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Co-infection of *Borrelia afzelii* and *Bartonella* spp. in bank voles from a suburban forest

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ABSTRACT

We report the molecular detection of *Borrelia afzelii* (11%) and *Bartonella* spp. (56%) in 447 bank voles trapped in a suburban forest in France. Adult voles were infected by significantly more *Borrelia afzelii* than juveniles ($p < 0.001$), whereas no significant difference was detected in the prevalence of *Bartonella* spp. between young and adult individuals ($p = 0.914$). Six percent of the animals were co-infected by both bacteria. Analysis of the bank vole carrier status for either pathogen indicated that co-infections occur randomly ($p = 0.94$, $CI_{95} = [0.53; 1.47]$). Sequence analysis revealed that bank voles were infected by a single genotype of *Borrelia afzelii* and by 32 different *Bartonella* spp. genotypes, related to three known species specific to rodents (*B. taylorii*, *B. grahamii* and *B. doshiae*) and also two as yet unidentified *Bartonella* species. Our findings confirm that rodents harbor high levels of potential human pathogens; therefore, widespread surveillance should be undertaken in areas where humans may encounter rodents.

Rodents are recognized as a major reservoir of pathogens such as tick-borne diseases, which can cause serious illness in livestock and humans [1]. Tick-borne diseases are extraordinarily diverse and are responsible for high morbidity and mortality rates around the world. *Ixodes ricinus*, the most widespread and abundant tick in Western Europe, accounts for significant numbers of human bites and is a major vector of pathogens responsible for rodent-borne disease [2,3].

In Europe, the most prevalent tick-borne disease is Lyme borreliosis, caused by bacteria belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex [4]. Particular species of rodents are known to be infected by certain *Borrelia* species. *Borrelia afzelii* has been described as specific to smaller rodent species, including the wood mouse (*Apodemus sylvaticus*) and bank vole (*Myodes glareolus*), while *B. burgdorferi* sensu stricto is found in other rodent hosts, such as the red squirrel (*Sciurus vulgaris*) and brown rat (*Rattus norvegicus*) [5–7]. These *Borrelia* species are known causative agents of human disease [8] but in addition, they can assume an aggravating role when co-infected along with *Babesia* sp., *Anaplasma* sp., *Ehrlichia* sp., and *Bartonella*

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sp. in Lyme disease [9,10]. However the prevalence of these co-infections remains unknown as their diagnosis is exceedingly difficult.

At least ten of the 26 *Bartonella* spp. are asymptomatically carried by rodents, and five of the rodent-specific *Bartonella* spp. (*B. grahamii*, *B. elizabethae*, *B. vinsonii* subsp. *arupensis*, *B. washoensis* and *B. tamiae*) have been identified as zoonotic agents [11]. These bacteria induce diverse and species-dependent diseases in humans, but mostly cause bacteremia, myocarditis, endocarditis, lymphadenitis and hepatitis [12]. *Bartonella* spp. are mostly transmitted to rodents by vectors such as fleas (*Ctenophthalmus nobilis nobilis* for voles) [13] and to a lesser extent, ticks (*I. ricinus*) [14].

In recent times, outdoor activities in suburban forests have been increasing, consequently providing excellent conditions for contact between rodents, arthropod vectors and humans. Thus for the exposed human population, there is an urgent need to conduct studies investigating the numbers of rodents carrying vector-borne zoonotic pathogens. Therefore this study was undertaken to obtain an initial assessment of the prevalence, diversity and co-infection rates of *B. burgdorferi* sensu lato and *Bartonella* spp. within a population of bank voles (*M. glareolus*), the most abundant diurnal murid species in the studied site, a highly visited suburban forest [15].

1. Material and methods

1.1. Study site, trapping and animal processing

The study was conducted in the Sénart Forest (3200 ha, 02°29′E, 48°40′N), which is located 22 km from Paris (France) between the months of March and October over a four-year period (2006–2009). This forest is essentially composed of broad-leaved trees dominated by oaks (*Quercus robus*, *Quercus pedunculata*) and European hornbeams (*Carpinus betulus*), with two other forest tree species, the sweet chestnut (*Castanea sativa*) and the lime tree (*Tilia vulgaris*). Infection prevalence was studied in 447 bank voles trapped within a grid system consisting of 104 geo-localized Sherman© baited traps. For all captured individuals, sex was determined and the age class (juvenile and adult) evaluated by coupling the date of capture and the body weight. For *B. burgdorferi* s.l. detection, ear biopsy was obtained by cutting a small piece from the ear of the trapped voles (maximum 3 mm^2) with scissors, then immediately storing the sample in 90% ethanol [7]. The bank voles were then sacrificed and their spleens removed under sterile conditions and stored at −80 °C until they were to be used for *Bartonella* spp. detection.

