Veterinary Parasitology 252 (2018) 22–28

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Babesia microti-like piroplasm (syn. *Babesia vulpes*) infection in red foxes (*Vulpes vulpes*) in NW Spain (Galicia) and its relationship with *Ixodes hexagonus*

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ARTICLE INFO

Keywords:
Babesia microti-like piroplasm
Theileria annae
Red foxes
Vulpes vulpes
Ixodes hexagonus
NW Spain

ABSTRACT

Piroplasmosis is caused by several species of protozoa such as the *Babesia microti*-like piroplasm (Bml), an emerging blood protozoan also known as *Theileria annae* or *Babesia vulpes*. Infection by Bml was first reported in dogs in Spain where it is endemic today. Recently, a high prevalence of Bml has been increasingly detected in red foxes (*Vulpes vulpes*) in European countries. The objective of this study was to determine infection levels of this parasite in foxes from Galicia, NW Spain, and ticks species infestation in these carnivores, where they are so far unknown. Samples of blood, spleen and ticks (if present) were taken from 237 hunted red foxes in the Galicia region. Blood smears were prepared for direct parasite observation, and spleen and tick samples were examined by nested PCR. Prevalences of Bml infection in Galician red foxes were estimated at 72% (171/237) by PCR and 38.23% (26/68) by direct observation. Among 837 ticks collected, the main tick identified was *Ixodes hexagonus* (present in 82.4% of the foxes) followed by *Ixodes ricinus* (12.3%), *Dermacentor reticulatus* (12.3%) and *Rhipicephalus sanguineus* sensu lato (3.5%). From 34 foxes testing positive for Bml, 616 ticks were collected: positive Bml PCR results were obtained in 55.6% (227/408) of ticks collected from 9 foxes, while the 208 ticks from the remaining 25 infected foxes returned negative PCR results. Given that canine piroplasmosis is endemic in this area, our observations point to the red fox as the main reservoir for Bml infection and the high proportion of *I. hexagonus* among ticks collected from red foxes suggests its likely role as vectors of *B. microti-like piroplasm* in this region. Further studies are needed for a better understanding of the link between the wild and domestic life cycles of this piroplasm.

1. Introduction

Piroplasms (Apicomplexa, Piroplasmida) are intra-erythrocytic protozoan parasites from genera *Babesia*, *Theileria* and *Cytauxzoon*, which can affect wild and domestic carnivores (Otranto et al., 2015). Based on the morphology of parasites in the red blood cells, piroplasms have been traditionally divided into large (3–5 µm) and small piroplasms (0.5–2.5 µm). Although all large forms reported to date belong to the genus *Babesia*, small *Babesia* spp. and *Theileria* spp. hardly be distinguished by light microscopy alone (need a specialist), and DNA-based molecular techniques are needed for their accurate identification at species level (Irwin, 2010). In many parts of the world, different species of large and small piroplasms have been identified morphologically and genetically in both domestic and wild canids (Cardoso et al., 2013).

The *Babesia microti*-like piroplasm (Bml), generally considered as synonym with *Theileria annae*, is a small piroplasm first described in dogs in Spain. Nowadays, Bml is considered to be endemic in the northwest of the country (Camacho et al., 2001; García, 2006; Miró et al., 2015). Other authors prefer the name *Babasia vulpes* because of the high prevalence reported in its natural host, the red fox (*Vulpes vulpes*), and the apparent lack of a pre-erythrocyte infection stage in lymphocytes (Baneth et al., 2015). It is currently unclear if the protozoan Bml belongs to the genus *Theileria* or *Babesia*, as there is still no

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https://doi.org/10.1016/j.vetpar.2018.01.011
Received 27 December 2017; Accepted 12 January 2018

evidence of an extra-erythrocyte infecting stage or of the lack of transovarial transmission in ticks (distinguishing features of *Theileria* spp.) (Miró et al., 2015). In this paper, we will refer to this piroplasm as *Babesia microti*-like (Bml).

