Molecular detection and identification of piroplasms (*Babesia* spp. and *Theileria* spp.) and *Anaplasma phagocytophilum* in questing ticks from northwest Spain

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Research

Keywords: Babesia venatorum, Babesia microti, Theileria sp. OT3, Anaplasma phagocytophilum, questing Ixodes ricinus, Spain

Posted Date: January 28th, 2020

DOI: https://doi.org/10.21203/rs.2.21993/v1

Version of Record: A version of this preprint was published at Medical and Veterinary Entomology on August 5th, 2020. See the published version at https://doi.org/10.1111/mve.12468.

Abstract

Background: Ticks can transmit a wide range of pathogens; some of them are regarded as emerging or re-emerging pathogens causing a significant impact on human and animal health.

Methods: In order to determine prevalence and zoonotic potential of *Anaplasma* spp., *Candidatus* Neoehrlichia mikurensis and piroplasms in questing ticks from north-western Spain, 1,056 *Ixodes ricinus*, nineteen *Dermacentor marginatus*, seventeen *Dermacentor reticulatus*, twelve *Ixodes frontalis* and a single *Ixodes acuminatus* were molecularly analysed. Also, 23 pools of *I. ricinus* larvae were investigated for the presence of piroplasms. *Anaplasma* and piroplasm DNA was firstly detected using two commercial qPCR assays. Samples positive to *Anaplasma* spp. were confirmed and identified at species level by sequence analysis of the gro*ESL,* 16S rRNA and *msp2* genes. Those samples negative to *Anaplasma* spp. gro*ESL* were pooled and processed to detect a fragment of the gro*ESL* gene of *Ca.* N. mikurensis. qPCR piroplasm positive samples were molecularly identified at the species level by partial sequencing of the 18S rRNA and *ITS1* genes.

Results: Four pathogen species were detected in individual *I. ricinus,* namely *Babesia venatorum* (1.5%), *A. phagocytophilum* (0.7%), *Babesia microti* (0.3%) and *Theileria* sp. OT3 (0.2%). *Babesia venatorum* was also identified in a single *I. ricinus* larvae pool (maximum likelihood estimation 0.6%) whereas no *I. ricinus* pools were positive to *Ca.* N. mikurensis. In addition, one *I. frontalis* (8.3%) tested positive to *A. phagocytophilum.*

Conclusions: Our results revealed that a low percentage of *I. ricinus* from northwest Spain were infected with *A. phagocytophilum* and piroplasms. Since a potentially pathogenic variant of *A. phagocytophilum* and two zoonotic *Babesia* species were detected, these results may have public health concern. Since the vector of *Theileria* sp. OT3 remains unknown, its identification in *I. ricinus* is especially interesting; nevertheless, further investigations are needed to unravel the role of *I. ricinus* in the transmission of this *Theileria* species.

Background

Ticks are considered, after mosquitoes, the second vector in terms of public health importance, being able to transmit a wide range of pathogens [1]. Although Lyme borreliosis and tick-borne encephalitis are the most common tick-borne diseases in Europe, the negative impact of other tick-borne pathogens causing diseases on human and animals should also be considered [2]. Many of these pathogens, including Anaplasma phagocytophilum, Candidatus Neoehrlichia mikurensis and some Babesia species such as Babesia venatorum and Babesia microti, are regarded as emerging or re-emerging pathogens with a significant impact on human health [3].

Ixodes ricinus has been identified as the main vector of A. phagocytophilum and Ca. N. mikurensis, showing prevalence up to 33.9% and 10.5%, respectively, in questing ticks throughout Europe [4, 5]. That tick species does not act as reservoir for both pathogens since no transovarial transmission has been currently reported [6]. In this respect, hedgehogs and other rodent species have been identified as suitable reservoir hosts for Ca. N. mikurensis [7, 8]. In contrast, the epidemiological cycle of A. phagocytophilum is complex due to the existence of some variants/ecotypes related to different hosts and vector species. Thus, analysis at different genetic markers should be performed in order to identify the genetic variants and to determine their zoonotic potential, being groEL and 16S rRNA two of the most used genes [9]. Up to date, four A. phagocytophilum ecotypes (I to IV) have been identified at the groEL gene, and the presence of a thymine in the nucleotide position 724 of this gene has been identified as a predictor of pathogenicity [10, 11]. In addition, more than ten variants have been described at the 16S rRNA gene, although only three (“A”, “B” and “W”) have been identified in cases of granulocytic anaplasmosis [12–14].

