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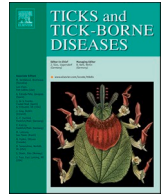
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Original article

High-throughput screening of pathogens in *Ixodes ricinus* removed from hosts in Lombardy, northern Italy

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ABSTRACT

Ticks are important vectors of many pathogens in Europe, where the most impactful species is *Ixodes ricinus*. Recently, the geographical distribution of this tick species has been expanding, resulting in an increased risk of human exposure to tick bites. With the present study, we aimed to screen 350 *I. ricinus* specimens collected from humans and wild animals (mainly ungulates), to have a broader understanding of the tick-borne pathogens circulating in the Lombardy region, in northern Italy. To do so, we took advantage of a high-throughput real-time microfluidic PCR approach to screen ticks in a cost-effective and time-saving manner. Molecular analysis of the dataset revealed the presence of four genera of bacteria and two genera of protozoa: in ungulates, 77 % of collected ticks carried *Anaplasma phagocytophilum*, while the most common pathogen species in ticks removed from humans were those belonging to *Borrelia burgdorferi* sensu lato group (7.6 %). We also detected other pathogenic microorganisms, such as *Rickettsia monacensis*, *Rickettsia helvetica*, *Neoehrlichia mikurensis*, *Babesia venatorum*, and *Hepatozoon martis*. Besides, we also reported the presence of the pathogenic agent *Borrelia miyamotoi* in the area (1.4 % overall). The most common dual co-infection detected in the same tick individual involved *A. phagocytophilum* and *Rickettsia* spp. Our study provided evidence of the circulation of different tick-borne pathogens in a densely populated region in Italy.

1. Introduction

Ixodid ticks (Acari: Ixodida) are worldwide-distributed obligate hematophagous ectoparasites, able to feed on most vertebrate species from all classes, except fishes (Guglielmo and Robbins, 2018; Parola and Raoult, 2001). During feeding, ticks can transmit different microorganisms (including bacteria, viruses, protozoa and helminths) to their hosts, causing diseases of both medical and veterinary importance (Boulanger et al., 2019; Parola and Raoult, 2001). Tick-borne pathogens (TBPs) are the main cause of vector-borne diseases in temperate areas, including North America and Europe, and thus have a strong impact on livestock-related economy and on public health (Rochlin and Toledo, 2020).

Ixodes ricinus is a generalist and ubiquitous parasite, capable of feeding on more than 300 vertebrate host species. It is the most widely distributed tick species in Europe, and, specifically, in northern Italy

(Bowman and Nuttall, 2008; Rizzoli et al., 2014). To our knowledge, other species of the genus *Ixodes* are rarely present in this area. In particular, *Ixodes inopinatus* has never been collected in northern Italy, although being reported in northwestern Africa, Iberian peninsula, and Central Europe (Chitimia-Dobler et al., 2018; Estrada-Peña et al., 2014; Kahl et al., 2021).

Ixodes ricinus is the tick species most frequently reported to bite humans, and is the vector of the most prevalent tick-borne zoonotic pathogens in Europe, namely spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex (mostly *B. burgdorferi* sensu stricto, *Borrelia afzelii* or *Borrelia garinii*), which cause Lyme borreliosis (LB) (Bowman and Nuttall, 2008; Stanek et al., 2012). In different European countries, the incidence of LB ranges from few reports to >100 cases per 100,000 individuals per year (Burn et al., 2023). In the last decade, another species of the genus *Borrelia*, namely *Borrelia miyamotoi*, has been identified as an infectious threat, causing a non-specific febrile

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illness distinct from LB (Cleveland et al., 2023; Kubiak et al., 2021). In immunocompromised patients, it can lead to severe disease with central nervous system infections and meningoencephalitis (Boden et al., 2016; Gandhi et al., 2022).