When dogs are clinically infected with Bml they show severe clinical signs such as pale mucous membranes, anorexia, apathy and fever. White blood cell counts indicate severe macrocytic/hypochromic regenerative anaemia and thrombocytopenia (Guitián et al., 2003; Miró et al., 2015). While the vector of this small piroplasm has not yet been identified, it is thought to be transmitted by ticks of the genus *Ixodes* like for many other small piroplasms. The hedgehog tick *Ixodes hexagonus* seems a likely candidate because endemic areas of Bml infection closely match its distribution range (Camacho et al., 2003). Apart from *I. hexagonus*, Bml DNA has also been detected in other tick species such as *Ixodes ricinus* (Lledó et al., 2010), *Ixodes canisuga* and *Rhipicephalus sanguineus* sensu lato (Solano-Gallego et al., 2016). Nevertheless, we still lack data to suggest their competence as vectors for Bml.

Over the last 10 years, Bml has been increasingly detected in carnivores and there have been reports of its presence in dogs and foxes across Europe and North America. Using molecular techniques, Bml has been also detected in dogs in Spain in regions outside Galicia, such as Asturias (Miró et al., 2015) or Barcelona (Tabar et al., 2009), and in also in other countries, including Portugal (Simões et al., 2011), Croatia (Beck et al., 2009), Sweden (Falkenö et al., 2013), France (René-Martellet et al., 2015), and the USA (Yeagley et al., 2009). However, in most of these studies, the travel history of the dogs was unknown. Some authors have reported cases of Bml infecting foxes in central and northern Spain (Criado-Fornelio et al., 2003; Gimenez et al., 2009), in some European countries (Portugal Croatia, Italy, Austria, Germany and Hungary) (Cardoso et al., 2013; Duscher et al., 2014; Najm et al., 2014; Zanet et al., 2014; Farkas et al., 2015), and in Canada and the USA (Birkenheuer et al., 2010; Clancey et al., 2010). Among all piroplasm species reported in Europe, Bml seems to show a strong preference for foxes. Thus, Bml is the main piroplasm species confirmed molecularly in foxes, while *Babesia canis* has been only rarely identified in these animals (Cardoso et al., 2013).

The red fox is the most common carnivore in Europe and shows a large distribution range and population size. Fox abundances in Europe depend on habitat, feeding, season, competition with other species, other diseases and control measures (e.g., hunting), as well as interactions among these factors.

Estimates for the region of Galicia (NW Spain) suggest an average population density of 2.7-foxes/km^2 in winter, with the highest densities (5.6 foxes/km^2) in peri-urban areas close to large human populations, such as industrial areas, farms, slaughterhouses and uncontrolled rubbish dumps (Fidalgo et al., 2009). In central and southern Spain, densities are lower than in NW Spain (0.8 and 2.5 fox/km^2, respectively) (Gortázar, 1997). Local fox abundances benefit from the presence of people. In urban areas, the availability of food and resting places, human tolerance and the absence of predators/competitors, make the red fox a very successful species (Otranto et al., 2015). The large region of Galicia is especially suitable in terms of its demographic and ecological characteristics for the high proliferation of foxes.

Some studies have reported the incidence and clinical management of Bml infection in domestic dogs in NW Spain (Camacho et al., 2001; Miró et al., 2015; Checa et al., 2017) as the only recognized endemic area in Europe. In contrast with the situation for domestic dogs, information on the occurrence and prevalence of piroplasms in red foxes in NW Spain is still limited.

The present study sought to examine using molecular tools the prevalence of Bml and to describe the tick species (Ixodidae) infesting red foxes in NW Spain.

2. Materials and methods

2.1. Study area, sampling and data collection

The dead bodies of 237 apparently healthy, wild red foxes found in Galicia (NW Spain) were included in this study. The foxes had been shot during the official hunting season (by members of the Federación Galega de Caza and other hunting societies in this region) in January and February of 2016. Not more than 6 h after the death of each animal, spleen and blood samples were obtained. During necropsy, blood or haemolyzed serum were collected using a sterile technique from the right atrium or chest cavity and placed in EDTA tubes for blood smears. Spleen samples were also collected with sterile instruments and placed in sterile tubes to detect Bml following genomic DNA isolation, PCR and sequencing. Spleen samples were kept at −20 °C and blood samples at 4 °C until further processing.

In a clinical file, we recorded: identification number, date, capture site, age, sex, and presence of visible ectoparasites. Clinical signs were also assessed at the time of sampling, such as changes in the colour of mucous membranes, body condition, skin condition or lymphadenomegaly.

Animal ages were estimated according to several factors: body development (complete or not), external appearance, development stage of genitals (external and internal) and dentition (presence, development and wear of teeth, periodontal disease). We established three age groups: immature or young, defined as individuals under 1 year old; adults, or foxes between 1 and 5 years old with reproductive capacity; and older adults foxes older than 5 years old, who showing greater wear of all teeth and often a varying extent of periodontal disease or even loss of teeth.