Ixodes ricinus has been also identified as the most important vector of the zoonotic Babesia divergens and B. venatorum in several European countries although it has been reported that other tick species such as Dermacentor reticulatus and Ixodes persulcatus could play a role as vectors [15]. In contrast, Ixodes trianguliceps is the main vector of B. microti which is considered the main etiological agent of human babesiosis in Northern America, although it was also reported in Europe [16]. Domestic cattle and wild ruminants such as roe deer are the major reservoirs of B. divergens and B. venatorum, respectively; in contrast, the main reservoir of B. microti are rodents [17].

Theileria spp. are transmitted by numerous ixodid species belonging to several genera such as Amblyomma, Haemaphysalis, Hyalomma and Rhipicephalus [18]. This piroplasm can infect wild ungulates and a wide range of domestic animals such as cattle, sheep, goats and horses, some of which can develop clinical disease [19]. However, no Theileria species have been currently identified as zoonotic [20].

Previous investigations carried out in questing I. ricinus from Spain reported A. phagocytophilum prevalence up to 20.5% [21]. Nevertheless, the number of studies using different genetic markers for characterizing A. phagocytophilum isolates is scarce [22, 23] and therefore information on both animal reservoirs and ecology of Anaplasma spp. in this country is still limited. As regards to Ca. N. mikurensis, only a single investigation was performed in Spain, showing a low prevalence (1%) in I. ricinus collected from cattle in northern areas [24]. In the same way, the information available on the presence of piroplasms in questing ticks from this country is limited [25].

Considering that a recent study has shown that I. ricinus, the main vector of some of these pathogens, is the predominant tick species in north-western Spain [26], the presence of both bacteria (Anaplasma spp. and Ca. N. mikurensis) and piroplasms (Babesia spp. and Theileria spp.) in questing ticks collected from that region was assessed using molecular methods. In addition, A. phagocytophilum positive samples were further analysed in order to identify the ecotypes/variants and to assess their possible pathogenicity for human. Findings of the current study provide useful information on the prevalence of these pathogens among tick species as well as their significance for public and animal health.

Methods

Study area, tick collection and identification

Field studies were conducted from November 2015 to October 2017 in three forest areas (mountain, coastal and plateau) of Galicia, a region located in the northwest of Spain (43°47'–41°49'N, 6°42'–9°18'W). The main weather features of each sampling area as well as the tick sampling protocols were previously reported [26]. During 24 months, tick collection was monthly performed by flagging along three selected 300 m transects; then, ticks were identified to species level using previously described morphological keys [27].

DNA extraction and detection of pathogens

A total of 1,056 I. ricinus (652 nymphs, 202 females and 202 males), twelve I. frontalis nymphs, seventeen D. reticulatus (ten females and seven males), nineteen D. marginatus (fifteen females and four males) and a single I. acuminatus male were individually processed to detect the presence of Anaplasma spp., Babesia spp. and Theileria spp. Since some piroplasm species could be transmitted transovarially, 23 pools including 165 I. ricinus larvae were also performed. Ixodes ricinus larvae were not frequently collected, but when found, all specimens were captured in a single location point and considered offspring of the same female tick; depending on the number of larvae collected in a single sampling, each pool consisted of between two and ten specimens.