1.2. DNA extraction

For *Borrelia* analysis, DNA from one ear biopsy per bank vole was extracted using the NucleoSpin® Tissue kit (Machery-Nagel, Düren, Germany) [7].

The presence of *Bartonella* spp. was detected by extracting DNA from spleen samples of each bank vole. First, a piece of the sample was ground in 500 μl of F-12 nutrient mixture medium (Invitrogen) and 250 μl of this mixture were placed in a Lysing Matrix E tube (MP Biomedical, Solon, USA). Then, 500 μl of hexadecyltrimethylammonium bromide (CTAB) extraction buffer and 500 μl of phenol–chloroform–isoamyl alcohol (25:24:1; pH 8.0) were added to the sample. Extraction buffer was prepared by mixing equal volumes of 10% (w/v) CTAB (Sigma–Aldrich, France) in 0.7 M NaCl with 240 mM potassium phosphate buffer, at pH 8.0. Samples were homogenized and lysed for 30 s at a speed setting of 6500 rpm in a Precellys 24 centrifuge (Bertin Technologies, Montigny-le-Bretonneux, France). The aqueous phase, containing nucleic acids, was separated by centrifugation at 16,000 × *g* for 5 min at 4 °C, and transferred to a new microcentrifuge 2 ml tube. Phenol was extracted by adding an equal volume of chloroform–isoamyl alcohol (24:1), mixing the tubes by inversion then centrifuging for 5 min at 16,000 × *g* (4 °C). The nucleic acids present in the aqueous phase were precipitated by adding two volumes of 30% (w/v) polyethylene glycol (Fluka Biochemica, Sigma–Aldrich, France) – 1.6 M NaCl solution for 2 h at room temperature. The tubes were then centrifuged for 10 min at 18,000 × *g* (4 °C). The pelleted nucleic acids were washed in ice-cold 70% (v/v) ethanol and then centrifuged at 10,000 × *g* for 3 min (4 °C). The ethanol was eliminated, and the pellets were air dried, and then resuspended in 50–200 μl of Tris–EDTA buffer (pH 7.4), depending on their size, and stored overnight at 4 °C.

1.3. PCR amplification and sequencing

PCR analysis for *B. afzelii* was carried out using DNA extracted from the ears, and *Bartonella* spp. detection was performed using DNA extracted from the spleen.

Each reaction was carried out in a total volume of 25 μl containing; 0.5 mmol/ml of each primer, 2.5 mM of each dNTP, 2.5 μl of 10× PCR buffer, 1 U of Taq DNA polymerase (Takara Biomedical Group, Shiga, Japan) and 5 μl of the DNA extract. The PCR was performed in a MyCycler thermocycler (Bio-Rad, Strasbourg, France). Negative and positive controls were included in each run, (water and then either genomic DNA of *B. burgdorferi* sensu stricto isolated in our laboratory or genomic DNA of *B. birtlesii* strain IBS 325).

The presence of *B. afzelii* in the extracted DNA has been detected using a PCR that targets the *rrs* gene (16S rRNA) [7]. The intergenic 16S(*rrs*)–23S(*rrlA*) spacer (IGS) was then used in this study to characterize the diversity of *B. afzelii* as previously described [16].

The presence of *Bartonella* spp. was determined by using a portion of the *gltA* gene encoding for the citrate synthase protein, previously shown to be a reliable tool for distinguishing *Bartonella* spp. and closely related genotypes [17]. The *gltA* fragment was amplified by PCR using primers specific to the *Bartonella* genus [18].

The amplification products were analyzed by electrophoresis on 1% agarose gels with 0.1 mg/ml of ethidium bromide and imaged under UV light. PCR products obtained from positive samples were then purified and sequenced in both directions by Eurofins MWG Operon (Germany).