2.2. Microscopy parasite detection

Giemsa-stained thin blood smears were examined by light microscopy (LM) to detect intraerythrocytic forms consistent with small *Babesia* merozoites. The smears were air dried, fixed in absolute methanol for 5 min, stained with 20% Giemsa and then observed by light microscopy (LM) using a x1000 magnification objective under immersion oil. All samples were examined by the same person.

Of 237 blood samples collected, blood smears were prepared from only 68. This was because blood could not always be collected in optimal conditions following animal death by hunting.

2.3. Tick collection and identification

Ticks obtained from foxes after the clinical exam were stored in 70% ethanol in individual vials for each fox. Ticks were identified to the species level and sexed using morphological keys (Gil Collado et al., 1979; Manilla and Iori, 1992; Estrada-Peña et al., 2004). Before to isolate DNA from the ticks, they were classified according to stage (adult, larvae or nymphs) and degree of feeding (adult engorged females, fed nymphs and fed larvae). In our study, due to economical constrains we decided to analyse only ticks collected from Bml-positive foxes.

2.4. DNA isolation

Genomic DNA was isolated from spleen (10 mg) and from ticks using the QIAamp® DNA mini kit (Qiagen®, USA). The DNA from the spleens was extracted as described by the manufacturer and eluted in molecular-grade water (200 µl) and stored at −20 °C until further use. To isolate DNA from the ticks some modifications to the original protocol were introduced. Briefly, each tick was washed in 70% ethanol and then once in distilled water. Using a clean scalpel and stainless steel beads (Werfren, Spain), the ticks were pressed against the sharp beads to spill their abdominal contents. Each sample was kept in 360 µl of the

Table 1
External and internal primer sets used to amplify a region of the *Babesia microti*-like (Bml) piroplasm 18S rRNA gene.

Primer name	Sequence (5′-3′)
BT1-F Universal *Babesia-Theileria*	5′GGTTGATCCTGCCAGTAGT 3′
BTH-1R Universal *Babesia-Theileria*	5′TTGCGACCATACTCCCCCCA 3′
BTFox1F (Bml)	5′AGTTATAAGCTTTTATACAGC 3′
BTFox1R (Bml)	5′CACTCTAGTTTTCTCAAAGTAAAATA 3′

tissue lysis buffer included in the kit and treated with 40 µl of proteinase K overnight at 56 °C. Subsequent steps were carried out according to the manufacturer's instructions (tissue protocol).

2.5. Nested PCR for bml detection in spleen and tick samples

Spleen and tick DNA samples were screened by specific PCR-based assays targeting the small ribosomal RNA subunit gene (18S rDNA), recently validated for the detection of Bml, using universal *Babesia-Theileria* primers BT1-F and BTH-1R (Criado-Fornelio et al., 2003) for the primary amplification round and Bml specific primers BTFox1F and BTFox1R for the second amplification round (Bartley et al., 2016) (Table 1). The reaction mixture was adapted from that previously described by Bartley et al. (2016) and amplification conditions were as follows; 2 µl of extracted DNA was added to a 23 µl reaction mixture containing 0.75 units of Tth Plus DNA polymerase 5U/µl (Biotools B&M Labs., S.A., Spain), 200 µM (each) deoxyribonucleotides (dATP, dTTP, dGTP, dCTP) (Biotools B&M Labs.), 10 pmol of each primer (Invitrogen, USA), 2.5 µl 10X PCR buffer and 1.5 mM MgCl2 (BiotoolS B&M Labs.). Negative (2 µl dH2O) and positive (*B. canis* and Bml DNA) control samples were included in each PCR assay. The identities of these positive controls had been previously confirmed by PCR amplification, sequencing and BLASTN analysis of the 18S rRNA gene. Amplification reactions for Bml specific nested PCR were carried out in a thermocycler GenAmp® PCR System 2700 (Applied Biosystems, Spain). 10 µl of PCR products were run on a 1.5% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA) and visualized with a dark reader trans-illuminator (Clare Chemicals, USA).

2.6. DNA sequencing

PCR products corresponding to the expected length were purified (15 µl) using a QIAquick Purification Kit (Qiagen®) as described by the manufacturer and sequenced at the sequencing service of the Genomics department, UCM, using an ABI Prism 3730 system (Applied Biosystems).