DNA was extracted from both individual and pooled ticks using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH®, Mannheim, Germany) following the manufacturer's instructions. Previously, tick tissues were disrupted using a MagNaLyser Instrument (Roche Diagnostic, Manheim, Germany) at 6,000 rpm during 60 seconds. Presence of Anaplasma and piroplasm DNA was firstly detected using two qPCR commercial kits (EXOone Anaplasma spp. and EXOone Piroplasms®, Exopol, Zaragoza, Spain). In order to identify the species present and their zoonotic potential, all qPCR-positive samples were selected and further analysed by specific conventional PCRs targeting the groESL, 16S rRNA and msp2 partial genes of Anaplasma spp. and the 18S rRNA and ITS1 partial genes of Babesia spp. and Theileria spp. following previously described protocols (Table 1). Finally, DNA from individual I. ricinus was pooled in groups of ten nymphs, females or males to detect Ca. N. mikurensis. Pools were then subjected to a conventional PCR targeting the groESL (Liz et al., 2002). Since this PCR also amplifies Anaplasma spp., qPCR-positive Anaplasma samples were not included in the pools.

Table 1
Primers and protocols used for detection and identification of Anaplasma spp., Candidatus Neoehrlichia mikurensis, Babesia spp. and Theileria spp.

Gene target	Primer name	Primer sequence 5'- 3'	Fragment size	Reference
GroESL of Anaplasma spp. and Candidatus Neoehrlichia mikurensis	HS1	AIT GGG CTG GTA ITG AAA T	1,297 bp	[57]
	HS6	CCI CCI GGI ACI AIA CCT TC		
	HS43	ATW GCW AAR GAA GCA TAG TC		
	HSVR	CTC AAC AGC AGC TCT AGT AGC		
16sRNA of Anaplasma spp.	ge3a	CAC ATG CAA GTC GAA CGG ATT ATT C	546 bp	[28]
	ge10r	TTC CGT TAA GAA GGA TCT AAT CTC C		
	ge9f	AAC GGA TTA TTC TTT ATA GCT TGC T		
	ge2	GGC AGT ATT AAA AGC AGC TCC AGG		
msp2 of Anaplasma spp.	msp2-3F	CCA GCG TTT AGC AAG ATA AGA G	334 bp	[59]
	msp2-3R	GCC CAG TAA CAA CAT CAT AAG C		
18srRNA of Babesia spp. and Theileria spp.	RIB-19	CGG GAT CCA ACC TGG TTG ATC CTG C	430 bp	[60, 61]
	RIB-20	CCG AAT TCC TTG TTA CGA CTT CTC		
	BAB-rumF	ACC TCA CCA GGT CCA GAC AG		
	BAB-rumR	GTA CAA AGG GCA GGG ACG TA		
ITS1 of Babesia spp. and Theileria spp.	BAITS1-F	CGAGTGATCCGGTGAATTATTC	600 bp	[39, 62]
	BAITS1-R	CCTTCATCGTTGTGTGAGCC		

Positive samples were sequenced in both directions using an ABI 3730xl sequencer (Applied Biosystems, Foster City, California, USA) at the Sequencing and fragment analysis Unit of the University of Santiago de Compostela. Sequences were aligned and edited using ChromasPro 2.1.4. (Technelysium, Brisbane, Australia) and consensus sequences were scanned against the GenBank database using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The zoonotic potential of A. phagocytophilum was studied comparing the sequences at groEL and 16S rRNA partial genes to reference sequences of A. phagocytophilum strains considered pathogenic for humans [28, 29].

Unique partial sequences obtained in this study were deposited in GenBank under accession numbers MK341070-MK341076 and MN726523.

Statistical analysis

Maximum likelihood estimation (MLE) was used to estimate the prevalence of piroplasms in I. ricinus larvae pools as previously reported [30]. The possible influence of some variables (I. ricinus development stage, sampling area, year and season of sampling) in the prevalence of the pathogens was analysed by Chi-square test; the level of significance was set at P values < 0.05. All statistical analyses were performed using the statistical software R 3.4.3 [31].