1.4. Phylogenetic analysis and construction of phylogenetic trees for gltA and IGS sequences

Sequences were analyzed using the sequence analysis software Lasergene (DNASTAR, Madison, WI, USA), which determined the consensus sequence for the amplified region of the *gltA* gene and the intergenic 16S(*rrs*)–23S(*rrlA*) spacer. Sequence data were aligned with those of typed strains of *Bartonella* and *Borrelia* species using CLUSTAL W software (included in MEGA 5). MEGA 5 software (http://www.megasoftware.net/) [19] was used to calculate the DNA sequence similarities (P-distance). Phylogenetic trees were constructed using the neighbor joining (NJ) method, combined with the Jukes-Cantor parameters method. Bootstrap analysis was carried out on 1000 replications. Sequences were also analyzed by the BLASTn algorithm [20] to identify the closest relative.

1.5. Data analysis

Co-infections by *B. afzelii* and *Bartonella* spp., as well as rodent age compared to the carrier-status for each pathogen, were statistically evaluated using the chi-squared test and the software Epi Info version 3.5.3 (CDC, Atlanta, GA, USA). A two-tailed Fisher's exact test was used when the expected numbers of observations were less than five. A p-value of <0.05 was considered to be statistically significant. The diversity of *Bartonella* spp. sequences was calculated using the Hunter and Gaston discrimination index [21].

2. Results

B. afzelii rrs was detected in 49 of the 447 bank voles (11%), and infected more adults than juveniles, at levels of 14.4% and 1.7% respectively ($p < 0.001$). IGS sequences were all identical (100% identity – accession number: JQ693994) and shared 93.8% identity with the *B. afzelii* IGS sequence amplified from *I. ricinus* collected in southern Sweden (accession number AY363694) [16]. The phylogenetic tree based on the IGS fragment showed that the unique *B. afzelii* sequence identified here is distant from all other IGS *B. afzelii* sequences amplified from rodents, ticks and humans from Sweden, UK and Latvia, with a pairwise sequence dissimilarity that ranged between 4.8% and 6.4% (Fig. 1).

The *Bartonella* spp. specific fragment of the *gltA* gene was amplified from 252/447 bank voles (56.4%). Prevalence rates were similar in juveniles (56.7%) as in adults (56.3%) ($p = 0.915$, $CI_{95} = [0.72; 1.34]$). Sequence analysis of *gltA* fragments revealed the presence of 32 different *gltA* genotypes (unique sequence variants with $\geq$1 nucleotide difference). The percentage identity of the *gltA* sequence with known *Bartonella* type strains ranged from 91 to 100%. Simpson's index was 0.85, indicating that a diverse range of *Bartonella* spp. *gltA* amplicons was found in the bank voles. A phylogenetic tree based on the partial sequence of the *gltA* gene is shown in Fig. 2. The 32 *gltA* genotypes were distributed in four clades along the *gltA* dendrogram, i.e., A, B, C and D (Fig. 2), and which are detailed as follows.

Of these 32 genotypes, 12 belonged to clade A (A32RCG, A42FCG, A60RCG, A252FCG, A286FCG, A343RCG, A351FCG, A364FCG, A479FCG A573FCG, A597FCG, A641RCG). They showed 95.9 to 98.8% similarity to *Bartonella taylorii* type strain (AF191502). Those genotypes were closely related (97–100% similarity) to *Bartonella* strains (AY435106, AY435113, AF391790 and GU338962) isolated from field mice (*Apodemus flavicollis*) live-trapped in Greece, Sweden and Poland [22–24].

Clade B contained 11 genotypes, 10 of which (A8RCG, A53RCG, A55RCG, A138RCG, AR183RCG, A186RCG, A191RCG, A306RCG, A321FCG, A373FCG) were related to a *Bartonella* strain (FJ946856) recovered from stray dogs in Thailand [25] with 96.2–100% similarity. One genotype (A188RCG) represented a unique sequence which differed from all previously described *Bartonella* strains or sequences (<95% identity).

Clade C contained seven genotypes. Six of which (A142R, A143R, A216F, A296F, A509F and A582R), exhibited the closest phylogenetic relationship (94–100%) to *B. grahamii* (Z70016), a bacterium isolated from various rodent species worldwide [26] and potentially pathogenic to humans [27]. One genotype from clade C (A199RCG) also represented an unique sequence which differed from all previously described *Bartonella* strains or sequences (<92% identity).

Clade D clustered two genotypes (A543R and A636F), both closely related to a *B. doshiae* type strain (Z70017) (97.9 and 98.8% respectively), originally isolated from rodents in the United Kingdom [28].

