Low prevalence of zoonotic Babesia in small mammals and Ixodes ricinus in Brittany, France

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Title: Low prevalence of zoonotic *Babesia* in small mammals and *Ixodes ricinus* in Brittany, France.

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Highlights

- Prevalences of *Babesia* spp. were investigated in small mammals and *Ixodes ricinus*.
- Only one Bank vole carried *B. microti* and 13 ticks carried *B. venatorum.*
- Exposure of humans to zoonotic *Babesia* is probably low in western France.

Summary

In order to evaluate the zoonotic risk due to *Babesia* spp., especially *B. microti*, we investigated their presence in 597 individuals of five small mammal species and in 2620 questing nymphs of *Ixodes ricinus* in rural landscapes of Western France (Brittany). Small mammals (rodents and shrews) are indeed suspected to be reservoir hosts for *B. microti*, and the tick *I. ricinus* is the vector of the three main zoonotic species in Europe, i.e. *B. divergens*, *B. venatorum* and *B. microti*.
Only one bank vole carried *B. microti* (genotype "Munich") and only 13 and 2 nymphs of *Ixodes ricinus* ticks carried *B. venatorum* and *B. capreoli* respectively.
According to these results, prevalences observed for zoonotic *Babesia* (0.17 % for small mammals and 0.50 % for ticks), indicate that exposure of humans to this infectious agent is probably low in western France.

Keywords: *Babesia*; *Babesia microti*; *Ixodes ricinus*; Zoonoses; Disease Reservoirs; Disease vectors.

Introduction

Emergence or re-emergence of tick-borne diseases is likely to be induced by land use changes and their consequences on the communities of vertebrate reservoirs and vectors (Lambin et al., 2010). Three species of *Babesia* of concern for human health are transmitted by Ixodid ticks in the Northern hemisphere: *B. microti*, *B. divergens* and *B. venatorum* (*Babesia* sp. EU1) (Gray et al., 2010; Schnittger et al., 2012). Transmitted by *Ixodes scapularis* ticks in Northern America, *B. microti* has been incriminated as an etiological agent in hundreds of cases, often in immunocompetent individuals. In Eurasia, autochthonous human cases are more rarely reported, and attributed to *B. divergens* or *B. venatorum*, but not to *B. microti* despite its presence on this continent. Both species cause mild to deadly babesiosis on splenectomized or otherwise immuno-compromised patients, and both are transmitted by *I. ricinus* ticks. Their vertebrate reservoirs are mainly domestic cattle and roe deer (*Capreolus capreolus*) (Devos and Geysen, 2004; Bastian et al., 2012). Adult females of *I. ricinus* acquire the parasites during blood meal and transmit them first transovarially to, and then transtadially within the next tick generation. Conversely, *B. microti*, also detected in vertebrate hosts and *I. ricinus* in Europe, is unable to be passed down transovarially. This infection is therefore acquired mainly by larvae feeding on infected small mammals, its main vertebrate reservoir (Gray et al., 2010). The predominant transmitter to humans of the three zoonotic *Babesia* species in Western Europe is therefore the nymphal stage of *I. ricinus*.

Material and methods

In a project (OSCAR) designed to study the exposure to infected *I. ricinus* ticks in rural landscapes, we sampled small mammals and questing *I. ricinus* nymphs in the “Zone Atelier Armorique” (https://osur.univ-rennes1.fr/za-armorique/) a Long Term Ecological Research (LTER) area located North-East of the Brittany region, France (48° 30′ N, 1° 32′W). Small mammals were trapped at 24 different locations (6 in core forest, 6 at forest edges and 12 at meadow/wood or meadow/hedgerow ecotones). Trapping sessions were carried out in May-June and October in 2012

and 2013. For each location, we set up linear trap lines of 34 baited live-traps spaced every 3 meters, checked after 24 and 48 h, for a total of 1632 trap nights each season. Ticks were sampled by the dragging method along 100-square metre transect subdivised in ten 10-square metre sub-transects (see Perez et al, 2016 for more details). The sampling sites, 24 for both small mammals and ticks, and 36 additional sites for only ticks, were located either in forest habitats or at woodland-grassland ecotones in various landscape contexts (ratio crops/grasslands, hedgerow network density). These ecotones are the most favorable habitats for *I. ricinus* and the maintenance of vector-host cycles. We described the prevalence of *Babesia*, at the species and strain levels, by detecting and sequencing a hypervariable region of the *Babesia* 18S rRNA gene in *I. ricinus* nymphs and in rodents. This marker allows discrimination of numerous *Babesia* species (Gray et al., 2010; Schnittger et al., 2012).

Small mammals were trapped in May-June and October in 2012 and 2013. They were euthanized by authorized experimenters (pentobarbital injection) in accordance with French law, dissected and their spleen removed to be stored at -20°C until further analysis. A total of 597 animals have been tested, including 441 *Apodemus sylvaticus*, 147 *Myodes glareolus*, 4 *Microtus agrestis*, 2 *Microtus subterraneus* and 3 *Sorex coronatus*. Questing nymphs were collected by dragging the vegetation with a white flannel blanket in spring 2012 and 2013. Ticks removed from the blanket were conserved in ethanol 70% and identified under a binocular microscope according to Pérez-Eid (Pérez-Eid, 2007). A total of 2620 *I. ricinus* nymphs were screened for *Babesia* spp.