Results

Seven I. ricinus (0.7%) and a single I. frontalis nymph (8.3%) resulted positive to Anaplasma qPCR. All qPCR-positive samples were also positive to all conventional PCRs performed. Using qPCR, piroplasm DNA was detected in 21 individual I. ricinus (2.0%) and in a single I. ricinus larvae pool (MLE = 0.6%); all these positives were also confirmed by 18S rRNA and ITS1 PCR. Anaplasma or piroplasm DNA was not found in the other tick species analysed (D. marginatus, D. reticulatus and I. acuminatus) and no Ca. N. mikurensis positive pools were detected. No ticks were positive to more than one pathogen.

Sequence analysis allowed the identification of A. phagocytophilum in all Anaplasma positive samples (Table 2). In addition, three piroplasm species, namely B. venatorum (16/21), B. microti (3/21) and Theileria sp. OT3 (2/21), were also identified (Table 2). Babesia venatorum was also identified in the single positive larvae pool (1/23; 4.3%; MLE = 0.62%). No significant differences in A. phagocytophilum or piroplasm species prevalences when considering the tick development stages, sampling areas, seasons and years of study (Table 2) were found.

Table 2

Prevalence of Anaplasma, Babesia and Theileria species in Ixodes ricinus from northwestern Spain when considering different variable

		Tick development stage			Sampling area			Season of sampling				Year of samp	
		Nymphs	Female	Male	Plateau	Mountain	Coastal	Spring	Summer	Autumn	Winter	Year 1	Year
Anaplasma spp.													
A. phagocytophilum	Ir	4/652 (0.61%)	2/202 (0.99%)	1/202 (0.50%)	1/272 (0.37%)	4/372 (1.08%)	2/412 (0.49%)	3/437 (0.69%)	1/259 (0.39%)	2/148 (1.35%)	1/212 (0.47%)	4/534 (0.75%)	3/52 (0.57
	If	1/12 (8.33%)	-	-	0/8 (0%)	0/2 (0%)	1/2 (50%)	0/2 (0%)	-	1/4 (25%)	0/6 (0%)	0/5 (0%)	1/7 (14.2
Babesia spp.													
B. venatorum	Ir	11/652 (1.69%)	3/202 (1.49%)	2/202 (0.99%)	3/272 (1.10%)	4/372 (1.08%)	9/412 (2.18%)	5/437 (1.14%)	5/259 (1.93%)	2/148 (1.35%)	4/212 (1.89%)	7/534 (1.31%)	9/52 (1.72
B. microti	Ir	3/652 (0.46%)	0/202 (0%)	0/202 (0%)	0/272 (0%)	0/372 (0%)	3/412 (0.73%)	0/437 (0%)	0/259 (0%)	2/148 (1.35%)	1/212 (0.47%)	3/534 (0.56%)	0/52 (0%)
TOTAL		14/652 (2.15%)	3/202 (1.49%)	2/202 (0.99%)	3/272 (1.10%)	4/372 (1.08%)	12/412 (2.91%)	5/437 (1.14%)	5/259 (1.93%)	4/148 (2.70%)	5/212 (2.36%)	10/534 (1.87%)	9/52 (1.72
Theileria spp.													
Theileria sp. OT3	Ir	2/652 (0.31%)	0/202 (0%)	0/202 (0%)	1/272 (0.37%)	1/372 (0.27%)	0/412 (0%)	2/437 (0.46%)	0/259 (0%)	0/148 (0%)	0/212 (0%)	0/534 (0%)	2/52 (0.38