Sequence chromatogram files were analyzed using Chromas 2.1.1 and imported into BioEdit v7.0.5 for editing, assembly and alignments. The sequences obtained were aligned with sequences available from GenBank using Clustal W and compared with additional piroplasm sequences available from GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine percentage identities of the generated sequences against published sequences.

2.7. Statistical analysis

Results were analyzed using the software package SAS version 9.4. Associations between Bml infection (detected by PCR) and remaining categorical variables such as age, sex, body condition, ticks and fox location were assessed using the Chi-squared test. Molecular results were compared with LM results through simple Kappa coefficients to assess agreement between these two diagnostic techniques. Significance was set at $p \leq 0.05$.

3. Results

3.1. Molecular diagnosis

Of the 237 spleen samples screened by Bml-specific PCR, 171 (72.2%) returned positive results. Of these, 14 samples were submitted for sequencing with BTFox1F and BTFox1R primers. All sequences showed 99–100% identity to several isolates of the *B. microti-like* piroplasm (*Babesia annae*, *Babesia cf. microti*, and *Babesia* Spanish dog isolate, accession numbers KT580785.1, KJ871351.1, and EU583387.1, respectively).

In this study, 816 ticks were collected from 50 foxes testing positive for Bml. Of these, 616 ticks from 34 foxes testing positive for Bml were analysed grouped together in 57 lots depending on their stage (individual adults, pools of up to 100 larvae or pools of 23 nymphs). Nine of these lots were Bml positive by specific nested PCR (BTFox1F/R): two lots of *I. hexagonus* adult engorged females (n = 2), four pools of *I. hexagonus* fed nymphs (n = 66), and three pools of *I. hexagonus* fed larvae (n = 159). Bml DNA were not detected in other ticks species analysed (*I. ricinus* and *D. reticulatus*) (Additional file). From 34 foxes testing positive for Bml, 616 ticks were collected: positive Bml PCR results were obtained in 55.6% (227/408) of ticks collected from 9 foxes, while the 208 ticks from the remaining 25 infected foxes returned negative PCR results. In addition, 6.4% (2/31), 23.5% (4/17) and 37.5% (3/8) of pools of adults, nymphs and larvae were Bml positive respectively.

3.2. Microscopy

Of the 237 blood samples obtained, only 68 were valid for light microscopy examination. Due to in the most of the foxes the blood sample after de death was not collected in sterile conditions or it was clotted.

Intraerythrocytic ring-shaped bodies, morphologically compatible with small piroplasms, were detected by LM in 26 of the 68 blood samples (38.2%).

We observed poor agreement between a positive LM and PCR result. The kappa value was low (0.213, fair agreement) indicating 54.4% agreement and 45.6% discrepancy. All LM positive blood smears were PCR positive. However, 31 foxes testing LM negative for Bml were PCR positive for the parasite.

3.3. Epidemiology

Foxes testing Bml positive were widely distributed across Galicia (Fig. 1). Thus, Bml was detected in 61% (36/59) of the foxes from the northwestern coast (A Coruña province), in 70.9% (56/79) of the foxes from the southwestern coast (Pontevedra province) and in 79.8% (79/99) of the foxes from northeastern Galicia (Lugo province). By geographical region, significant differences appeared between the Lugo and A Coruña provinces ($p = 0.037$), the prevalence of Bml being the greatest in foxes from Lugo (79.8%).

Of the 237 foxes examined, 125 females and 112 males, 67 were classified as young, 143 as adults and 27 as older adult foxes. Body condition scores (out of 5 points) in 197 foxes ranged from 1 to 4, with a median of 2.9 points. Based on Bml specific PCR results, 95 (76%) females and 75 (67.9%) males were positive. Among these foxes testing positive, 52 (77.6%) were young, 102 (71.3%) were adults and 17 (63%) were older adults. Body condition scores in Bml infected foxes were 1–2 in 41 (74.5%), 3 in 76 (71.7%) and 4 in 20 (55.6%) animals. There were no significant differences in Bml prevalence between genders ($p = 0.16$), and among age groups ($p = 0.34$) or body condition score groups ($p = 0.21$).

Of 837 ticks of four different species collected, 72% were larvae, 21.5% were nymphs and 6.5% were adults. Minimum and maximum numbers of ticks in a single fox were one and 212 ticks, respectively.