Genomic DNA was extracted from individual ticks using ammoniac lysis (Morán-Cadenas et al., 2007), and from spleen tissue using the kit Macherey NucleoSpin Tissue (Macherey Nagel, Germany). The detection of *Babesia* spp. and *B. microti* was achieved by nested PCR of the 18S rRNA gene. Different primers were used to amplify *Babesia* spp. from ticks and *B. microti* from small mammals, as we experienced false positive amplifications with generic primers on small mammal spleen extracts (Table 1) (Persing et al 1992; Welc-Falęciak et al., 2007; Malandrin et al., 2010). The PCR conditions were: final volume 30 µl containing 0.33 mM dNTPs (Eurobio, France),

1 X PCR buffer, 1 U Extaq Takara (Ozyme, France), 1 µM each primer and deionized water. In a separate room, 7 µl of DNA extract were added. PCR steps: 98°C 5 min - 40 cycles of (98°C 30 s, selected annealing temperature 30 s, 72°C 30 s) - final elongation 72°C 5 min. A sample of 10 µl amplicons (diluted 1:100) of the first PCR step was used as template for the nested PCR. Genomic DNA from *Theileria ovis* (accession number: FJ603460) and *B. microti* (reference sequence XR_001160982.1 strain R1) were used as positive controls. Positive amplicons were purified using ExoSAP-IT (Ozyme, France) and sent for Sanger conventional sequencing (GATC, Germany). Sequences were assigned to species using BLASTn identity searches in GenBank and a detailed analysis of nucleotide substitutions with reference sequences.

Results and discussion

We found a low prevalence of infection by *Babesia* species in rodents and in questing *I. ricinus* nymphs (Table 2). Three species were detected, *B. microti* in rodents, *B. venatorum* and *B. capreoli* in ticks. The latter is non-zoonotic, closely related to, but different from, the zoonotic *B. divergens* (Gray et al., 2010; Malandrin el al., 2010). Roe deer is the vertebrate reservoir of both *B. venatorum* and *B. capreoli*, and an important blood source for adult *I. ricinus*, who acquire these parasites and transmit them transovarially. Their detection in questing nymphs in such a biotope frequented by roe deer could therefore be expected. The zoonotic *B. divergens* was not detected in nymphs despite the occurrence of sporadic babesiosis in cattle in the area.

B. microti was detected only in the spleen of one *Myodes glareolus* in autumn 2012 in a forest habitat (Table 2). This sequence (Genbank accession number KX758442) was of type "Munich", which has only once been suggested to be the cause of a case of low grade human babesiosis (Arsuaga et al., 2016). Recent phylogenetic work on *B. microti* discriminated 4 lineages ("USA-type" also called "Gray", "Munich", "Kobe" and "Hobetsu") (Schnittger et al., 2012). Only the North-American variants of the USA-type are known to be associated with frequent detrimental outcomes in humans, to the point that it has become a nationally notifiable condition in the USA. In

Europe, the only reported severe case of autochthonous *B. microti* involved the USA-type, and transfusion was the most probable source of infection (Hildebrandt et al., 2007).

Rodent and shrew reservoir species for *B. microti* (*Apodemus* spp., *Microtus* spp., *Myodes* spp., *Sorex* spp.) are highly dependent on the biotope and have apparently variable reservoir efficiency (Welc-Falęciak et al., 2008). *Microtus* species are considered as the main *B. microti* reservoir in Europe, but they prefer open habitats and were underrepresented in our study focused on ecotones (1.0% of the rodents), although they reach 9.5% to 28.2% in barn owl pellets in agricultural landscapes of this region (Groupe Mammalogique Breton, 2015). In North-East Poland, *B. microti* (USA-type) prevalence in small mammals can reach 35 % in *Microtus arvalis* in fallow land landscapes (Welc-Falęciak et al., 2008), but small mammal and tick communities are quite different between Western and Eastern Europe (Atlantic versus Continental biogeographical area). It has been shown that *I. trianguliceps* (with all 3 developmental stages feeding on small mammals) is the main vector for *B. microti*, while *I. ricinus* (with larvae feeding on rodents and nymphs and adults on larger vertebrates) would only serve as a bridge vector (Bown et al., 2008). So, although at least one *B. microti* variant may be enzootic in rodents in Western France, its apparent absence in *I. ricinus* in our study leads to think that human exposure to zoonotic *Babesia* in this area is very low. Combined serological and PCR screening of blood donors may give a more accurate estimation of exposure to contamination by tick bites, and may help to assess risks for transfusion patients.

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Table 1. PCR amplification conditions for *Babesia* spp. detection

		Target DNA	
		Babesia spp. amplification	*B. microti* specific amplification
First PCR			
Primer names	forward	BTh18S up[a]	CRYPTOFL[c]
	reverse	CryptOR[b]	CRYPTORN[c]
Annealing temperature		61°C	58°C
Expected amplicon size		1650 bp	1770 bp
Nested PCR			
Primer names	forward	BabGF2[d]	Bab1[e]
	reverse	BabGR2[d]	Bab4[e]
Annealing temperature		61°C	58°C
Expected amplicon size		560 bp	238 bp

[a] 5'- GGG CTA ATA CAW GTT CGA G- 3' this study, [b] 5' – GAA TGA TCC TTC TGC AGG TTC ACC TAC – 3' this study, [c] (Welc-Falęciak et al., 2007), [d] (Malandrin et al., 2010), [e] (Persing et al., 1992)

Table 2. *Babesia* spp. detection in small mammals and ticks. Results of PCR and sequencing of *Babesia* spp. 18S rRNA gene amplified from 597 small mammals and 2620 questing *I. ricinus* nymphs sampled in rural landscapes in 2012 and 2013 in North-eastern Brittany, France.

	Positive/tested	
Year	2012	2013
Small mammals		
B. microti (spring)	0/208	0/30
B. microti (autumn)	1/283	0/76
Questing *I. ricinus* nymphs (spring)		
B. microti	0/1215	0/1405
B. venatorum	8/1215	5/1405
B. capreoli	0/1215	2/1405