

Tick-borne pathogens in ticks from urban and suburban areas of north-western Spain: Importance of *Ixodes frontalis* harbouring zoonotic pathogens

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Funding information

Xunta de Galicia, Grant/Award Number: GRC2019/04

Abstract

To identify the questing tick populations in urban and suburban areas from the city of Lugo (NW Spain), ticks were collected monthly by flagging. The presence of *Borrelia* spp., *Rickettsia* spp. and *Anaplasma phagocytophilum* also was determined by polymerase chain reaction (PCR) and sequence analysis. Overall, 342 questing ticks were collected; the tick abundance was higher in suburban (95.9%) than in urban areas (4.1%). *Ixodes frontalis* was the most abundant (86.5%); 88.5% were larvae, 11.1% nymphs and 0.3% adults. All development stages of *I. ricinus* (7.3%) and adults of *Rhipicephalus sanguineus* sensu lato (5.8%) and *Dermacentor reticulatus* (0.3%) were found. *Rickettsia* spp. (31.9%) was more prevalent than *Borrelia* spp. (2.7%); no ticks were positive to *A. phagocytophilum*. Six *Rickettsia* species were identified (*R. slovaca*, *R. monacensis*, *R. massiliae*, *R. raoultii*, *R. sibirica* subsp. *mongolitimonae* and *R. aeschlimanii*); *Candidatus Rickettsia rioja* and two novel *Rickettsia* species also were detected. In addition, *Borrelia turdi* (1.8%) and *B. valaisiana* (0.9%) were identified in *Ixodes* ticks. This is the first report of *R. slovaca* in *R. sanguineus* s.l. and of *R. monacensis*, *R. raoultii*, *R. slovaca*, *R. sibirica* subsp. *mongolitimonae* and *Ca. R. rioja* in *I. frontalis*. Since most of the pathogens detected are zoonotic, their presence in these areas may have implications for public health.

KEYWORDS

Anaplasma phagocytophilum, *Borrelia*, *Dermacentor*, *Ixodes*, *Rhipicephalus*, *Rickettsia*, urban areas

INTRODUCTION

Ticks are one of the most important vectors of pathogens in the northern hemisphere (Jongejan & Uilenberg, 2004). In the last decades, the incidence of tick-borne diseases has increased posing important economic and medical consequences (Lippi et al., 2021). Lyme borreliosis (LB), tick-borne encephalitis and rickettsiosis are considered the most prevalent tick-borne diseases in Europe (Estrada-Peña et al., 2018; Oteo & Portillo, 2012). In addition, some tick-borne

pathogens are considered emergent such as *Anaplasma phagocytophilum*, the causative agent of the granulocytic anaplasmosis (Stuenkel et al., 2013). These pathogens circulate in complex cycles involving different tick and vertebrate species, which can have different roles in the maintenance, transmission and dispersion of the pathogens (Estrada-Peña et al., 2018).

The causative agents of LB are spirochetes within the group *Borrelia burgdorferi* sensu lato (s.l.) (Baranton et al., 1992). Among them, three genospecies are primarily responsible for LB in Europe, namely

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B. afzelii, *B. garinii* and *B. burgdorferi* sensu stricto (s.s.) (Margos et al., 2011). The pathogenicity of other genospecies such as *B. valaisiana*, *B. lusitaniae* and *B. spielmani* remains unknown (Estrada-Peña et al., 2018). In Europe, *Ixodes ricinus* is their major vector (Margos et al., 2009), although other *Ixodes* species might be important for the maintenance of this pathogen in animal-tick-animal cycles (Estrada-Peña et al., 2017; Heylen et al., 2014). It has been suggested that transovarial transmission is rather inefficient for *B. burgdorferi* s.l. in the tick or of low epidemiological importance (Hauck et al., 2020); thus, ticks are not considered reservoirs of these pathogens, a role mainly played by vertebrate species (Estrada-Peña et al., 2018).

Most cases of rickettsiosis diagnosed in Europe are caused by spotted fever group *Rickettsia* species (Parola & Raoult, 2001). In this way, *Rickettsia conorii* subsp. *conorii* is the main aetiological agent of the Mediterranean Spotted Fever (MSF), endemic in southern areas; *R. slovaca* and *R. raoultii* are associated with the Dermacentor-Borne Necrosis Erythema Lymphadenopathy (DEBONEL), which is the second most prevalent rickettsiosis in Europe (Oteo & Portillo, 2012). *Rhipicephalus sanguineus* sensu lato (s.l.) and *Dermacentor* spp., respectively, are considered the main vectors, reservoirs and amplifying hosts of the *Rickettsia* species involved in both syndromes (Parola & Raoult, 2001). However, other tick species such as *I. ricinus* are recognized vectors and reservoirs of other less-known rickettsiosis such as MSF-like syndrome caused by *R. helvetica* and *R. monacensis* (Oteo & Portillo, 2012).

Although LB and SF rickettsiosis are the most common tick-borne diseases in Spain, the impact of other tick-borne pathogens such as *A. phagocytophilum* should also be considered due its potential pathogenicity for human and other animals (Stuen et al., 2013). As no transovarial transmission of *A. phagocytophilum* has been demonstrated in *I. ricinus*, its main vector in Europe (Stuen et al., 2013), vertebrate reservoir hosts are necessary for maintaining its sylvatic cycle (Di Domenico et al., 2016).