The second most common tick-borne disease (TBD) in the European Union is tick-borne encephalitis (TBE), caused by a member of the *Flavivirus* genus (TBE virus, European subtype). TBE is currently endemic in 27 European countries, counting for thousands of human cases every year (Phipps and Johnson, 2022; Ruzek et al., 2019). The clinical signs range from mild flu-like symptoms to fatal neurological infections (Riccardi et al., 2019). TBE is irregularly distributed but continuously expanding in Eastern and Central Europe, and, so far, it has only been detected in the northeastern regions of Italy (Panatto et al., 2022; Riccardi et al., 2019). *Ixodes ricinus* transmits several other relevant TBPs, including bacteria such as *Rickettsia* spp. from the spotted fever group (particularly *Rickettsia helvetica* and *Rickettsia monacensis*) (Parola et al., 2013), *Anaplasma* spp. (most of which are relevant to livestock, with *Anaplasma phagocytophilum* being the only species capable of infecting humans) (Rymaszewska and Grenda, 2008), and the recently discovered opportunistic pathogen, *Neoehrlichia mikurensis* (Gyntheresen et al., 2023; Wass et al., 2019). So far ticks rarely transmit protozoans like *Babesia* spp. (Hildebrandt et al., 2021).

Recently, the distribution area (both altitude and latitude) of *I. ricinus* and its density have increased due to climate changes, fragmentation of habitats, and increase of availability of mammalian hosts (Jore et al., 2014; Medlock et al., 2013). This leads to an increased risk of tick bites for both humans and domestic animals, and thus to the spread of TBPs and TBDs. Therefore, efficient surveillance methods need to be implemented in order to assess the circulation of pathogens, to establish the infection rates in ticks, and to monitor cases in humans, as well as in wildlife reservoirs, that can be used as sentinels (Garcia-Vozmediano et al., 2022; Hai et al., 2014; Kazimírová et al., 2018).

To this purpose, a time- and cost-effective molecular screening method has been developed using microfluidic real-time PCRs, which enables the efficient high-throughput detection of numerous TBPs simultaneously, using only a few microlitres of sample and of reagents (Michelet et al., 2014; Migné et al., 2021). Such an approach was profitably applied to TBP screening in multiple countries from five continents (Banović et al., 2021; Boularias et al., 2021; Ghafar et al., 2023, 2020; Klitgaard et al., 2019; Lejal et al., 2019; Sprong et al., 2019; Zintl et al., 2017). In particular, it has provided an unprecedented possibility for high-throughput and cost-effective assessment of TBP burden (e.g., (Borşan et al., 2021)), even allowing for the first detection of pathogens in unexpected/unknown areas (Borşan et al., 2021; Defaye et al., 2022; Gondard et al., 2020).

In Italy, especially in its northeastern area, several studies have been conducted throughout the years both on questing ticks and on ticks collected from wild animals. However, most studies were focused on one or few pathogens, and, to our best knowledge, none of them employed high-throughput real-time microfluidic PCR (Battisti et al., 2019; Bertola et al., 2021; Castro et al., 2015; Corrain et al., 2012; Ebani et al., 2015; Otranto et al., 2014; Pintore et al., 2015).

In this study, we aimed to perform the first Italian high-throughput survey of TBPs by microfluidic real-time PCR on 350 *I. ricinus* ticks collected in 2022 from humans and wild animals throughout the territory of Lombardy (northern Italy). This is the most populous and most densely populated Italian region (around 10 million of people, March 2023-ISTAT update). Furthermore, a significant portion of its territory (27 %) consists of protected natural parks and reserves (source: Lombardy region official website, 2023), where populations of large wild mammals, such as roe deer, are rising. Human activities in the same areas are in expansions, resulting in exponential growth of the risk of contracting TBDs (Rizzoli et al., 2014). Therefore, Lombardy represents an ideal and pivotal study case for a comprehensive surveillance of TBPs and TBDs in Italy, as well as from a Europe-wide perspective.