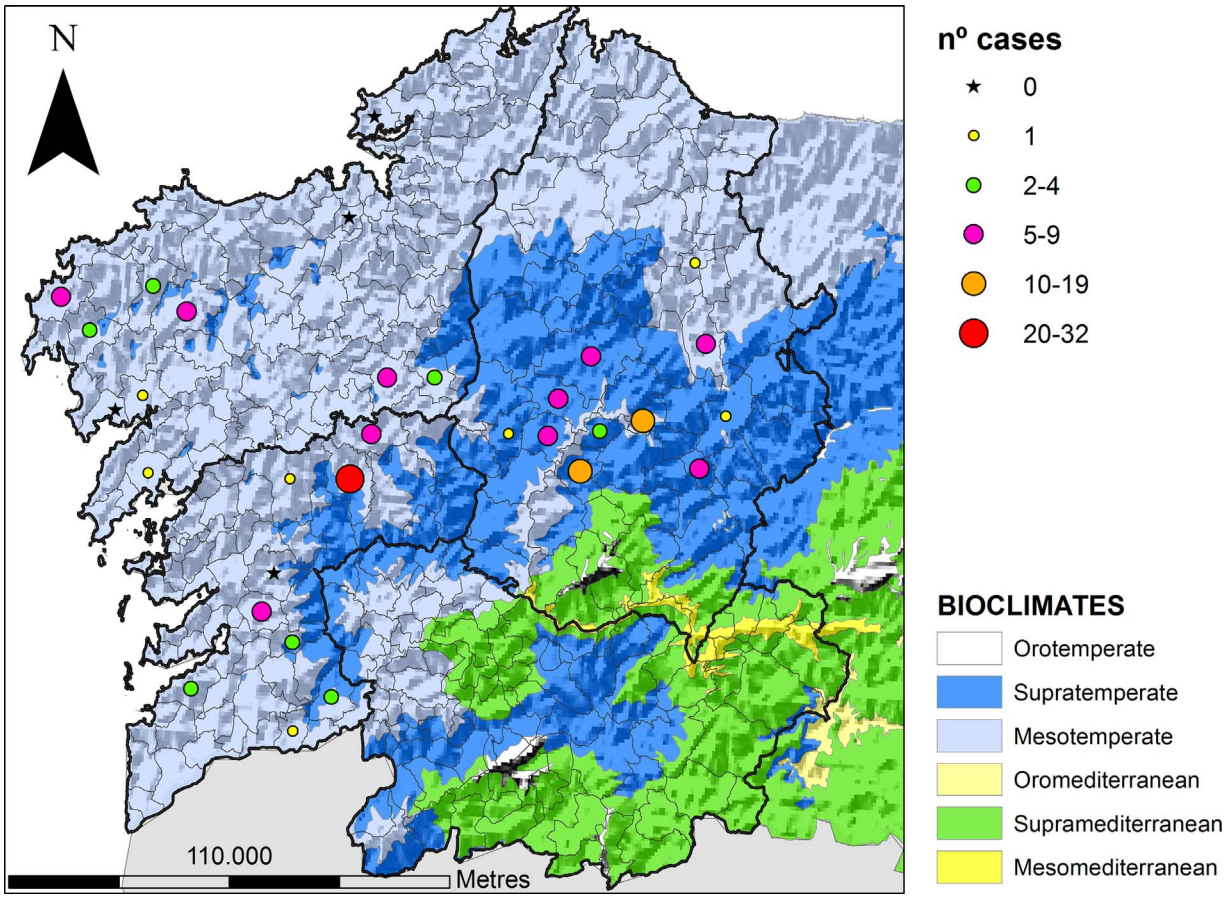

Fig. 1. Geographical distribution of Bml in red foxes in NW Spain (Galicia). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Orotemperate = T 3–7 °C; m −7 to −4 °C; M 0–3 °C
Supratemperate = T 7–12 °C; m −4 to 2 °C; M 3–10 °C
Mesotemperate = T > 12 °C; m > 2 °C; M > 10 °C
Oromediterranean = T 4–8 °C; m −7 to −4 °C; M 0–3 °C
Supramediterranean = T 8–13 °C; m −4 to −1 °C; M 3–8 °C
Mesomediterranean = T 13–17 °C; m −1 to 5 °C; M 8–14 °C
Temperature parameters: T = average monthly temperature; m = average temperature of the minimums of the coldest month; M = average temperatures of the maximums of the coldest month.

