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**Original article**

# Evidence of *Bartonella* sp. in questing adult and nymphal *Ixodes ricinus* ticks from France and co-infection with *Borrelia burgdorferi* sensu lato and *Babesia* sp.

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**Abstract** – Ticks are known vectors for a wide range of pathogenic microorganisms. Their role in the transmission of some others is so far only suspected. Ticks can transmit multiple pathogens, however, little is known about the co-existence of these pathogens within questing ticks. We looked for the presence of DNA from three micro-organisms, *Bartonella* sp., *Borrelia burgdorferi* sensu lato and *Babesia* sp. which are known or suspected tick-borne pathogens, using a cohort of 92 questing *Ixodes ricinus* ticks collected from pastures in northern France. DNA was extracted from each individual tick and the presence of the three pathogens was investigated using Polymerase Chain Reaction (PCR) amplification. Nine among 92 samples (9.8%) demonstrated PCR products using *Bartonella* specific primers, 3 among 92 (3.3%) using *Borrelia burgdorferi* sensu lato specific primers and 19 among 92 (20.6%) using *Babesia* specific primers. Seven among 92 samples (7.6%) were PCR positive for at least two of the pathogens and one sample was positive for all three. Adult ticks (12/18; 67%) showed significantly higher infection rates compared to nymphs (11/74; 15%) for all three pathogens ($P < 0.001$). This study is the demonstration of the simultaneous presence of *Bartonella* sp., *Borrelia burgdorferi* sensu lato and *Babesia* sp. in questing *Ixodes ricinus* ticks.

**questing ticks / co-infection / PCR detection**

## 1. INTRODUCTION

Tick-borne diseases are of great medical and veterinary importance [41]. Ticks can transmit a large spectrum of pathogens including bacteria, viruses and parasites with a significant number of these pathogens being agents of emerging infectious diseases.

*Bartonella* species are emerging arthropod-borne pathogens that cause severe disease in humans and animals [1, 7]. They have been detected in diverse mammalian

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hosts such as cats, dogs, rodents, and most recently in ruminants [7, 10, 14]. *B. bacilliformis* is known to be transmitted by the sand fly [1], *B. quintana* by the human body louse [28] and *B. henselae* by the cat flea [13]. The vector involved in transmission of *Bartonella* in ruminants remains unknown, although ticks have been suspected [11]. *Bartonella* DNA has been detected in engorged *Ixodes* sp. in Europe [37, 39] while the presence of DNA from *Bartonella* species has been demonstrated in questing *Ixodes pacificus* ticks in California [11]. Furthermore, there are several reports of co-segregation of *Bartonella* sp. with known tick-borne pathogens [4, 16, 24, 39], emphasising the putative role of ticks in *Bartonella* transmission. It has been shown that *Bartonella* co-segregates in ticks with *Borrelia burgdorferi* sensu lato [21, 39], the causative agent of Lyme borreliosis and co-segregation of *Bartonella* with protozoan parasites of the genus *Babesia* is also suspected [21, 24, 42]. Considering that *Bartonella* sp. are emerging human pathogens and that ticks can transmit a large spectrum of pathogens to humans, it is, therefore, of high importance to determine the role of ticks in *Bartonella* transmission.

Lyme borreliosis is the most significant human vector-borne disease in Europe and the United States [2, 29] which also occurs in cattle [8, 43]. *Babesia* sp., the causative agents of babesiosis are transmitted by ixodid ticks and are capable of infecting a wide variety of vertebrate hosts including humans and cattle [20]. Co-infections of *Borrelia burgdorferi* sensu lato with *Bartonella henselae* have been found in patients presenting symptoms of atypical neuroborreliosis [16, 35] while mice have been found to be naturally co-infected with *Borrelia burgdorferi, Babesia microti* and a novel *Bartonella* sp. in the USA [21].