2. Materials and methods

2.1. Study area, tick collection and DNA extraction

The study area encompassed 87 municipalities in the Lombardy region, northern Italy (Fig. 1). All the examined ticks were obtained from deceased (either natural deaths, or animals killed in population control plans) wild animals (mainly ungulates) and private citizens, and conferred to the Zooprophyllactic Institute of Pavia from January to December 2022. Tick species were determined using taxonomic keys based on morphological features (Manilla, 1998), and only females and nymphs of *I. ricinus* were included in this study, for a total of 350 ticks. Among those, 113 ticks (96 females and 17 nymphs) were sampled from ungulates ($n = 36$), such as roe deer (*Capreolus capreolus*, $n = 19$), red deer (*Cervus elaphus*, $n = 11$), chamois (*Rupicapra rupicapra*, $n = 5$) and mouflon (*Ovis musimon*, $n = 1$). Other 170 specimens (34 females and 136 nymphs) were removed from humans, whereas 17 specimens (all females) were removed from Carnivora hosts, such as badger (*Meles meles*, $n = 1$), wolf (*Canis lupus*, $n = 1$) and stone marten (*Martes foina*, $n = 1$). The hosts remain unknown for 50 ticks (25 females and 25 nymphs). DNA from single specimens was extracted immediately after morphological identification using NucleoSpin® Tissue kit (Macherrey-Nagel, Germany), following manufacturer's instructions.

2.2. DNA pre-amplification and high-throughput microfluidic real-time PCR

All samples underwent a pre-amplification step by PCR with the Preamp Master Mix (Standard BioTools, USA) following Michelet et al. (2014). This step was designed to optimise the sensitivity of the microfluidic PCR for pathogen detection. All the primers used for pathogen detection (Supplementary Table 1) were pooled, with a final concentration of 0.2 μ M each (Supplementary Table 1). The reaction was performed combining 1.25 μ l of DNA for each sample, 1.25 μ l of primer pool mix, 1 μ l of PreAmp Master Mix and 1.5 μ l of distilled water, reaching a final volume of 5 μ l. PCR protocol was as follows: 95 °C for 2 min, 14 cycles at 95 °C for 15 s, 60 °C for 4 min. Pre-amplified samples were diluted 1:10 and then stored at -20 °C until further analysis.

To detect the main tick-borne pathogens, a high-throughput microfluidic real-time PCR was performed on a BioMark™ real-time PCR system (Standard BioTools, USA), using the 48.48 Dynamic Array™ (Standard BioTools, USA). This chip includes two sets of 48 inlets, allowing loading of (up to) 48 samples and 48 PCR assays mixtures. All 2304 sample-assay combinations are obtained and tested simultaneously by the automated microfluidic system, requiring only few total microlitres for each sample and each assay (see Michelet et al., 2014 for details). Amplifications were performed as in Michelet et al. (2014). Briefly, the TaqMan Gene expression master mix (Applied Biosystems, USA) and TaqMan probes labelled with 6-carboxy-fluorescein (FAM) and with black hole quencher (BHQ1) were used following the manufacturer's instructions. The thermal cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. On each 48.48 Dynamic Array™ chip, a negative control (water) was added, as well as an internal "inhibition control", represented by DNA of *Escherichia coli*, to be tested by specific primers and probe. All primers and probes used are listed in (Supplementary Table 1).

2.3. Validation of the results by PCR and DNA sequencing

Following Michelet et al. (2014), in order to evaluate and possibly refine the results obtained with the BioMark™ system, conventional PCRs were performed on a subset of representative positive samples, in order to validate the results obtained with the BioMark™ system. This representative subset included at least 20 % of all positives to each pathogen. Different primers were used for each microorganism