Ixodes hexagonus larvae were the predominant forms detected. These ticks were identified as *I. hexagonus* (90.7%), *I. ricinus* (1.3%), *Dermacentor reticulatus* (1.3%), *Rhipicephalus sanguineus* (0.3%) and *Ixodes* spp. (6.3%) (Fig. 2).

Out of 139 of the 237 foxes that could be externally examined, 57 harboured ticks (41%) (Table 2). While 87.7% (50/57) of foxes with ticks were Bml positive, 76.8% (63/82) of foxes without ticks infestation proved Bml positive. No significant differences in Bml infection were found according to tick infestation ($p = 0.1$). *Ixodes hexagonus* was the most abundant tick among foxes (82.4%; 47/57) and was identified in 41 of the 50 Bml infected foxes (82%). However, no significant relationship was observed between infected foxes and the presence of *I. hexagonus* ($p = 0.8$). Less abundant among the foxes were *D. reticulatus* (12.3%; 7/57), *I. ricinus* (12.3%; 7/57) and *R. sanguineus* s.l. (3.5%; 2/57). In addition, 5.2% of the foxes had *Ixodes* spp. larvae (not identified to species level).

4. Discussion

The findings of this study clearly indicate the wide presence of Bml DNA in red fox population of NW Spain. Hunted animals testing positive for this piroplasm were found across all regions of Galicia except Ourense province, where foxes were not sampled.

As far as we are aware, this is the first study to address Bml infection in wild foxes in Galicia. This NW region of Spain was the first area where Bml infection was reported to cause severe disease in dogs (Camacho et al., 2001; García, 2006). Subsequent infected dogs were reported from Croatia and Portugal (Simões et al., 2011; Farkas et al., 2015). Today, NW Spain is the main area considered endemic of Bml infection in dogs, which is rarely detected in neighbouring areas (Miró et al., 2015). Interest in this emerging parasite among wild carnivores has recently mounted (Clancey et al., 2010; Cardoso et al., 2013; Baneth et al., 2015). Bml infection in foxes was first described in two separate studies conducted in northern (Burgos province) and central Spain (Guadalajara province), where 20% (1/5) and 50% (5/10) of foxes tested proved positive, respectively (Criado-Fornelio et al., 2003; Gimenez et al., 2009). More recently, in a study carried out in two regions of northern Spain (Basque country and Asturias region), Bml was the only haemoparasite detected in 35.4% (17/48) of the red foxes analysed (Barandika et al., 2016). However, these figures may not reflect current prevalences across Spain, as very low numbers of foxes were sampled. In this larger study, we detected a prevalence of Bml in red foxes of 72.2% (171/237), which is higher than the prevalences reported in previous Spanish studies and more similar to that reported for foxes in Portugal (69.2%) (Cardoso et al., 2013).