Ir, Ixodes ricinus; If, Ixodes frontalis

Nucleotide sequences of A. phagocytophilum showed a 99.3–100% homology when compared to reference sequences at groESL, 16S rRNA and msp2 genes. Sequence analysis at the groESL gene showed that, of the eight positive samples, three were included in clade I and five in clade II (Table 3). Most of the sequences obtained for the groESL gene (5/8) were identical to other deposited GenBank sequences originated from roe deer captured in France (KJ832450) and Czech Republic (AY22046), and from I. ricinus collected from a bird (KF031393) and a roe deer (EU552912) from Italy. Nevertheless, all A. phagocytophilum groESL sequences showed up to 55 polymorphisms with respect to sequence U96728, associated with human anaplasmosis [29] (Table 3). The study of 16S rRNA partial gene of A. phagocytophilum (Table 4) revealed that six out of eight isolates were included within variants "W", "X" and "Y", whereas two did not coincide with any known variant. Most 16S rRNA sequences (5/8) were identical to sequences obtained from a roe deer in Czech Republic (EU839847), a moose in Sweden (KC800983) and I. ricinus feeding on a wolf (KY404195) and a bird (JN181070) in Italy and Norway, respectively (Table 4). All these sequences showed one to three single nucleotide polymorphisms with regards to variant "B" strain U02521 which was identified in clinical cases [12]. Finally, all msp2 sequences were identical to A. phagocytophilum sequences obtained from questing I. ricinus ticks from Ukraine (KX591651) and Italy (JQ669948) as well as from a dog from Poland (DQ519568) and from the A. phagocytophilum laboratory strain Norway variant 2 (CP015376).

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groEL nucleotide differences of Anaplasma phagocytophilum positive samples from questing ticks in north-western Spain when com

Clade, tick development stage and ID			groEL nucleotide position (bp)																												
			29	60	282	285	291	296	324	327	381	450	465	537	615	627	690	697	705	720	721	723	724	729	732	741	744	756	768	780
U96728[a]			A	C	A	G	A	G	T	A	T	G	C	A	A	A	A	C	C	A	C	G	T	A	A	C	C	C	A	G
I. ricinus																															
I	N	GV320	G	T	G	A	-	A	G	G	-	A	T	G	G	G	G	T	A	G	A	A	G	T	G	-	T	T	G	A
I	M	GV348	-	-	-	A	G	-	-	-	C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
II	F	GV036	-	-	G	A	-	-	-	-	-	A	-	-	-	-	-	-	A	G	A	A	G	T	-	T	-	T	-	-
II	F	GV450	-	-	G	A	-	-	-	-	-	A	-	-	-	-	-	T	A	G	A	A	G	T	-	T	-	T	-	-
II	N	GV566	-	-	G	A	-	-	-	-	-	A	-	-	-	-	-	-	A	G	A	A	G	T	-	T	-	T	-	-
II	N	GV567	-	-	G	A	-	-	-	-	-	A	-	-	-	-	-	-	A	G	A	A	G	T	-	T	-	T	-	-
II	N	GV898	-	-	G	A	-	-	-	-	-	A	-	-	-	-	-	-	A	G	A	A	G	T	-	T	-	T	-	-
I. frontalis																															
I	N	GV1104	G	T	G	A	-	A	G	G	-	A	T	G	G	G	G	T	A	G	A	A	G	T	G	-	T	T	G	A

[a]Pathogenic strains; [b]Novel sequences obtained in this study; N, nymph; M, Male; F, female

Table 4

16S rRNA nucleotide differences of Anaplasma phagocytophilum positive samples from questing ticks in north-western Spain when compared to reference sequences related to clinical cases. Hyphens indicate the presence of the same nucleotide as in the reference sequence

Variant, tick development stage and ID			16S rRNA nucleotide position (bp)							Sequence showing 100% homology
			75	76	77	78	82	84	376	
B		U02521[a]	T	A	A	A	T	G	G	
I. ricinus										
X	F	GV036	-	G	-	-	-	A	-	KY404195
X	N	GV567	-	G	-	-	-	A	-	JN181070
X	N	GV898	-	G	-	-	-	A	-	MK341075[b]
Y	F	GV450	-	G	-	-	-	-	-	KC800983
Y	N	GV566	-	G	-	-	-	-	-	KC800983
W	M	GV348	-	-	-	-	-	A	-	EU839847
-	N	GV320	C	-	-	-	C	A	-	MK341074[b]
I. frontalis										
-	N	GV1104	C	-	-	-	C	A	-	MK341076[b]