Tick-borne pathogens circulate in enzootic cycles, involving different tick species and suitable animal hosts (Parola & Raoult, 2001). However, other variables such as climate and social factors are involved in the epidemiology of these pathogens (Estrada-Peña et al., 2012). For this reason, effective surveillance for monitoring those tick-borne pathogens affecting humans and other animals is needed (Parola & Raoult, 2001). It is worth noting that most studies on questing ticks to date were performed in woodland areas far away from the major population centres; in consequence, the detection of high prevalences in ticks from these areas does not always involve a real risk of infection for humans and domestic animals (Estrada-Peña & de la Fuente, 2014). Previous studies on questing ticks collected from the vegetation in urban and suburban areas reported a high risk of acquiring tick-borne pathogens in cities where micromammals and birds may play an important role in the maintenance of tick populations (Hansford et al., 2022); this could be related to the high density of these animals in urban areas when compared to natural areas as they have few or no natural predators in urban areas (Estrada-Peña et al., 2017).

Although data on the presence of ticks and tick-borne pathogens in urban and suburban areas from European countries are available, information from Spain is limited. For these reasons, the main objectives of the present study were: (i) to identify the questing tick populations in urban and suburban areas from the city of Lugo (north-western Spain) and to establish tick phenology during a one-year period and (ii) to determine the prevalence of *Borrelia* spp., *Rickettsia* spp. and *A. phagocytophilum* in the collected ticks.

MATERIALS AND METHODS

Study area and tick collection

Field studies were conducted in eight green areas of the city of Lugo (43°00'42"N 7°33'26"W), located in Galicia (north-western Spain), from November 2020 to October 2021. Four sampling points were located in urban areas and four in suburban areas, which are primarily residential areas less-densely populated than urban commercial areas (Figure 1). Urban areas three and five and suburban areas one, six and eight were public parks. Urban areas four and seven corresponded to a green area close to the street and a dog park in the city centre, respectively. All these areas were mainly composed by grasses. Finally, suburban area two was a not-urbanized unused wasteland mainly composed by grass species (*Poa pratensis*, *Dactylis glomerata*, *Lolium perenne* and *Festuca pratensis*), ferns (*Polypodium vulgare*) and shrubs (*Genista hirsuta* and *Calluna vulgaris*).

Ticks were collected monthly by flagging method along a constant single 300 m transect in each sampling area. The 1 m² flannel flag (1 × 1 m) was checked every 2–5 m depending on the number of ticks collected; if at least one specimen was observed when the flag was checked, the flag was checked every 2 m; if no specimens were present, the flag was checked every 5 m. Collected ticks were removed from the flag using tweezers and stored in 70% ethanol. All larvae adhering to the flag in a clumped manner were defined as a 'nest' originating from a single female (Hauck et al., 2020).

Tick species identification

In the laboratory, ticks were identified to species level using previously described morphological keys (Estrada-Peña et al., 2017). A subset of each tick species was analysed using molecular methods to confirm the morphological identifications. DNA was extracted from individual ticks using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH®) following the manufacturer's instructions. Before DNA extraction, tick tissues were disrupted using a MagNaLyser Instrument (Roche Diagnostic) at 6000 rpm during 60 s. A 460 bp fragment of the 16S rRNA gene of ticks was amplified using previously reported PCR protocols (Norris et al., 1996). Selected fragments were purified and sequenced on an ABI 3730xl sequencer (Applied Biosystems) at the Sequencing and Fragment Analysis Unit of the Santiago de Compostela University. Sequences were aligned