Although it is suspected that the *Ixodes* sp. tick may transmit multiple pathogens [4, 9, 27], limited work has been carried out on co-infection with multiple pathogens in questing ticks. Testing questing ticks is of greater interest than testing engorged ticks, since the identification of bacterial DNA relates to exposure during the previous blood meal before molting, whereas bacterial DNA identified in engorged ticks may only reflect upon the presence of that organism in the blood of the host.

Clinical surveys show that subjects co-infected by several tick-borne pathogens present more severe symptoms and a longer duration of illness [4]. Co-infection rates among human patients with a confirmed tick-borne infection have been reported to be as high as 39% [4]. However, data on the co-segregation of these tick-borne pathogens in ticks is still lacking.

Our goal was to determine the infection rates of *Bartonella* sp., *Borrelia burgdorferi* sensu lato and *Babesia* sp. in a cohort of nymphal and adult questing *Ixodes ricinus* ticks tested individually using specific Polymerase Chain Reaction (PCR) primer sets, in order to evaluate the potential vector role of *Ixodes ricinus* for *Bartonella* sp. and to establish the simultaneous presence of *Borrelia burgdorferi* and/or *Babesia* sp. DNA in ticks infected with *Bartonella* sp.

## 2. MATERIALS AND METHODS

### 2.1. Tick samples

This study was conducted on 94 questing *Ixodes ricinus* ticks including 76 nymphs, and 18 adults (9 males and 9 females) collected by flagging two neighbouring pastures during the spring of 2002 in the north of France (Lille area). Each sample was kept individually in absolute ethanol immediately after collection. Prior to extraction, the ticks were taxonomically classified. Each tick was treated individually.

### 2.2. DNA extraction

The ticks were crushed by shaking with a bead beater (mixer mill MM301, Qiagen, Hilden, Germany) and DNA was extracted

as previously described [19]. The efficiency (100%) of DNA extraction was confirmed for 92 *Ixodes ricinus* samples by PCR amplification of the 16S mitochondrial gene using tick-specific primers. Two nymphal samples, for which no amplification products were obtained, were excluded from the study.

## 2.3. PCR amplification

All PCR reactions were performed in a Genamp thermocycler (Applied biosystem, Courtabœuf, France). Each reaction was carried out in 50 μL volume containing 0.5 μmol/μL of each primer, 2.5 mM of each dNTP, 5 μL of 10X PCR buffer, 1 U of *Taq* DNA polymerase (Takara biomedical group, Shiga, Japan) and 5 μL of the DNA extract. Negative (sterile water) and positive controls were included in each experiment.

The efficiency of tick DNA extraction was evaluated by amplification of a fragment of the tick mitochondrial 16S rRNA gene (ribosomal DNA [rDNA]) using tick-specific primers [6]. The presence of *Borrelia burgdorferi* sensu lato, *Bartonella* sp. or *Babesia* sp. DNA in tick DNA extracts was tested by PCR using specific primers for each of the pathogens [32–34, 39]. The primer sets used in this study and PCR conditions are listed in Table I.

DNA electrophoresis was carried out in 2% agarose gels containing ethidium bromide, and DNA fragments were visualised under ultraviolet light. Each reaction was performed at least twice.

## 2.4. Sequencing and sequence analysis

All amplified fragments using *Bartonella* sp. primers were sent for sequencing to GENOMEXPRESS (Meylan, France). The DNA amount was sufficient to allow sequencing for one of the nine *Bartonella*-positive samples. The sequence, derived from this sample, was compared with known sequences listed in the GenBank nucleotide sequence databases. The BLAST search option of the National Center for Biotechnology Information (NCBI) (internet site http://www.ncbi.nlm.nih.gov) was used to confirm the origin of the sequence.

## 2.5. Statistical analysis

Chi-square for trends and the Fisher exact test for dichotomous variables were performed on Epi-Info version 3.2 (CDC, Atlanta, GA, USA) and SAS/stat® (version 8.02) softwares. $P < 0.05$ was regarded as significant.