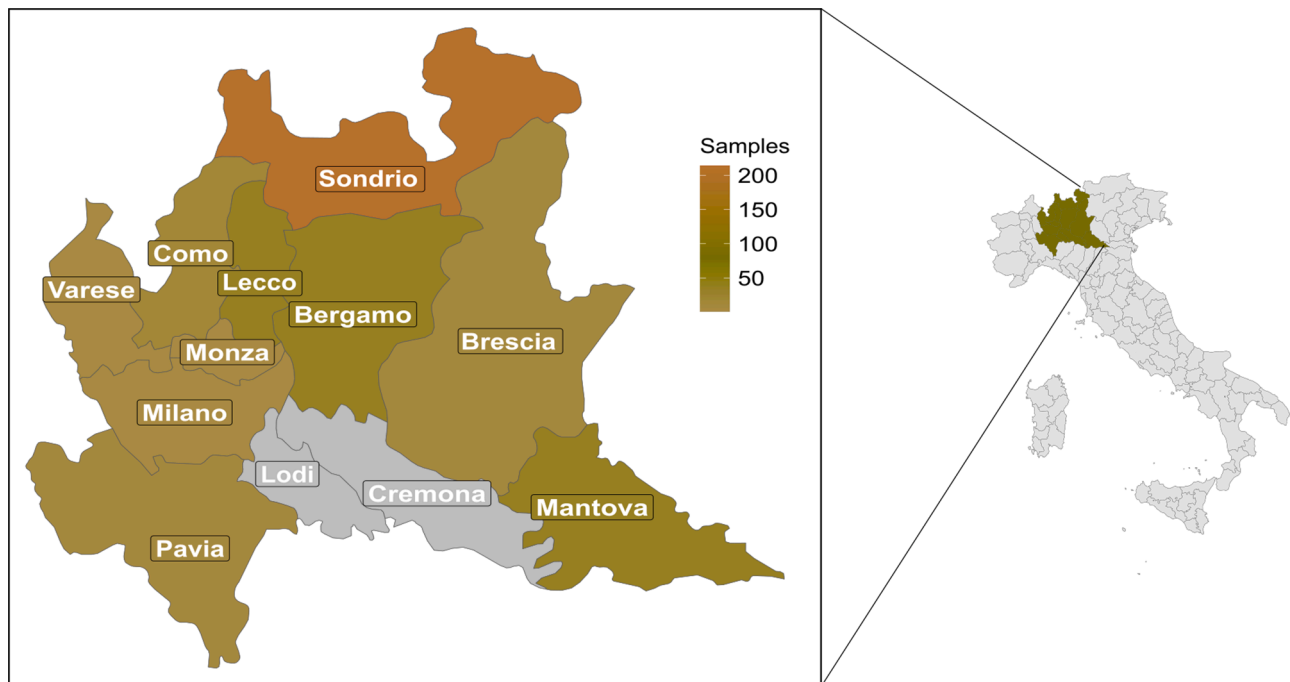


Fig. 1. Map of the Lombardy region (northern Italy) subdivided into provinces. For each province, the total tick individuals retrieved are shown as heatmap. No sample was obtained from the provinces of Lodi and Cremona.

compared to those in the microfluidic real-time PCR, following published thermal protocols (Supplementary Table 2). The amplicons were bidirectionally sequenced by Eurofins (Germany), and then sequencing results were inspected and processed using the BioEdit software (Ibis Biosciences, Carlsbad). The results were compared with the NCBI nucleotide database using BLASTN. In parallel, 31 samples that were positive to the genus-specific microfluidic PCR assay for *Rickettsia*, but negative to all species-specific assays, were tested by the conventional PCR as described above, in order to be characterised at the species level.

2.4. Statistical analysis

The prevalence of the most common pathogens detected (*A. phagocytophilum*, *B. burgdorferi sensu lato* and *Rickettsia* spp.) across different hosts (humans, ungulates, and Carnivora, excluding the 50 ticks with unknown hosts) and developmental stages (females and nymphs) was assessed using the Chi-square test or the Fisher's exact test, when appropriate. Co-infections between pairs of the same three most common pathogens were tested in the same way. Differences were

Table 1

Number and percentages (in brackets) of ticks tested positive to different tick-borne pathogens, divided by vertebrate host and tick stage (*F*=females; *N*=nymphs). Assays representing a further characterization of wider assays are shown not in bold (e.g., the assays for the five species representing the *B. burgdorferi sensu lato* complex).