[a]Pathogenic strains; [b]Novel sequences obtained in this study; N, nymph; M, Male; F, female

All piroplasm 18S rRNA sequences were identical to others previously reported. Thus, B. venatorum sequences showed a 100% homology to that obtained from an I. ricinus tick in Spain (KM289158), B. microti sequences were identical to that from a USA human pathogenic lineage (XR_002459986) whereas Theileria sp. OT3 sequence matched with that from sheep in China (KF470868). In addition, all B. venatorum ITS1-sequences were identical to that obtained from an I. ricinus in Germany (HM113372). In contrast, B. microti ITS1-sequences (MN726523) showed a maximum similarity of 96% with other B. microti sequences (AF510197-AF510198) and a 97% homology with a sequence deposited as Babesia muratovi (AF510202). Although Theileria sp. OT3 ITS1-sequence had bad quality and could not be properly corrected, the percentage of identity with other Theileria sp. OT3 sequences (KF470865 to KF470867) was 90%.

Discussion

Our results revealed the presence of Anaplasma, Babesia and Theileria species in questing ticks from the northwest of Spain although the prevalence found were lower than 2%.

The percentage of I. ricinus positive to A. phagocytophilum was similar to those (0.4%-0.6%) reported in questing specimens of this tick species from some European countries such as Hungary, Slovenia and The Netherlands [2, 32, 33]. However, our data contrast with previous investigations performed in northern Spain where the percentage of A. phagocytophilum positive questing I. ricinus ranged from 5.6–20.5% [21]. Similarly, prevalence ranging between 1.9% and 23.6% were detected in questing I. ricinus collected in most European countries [1, 5, 13, 34]. These noticeable differences among the prevalence of A. phagocytphilum in I. ricinus throughout Europe may be mainly related to the presence and abundance of reservoirs and susceptible hosts in these areas, although other variables such as the season of study or the number of ticks analysed must be also considered.

A high heterogeneity of A. phagocytophilum groEL-sequences was found (Table 3). Nevertheless, all those single nucleotide polymorphisms do not imply changes in the amino acid sequence except for a single sequence belonging to clade I (GV348) which showed a thymine at the nucleotide position 724 resulting in a change in the codified amino acid (Ala to Ser). This mutation has been observed in A. phagocytophilum variants associated to human or animal clinical cases [10, 35]. In addition, three different variants of A. phagocytophilum were detected at the 16S rRNA gene (Table 3). The most prevalent variants found, “Y” and “X”, are currently considered apathogenic [12, 14]; both variants were the most frequently detected in questing ticks and ticks feeding on both red deer and roe deer from Germany [14] as well as in roe deer from Spain [23]. Variant “W” has been previously found on I. ricinus and some mammalian species, mainly domestic and wild ungulates and it has been identified as pathogenic for cattle and sheep [12, 14, 36, 37]. This variant was detected in sample GV348, so both molecular markers (groEL and 16S rRNA) indicate that this strain may have zoonotic potential [10, 35].

Previous studies performed in Spain have reported the presence of A. phagocytophilum human-pathogenic strains in both questing I. ricinus and blood from roe deer [22–24] and human anaplasmosis cases have been also reported in this country [22]. Although sequence analysis at groEL and 16S rRNA genes does not provide complete information about A. phagocytophilum ecotypes [38] these results provide useful information about A. phagocytophilum pathogenic potential.

Although Ca. N. mikurensis has been found in free-living I. ricinus from several European countries with prevalence ranging from 0.1–24.2% [4, 8, 39], this pathogen was not detected in the present study. In Spain, in fact, Ca. N. mikurensis was only identified in two feeding I. ricinus males collected from a cow in a northern area [24]; however, neither positive questing ticks nor human cases were currently reported [24]. Since a significant number of I. ricinus was analysed in the present study, our results suggest that Ca. N. mikurensis is not present or exists with a very low percentage in free-living ticks from north-western Spain. However, further studies are needed since spreading of tick-borne diseases depends on environmental, socio-economic and demographic factors, among others [40].

