; Co-infection; Bacterial pathogens; Protozoan parasites; MALDI-
25 TOF MS.

26 **Abstract**

27 Fifty nymphal *Ixodes ricinus* ticks collected in Alsace, France, identified by morphological
28 criteria and using MALDI-TOF MS, were tested by PCR to detect tick-associated bacteria and
29 protozoan parasites. Seventy percent (35/50) of ticks contained at least one microorganism;
30 26% (9/35) contained two or more species. Several human pathogens were identified
31 including *Borrelia burgdorferi* s.s. (4%), *Borrelia afzelii* (2%), *Borrelia garinii* (2%),
32 *Borrelia valaisiana* (4%), *Borrelia miyamotoi* (2%), *Rickettsia helvetica* (6%) and “*Babesia*
33 *venatorum*” (2%). *Bartonella* spp. (10%) and a *Wolbachia* spp. (8%) were also detected. The
34 most common co-infections involved *Anaplasmataceae* with *Borrelia* spp. (4%),
35 *Anaplasmataceae* with *Bartonella* spp. (6%) and *Anaplasmataceae* with *Rickettsia* spp. (6%).
36 Co-infection involving three different groups of bacteria was seen between bacteria of the
37 family *Anaplasmataceae*, *Borrelia* spp. and *Bartonella* spp. (2%). Results highlight the panel
38 of infectious agents carried by *Ixodes ricinus*. Co-infection suggests the possibility of
39 transmission of more than one pathogen to human and animals during tick blood feeding.

40

41 **Introduction**

42 *Ixodes ricinus*, the European sheep tick, is widely found in temperate regions of the northern
43 hemisphere. It has a high attraction to biting humans (Parola and Raoult, 2001) and well
44 known in Europe as the vector of *Borrelia burgdorferi* sensu lato (s.l.), the etiologic agent of
45 Lyme disease (Stanek et al., 2012), tick-borne encephalitis (Amicizia et al., 2013) and
46 granulocytic anaplasmosis (Jin et al., 2012). The Alsace and Meuse areas, in north-eastern
47 France, are the most endemic regions of the country with more than 100 cases per 100,000
48 inhabitants between 2009 and 2011 (HCSP, 2014). Moreover, *I. ricinus* ticks have been
49 reported to harbour or transmit other emerging human pathogens such as *Rickettsia helvetica*,
50 *Rickettsia monacensis* and *Babesia divergens* (Rizzoli et al., 2014). The emergence of tick-
51 borne diseases in the past decade is worrying (Silaghi et al., 2016) and has underlined the
52 need to strengthen epidemiological and vector surveys. A recent study conducted in the region
53 of the French Ardennes on *I. ricinus* revealed high microorganism co-infection rates in adult
54 ticks, raising questions about the possible co-transmission of these agents to humans or
55 animals, and the potential consequences for human and animal health (Moutailler et al.,
56 2016). The aim of the present work was to test nymphal *I. ricinus* stage infections by *Borrelia*
57 spp. and other associated microorganisms circulating in an area of the Alsace region using
58 molecular tools.

59

60 **Materials and methods**

61 Ticks were collected using the drag flag technique (Parola and Raoult, 2001) between May
62 and June 2014 in the region of Murbach: N 47.918961 ; E7.210436 ; Altitude 520 m (Haut-
63 Rhin, Alsace, France) in the frame of the study of Goldstein et al. 2018 (Goldstein et al.,
64 2018). A convenient sample of 50 nymphs were selected for the current study, to launch a
65 collaboration between two reference centers in France. Each specimen was morphologically