Pathogens / Tick stage	Humans (<i>n</i> = 170)			Ungulates (<i>n</i> = 113)			Carnivores (<i>n</i> = 17)	Unknown (<i>n</i> = 50)		
	F (%)	N (%)	TOT (%)	F (%)	N (%)	TOT (%)		F (%)	N (%)	TOT (%)
<i>B. burgdorferi sensu lato</i>	5/34 (15)	8/136 (5.9)	13/170 (7.6)	0	0	0	0	3/25 (12)	1/25 (4)	4/50 (8)
<i>B. burgdorferi sensu stricto</i>	1/34 (3)	0	1/170 (0.6)	0	0	0	0	0	0	0
<i>B. garinii</i>	2/34 (6)	2/136 (1.5)	4/170 (2.4)	0	0	0	0	1/25 (4)	0	1/50 (2)
<i>B. afzelii</i>	1/34 (3)	5/136 (3.7)	6/170 (3.5)	0	0	0	0	2/25 (8)	1/25 (4)	3/50 (6)
<i>B. valaisiana</i>	0	1/136 (0.7)	1/170 (0.6)	0	0	0	0	0	0	0
<i>B. lusitanae</i>	1/34 (3)	0	1/170 (0.6)	0	0	0	0	0	0	0
<i>B. miyamotoi</i>	1/34 (3)	0	1/170 (0.6)	2/96 (2)	0	2/113 (1.8)	0	0	1/25 (4)	1/50 (2)
<i>A. phagocytophilum</i>	1/34 (3)	6/136 (4.4)	7/170 (4.1)	82/96 (85)	5/17 (29)	87/113 (77)	0	8/25 (32)	0	8/50 (16)
<i>N. mikurensis</i>	1/34 (3)	3/136 (2.2)	4/170 (2.4)	1/96 (1)	1/17 (6)	2/113 (1.8)	0	0	0	0
<i>Rickettsia</i> spp.	4/34 (12)	3/136 (2.2)	7/170 (4.1)	15/96 (16)	0	15/113 (13.3)	2/17 (12)	7/25 (28)	14/25 (56)	21/50 (42)
<i>R. helvetica</i>	2/34 (6)	1/136 (0.7)	3/170 (1.8)	2/96 (2)	0	2/113 (1.8)	0	2/25 (8)	7/25 (28)	9/50 (18)
<i>R. monacensis</i>	2/34 (6)	2/136 (1.5)	4/170 (2.4)	13/96 (14)	0	13/113 (11.5)	2/17 (12)	5/25 (20)	7/25 (28)	12/50 (24)
<i>B. venatorum</i> EU1	1/34 (3)	0	1/170 (0.6)	0	0	0	0	0	0	0
<i>H. martis</i>	0	0	0	0	0	0	5/17 (29)	0	0	0

considered statistically significant at p -value < 0.05. All tests were performed using R.

3. Results

We performed a study on a set of 350 *I. ricinus* specimens collected from humans and dead wild animals in the Lombardy region (Italy), analysing the number of individuals carrying tick-borne pathogens. The results for humans, ungulates, carnivores, and unknown hosts are presented below (Tables 1 and 2; for details see Supplementary Table 3), followed by statistical comparisons.

3.1. Ticks collected from humans

A total of 170 *I. ricinus* (34 females and 136 nymphs) removed from humans were examined. Among these, 26 carried at least one pathogen group (15 %) (Tables 1 and 2). The most common pathogen group was *B. burgdorferi* sensu lato complex (specifically *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto, *Borrelia lusitaniae*, and *Borrelia valaisiana*), being detected in 5/34 in females (15 %) and in 8/136 nymphs (5.9 %). A relevant proportion of ticks hosted *Rickettsia* spp. (*R. monacensis* and *R. helvetica*), namely 4/34 females (3 %) and 3/136 nymphs (3/136). We also detected *A. phagocytophilum* in females (1/34; 3 %) and nymphs (6/136; 4.4 %), as well as *N. mikurensis* in 1/34 females (3 %) and 3/136 nymphs (2.2 %). One female carried *B. miyamotoi* (0.6 %) and another female specimen harboured *Babesia venatorum* (0.6 %). No other pathogen, such as *Coxiella burnetii*, *Bartonella* spp., *Francisella tularensis*, *Ehrlichia* spp., *Anaplasma* other than *A. phagocytophilum*, or *Theileria* was detected.

3.2. Ticks collected from ungulates

A total of 113 feeding ticks, 96 females and 17 nymphs, were sampled from ungulates, i.e. red deer, roe deer, chamois, and mouflon. The vast majority of females carried *A. phagocytophilum*, (82/96; 85 %), while this pathogen was detected only in relatively fewer nymphs (5/17; 29 %). *Rickettsia* spp. were found only in females (15/96; 16 %), specifically *R. monacensis* (13/96; 14 %) and *R. helvetica* (2/96; 2 %). In contrast with human-associated ticks, *B. burgdorferi* sensu lato was absent, however, two females carried *B. miyamotoi* (2 %). One female and one nymph harboured *N. mikurensis* (1 % and 6 %, respectively). No other tested pathogen was detected (Tables 1 and 2).