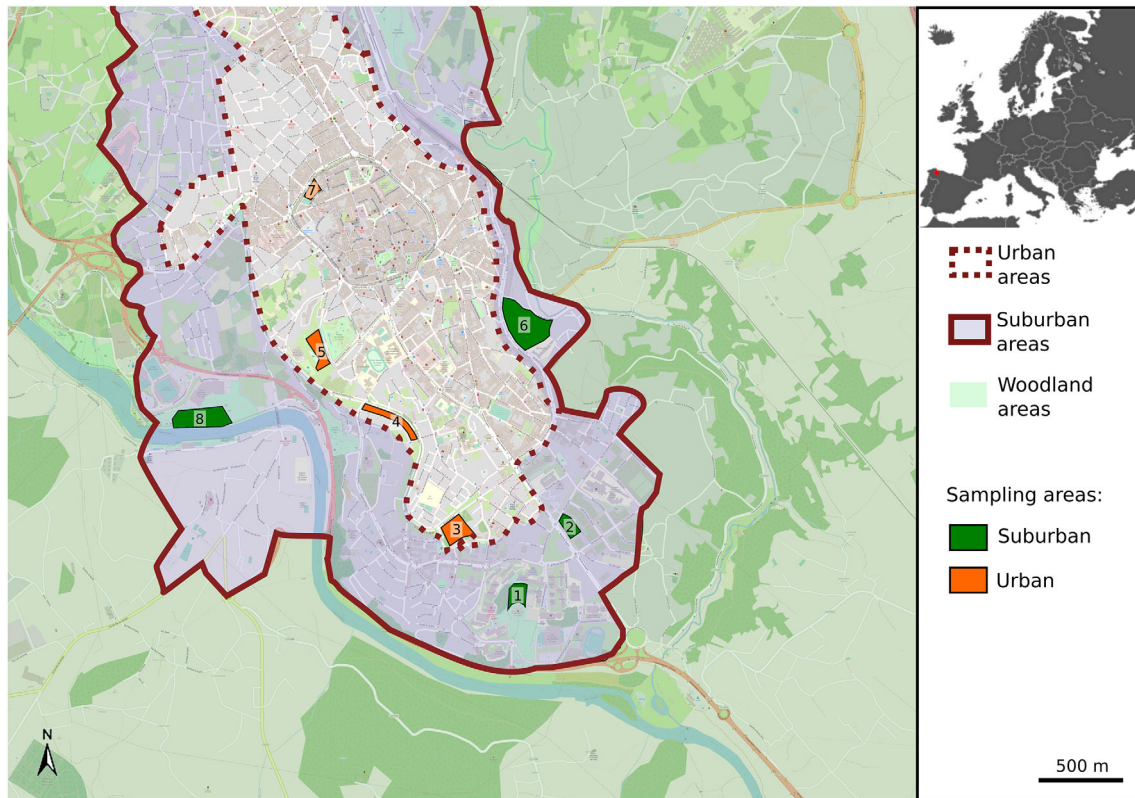


FIGURE 1 Location of the eight tick sampling points in the city of Lugo (north-western Spain).

and edited using ChromasPro (Technelysium) and consensus sequences were then compared to the sequences available in GenBank database using BLAST.

DNA extraction and pathogen detection

DNA was individually extracted from 26 adults, 56 nymphs and 17 larvae using a commercial kit as previously indicated. In addition, 49 *I. frontalis* larvae originating from two larvae nests composed of 14 and 232 ticks, respectively, were analysed in 14 pools (seven pools of two larvae for the first larvae nest and seven pools of five larvae for the second). DNA from larvae pools was extracted as previously indicated.

The presence of *Borrelia* spp., *Rickettsia* spp. and *A. phagocytophilum* in tick samples was detected molecularly using primers and protocols previously described. The presence of *Borrelia* spp. and *A. phagocytophilum* was studied using PCR assays targeting the genes encoding for the flagellin protein (Stromdahl et al., 2003) and *groEL* heat-shock protein, respectively (Liz et al., 2002). Those samples positive to *Borrelia* spp. were further analysed at the *gfpQ* gene (Hovius et al., 2013). In addition, *Rickettsia* DNA was detected by amplifying fragments of two genes encoding for the major outer membrane proteins *rOmpA* and *rOmpB* (Choi et al., 2005; Regnery et al., 1991).

DNA of *B. burgdorferi* s.l., *A. phagocytophilum* and *R. amblyommii* and nuclease free water were included as positive and negative controls in each PCR assay.

PCR products were processed and sequenced as previously indicated; finally, the obtained sequences were scanned against the GenBank database. Unique sequences identified in this study were deposited in GenBank under accession numbers ON859976-ON859999.

A phylogenetic analysis was carried out using MrBayes 3.2.7 software by Bayesian approach with Markov Chain Monte Carlo sampling (10,000,000 generations sampling every 1000 steps). A General Time Reversible substitution model with gamma-distributed rate variation across sites (GTR + G) and a Hasegawa-Kishino-Yano substitution model with gamma-distributed rate variation across sites (HKY + G) were used for the analysis of tick sequences at the *rOmpA* and *rOmpB* partial genes, respectively. The models were selected based on Akaike information criterion (AIC) value using the free software jModelTest v.2.1.10. The tree was visualized and edited using FigTree 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Statistical analysis

The influence of the sampling season and the studied area (urban and suburban) on the total number of collected nymphs and adults was assessed using a test of equal or given proportions. The possible influence of some variables (tick development stage, sampling area and season of sampling) on the prevalence of *Rickettsia* spp. was analysed by multivariate analysis using a multiple logistic regression model

TABLE 1 Number of tick specimens collected in the city of Lugo (north-western Spain) when considering the tick species, stage of development, sampling area and season.

Area	<i>Ixodes frontalis</i>				<i>Ixodes ricinus</i>				<i>Rhipicephalus sanguineus</i> s.l.			<i>Dermacentor reticulatus</i>		Season				Total	
	L	N	F	T	L	N	F	T	F	M	T	M	T	Sp	Su	Au	Wi		
1	SU	20	11	-	31	-	-	-	0	5	3	8	-	0	9	-	22	8	39
2	SU	0	11	1	12	-	-	-	0	11	4	15	-	0	14	-	12	1	27
3	U	1	2	-	3	-	-	-	0	-	-	0	-	0	-	-	1	2	3
4	U	-	-	-	0	-	-	-	0	-	-	0	-	0	-	-	-	-	0
5	U	8	-	-	8	-	2	1	3	-	-	0	-	0	2	1	7	1	11
6	SU	1	1	-	2	1	21	-	22	-	-	0	-	0	13	0	5	6	24
7	U	-	-	-	0	-	-	-	0	-	-	0	-	0	-	-	-	-	0
8	SU	232	8	-	240	-	-	-	0	-	-	0	1	1	1	-	235	5	241
Total		262	33	1	296	1	23	1	25	16	7	23	1	1	39	1	282	23	345

Abbreviations: Au, autumn (samplings from October to December); F, females; L, larvae; M, males; N, nymphs; Sp, spring (including the samplings performed from April to June); SU, suburban; Su, summer (samplings from July to September); T, total; U, urban areas; Wi, winter (samplings from January to March).