66 identified using standard taxonomic keys (Hylliard,1996). Proteomic identification using
67 MALDI-TOF MS (Matrix-assisted laser desorption/ionization mass spectrometry) was
68 conducted as previously described (Yssouf et al., 2013). DNA from these specimens was then
69 extracted using the EZ1[®] Tissue Kit and BioRobot[®] EZ1[®](QIAGEN, Hilden, Germany)
70 according to the manufacturer's instructions. To assess the presence of the DNA of common
71 microorganisms in ticks, all extracted DNA were screened by real-time polymerase chain
72 reaction (qPCR), targeting specific genes of *Borrelia* spp. (*16S rRNA*), *Rickettsia* spp. (*gltA*),
73 *R. helvetica* (*rfbE*), *Bartonella* spp. (*ITS*), *Bartonella henselae* (*Pap31*), Anaplasmataceae
74 (*23S*), *Anaplasma phagocytophilum* (*polA*), *Coxiella burnetii* (*IS30A*) and *Babesia* spp.
75 (*18SrDNA*), as previously described (Aubry et al., 2016). Negative controls (qPCR mix and
76 DNA of uninfected *Rhipicephalus sanguineus* from laboratory colony) and positive controls
77 (*Borrelia crocidurae*, *Bartonella elizabethae*, *R.monacensis*, *A. phagocytophilum*, *Babesia*
78 *microti* and *C. burnetii* DNA originating from laboratory collection) were used in each
79 respective qPCR. Ticks analyzed by qPCR were considered to be positive when the cycle
80 threshold (Ct) value was less than or equal to 36, as used in previous studies (Aubry et al.,
81 2016). All ticks which were identified as being positive by qPCR were confirmed by
82 conventional PCR amplifications and sequencing. Sequences were analyzed as described
83 previously (Aubry et al., 2016).

84

85 **Results**

86 All specimens were morphologically identified as the nymphal stage of *I. ricinus*. The
87 protein spectra profiles from the MALDI-TOF MS analysis of tick legs were consistent and
88 reproducible with high-intensity peaks in the range of 2–20 kDa (Additional file1). Of them,
89 34 tick specimens obtained log-score values (LSVs) higher than 1.8 when they were tested
90 against the home-made reference MS spectra database. These LSVs (>1.8) considered as

91 correctly-identified specimens, corresponded to ticks from *I. ricinus*. For the remaining 16
92 ticks, the intensity and diversity of MS spectra were insufficient for relevant analysis.

93 Nearly 70% (35/50) of the *I. ricinus* collected were infected by at least one of the
94 microorganisms tested by the qPCR (Additional file2). A total of 27/50 (54%) specimens
95 harboured bacteria from the *Anaplasmataceae* family. Positive samples were successfully
96 amplified through the standard PCR using primers targeting 23S rRNA. However, after the
97 sequencing, only four high-quality sequences were obtained. The sequences of the remaining
98 23 amplicons, were of poor quality. Blast analyses of the four high-quality sequences show
99 99.6% identity with “*Wolbachia* endosymbiont of *Drosophila incompta*” (GenBank accession
100 no. CP011148). *Borrelia* spp. DNA was found in 20% (10/50) of the ticks. Sequencing
101 analyses targeting the 16S rRNA gene long fragment led to the identification of seven
102 sequences including two *B. burgdorferi* s.s. (GenBank accession no. CP019767 with 99.32
103 and 100% identity), two *B. valaisiana* (GenBank accession no. NR_036807, with 100%
104 identity), one *B. afzelii* (GenBank accession no. KX688604, with 100% identity), one *B.*
105 *garinii* (GenBank accession no. DQ147793, with 99.74% identity) and one *B. miyamotoi*
106 (GenBank accession no. JF951382, 100% identity). The three remaining sequences of *Borrelia*
107 spp. were of poor quality and were removed from analyses. Three ticks were found to be
108 positive for *Rickettsia* spp. *R. helvetica* was found in these three ticks using the *R. helvetica*-
109 specific qPCR targeting the *rflE* gene.

110 *Bartonella* spp. screening targeting the ITS gene found five positives. These five positive
111 samples were only successfully amplified using primers targeting the long fragment of the
112 *ftsZ* gene. None of the *rpoB*, *gltA* or ITS genes were amplified. The *ftsZ* sequences obtained
113 from these five positive samples had less than 1% mismatches. Blast analyses showed 91.9%
114 identity with uncharacterized *Bartonella* sp. strain B44608 (KY679153) and *B. vinsonii*
115 subsp. *vinsonii* (AF467757) and 91.3% identity with *B. vinsonii* subsp. *berkhoffii*

116 (KU292573). Phylogenetic analyses showed that this potentially new *Bartonella* sp. forms a
117 separate clade between *Bartonella ancashensis* and the other *Bartonellaceae* species despite
118 belonging to the latter (Figure 1).