3.3. Ticks collected from Carnivora

We also analysed 17 adult females of *I. ricinus* sampled from a few specimens of Carnivora, namely wolf, badger, and stone marten. Some wolf-associated ticks carried *R. monacensis* (2/17 of all ticks from carnivores; 12 %), while *Hepatozoon martis* 5/17 was found in all stone marten-associated ticks (5/17 of all ticks from carnivores; 29 %; Supplementary Table 3). No other pathogens were detected, in particular no

Table 2

Overall detected number and percentage (in brackets) of *I. ricinus* ticks positive to each pathogen group, considering females and nymphs collected from all host species.

Pathogen	Overall positive females (%)	Overall positive nymph (%)	Overall total positives (%)
<i>B. burgdorferi</i> sensu lato	8/172 (4.7)	9/178 (5.1)	17/350 (4.8)
<i>B. miyamotoi</i>	3/172 (1.7)	1/178 (0.6)	4/350 (1.1)
<i>A. phagocytophilum</i>	91/172 (53.0)	11/178 (6.2)	102/350 (29.1)
<i>N. mikurensis</i>	2/172 (1.2)	4/178 (2.2)	6/350 (1.7)
<i>Rickettsia</i> spp.	28/172 (16.3)	17/178 (9.5)	45/350 (12.8)
<i>B. venatorum</i> EU1	1/172 (0.6)	0	1/350 (0.3)
<i>H. martis</i>	5/172 (2.9)	0	5/350 (1.4)

Anaplasma spp. or *Borrelia* spp (Table 1).

3.4. Ticks collected from unknown hosts

As regards the other samples, 50 ticks (25 females and 25 nymphs) were collected from unknown hosts. In those ticks, we detected several of the same pathogens which we frequently found in ticks collected from humans, ungulates and Carnivora. *Rickettsia* spp. were overall very common, in particular *R. monacensis* and *R. helvetica*. Interestingly, they were more frequently found in nymphs (14/25; 56 %) than in females (7/25; 28 %), in contrast with what we observed in ticks sampled from humans and ungulates. *Anaplasma phagocytophilum* was also frequently identified in females (5/25; 32 %), but never in nymphs (0/25). Ticks also harboured *B. burgdorferi* sensu lato, specifically *B. afzelii* and *B. garinii*, with higher occurrence in females (3/25; 12 %) than in nymphs (1/25; 4 %). A single nymph carried *B. miyamotoi* (4 %) (Table 1).

3.5. Co-infections

Overall, 100/172 females (58.1 %) and 29/178 nymphs (16.3 %) tested positive for a single pathogenic microorganism. Co-infections of two pathogens were detected in 21 specimens (15 females: 8.7 %, and 6 nymphs: 3.3 %), and only two females (1.2 %) and one nymph (0.6 %) carried three agents (Table 3). Almost all the pathogens detected were found at least once as co-infections, with the exceptions being *H. martis* and the three least represented species of the *B. burgdorferi* sensu lato complex (i.e., *B. burgdorferi* sensu stricto, *B. valaisiana* and *B. lusitaniae*). The most frequent co-occurrence was between *A. phagocytophilum* and *R. monacensis* (in total 13 ticks, 11 as dual infections, two triple infections, with *B. garinii* and *N. mikurensis*, respectively).

3.6. Statistical comparisons of pathogen occurrence

For the statistical analyses, we focused only on the most frequently detected pathogens, *A. phagocytophilum*, *B. burgdorferi* sensu lato, and *Rickettsia* spp. Thus, we investigated the differences in the proportion of positive ticks to each pathogen depending on tick life stage and tick host. For *A. phagocytophilum*, we observed a statistically significant difference in the proportion of positive ticks between the tick life stages (females or nymphs; $p < 0.01$, Chi-square test) and between different tick hosts (humans, ungulates, and Carnivora; $p < 0.01$, Chi-square test). Accordingly, *A. phagocytophilum* appears to be more significantly associated with wild ungulates and with adult females of *I. ricinus*. For *B. burgdorferi*

Table 3

Tick-borne pathogen combinations found in *I. ricinus* ticks.