The most frequently found *Bartonella* were genotypes belonging to clade A (closely related to *B. taylorii* type strain) detected in 58.8% (148/252) of the animals. Less frequent were genotypes from clade B in 21.4% (54/252) of the animals, genotypes A142R, A143R, A216F, A296F, A509F and A582R (from clade C and related to *B. grahamii*) in 16.3% (41/252) of animals, and genotypes of clade D (related to *B. doshiae*) in 2.8% (7/252) of animals. The two novel *Bartonella* genotypes A188R and A199R (from clades B and C) were each detected in only one specimen.

Co-infections were detected in 27/447 bank voles (6%) screened for the presence of *B. afzelii* and *Bartonella* spp. No trends of association or exclusion between the two bacteria were observed ($\chi^2 = 0.4$, $p = 0.849$, $CI_{95} = [0.53; 1.47]$).

Co-infections occurred between *B. afzelii* and different *Bartonella* genotypes distributed throughout all clades. The *Bartonella* genotypes implicated in co-infections were, A351F and A364F (from clade A), closely related to *B. taylorii*; A373F, A8R and A53R, A188R belonging to clade B; A216F from clade C, which showed 100% relatedness to *B. grahamii* type strain, and A636F (clade D), with similarity to *B. doshiae*.

3. Discussion

This work is the first demonstration that bank voles are significantly infected with diverse *Bartonella* spp. (56.4%) and to a lesser extent with a single *B. afzelii* strain (11%), and also demonstrate a co-infection rate of 6%.

B. afzelii has already been shown to be present in rodents and some insectivore species at similar levels [4,29]. In our study, we identified one single genotype in bank voles in the geographical region studied. Interestingly, introduced Siberian chipmunks (*Tamias sibiricus*) and *I. ricinus* ticks

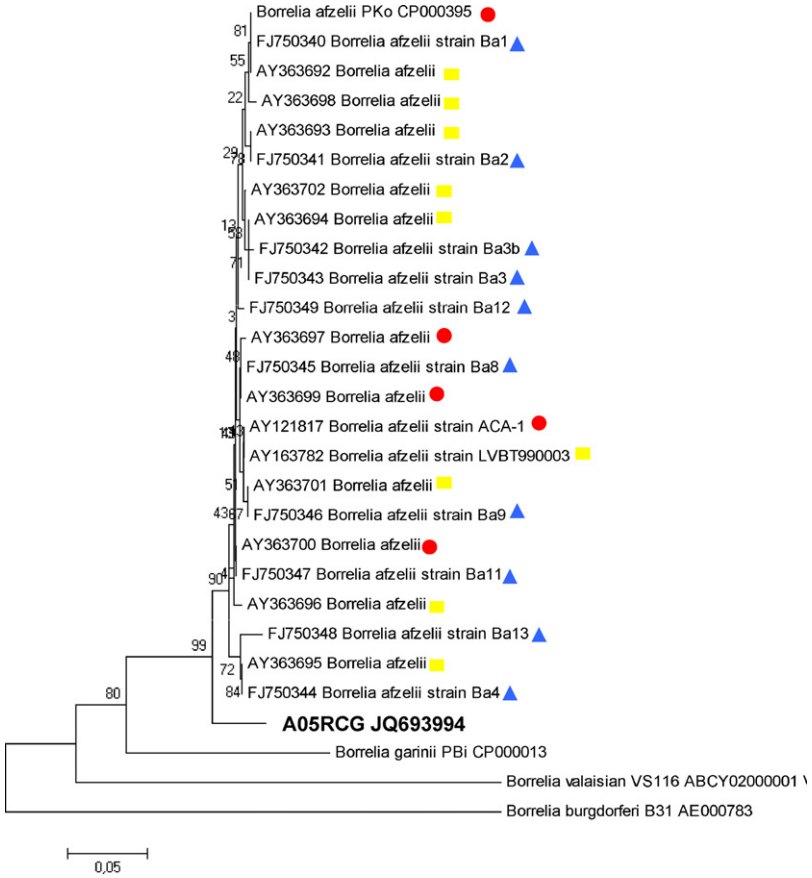

Fig. 1. Phylogenetic relationships between the unique *Borrelia afzelii* genotype detected in the population of bank voles from the Sénart forest (France) and several *Borrelia* spp. type strains, based on the sequence of the intergenic 16S(*rrs*)–23S(*rrlA*) spacer (IGS). The phylogenetic tree was constructed by the neighbor-joining method. Bootstrap analysis was carried out on 1000 replications; values are indicated at the nodes. Genbank accession numbers are indicated for each sequence used. The sequence of *Leptospira biflexa* strain Patoc 1 (accession number: NC010502) was chosen as an outgroup in the phylogenetic tree. Symbols indicate the host origin of the amplified IGS sequence: (●) humans, (▲) rodents, (■) ticks. All sequences came from southern Sweden expect PKO (CP000395) isolated in UK and LVBT990003 (AY163782) isolated in Latva.