119 Lastly, from one tick the DNA of *Babesia* spp. was amplified using primers and probes
120 targeting the 18S rDNA specific. A fragment of the *18S rRNA* gene was then amplified using
121 conventional PCR primers. The resulting sequence was blasted and presented 100% identity
122 with *Babesia* sp. “venatorum” (KU204792) and 98% with *B. odocoilei* (KC460321) detected
123 in elk blood in Canada (Figure 2). None of the tested ticks was positive for *A.*
124 *phagocytophilum*, *B. henselae* or *C. burnetii*.

125 The overall prevalence of the pathogens found in this study was as follows: *B. burgdorferi* s.s.
126 (4%), *B. afzelii* (2%), *B. garinii* (2%), *B. valaisiana* (4%), *B. miyamotoi* (2%), *R. helvetica*
127 (6%), “*B. venatorum*” (2%), *Bartonella* spp. (10%) and *Wolbachia* spp. (8%).

128 Co-infection between two or three microorganisms was observed among the positive ticks.
129 Indeed, co-infection by two microorganisms was found in 23% (8/35) of positive ticks. These
130 include *Borrelia* spp. with *Anaplasmataceae* (2/35) and *Bartonella* spp. with
131 *Anaplasmataceae* (3/35), followed by *Rickettsia* spp. with *Anaplasmataceae* (3/35). Co-
132 infection with three microorganisms was found in 3% (1/35) of positive ticks. This multiple
133 co-infection was found between *Borrelia* spp., *Bartonella* spp. and *Anaplasmataceae*
134 (1/35)(Additional file2).

135

136 **Discussion**

137 Results reported in the present study indicate that *I. ricinus* ticks collected in Alsace, France
138 were infected with multi-organism bacteria and protozoa (70%). However, the limited number
139 of tested nymphs cannot reflect a complete epidemiological picture of all bacteria and

140 protozoa circulating in or between *I. ricinus* ticks in this region. Thus, the full spectrum of
141 bacteria infecting the collected *I. ricinus* has not been identified. That said, the results
142 reported here present additional knowledge about *I. ricinus* associated microorganisms
143 circulating in the north-eastern region of France.

144 The high rate of infection (70%) was previously reported in this same region (Ferquel et al.,
145 2006) and in another neighbouring region (Moutailler et al., 2016). This study confirmed the
146 presence of a broad spectrum of *Borrelia* species belonging to the Lyme disease group,
147 namely: *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. valaisiana*, already reported in this
148 region (Ferquel et al., 2006; Moutailler et al., 2016). We also detected emerging pathogens
149 such as *B. miyamotoi* and *R. helvetica* as well as a probable symbiotic bacteria belonging to
150 the *Wolbachia* genus that has 99.6% identity with “*Wolbachia* endosymbiont of *Drosophila*
151 *incompta*”. Protozoan parasites belonging to the *Babesia* genus were detected in 2% of ticks.
152 However, phylogenetic analysis of the *18S rRNA* gene revealed that the *Babesia* detected here
153 is part of a group of “*B. venatorum*” strains that form a separate clade. These strains have
154 been detected in China (Jiang et al., 2015) and other European countries, such as Italy
155 (Cassini et al., 2010), Slovenia (Duh et al., 2005), the Netherlands (Kik et al., 2011) and the
156 Czech Republic (Venclikova et al., 2015), being isolated from *I. ricinus* ticks, roe deer or
157 both. *Babesia* “*venatorum*”, previously called *Babesia* sp. EU1, is an emerging human and
158 animal pathogen causing clinical features similar to those of *B. microti* in humans but its
159 typical clinical course is unknown (Jiang et al., 2015). In France, it was isolated by culture
160 from infected roe deer in the region of Deux Sèvres (Bonnet et al., 2007) and detected in *I.*
161 *ricinus* ticks collected in the Île-de-France region (Paul et al., 2016) and in Brittany with a
162 low prevalence of 0.65% and 0.35% in 2012 and 2013, respectively (Jouglin et al., 2017). A
163 study conducted in a neighbouring region did not detect “*B. venatorum*” in *I. ricinus* ticks
164 (Moutailler et al., 2016), suggesting a low prevalence or a recent introduction to the area.