(Hosmer et al., 1989). Factors were eliminated from the initial model using a method based in AIC value until the best model was built. All pairwise interactions were evaluated. Odds ratio were computed by raising 'e' to the power of the logistic coefficient over the first category of each factor (reference category).

The level of significance was set at p values <0.05. All statistical analyses were performed using the statistical software R 4.1.2.

RESULTS

Questing tick species identified

A total of 345 ticks were collected during the study (Table 1). Ticks were found in all suburban sampling areas and in two out of four urban areas (Table 1). A higher number of ticks was collected in suburban (331/345; 95.9%) than in urban areas (14/345; 4.1%). Significant differences in the total number of both nymphs and adults ($\chi^2 = 122.95$, $p < 0.001$) were detected between suburban (77/82; 93.9%) and urban (5/82; 6.1%) areas.

Four tick species were identified, *I. frontalis* being the most abundant (296/345; 85.8%) followed by *I. ricinus* (25/345; 7.2%) and *R. sanguineus* s.l. (23/345; 6.7%); a single specimen of *D. reticulatus* was found (1/345; 0.3%). The number of the different development stages collected for each tick species is summarized in Table 1, including 246 *I. frontalis* larvae found in two nests, one in the suburban area 1 (14 larvae) and other in suburban area 8 (232 larvae). Morphological identification was confirmed molecularly in all cases. *I. frontalis* sequences showed a percentage of identity ranging from 99.8% to 100% when compared to the deposited sequences MF370645 and KJ414454 obtained from *I. frontalis* collected on vegetation in Portugal (Santos et al., 2018) and on birds in Belgium (Heylen et al., 2014), respectively. In addition, *I. ricinus* and *D. reticulatus* sequences were identical to MH645522 and MH645514,

respectively, obtained from questing ticks captured in north-western Spain (Remesar, Díaz, Venzal, Pérez-Creo, et al., 2019). Finally, sequences of *R. sanguineus* s.l. were identical to MZ420717 obtained from that tick species feeding on wild rabbits in Spain (Remesar, Castro-Scholten, et al., 2021).

A higher diversity of tick species was detected in suburban than in urban areas (Table 1); thus, all the species identified were found in suburban areas whereas only *Ixodes* spp. ticks were captured in urban areas. *I. frontalis* was the most abundant species since 33 nymphs and one adult were collected; this species was also widely distributed since it was detected in all suburban areas and in two urban areas (Table 1). In contrast, *I. ricinus* (23 nymphs and one adult) and *R. sanguineus* s.l. (23 adults) were only detected in two sampling areas (one urban and one suburban areas for *I. ricinus* and two suburban areas for *R. sanguineus* s.l.), and the only *D. reticulatus* specimen collected was found in one suburban area (Table 1). All development stages were only collected for *Ixodes* ticks, although no males were found. *I. frontalis* larvae were the most abundant stage of development followed by nymphs; only a single female was found (Table 1). It is worth noting that most *I. frontalis* larvae (93.9%) were found in two larval nests. For *I. ricinus*, nymphs were predominant and only one larvae and one female were identified (Table 1). Only adults of *R. sanguineus* s.l. and a single male of *D. reticulatus* were detected (Table 1).

Ticks were found in all seasons, especially in autumn, when all the larvae were collected (Table 1 and Figure 2). It is worth noting that no ticks were collected in June, August and September; in addition, only a single nymph of *Ixodes* spp. was detected in January, October and July (Table 1 and Figure 2). *I. frontalis* nymphs were found from October to April; its density showed a bimodal pattern with peaks in November and February (Table 1 and Figure 2). In contrast, *I. ricinus* nymphs were detected from February to April showing a unimodal pattern, peaking in April. The only adult specimen of *I. frontalis* was found in November and the only adult specimen of *I. ricinus* was found in May (Table 1 and Figure 2). All the specimens of *R. sanguineus* s.l.