collected in the same site and during the same period as our study were found to harbor three different species in the *B. burgdorferi* complex: *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*, suggesting that bank voles are exclusively tolerant to *B. afzelii* [7,30]. At various European sites, *B. afzelii* strains have been shown to exhibit restricted movement between geographic regions [31]. Moreover, Hellgreen and colleagues [32] showed that three different genetic loci of *B. afzelii* increased in stability as the geographical scale decreased, despite the occurrence of frequent recombination events. This suggests that specific *B. afzelii* strains are only associated with specific geographical regions, due to physical barriers and limited rodent movement. This could explain the low diversity of *B. afzelii* found in this study, as well as the differences between the *B. afzelii* genotypes reported here compared to those detected in other European countries [32].

Concerning *Bartonella* spp., the rodent infection rate observed in our study was similar to levels reported in many studies from different geographical areas: 52.4% in western USA [33], 62% in the United Kingdom [34], and 57% in Canada [35]. Conversely, studies conducted in southern China and northern USA revealed *Bartonella* rodent infection rates exceeded 80% [36,37]. The presence of such high levels of *Bartonella* in rodent communities is still unexplained. In all these studies, as in ours, rodent-associated *Bartonella* species were genetically very diverse.

Our results also agree with those of Inoue and colleagues [26], who demonstrated that the different *B. grahamii* strains sampled from all over the world tend to be associated with a geographical region on a large scale, but that a strict association of strains with a specific rodent host did not seem to occur. In the bank vole population studied here, the genotypes from clade B, (except A188R) which formed a well-demarcated cluster (Fig. 2), were similar to a *Bartonella* sequences recovered from the blood of stray dogs in Thailand [25] and might represent a new species. Two unique genotypes, A188R from clade B and A199R from clade C showed less than 96% similarity with known *Bartonella* strains or sequences, suggesting the presence of two new species according to the criteria specified by La Scola et al. [17]. The exact taxonomic positions of these *Bartonella* genotypes now need to be further characterized by MLST or MLSA.