165 *Bartonella* spp. was found in 10% of collected ticks. Only five positive samples in the qPCR
166 were amplified using the primers targeting the *ftsZ* gene. The phylogenetic tree showed that
167 this unique sequence of *Bartonella* sp. forms a separate clade between *B. ancashensis* and
168 other *Bartonellaceae* species. Unfortunately, we did not successfully amplify this *Bartonella*
169 sp. using other genes (La Scola et al., 2003). The lack of this additional data precludes the
170 complete description of this potentially new *Bartonella* species. *Bartonella* associated with *I.*
171 *ricinus* was previously reported (Chomel & Kasten 2010). DNA of *B. henselae*, the agent of
172 cat scratch disease was detected in 17.6% of ticks in the French Ardennes (Moutailler et al.,
173 2016) and in 9.8% of ticks collected in northern France (Halos et al., 2005). Interestingly, in
174 France, *B. henselae*, *B. doshiae*, *B. tribocorum* and *B. schoenbuchensis* were isolated from
175 blood samples of patients with poorly qualified syndromes after tick bites (Vayssier-Taussat
176 et al., 2016).

177 Co-infections with two or three microorganisms were identified in 18% of *I. ricinus*
178 nymphs (9/50), which accounted for 26% (9/35) of all infected nymphs. This co-infection
179 could be the result of co-feeding or feeding on an infected host by several pathogens
180 (Raileanu et al., 2017). *Anaplasmataceae* were involved in almost all combinations of co-
181 infections. The association of three bacteria was seen between *Anaplasmataceae*, *Borrelia*
182 spp. and *Bartonella* spp. (2%). In France, *B. burgdorferi* s.l. and *Bartonella* spp. have
183 previously been simultaneously detected in 1.3% of nymphs collected in Lille (Halos et al.,
184 2005). In other European countries, the prevalence of co-infection with different bacteria in *I.*
185 *ricinus* nymphs has been reported. Indeed, the rate reported in Romania was 17.1% (Raileanu
186 et al., 2017); in Italy, the prevalence was 5.71% (Otranto et al., 2014), whereas in western
187 Switzerland 4.6% of the nymphal *I. ricinus* collected were found to be infected with several
188 microorganisms (Lommano et al., 2012). In Spain, one nymph was co-infected with *B.*
189 *valaisiana* and *Rickettsia* sp. (Palomar et al., 2012). It appears that differences in co-infection

190 found in *I. ricinus* depend on the local epidemiology of infectious diseases and the availability
191 of host reservoirs of those bacteria.

192 Co-infections by tick-borne pathogens in humans have been reported. These occurred
193 from the bite of a single tick infected by several pathogens, or repetitive bites of several
194 infected ticks (Diuk-Wasser et al., 2016; Krause, 2002). Co-infections in humans mostly
195 involved Lyme borreliosis with either human anaplasmosis or babesiosis (Swanson et al.,
196 2006). However, in Europe, co-infection with *Borrelia* spp. , *A. phagocytophilum* and *Babesia*
197 spp. was observed in patients with tick-borne encephalitis in Poland (Moniuszko et al., 2014).
198 Co-infection with multiple pathogens may lead to an increase in the severity of the disease
199 and the development of an uncharacterized illness (Stanek et al., 2012). The risk of infections
200 in people with a tick-borne pathogen other than Lyme borreliosis or tick-borne encephalitis
201 after a bite from a co-infected tick is 2.5% (Hofhuis et al., 2017; Jahfari et al., 2016).

202 In the present study, we used MALDI-TOF MS to confirm the morphological
203 identification of ticks we tested. Applying this technology, 68% (34/50) of ticks, for all of
204 which a high-quality spectrum had been obtained, were successfully identified as *I. ricinus*.
205 This innovative proteomic approach was successfully applied in identifying laboratory-reared
206 ticks using only legs (Rothen et al., 2016; Yssouf et al., 2013), as well as ticks collected in the
207 field (Kumsa et al., 2016). However, for some specimens, low-quality spectra were obtained.
208 This may be due to several reasons including the storage method, homogenization method or
209 the size of the nymph legs which are smaller than adult legs.

210 In conclusion, this study has led to the identification of several zoonotic pathogens
211 including an uncharacterized *Bartonella* sp. carried by *I. ricinus* nymphs in the region of
212 Alsace, northern France. A high level of co-infection was revealed in the ticks collected in
213 this area, despite the small number of tested specimens. Infection or co-infection by multiple-

214 organisms suggests the need to increase our vigilance to avoid tick bites, especially by

215 nymphs which are more difficult to detect than adults.