The *Babesia microti*-like piroplasm is increasingly being detected in

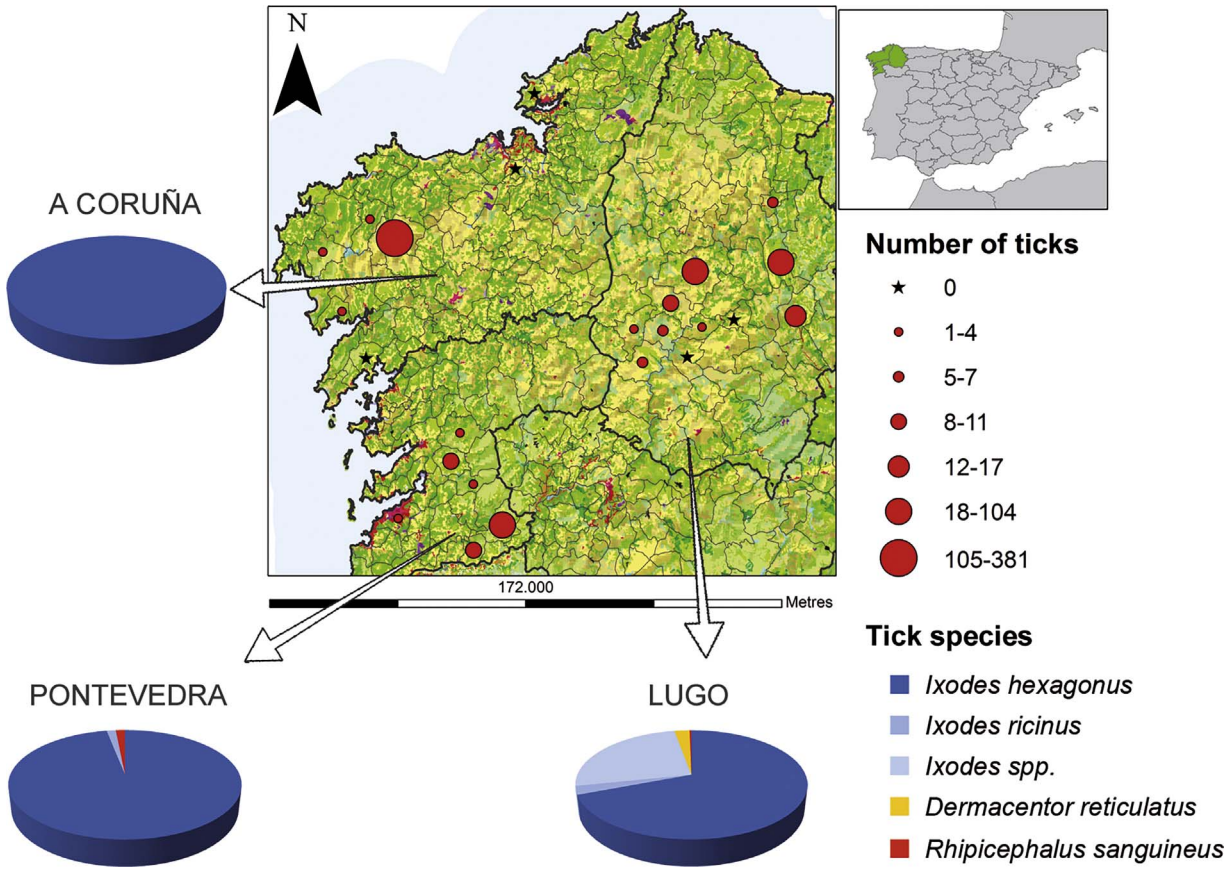

Fig. 2. Geographical distribution of ticks collected from red foxes (*Vulpes vulpes*) in three provinces of Galicia (NW Spain). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

foxes in Europe and has been reported in 50% of foxes in Austria (Duscher et al., 2014), 46.4% in Germany (Najm et al., 2014), 20% in Hungary (Farkas et al., 2015), 14.6% in Great Britain (Bartley et al., 2016), 5.2% in Croatia (Dezdek et al., 2010), and 0.98% in Italy (Zanet et al., 2014). Furthermore, the protozoan has also been detected in foxes in North America (Birkenheuer et al., 2010; Clancey et al., 2010). These findings suggest that red foxes are an important reservoir of Bml in Spain and other European countries and also in the USA.

Our epidemiological data indicated no correlation between Bml infection and age, sex or body condition of the red foxes examined. Bartley et al. (2016) described a higher percentage of infected male than female foxes, while our data point out to more affected females, though differences were not significant in either case. Positive fox ages (i.e., younger animals) are in agreement with observations by Zanet et al. (2014) in foxes in Italy. Other authors have also reported a greater risk of Bml infection in younger dogs in endemic areas (Miró et al., 2015). Hunting dogs are in close contact with red foxes in areas with a high density of this wild carnivore such as NW Spain. Both animal species share the same habitat and can have direct contact during hunting activities. In effect, a higher prevalence of Bml has been described in hunting than in companion or guard dogs (Guitián et al., 2003; Miró et al., 2015). The transfer of parasites from wild to domestic carnivores and vice versa mostly depends on the ecological variants that characterize a given area. For example, hunting could promote spill-over of parasitic infections from domestic canids and felids to wild populations (Otranto et al., 2015).

In our study, four tick species were found infesting foxes: *I. hexagonus*, *I. ricinus*, *D. reticulatus* and *R. sanguineus* s.l. However, by far the most numerous ticks found in the red foxes in NW Spain were immature and adult stages of *I. hexagonus*. These findings are similar to those of another study in northern Spain in which red foxes were mostly parasitized by *I. hexagonus* (Barandika et al., 2016). Similar prevalences of

Table 2
Babesia microti-like infection and tick infestation in red foxes (*Vulpes vulpes*) as determined by specific PCR in spleen and tick samples.