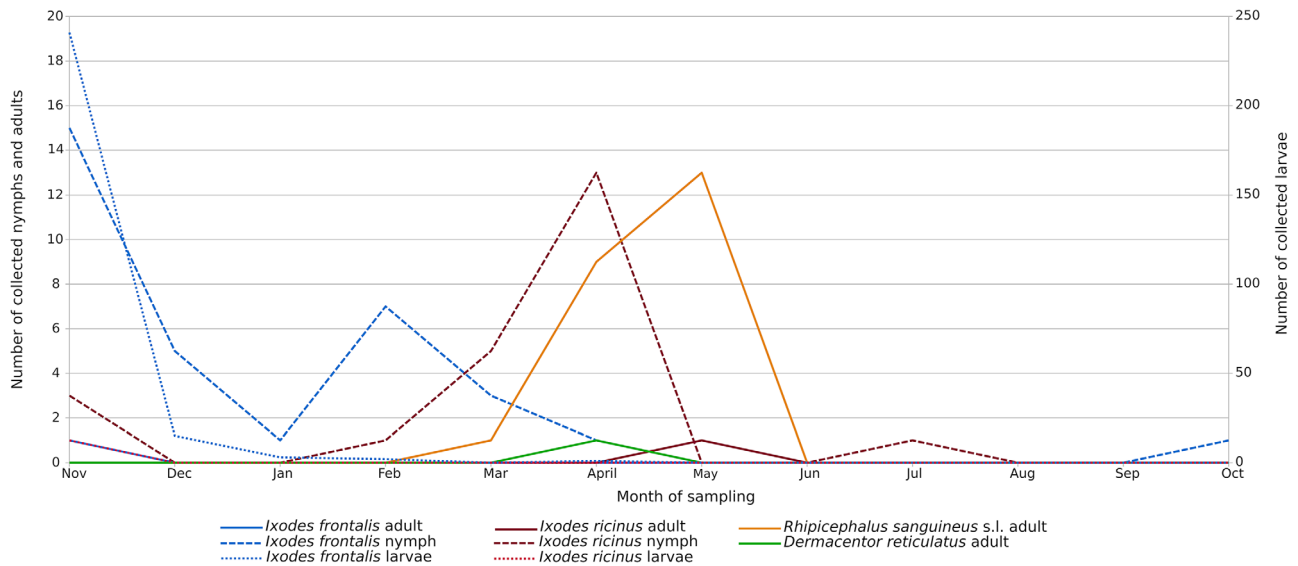


FIGURE 2 Number of ticks collected in the city of Lugo (north-western Spain) when considering the tick species, stage of development and month of sampling. The data series of nymphs and adults was aligned to the primary Y axis and larvae data series to the secondary Y axis.

were found from March to May and the male of *D. reticulatus* in April (Table 1 and Figure 2).

Pathogens detected in questing ticks

Three out of 113 (2.7%) individual tick samples were positive to *Borrelia* spp. Two *Borrelia* species were identified in *Ixodes* ticks: *B. turdi* was detected in two (1.8%; 2/113) *I. frontalis* specimens (one female and one nymph) and *B. valaisiana* in a single (0.9%; 1/113) *I. ricinus* nymph (Table 2). Both *fla* and IGS sequence data of *Borrelia*-positive isolates are summarized in Table S1.

In addition, 31.9% of samples were positive to *Rickettsia* spp. (36/113). Positive samples to this pathogen genus were detected in 17 (26.6%) *I. frontalis* (two individual and two larvae pools and 13 nymphs), 10 (40%) *I. ricinus* nymphs and in nine (39.1%) *R. sanguineus* s.l. adults (Table 2). Most *Rickettsia*-positive ticks were detected in winter (34.8%; 8/23) and autumn (34.0%; 17/50), whereas a percentage of 28.2% was detected in spring (11/39); no *Rickettsia*-positive ticks were detected in summer, but it must be noted that only one tick was collected. Positive ticks to *A. phagocytophilum* were not found. Logistic regression results showed that the prevalence of *Rickettsia* spp. was significantly higher in *I. ricinus* than in *I. frontalis* and in nymphs than in larvae (Table 3). In addition, significant differences also were observed regarding the sampling season; thus, the risk of being infected by *Rickettsia* spp. was 8.1 and 24.2-fold higher in winter and autumn, respectively, than in spring (Table 3).

rOmpA and *rOmpB* sequence data of *Rickettsia*-positive isolates is summarized in Table S1; sequence analysis allowed the identification of six validated and one *Candidatus* *Rickettsia* species (Table 2). *R. slovaca* was the most prevalent, followed by *R. monacensis*, *R. massiliae*, *R. raoultii* and *Candidatus* *Rickettsia rioja* (Table 2). *R. sibirica mongolitimonae* and co-infections *R. massiliae/R. aeschlimanii* and *R. massiliae/R.*

monacensis were detected in one sample each (0.9%; 1/113). In addition, two novel *Rickettsia* species were detected: one novel species was detected in a single *I. ricinus* nymph (4.35%; 1/113) and the other in a single *R. sanguineus* female in co-infection with *R. massiliae* (4.35%; 1/113). Phylogenetic analysis at *rOmpA* and *rOmpB* genes showed that both novel *Rickettsia* species were located outside the clusters including the main *Rickettsia* species (Figures 3 and 4). The highest diversity of *Rickettsia* species was detected in *I. ricinus*, which was positive to all the species identified, with the exception of *R. sibirica* subsp. *mongolitimonae*. Five and three *Rickettsia* species were identified in *I. frontalis* and *R. sanguineus* s.l., respectively, and co-infections were only detected in *I. ricinus* and *R. sanguineus* s.l. (Table 2). It is also worth noting that two *Rickettsia* species were detected in *I. frontalis* larvae, *R. slovaca* and *Ca. R. rioja* (Table 2).