216

217 **Competing interests**

218 The authors declare that they have no competing interests

219

220 **Author contribution**

221 Conceived and designed the experiments: NA, AL, PP. Performed the experiments: NA.

222 Analyzed the data: NA, AL, PP. Contributed reagents/materials/analysis tools: NB, DR, HD,

223 OM. Drafted the paper: NA. Critically reviewed the paper: PP, AL, NB, BJ.

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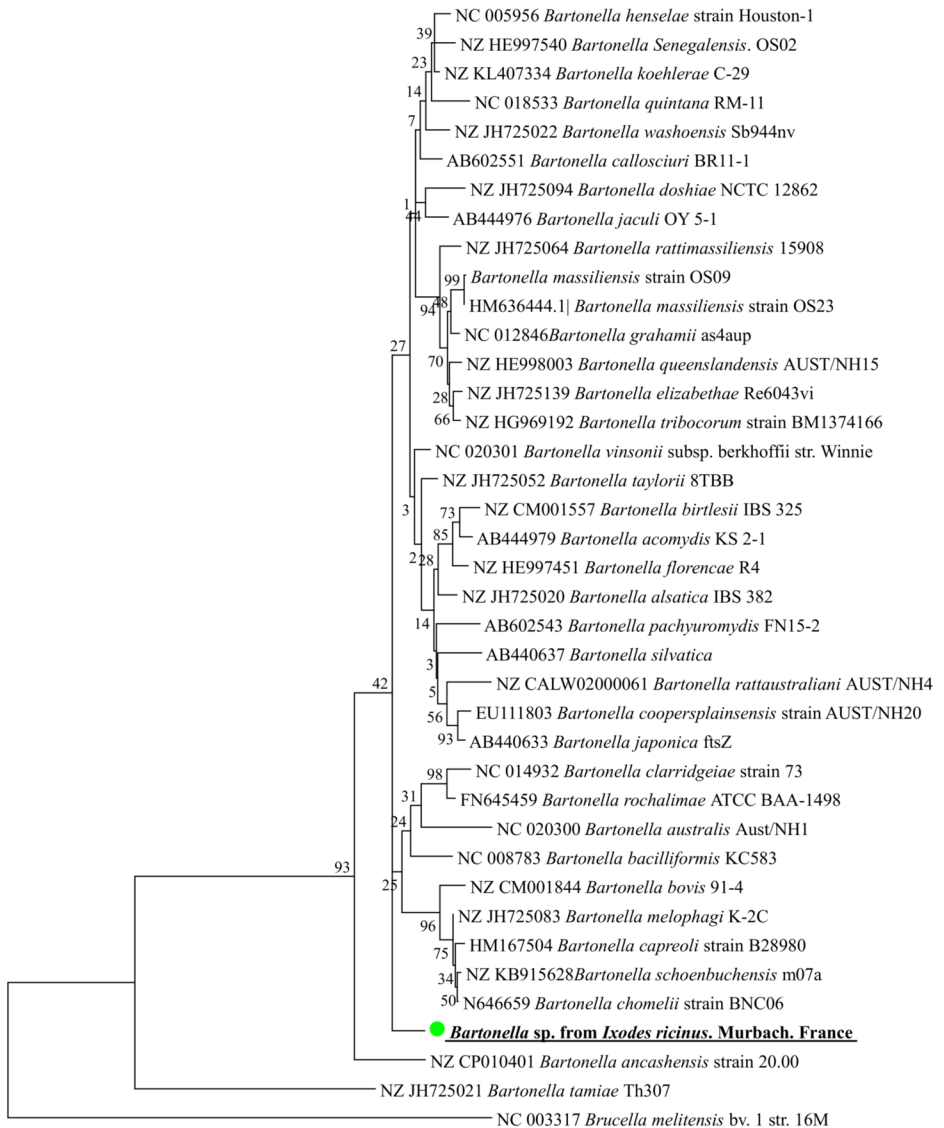
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364 **Figure legends**