Ticks species	Foxes		Ticks							
	No.	(PCR +)	Total		From positives foxes		Analysed		Positive results	
			No.	Stages	No.	Stages	No.	Stages	No.	Stages
I. hexagonus	47	41	759	32F, 1M, 177N, 549L	741	28F, 1M, 163N, 549L	606	22F, 146N, 438L	227	2F, 66N, 159L
I. ricinus	7	7	11	7F, 3N, 1L	11	7F, 3N, 1L	5	4F, 1N	0	0
Ixodes spp.	3	3	53	53L	53	53L	nd	nd	nd	nd
D. reticulatus	7	6	11	8F, 3M	10	7F, 3M	5	5F	0	0
R. sanguineus s.l.	2	1	3	2F, 1M	1	1F	nd	nd	nd	nd
Total	57[a]	50[a]	837	49F, 5M, 180N, 603L	816	42F, 4M, 166N, 603L	616	31F, 147N, 438L	227	2F, 66N, 159L

[a] Including foxes harbouring more than one tick species. F, adult female; M, adult male; N, nymph; L, larvae.

this tick have been provided by large studies conducted in Romania and in countries with a moist Atlantic climate (UK or northern Germany) (Sándor et al., 2017). Climate and environmental conditions in northern and NW Spain (humid climates) are capable sustaining high burdens of ixodid ticks (Barandika et al., 2006). Based on matching areas of high Bml and *I. hexagonus* prevalences in Spain, this tick has been suggested as the vector of Bml (Camacho et al., 2003). In our study, the high proportion of *I. hexagonus* among ticks collected from red foxes suggests its likely role as vectors of *B. microti*-like piroplasm in this region. However, this tick species is not known to occur in other European countries where Bml infection have been reported in foxes such Croatia (Beck et al., 2009) or the USA. This raises the possibility of vectors other than *I. hexagonus*. Further studies are needed to identify other potential vectors or transmission routes for Bml particularly in regions in which *I. hexagonus* is absent.

We examined Bml infection by both PCR and LM in spleen and blood samples obtained from 68 foxes in NW Spain. Our results indicate poor agreement between direct observation of the parasite and PCR (54.4% of the samples). Miró et al. (2015) noted moderate agreement (Kappa value: 0.6680) between LM and PCR in samples from dogs tested in the clinical phase of Bml infection when microscopy observation of piroplasms is easier than in animals showing low parasitaemia levels due to chronic disease. By LM, we were only able to detect intraerythrocytic ring-shaped bodies that were morphologically compatible with small piroplasms in 26 (38.2%) of the 68 blood samples. In contrast, Bml DNA was detected by PCR in 57 (83.8%) of the 68 blood samples. In a similar study, PCR was able to detect the parasite both at an earlier stage of infection or at later stages, when parasitaemia levels are low and Giemsa-stained thin blood smears return negative (Fukumoto et al., 2001). Indeed, the molecular approach has been proposed as the gold standard method for the detection of small piroplasm infections (Miró et al., 2015). Accordingly, using the molecular diagnostic test, a larger number of foxes positive for Bml infection were detected (57/68, 83.8%) in animals tested by both techniques.

All positive blood smears showed a low parasitemia level. This could be explained by the absence of clinical signs compatible with Bml infection noted in the foxes testing positive. All the foxes externally examined showed good clinical status in agreement with previous studies (Cardoso et al., 2013). To date, there are no available data regarding clinical impacts on foxes, and more studies would be useful to define the clinical impact of Bml infection in red foxes.

5. Conclusions

Our findings indicate that *B. microti*-like piroplasm is widespread among red foxes in NW Spain, and suggest that prevalences of Bml infection in foxes are somewhat higher than those recorded in dogs from the same region.

Our observations also point out to *I. hexagonus* as a most likely vector for the spread of Bml to foxes and other carnivores including dogs in NW Spain. However, we should not underestimate the possibility that other ticks or transmission modes may play a major role in Bml transmission in other regions where this tick species is absent. Owing to its suburban habitat, adaptive capacity and broad geographic distribution, the red fox is the key in the spread of Bml throughout Europe.

Acknowledgments

The red foxes used in this study were provided by the Wildlife Recovery Centres of Galicia, Dirección Xeral de Patrimonio Natural (Xunta de Galicia, Spain) ans by Federación Galega de Caza.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetpar.2018.01.011.

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