DISCUSSION

The detection of ticks and tick-borne pathogens in urban and suburban areas is of increasing concern. Although tick densities are usually lower in urbanized areas than in non-urban woodland environments (Hauck et al., 2020), the presence of tick populations in urban and suburban areas could pose an increased risk of tick-bites since people and companion animals usually develop their activities in these areas (Estrada-Peña et al., 2017). Therefore, the presence of zoonotic pathogens in ticks from urbanized areas may involve implications for public health.

Questing tick species identified

Our data reveal the presence of a considerable number of ticks in urban and suburban areas of the city of Lugo, although tick abundance was lower than that previously reported, using the same collection

TABLE 3 Logistic regression model for the prevalence of *Rickettsia* spp. in questing ticks collected in green areas from the city of Lugo (NW Spain).

	Estimate	Z-value	p-Value	OR	95% CI
(Intercept)	-5.0397	-4.195	<0.001	-	-
Tick species					
<i>Ixodes frontalis</i>	-	-	-	-	-
<i>Ixodes ricinus</i>	1.8535	2.252	0.024	6.38	1.40–37.86
Tick development stage					
Larvae	-	-	-	-	-
Nymph	1.8839	2.795	0.005	6.58	1.90–27.86
Sampling season					
Spring	-	-	-	-	-
Autumn	3.1845	3.033	0.00242	24.16	3.57–229.19
Winter	2.0853	2.229	0.02580	8.05	1.45–61.25

protocol in constant 300 m transects, in forest areas from the same region (Remesar, Díaz, Venzal, Prieto, et al., 2019); thus, the number of specimens yearly collected in each sampling area ranged from 0 to 241 in the present study, but increased to 500–2000 in forest areas (Remesar, Díaz, Venzal, Prieto, et al., 2019). Previous studies carried out in Europe indicated that overall tick densities are low in urban areas, but noticeable variations mainly related to habitat types and urban gradient can be detected (Hansford et al., 2022). In this regard, a significantly higher number of ticks were found in suburban areas than in urban areas. These results are consistent with previous investigations performed in urban environments reporting the highest tick densities in woodland habitats such as wide centrally located parks or urban fringes (Hauck et al., 2020; Stańczak et al., 2015). Although the tick density is influenced by the urban gradient, some results are conflicting and suggest that other variables such as the tick species and the presence of wild animals acting as maintenance hosts for tick populations must be considered (Hansford et al., 2022).

Although the urban zones sampled in the present study have bird and micromammal populations, they are isolated from the main woodland areas and therefore wild large mammals have no access to these zones. This fact may have a major role in the presence and number of *I. ricinus* collected in a particular area; thus, wild large mammals such as roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*), which can host all the stages of development of this tick species (Estrada-Peña et al., 2017), are common in the forest area close to suburban sampling area 6 where *I. ricinus* was mainly found. Nevertheless, the presence and abundance of wild ungulates cannot explain the predominance and abundance of *I. frontalis*, which is considered a passerine tick (Estrada-Peña et al., 2017). In this regard, it has been suggested that the abundance of *I. frontalis* could be higher in areas acting as roost sites for birds (Plantard et al., 2021). In the present study, this tick species was mostly found in suburban areas with high vegetation and tree density (sampling area 8) or with a high bramble density (sampling areas 1 and 2) where birds can nest and are protected from predators. With regard to *R. sanguineus* s.l. and *D. reticulatus*, only adults were collected since their immature stages show

endophilic behaviour and, therefore, are not usually captured by flagging (Estrada-Peña et al., 2017). *Rhipicephalus sanguineus* s.l. was only captured in the two suburban areas closest to the main urbanized zone; these results are surprising since this tick is traditionally associated to dogs and humans and consequently it would be more likely to be found in urban areas (Shimada et al., 2003). It has been previously suggested that the use of ectoparasiticides may play a significant role in its absence in the selected urban areas (Lorusso et al., 2010).

Our results also indicate that the tick species identified showed different activity patterns. In this way, *I. frontalis* showed a bimodal activity pattern with the highest peak in autumn (November) when the only *I. frontalis* adult as well as the highest number of nymphs were captured. However, it is worth noting that the pattern observed in the present study is based on reduced number of individuals ($n = 33$ nymphs spread among two different sites). These data are consistent with previous studies indicating that all the development stages can be found from late autumn to early-winter showing an absence of activity in summer (Plantard et al., 2021). *I. ricinus* showed an activity peak in late spring-early summer; although different seasonal patterns have been observed in Europe (Estrada-Peña et al., 2017), our results are in line with previous studies carried out in forest areas from Spain (Remesar, Díaz, Venzal, Pérez-Creo, et al., 2019). Finally, *R. sanguineus* s.l. was only detected between March and May, agreeing with previous studies reporting a reduced activity of this tick during winter in southern Europe (Dantas-Torres, 2010).