365 **Figure 1. Phylogenetic tree showing the position of *Bartonella* sp. amplified from**
366 ***I. ricinus* in this study compared to other species.** The sequences of the *Bartonella* sp.
367 obtained in this study were aligned with *Bartonellaceae* family sequences available on
368 GenBank using CLUSTALW implemented on BioEdit v3 (Hall, 1999). The sequence of *ftsZ*
369 gene was first aligned, gaps and missing data were eliminated. The phylogenetic tree was
370 constructed using MEGA7 (Kumar et al., 2016). The evolutionary history was inferred by
371 using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The
372 percentage of trees in which the associated taxa clustered together is shown next to the
373 branches. Initial tree(s) for the heuristic search were obtained automatically by applying
374 Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the
375 Maximum Composite Likelihood (MCL) approach and then selecting the topology with
376 superior log likelihood value. A discrete Gamma distribution was used to model evolutionary
377 rate differences among sites (4 categories (+G, parameter = 0.3117)). The tree is drawn to
378 scale, with branch lengths measured in the number of substitutions per site. The analysis
379 involved 39 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.
380 All positions containing gaps and missing data were eliminated. There was a total of 335
381 positions in the final dataset.

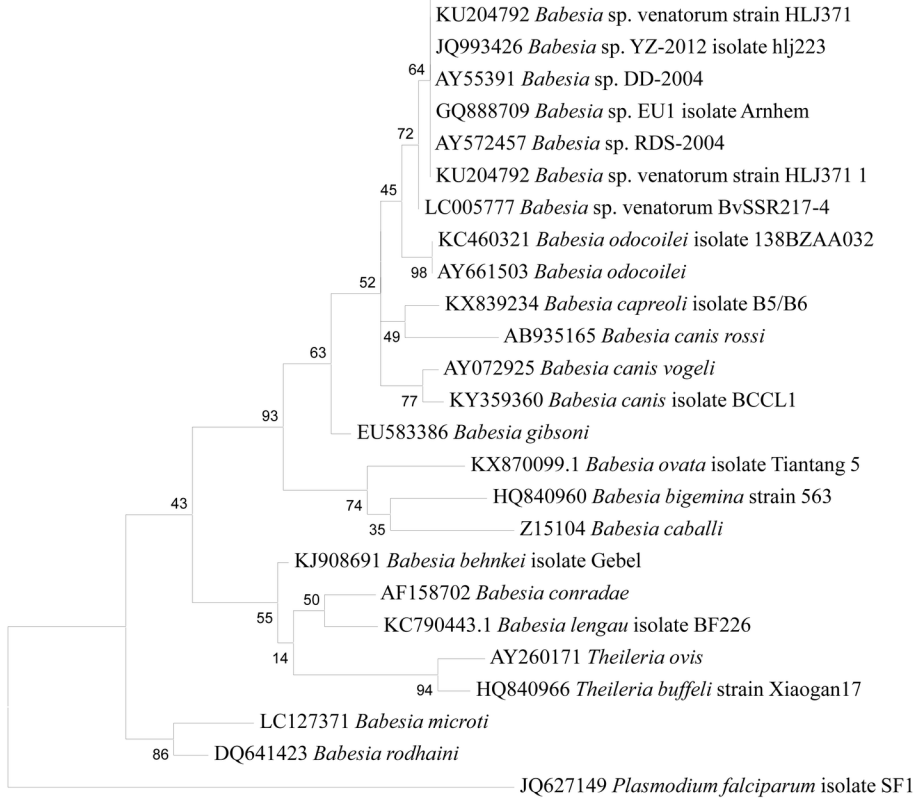
382 **Figure 2. Phylogenetic tree showing the position of *Babesia* sp. amplified from *I. ricinus***
383 **in this study compared to other species.** The sequence of the *Babesia* sp. obtained in this
384 study was aligned with *Babesiidae* family sequences available on GenBank using
385 CLUSTALW implemented on BioEdit v3 (Hall, 1999). The sequence of *18S* gene was first
386 aligned, gaps and missing data were eliminated. The phylogenetic tree was constructed using
387 MEGA7 (Kumar et al. 2016). The evolutionary history was inferred by using the Maximum

388 Likelihood method based on the Hasegawa-Kishino-Yano model. The tree with the highest
389 log likelihood (-1164.9283) is shown. The percentage of trees in which the associated taxa
390 clustered together is shown next to the branches. Initial tree(s) for the heuristic search were
391 obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of
392 pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach,
393 and then selecting the topology with superior log likelihood value. The tree is drawn to scale,
394 with branch lengths measured in the number of substitutions per site. The analysis involved
395 26 nucleotide sequences. All positions containing gaps and missing data were eliminated.
396 There were a total of 192 positions in the final dataset.



0.2

● **Babesia sp. from Ixodes ricinus . Murbach. France**



0.050