# 3. RESULTS

## 3.1. Specific detection of *Bartonella* sp. DNA in questing *Ixodes ricinus* ticks

Amongst the 92 samples, 9 (9.8%), including 6 adults (33% of the adult ticks) and 3 nymphs (4% of the nymphs) showed an amplified fragment of the expected size for specific amplification of the *Bartonella gltA* gene (Tab. II). Sequence analysis of the fragment obtained from 1 adult sample (registered in Genbank under the accession number: AY568725) showed 96% identity with the *gltA* gene of *B. schoenbuschensiis* (Genbank accession number: AJ564633). Adult ticks were significantly ($P = 0.0015$) more likely to be infected than nymphs (Tab. II).

## 3.2. Specific detection of *Borrelia burgdorferi* sensu lato and *Babesia* sp. DNA in ticks

Visualisation of a 170 bp. amplified fragment of *B. burgdorferi* sensu lato 23S-5S spacer region was obtained for 3 (3.3%) of the 92 samples, of which 2 were adult ticks (11% of the adult ticks) and one was a nymph (1.3% of the nymphs) (Tab. II). No statistically significant difference was detected in prevalence of infection between the adults and nymphs.

Amongst the 92 samples, 19 samples (20.6%), including 10 adults (56% of the adult ticks) and 9 nymphs (12% of the

**Table I.** Oligonucleotide primers used in this study.

| Organism | Template | | Primer sequences 5’-3’ | Product size bp | Targeted gene | PCR conditions | Reference |
|---|---|---|---|---|---|---|---|
| Tick | TQ16S+1F | F | CTGCTCAATGATTTTTTAAATTGTGTGG | 320 | ADNr 16S mitochon-drial | Denaturation: 94 °C 8 min<br>Hybridization: 10 cycles:<br>92 °C 1 min, 48 °C 1 min, 72 °C 1 min 30 s<br>32 cycles<br>92 °C 1 min, 54 °C 1 min, 72 °C 1 min 30 s<br>Extension: 72 °C 10 min | [6, 19] |
| | TQ16S-2R | R | ACGCTGTTATCCCTAGAG | | | | |
| *Bartonella* sp. | 781 | F | GGGGACCAGCTCATGGTGG | 356 | Citrate synthase | Denaturation: 90 °C 8 min<br>Hybridization: 35 cycles<br>93 °C 20 s, 54 °C 30 s, 72 °C 30 s<br>Extension: 72 °C 5 min | [32] |
| | 1137 | R | AATGCAAAAAGAACAGTAAACA | | | | |
| *Borrelia burgdorferi* sensu lato | 5SCB | F | GAGAGTAGGTTATTGCCAGGG | 226 | 23S- 5S spacer | Denaturation: 95 °C 8 min<br>Hybridation: 30 cycles<br>94 °C 1 min, 50 °C 1 min, 72 °C 1 min<br>Extension: 72 °C 5 min | [39] |
| | 23SN2 | R | ACCATAGACTCTTATTACTTTGACCA | | | | |
| | BORVRigs | R | AATAATATATATCTTTGTTTAAT | 170 | | | This study |
| *Babesia* sp. Set 1 | PIRO A | F | AATACCCAATCCTGACACAGG | 408 | 18S Small sub-unit ribosomal DNA | Denaturation: 95 °C 8 min<br>Hybridization: 30 cycles<br>94 °C 1 min, 52 °C 1 min, 72 °C 1 min<br>Extension: 72 °C 10 min | [33] |
| | PIRO B | R | TTAAATACGAATGCCCCCAAC | | | | |
| Set 2 | bab1 | F | CTTAGTATAAAGCTTTTATACAGC | 230 | 18S Small sub-unit ribosomal DNA | Denaturation: 94 °C 1 min<br>Hybridization:<br>5 cycles<br>94 °C 1 min, 49 °C 1 min, 72 °C 1 min<br>30 cycles<br>94 °C 1 min, 53 °C 1 min, 72 °C 1 min<br>Extension: 72 °C 10 min | [34] |
| | bab4 | R | ATAGGTCAGAAACTTGAATGATACA | | | | |