Pathogens detected in questing ticks

Two out of the three analysed pathogens, *Borrelia* spp. and *Rickettsia* spp., were detected in the ticks collected in green areas from the city of Lugo. *Borrelia* DNA was only detected in a low percentage of *I. ricinus* and *I. frontalis* nymphs as well as in the only *I. frontalis* female analysed. It is worth noting that no data on the prevalence of *Borrelia* spp. in questing *I. frontalis* is currently available whereas this pathogen

was detected in 0% to 50% *I. frontalis* feeding on wild birds from Northern Spain (Estrada-Peña et al., 1995; Palomar et al., 2017); although no successful transmission of *B. burgdorferi* s.l. by *I. frontalis* nymphs was demonstrated in the great tit (*Parsus major*) (Heylen et al., 2014), further studies are needed to determine its vector competence (Estrada-Peña et al., 2017). The percentage of *I. ricinus* positive to *Borrelia* spp. was lower than that previously reported (8.1%–18.6%) in woodland areas from the same region (Díaz et al., 2017; Remesar, Díaz, Venzal, Prieto, et al., 2019). In this regard, it has been reported that climate, density of reservoirs, land use, and human actions on landscape can locally affect the prevalence of *Borrelia* spp. in questing ticks (Estrada-Peña & de la Fuente, 2014).

Two *Borrelia* species were identified, *B. valaisiana* in *I. ricinus* and *B. turdi* in *I. frontalis*. The former was previously reported in questing *I. ricinus* from the same study region (Díaz et al., 2017; Remesar, Díaz, Venzal, Prieto, et al., 2019) and *B. turdi* was also identified together with *B. afzelii* and *B. garinii* in *I. frontalis* feeding on wild birds from Spain (Estrada-Peña et al., 1995; Palomar et al., 2017). It has been reported that the distribution of *B. burgdorferi* s.l. genospecies is related to the presence of particular reservoirs (Estrada-Peña et al., 2011). Since *B. valaisiana* and *B. turdi* have been associated to birds (Palomar et al., 2017), our data suggest that micromammals and lizards are not playing an important role in the maintenance of *Borrelia* spp. in green areas from Lugo city; in contrast, wild birds may have an important role in the introduction of these spirochetes from the woodland.

Rickettsia spp. was very common in ticks from public parks in the city of Lugo and was detected in a high percentage (26.6%–40%) of *I. frontalis*, *I. ricinus* and *R. sanguineus* s.l. Our results are higher than those (10%–21.7%) found in *I. frontalis* feeding on birds from Greece and Italy (Diakou et al., 2016; Wallménus et al., 2014). Although *Rickettsia* DNA also has been detected in *I. frontalis* feeding on birds from other European countries such as Germany, no data is available to allow the calculation of a prevalence (Wimbauer et al., 2022). In contrast, there is extensive information on the presence of *Rickettsia* spp. in questing *I. ricinus*, and prevalences found in woodland areas from the same study region (20.7%) as well as the mean prevalence reported in Europe (18%) are lower than that detected in the present study (Milhano et al., 2010). In Europe, information on the prevalence of *Rickettsia* spp. in questing *R. sanguineus* s.l. is limited and restricted to Portugal (2.8%; Milhano et al., 2010) and France (27.8%; Parola et al., 2008), but data on *R. sanguineus* s.l. feeding on wild and domestic animals are more extensive, ranging from 0% to 50% (Fernández-Soto et al., 2006; Parola et al., 2008; Remesar, Castro-Scholten, et al., 2021). The prevalence of *Rickettsia* spp. was significantly higher in autumn and winter (Table 3), agreeing with previous studies performed in Spain (Remesar, Díaz, Portillo, Santibáñez, et al., 2019) and coinciding with the activity peak of *I. frontalis* and the emergence of questing *I. ricinus*.

Six *Rickettsia* spp. and Ca. R. rioja were identified; in addition, two novel *Rickettsia* sp. were detected. Our data reveal that *I. frontalis* can harbour a wide range of *Rickettsia* species, being the first report of *R. slovacica*, *R. monacensis*, *R. raoultii*, *R. sibirica* subsp. *mongolitimoniae*

and Ca. R. rioja in this tick. In addition, *R. aeschlimanii*, *R. monacensis*, *R. helvetica* and a not identified *Rickettsia* sp. were found in *I. frontalis* collected from wild birds in Greece, Italy and Germany (Diakou et al., 2016; Wallménus et al., 2014; Wimbauer et al., 2022). In addition, *R. slovacica* and Ca. R. rioja were detected in questing *I. frontalis* larvae suggesting the transovarial transmission of both pathogens in this tick species. With regard to *I. ricinus*, only *R. massiliae* and *R. aeschlimanii* were detected in this tick; these species are different to those found in questing *I. ricinus* from the same region, that is *R. monacensis*, *R. raoultii*, *R. slovacica*, *R. felis* and Ca. R. rioja (Remesar, Díaz, Portillo, Santibáñez, et al., 2019). One of the two novel *Rickettsia* species detected in this study was identified in a single *I. ricinus* nymph; the large phylogenetic distance with well characterized species suggests that it may be considered as a distinct species of *Rickettsia* (Figure 4). In addition, the phylogenetic analysis at the *rOmpB* gene showed a close relationship between our sequence and those from other rickettsial endosymbionts of *Haemaphysalis* spp. (Portillo et al., 2008); nevertheless, the limited number of ticks infected with this *Rickettsia* sp. suggests that it may not be an endosymbiont. *R. massiliae*, *R. slovacica* and *R. monacensis* were identified in *R. sanguineus* s.l., which is considered the main vector and reservoir of *R. massiliae* and *R. conorii* subsp. *conorii* (Milhano et al., 2010; Oteo & Portillo, 2012; Parola et al., 2008). Although *R. slovacica* is traditionally associated to *Dermacentor* spp., there are few reports of this *Rickettsia* spp. in *Rhipicephalus bursa* collected from sheep and wild boars (Cicculli et al., 2019; Kachrimanidou et al., 2010). In addition, the other novel *Rickettsia* species found in this investigation was detected in *R. sanguineus* s.l.; it was genetically very similar (99.8%) to *Candidatus Rickettsia africaustralis* (KT835150.1) obtained from *Mus musculus* in South Africa (Essbauer et al., 2018).