The prevalence of Babesia spp. found in questing I. ricinus from the studied area was consistent with those found in previous studies carried out in Spain where a 0.5% of I. ricinus was positive [25]; in contrast, the percentage of I. ricinus positive to Theileria was lower than that found in the previous study (8.3%) [25]. Our data confirm the results reported in other European countries, since the prevalence of both pathogens is usually around 2% in questing I. ricinus [1, 41, 42]. Exceptionally, higher Babesia prevalences have been detected in questing ticks, even above 50%, that could be the consequence of high-density tick populations in sampled areas [43]. The piroplasm species identified in the present study and their diversity were different to those previously reported in I. ricinus from Spain [25] where the most prevalent piroplasms were Theileria ovis followed by Theileria sp. OT3, Theileria annulata and Theileria equi-like; the presence of Babesia spp. was limited since a single isolate of B. caballi, Babesia bigemina, Babesia ovis and Babesia major were identified. These differences could be related to the existing population host in the studied areas [42] since that investigation [25] was performed in a mountainous area from northern Spain where ovine livestock is abundant.

Both Babesia species identified in the present study are considered zoonotic as well as emerging pathogens with special interest in human health. Babesia venatorum, the most prevalent piroplasm in the present study, is frequent in its main vector, I. ricinus [44]. The prevalence found in our study was consistent with those reported (0.3% − 1%) in I. ricinus from Europe [41, 42]. Babesia microti has been detected in European I. ricinus, which could play an important role in the transmission and maintenance of this Babesia species with a prevalence ranging from 0.5–3% [41, 42]. Although B. microti was recently detected in an immunocompetent patient in Spain [45], it is worth noting that not all B. microti can infect humans. Four lineages of B. microti have been described [46] and only some variants of the USA-type are associated with human disease; thus, it has been suggested that most European cases of babesiosis caused by B. microti may be imported [47]. For this reason, the finding of B. microti in questing I. ricinus from north-western Spain may have a limited impact on human health.

It was suggested that both Babesia species showed a clear distribution pattern in I. ricinus from Europe [15]. Thus, I. ricinus from Eastern Europe are more frequently infected with B. microti, whereas B. venatorum infection is more common in ticks from western and northern Europe; Germany is considered a transitory area where I. ricinus presents similar rates of infection by both pathogens [15]. This distribution has been related to the distribution of their main vectors and reservoirs in these areas since B. venatorum have been also detected in some wild ungulates such as roe deer (Capreolus capreolus) [48, 49] and mouflons (Ovis aries musimon) [36] and B. microti is closely related to the distribution of its main host, some Microtus species such as Microtus agrestis which is a more specialist species than deer [50].

Only two I. ricinus ticks were positive to Theileria sp. OT3 (0.2%), being the second report of this pathogen in I. ricinus ticks [25]. Since its main vector remains unknown [51], further studies to determine the role of I. ricinus in the transmission of this piroplasm should be performed. This piroplasm has been detected in European wild ungulates such as roe deer, red deer (Cervus elaphus), fallow deer (Dama dama) and chamois (Rupicapra rupicapra) [45, 52] as well as in

domestic ruminants such as sheep and goats [48]; however, it has been previously detected only in questing Haemaphysalis punctata and I. ricinus with prevalences of 3.6% and 1.6%, respectively [25].