**Table II.** Number of samples from 92 questing *Ixodes ricinus* ticks showing specific PCR amplification of the gene of the citrate-synthase (*gltA*) of *Bartonella* sp., the 23S-5S spacer of *Borrelia burgdorferi* sensu lato or the gene of the small ribosomal sub-unit (SS-rDNA) of *Babesia* sp. and number of samples showing simultaneous specific PCR amplification of 2 or 3 of the targeted micro-organisms.

| *Ixodes ricinus* samples | Detection of pathogen DNA<br>Number of infected ticks / number of samples (%) | | | Co-detection of pathogen DNA<br>Number of infected ticks / number of samples (%) | | | | | Number of coinfected ticks / number of infected ticks (%) |
|---|---|---|---|---|---|---|---|---|---|
| | *Bartonella* sp. | *Babesia* sp. | *Borrelia burgdorferi* s.l. | *Bartonella* / *Borrelia* | *Bartonella* / *Babesia* | *Borrelia* / *Babesia* | *Bartonella* / *Borrelia* / *Babesia* | Total number of co-infected ticks | |
| Females | 3/9 (33) | 6/9 (67) | 1/9 (11) | 0/9 (0) | 3/9 (33) | 1/9 (11) | 0/9 (0) | 4/9 (44) | 4/6 (67) |
| Males | 3/9 (33) | 4/9 (44) | 1/9 (11) | 0/9 (0) | 0/9 (0) | 0/9 (0) | 1/9 (11) | 1/9 (11) | 1/6 (17) |
| Nymphs | 3/74 (4) | 9/74 (12) | 1/74 (1.3) | 1/74 (1.3) | 1/74 (1.3) | 0/74 (0) | 0/74 (0) | 2/74 (2.7) | 2/11 (18) |
| Total | 9/92 (9.8) | 19/92 (20.6) | 3/92 (3.3) | 1/92 (1) | 4/92 (4) | 1/92 (1) | 1/92 (1) | 7/92 (7.6) | 7/23 (31) |

nymphs), showed a 400 bp amplified fragment of *Babesia* sp. SS-rDNA gene using a first set of primers. The presence of babesial DNA in the sample was further confirmed by a second PCR amplification of a 230 bp fragment of the SS-rDNA gene using a second set of *Babesia* sp. primers (set 2, Tab. I). All 19 positive samples showed an amplified fragment of the expected size (Tab. II). Adult ticks were almost ten times more likely to be PCR positive for Babesial DNA than nymphs (Odds ratio: 9.03; 95% confidence intervals: 2.42–33.69; $P < 0.001$).

Overall, 7 (7.6 %) of the ticks were found to be co-infected with at least two pathogens, accounting for 30% of the 23 infected ticks (Tab. II). Among the three *Borrelia burgdorferi* sensu lato positive samples, all were co-infected. The positive nymph was also found to be PCR positive for *Bartonella* sp., one of the two positive adult ticks was also found to be PCR-positive for *Babesia* sp. and the second one was also positive for the two other agents tested (Tab. II). Among the 19 *Babesia* sp. positive samples, three adults and one nymph were also found to be PCR positive for *Bartonella* while one adult was PCR positive for *Borrelia burgdorferi* sensu lato (Tab. II). Among the adult ticks, the prevalence of co-infection was higher in female ticks (4/9; 44%) than in male ticks (1/9; 11%), but that difference was not statistically significant.

## 4. DISCUSSION

The present study provided evidence for the common co-existence of various tick-borne pathogens in Ixodid ticks and for the first time, the simultaneous presence of *Bartonella* sp., *Borrelia burgdorferi* sensu lato and *Babesia* sp. DNA in the same tick.