It is worth noting that most of the pathogens detected in the present study are considered zoonotic. In fact, *B. valaisiana* belongs to *B. burgdorferi* s.l. group, being associated to LB (Godfröid et al., 2003). In addition, *R. slovacica*, *R. raoultii* and Ca. R. rioja are the causal agents of DEBONEL and *R. monacensis*, *R. massiliae*, *R. aeschlimanii* and *R. sibirica* subsp. *mongolitimoniae* are the etiological agents of the MSF-like (Oteo & Portillo, 2012). *Anaplasma phagocytophilum* was not detected in the studied tick population agreeing with previous studies reporting a low prevalence in *I. ricinus* (0.4%) in woodland areas from the same region (Remesar, Prieto, et al., 2021). Regarding the novel *Rickettsia* spp., further studies are needed for completing their characterization and for evaluating their pathogenicity.

CONCLUSIONS

The present study reveals that tick populations with different phenologies inhabit urban and suburban areas from the city of Lugo, implying a risk for tick-bites. The activity peak of *I. frontalis* was in autumn whereas those of *I. ricinus* and *R. sanguineus* s.l. were in spring. Our data also involve significant implications for public health since a number of those *Rickettsia* and *Borrelia* species detected in the ticks from green areas of the city are considered zoonotic; in addition, *Rickettsia*

spp. in *I. frontalis* and *I. ricinus* was reported with noticeable prevalence.

Our results are of great relevance from an epidemiological point of view since investigations on ticks and tick-borne pathogens from urban areas are limited. In addition, no data on pathogen detection in questing *I. frontalis* are currently available; thus, this is the first report of *R. monacensis*, *R. raoultii*, *R. slovacica*, *R. sibirica* subsp. *mongolitimonae* and *Ca. R. rioja* in this tick species. In addition, the detection of *R. slovacica* and *Ca. R. rioja* in *I. frontalis* questing larvae suggests their transovarial transmission in this tick species. This work also provides the first report of *R. slovacica* in *R. sanguineus* s.l. and the detection of two novel *Rickettsia* spp. in *I. ricinus* and *R. sanguineus* s.l. All these findings suggest that some *Rickettsia* species may be not tick species-specific; in consequence, further studies are needed for assessing the vector competence of these ticks.

AUTHOR CONTRIBUTIONS

Susana Remesar: Conceptualization; investigation; resources; writing – original draft; writing – review and editing. **Raquel Matute:** Resources. **Pablo Díaz:** Conceptualization; writing – original draft; writing – review and editing. **Néstor Martínez-Calabuig:** Investigation; resources. **Alberto Prieto:** Investigation. **Jose Manuel Díaz Cao:** Investigation. **Gonzalo López Lorenzo:** Investigation. **Gonzalo Fernández:** Writing – review and editing. **Ceferino Manuel López:** Writing – review and editing. **Rosario Panadero:** Writing – review and editing. **Pablo Díez-Baños:** Writing – review and editing. **Maria Patrocinio Morrondo:** Funding acquisition; writing – review and editing. **David García-Dios:** Investigation.

ACKNOWLEDGEMENTS

This research was supported by the Programme for Consolidating and Structuring Competitive Research Groups (GRC2019/04; Xunta de Galicia, Spain). The structured reflexivity statement is not applicable to this manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

TABLE S1. *fla*, *IGS*, *rOmpA* and *rOmpB* sequence data of *Borrelia* spp. and *Rickettsia* spp. isolates found in ticks from urban and suburban areas from the city of Lugo (NW Spain). The closest reference sequences deposited in GenBank and the isolation source, country and percentage of identity of the deposited sequences were also included.

How to cite this article: Remesar, S., Matute, R., Díaz, P., Martínez-Calabuig, N., Prieto, A., Díaz-Cao, J.M. et al. (2023) Tick-borne pathogens in ticks from urban and suburban areas of north-western Spain: Importance of *Ixodes frontalis* harbouring zoonotic pathogens. *Medical and Veterinary Entomology*, 1–12. Available from: <https://doi.org/10.1111/mve.12648>