The present study provides data on the presence of A. phagocytophilum, Ca. N. mikurensis and piroplasms in I. frontalis and I. acuminatus. In this respect, available information on the prevalence of pathogens in those tick species is limited and restricted to a low number of specimens. Our results are consistent with the absence or low prevalence of pathogens previously reported in I. frontalis specimens collected from birds and nest boxes since it shows endophilic behaviour and all its life stages feed mainly on birds [53]; thus, only the 2.3% and 3.6% of the specimens analysed were positive to Anaplasma bovis and A. phagocytophilum, respectively [54] and Ca. N. mikurensis has been also detected in one I. frontalis specimen feeding on a common blackbird (Turdus merula) from Russia [55]. In addition, only one investigation analysed the presence of piroplasms in I. frontalis but no positive specimens were detected [56]. It is worth noting that the close relationship between this tick species and birds hampers the transmission of those pathogens to both humans and other animals [53] suggesting a low impact on human health. Regarding I. acuminatus, it has been reported that may be involved in the endophilic cycle of some pathogens such as Borrelia afzelii, Borrelia valaisiana, Coxiella burnetii, Francisella tularensis and Rickettsia helvetica [35], although there is a lack of information concerning the vectorial capacity of this tick species [53] and no association with Anaplasma spp. or Ca. N. mikurensis has been reported up-to-now. Only one study on the presence of Babesia DNA in questing I. acuminatus was performed, although only three specimens were tested and all were negative [57].

Anaplasma phagocytophilum and Ca. N. mikurensis infection has been also reported in Dermacentor ticks such as Dermacentor reticulatus [8]; in contrast, only the former pathogen was currently identified in D. marginatus [1]. However, the vector capacity of both Dermacentor species for the transmission of A. phagocytophilum and Ca. N. mikurensis remains unknown [8]. Current available data suggest a lower prevalence of both pathogens in Dermacentor spp. than in I. ricinus; since our data show a low percentage of positive I. ricinus it is reasonable that no positive Dermacentor spp. were found. Nevertheless, further studies are needed to determine the real situation of both pathogens in Dermacentor ticks from NW Spain. Although both Dermacentor species are competent vectors of some Babesia and Theileria species such as B. caballi and T. equi [58], no specimens resulted positive. Some authors have detected Dermacentor positive to Babesia spp. and Theileria spp. in some European countries such as Slovakia, France and Poland with prevalences up to 5% [1]. In Spain, Babesia and Theileria positive questing Dermacentor were previously reported [25] and seven piroplasm species were identified, namely Theileria equi, Theileria sp. OT1, Theileria annae, Babesia canis, Babesia bigemina, Babesia divergens and Babesia caballi-like; however, the number of processed Dermacentor spp. was higher (n = 97) than in our study. In addition, although B. microti has been previously detected in questing D. reticulatus ticks [15], the role of this tick species as a vector of this piroplasm is currently unknown.

Conclusions

Our results revealed that a low percentage of I. ricinus from northwest Spain were infected with A. phagocytophilum and piroplasms, while Ca. N. mikurensis was not detected in I. ricinus pools. Nevertheless, one of the A. phagocytophilum positive samples was similar to a variant associated to human and/or animal clinical cases through the study of groEL and 16S rRNA genes. In addition, all the Babesia species detected (B. venatorum and B. microti) are considered pathogenic for humans. Thus, these data suggest that there is a risk of acquiring zoonotic A. phagocytophilum and piroplasms in the studied area. In addition, the finding of Theileria sp. OT3 in questing I. ricinus is especially interesting since its main vector has not been currently identified; consequently, further investigations are needed to unravel the role of I. ricinus in the transmission of this Theileria species.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors of the manuscript have read and agreed to its content, approving the text for submission.

Availability of data and material

All data are presented in the manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by a Postdoctoral Research Grant (Xunta de Galicia, Spain) to Susana Remesar and funds from the Program for Consolidating and Structuring Competitive Research Groups (ED431C 2019/04, Xunta de Galicia, Spain).

Authors' contributions

PDB and PM established the final methods and design. CML, GF and PD assisted with the preliminary design of the study. PD, SR, and AP collected the ticks. SR and DGD identified the ticks. SR and AP performed the PCRs. CML conducted the statistical analysis. SR, PD, and RP prepared the first paper draft. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Eva Cabanelas for her invaluable help in collecting ticks.

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