Our study confirmed the presence of *Bartonella* sp. DNA in European questing ticks since 9.8% of the samples were found to be positive by PCR for *Bartonella* sp. supporting the hypothesis that *Bartonella* bacteria can be carried by ticks. The partial sequence of the citrate-synthase (*gltA)* gene obtained from one sample was related to *Bartonella schoenbuschensis* (96% similarity) which infects the roe-deer (*Capreolus capreolus*) in Europe [14]. The presence of *Bartonella* in ruminant blood at very high infection levels has been previously reported [10]. Since the DNA of ruminant-related *Bartonella* species has already been detected in Ixodid ticks, they have been suspected to be a potential source of infection for ruminants [11, 39]. Considering that *Ixodes* ticks feed once per stage, the presence of *Bartonella* in questing ticks at different developmental stages would suggest the possibility of trans-stadial transmission of these bacteria, potentially related to transmission to mammalian hosts.

The analysis of the same tick cohort for co-infection with other pathogens revealed that 3.3% of the ticks were PCR positive for *Borrelia burgdorferi* sensu lato and 20.6% for *Babesia* sp. The prevalence of 3.3% of *B. burgdorferi* sensu lato PCR-positive ticks is relatively low compared with other studies probably due to the ecological habitat from where the samples were collected. Reported prevalences of *B. burgdorferi* sensu lato in ticks vary from 1.3% in Switzerland [5] to 55% in areas of Finland [23] with an average between 10 and 20% [9, 22, 29, 36]. Here, we investigated a tick population living on pastures. Since reservoirs for Lyme borreliosis are often forest living rodents, the prevalence of Lyme spirochetes is naturally higher in wood or fields near wood areas than in pastures [18, 38]. However, the presence of *Borrelia burgdorferi* sensu lato DNA in ticks collected on pastures strongly suggests that the risk for borreliosis is not limited to decidual forest areas.

Twenty-one percent of questing *Ixodes ricinus* ticks were PCR positive for *Babesia* sp., which correlates with infection rates described in other studies: between 6.2% and 62% of babesial infection in questing [3, 15], or engorged ticks [17]. Although the particular species of *Babesia* was not determined by sequencing, we confirmed that

the amplification was specific to *Babesia* sp. by using two different sets of primers which were described to be specific to the *Babesia* genus [33, 34].

Interestingly, adult ticks showed significantly higher infection rates compared to nymphs for all three pathogens. Previous studies have already shown different ranges of prevalence between adults and nymphs, for *Borrelia burgdorferi* [9, 22], for *Babesia* sp. [26] and *Bartonella* sp. [12]. One hypothesis explaining this difference is the number of potentially infected meals which is higher for adults than for nymphs (two blood meals for adults versus one for nymphs). This could either be related to the existence of stage-specific factors, favouring the acquisition of the pathogen by the tick at a special stage as previously shown for other vector arthropods such as tsetse flies [31]. It is noteworthy that despite the lack of a statistically significant difference in infection rates between males and females for any of the targeted pathogens, females were co-infected more often (44%) than males (11%).

Up to 7.6% of the ticks were co-infected with at least two pathogens and 30% of the infected ticks were co-infected (Tab. II). *Borrelia burgdorferi* and *Bartonella* sp. have already been simultaneously detected in ticks collected on wild ruminants [39] whereas the coexistence of DNA of *Borrelia burgdorferi* sensu lato and *Babesia microti* in *Ixodes ricinus* ticks showed rates ranging from 2% to 19% [4, 40]. Our results emphasise the fact that the co-existence of pathogens is not a rare event in ticks. This co-existence appears to be linked with co-segregations or co-infections in mammals including humans [4, 16, 21, 27, 30, 42]. An increasing number of studies suggest that symptoms attributed to one tick-borne disease are not due to a single pathogen [25]. For example, patients with ongoing symptoms attributed to chronic Lyme disease have been shown to be co-infected with *Bartonella* sp. [16, 35] or *Babesia* sp. and *Ehrlichia* sp. [25]. Immunoserologic evidence of co-infection with *B. burgdorferi* sensu lato and *Babesia* sp. among individuals in tick-endemic areas is also well documented [27, 30].

This study confirms the fact that the coexistence of *Bartonella* sp. and/or *Babesia* sp. and/or *Borrelia burgdorferi* sensu lato, occurs in ticks. Factors affecting tick co-infections and the risk to humans posed by such co-infections in terms of therapy will need further studies.

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