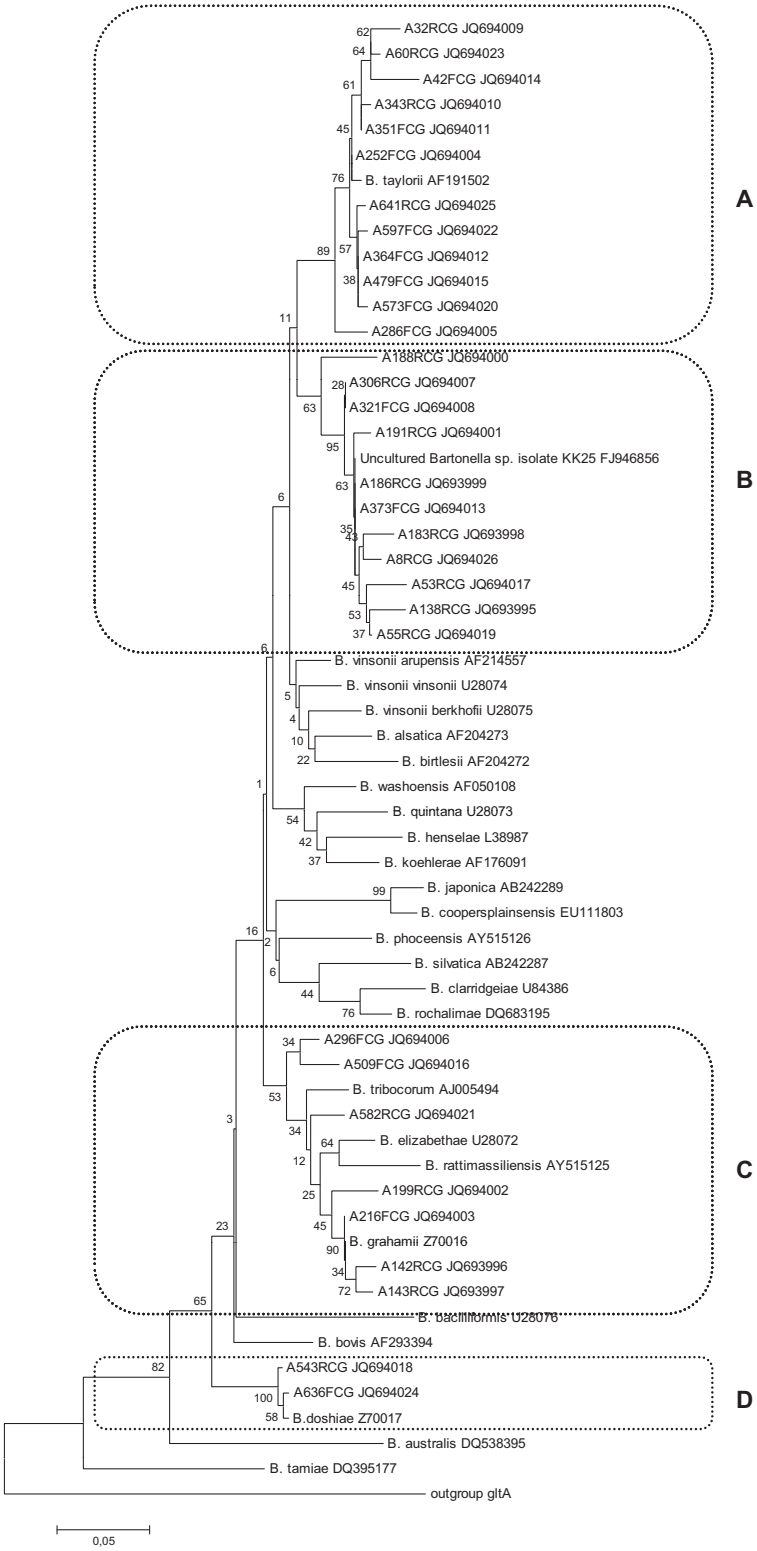

Fig. 2. Phylogenetic relationships among partial citrate synthase sequences (*gltA*) from the 32 *Bartonella* genotypes examined here and from known *Bartonella* species. The phylogenetic tree was constructed by the neighbor-joining method. Bootstrap values (percentage of 1000 replications) are indicated at the nodes. Genbank accession numbers are indicated for each sequence used. The sequence of *Brucella melitensis* strain 16 M (accession number: NC003317) was chosen as an outgroup in the phylogenetic tree. The four clades that contained the genotypes recovered here are highlighted by boxes and labelled as follows: A, B, C, and D.

We found a significant difference in the prevalence of *B. afzelii* between young and adult individuals. Juvenile bank voles are known to have lower levels of tick infestation compared to adults [38], most likely explained by the fact that juveniles inhabit a more confined physical environment, thereby limiting their exposure to *I. ricinus* [4]. In contrast to *B. afzelii*, we did not find any difference in *Bartonella* spp. prevalence in young and adult individuals. Hence, we hypothesize that the lack of difference between juveniles and adults suggests a similar exposure of both age groups to fleas, the major rodent *Bartonella* spp. vector, due to intimate contact between parents and their offspring in the burrow.

Wild populations are generally infected by more than one parasite type, which can result in different interactions between the parasites themselves, but also between the parasites and the host. Recent studies in a natural population of field voles (*Microtus agrestis*), demonstrated that the presence of cowpox virus increased vole susceptibility to *B. taylorii* infection, as well as the duration of bacteremia. In contrast, *Babesia microti* and *Anaplasma phagocytophilum* had a negative effect on *B. taylorii* infection probably due to an immune mediated response (cross immunity) and/or a competition for resources [39]. Six percent of the 447 bank voles screened here were co-infected with *Borrelia afzelii* and *Bartonella* genotypes. Considering that the *Bartonella* spp. bank vole infection rate with was around 56% in our study, half of the *Borrelia*-infected rodents were also infected with diverse *Bartonella* spp. suggesting that co-infections by the two bacteria occur randomly and are not correlated.

To our knowledge, this is the first study to assess the prevalence of both *Bartonella* and *Borrelia* species in rodents in a suburban forest, providing supplementary data about the geographic distribution and diversity of these vector-borne zoonotic bacteria. We also demonstrate the coexistence of both zoonotic pathogens in bank voles, but without apparent interactions. These preliminary data call for additional investigations to determine the impact of such pathogens on human health, especially when disease symptoms persist after exposure to rodents/arthropods for which no etiological agent has been identified.

Conflict of interests

The authors declared that they have no competing interests.

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