TBP combination	Stage	Hosts	Tot
<i>A. phagocytophilum</i> + <i>R. helvetica</i>	2 Females	Roe deer (2)	2
<i>A. phagocytophilum</i> + <i>R. monacensis</i>	11 Females	Roe deer (2), Red deer (3), Chamois (1) and unknown (2)	11
<i>A. phagocytophilum</i> + <i>B. miyamotoi</i>	1 Female	Roe deer (1)	1
<i>A. phagocytophilum</i> + <i>N. mikurensis</i>	1 Nymph	Roe deer (1)	1
<i>B. afzelii</i> + <i>R. helvetica</i>	2 Nymphs, 1 Female	Human (1) and unknown (2)	3
<i>B. afzelii</i> + <i>N. mikurensis</i>	2 Nymphs	Human (2)	2
<i>B. garinii</i> + <i>R. monacensis</i>	1 Female	Unknown (1)	1
<i>B. miyamotoi</i> + <i>R. monacensis</i> + <i>B. venatorum</i>	1 Female	Human (1)	1
<i>B. garinii</i> + <i>A. phagocytophilum</i> + <i>R. monacensis</i>	1 Nymph	Human (1)	1
<i>A. phagocytophilum</i> + <i>N. mikurensis</i> + <i>R. monacensis</i>	1 Female	Roe deer (1)	1

sensu lato, we observed a significant difference in the number of positive ticks depending on tick hosts ($p < 0.01$; Fisher test), while no statistically significant difference was found between nymphs and adults ($p > 0.05$; Chi-square test). As regards the genus *Rickettsia*, we also noted a difference in number of positive ticks to these pathogens between the different tick hosts ($p = 0.012$; Fisher test), while no significant difference between tick life stages was observed ($p > 0.05$; Chi-square test). Concerning co-infections, no significant differences were observed for the *Rickettsia* spp. and *B. burgdorferi* sensu lato or *Rickettsia* spp. and *A. phagocytophilum* pairs as compared with co-occurrence only determined by chance ($p > 0.05$; Chi-square tests), while a significant difference was found for the *B. burgdorferi* sensu lato and *A. phagocytophilum* ($p = 0.029$; Chi-square tests).

4. Discussion

Ixodes ricinus is currently expanding its geographical range across Europe (Medlock et al., 2013), posing an increased threat of tick bites for people and domestic animals (Medlock et al., 2013). Moreover, in northern Italy the roe deer population has been on the rise in recent years, providing favourable conditions for the expansion of the *I. ricinus* populations, since this tick species thrives on roe deer (Pasquale et al., 2019; Pfäffle et al., 2013). Thus, constantly updated surveillance programs are fundamental to have a broader awareness of TBPs circulation in order to adopt proper prevention and control strategies.

In this study, we performed a high-throughput screening of pathogens in 350 ticks collected from humans, ungulates (including roe deer), and other wild animals in the Lombardy region (northern Italy). Ticks were morphologically identified as *I. ricinus*. Although not ruled out, misidentification of individuals belonging to related species such as *I. inopinatus* seems unlikely. To our knowledge, *I. inopinatus* was never reported in the study area, and its presence is questionable also in other European countries (Rollins et al., 2023). Subsequently, we performed a high-throughput screening of pathogens, employing a previously established and validated protocol (Michelet et al., 2014). Accordingly, we detected representatives of four genera of pathogenic bacteria (*Borrelia*, *Anaplasma*, *Rickettsia*, and *Neoehrlichia*) and two of pathogenic protozoa (*Babesia* and *Hepatozoon*).

The occurrence of *B. burgdorferi* sensu lato complex was approximately 5 % in females and nymphs. This is lower than previous studies on questing ticks collected in the same area (ranging between 10 and 18 %) (Cafisoet al., 2021; Pistone et al., 2010; Zanzani et al., 2019) but in line with studies on ticks collected from humans (Beltrame et al., 2018; Garcia-Vozmediano et al., 2021; Otranto et al., 2014). Our results confirm previous reports on *I. ricinus* infected with *B. garinii* and *B. afzelii*, indicating that these are the most common genospecies in Italy (Strnad et al., 2017). In our dataset, *B. burgdorferi* sensu lato-positive ticks were collected from humans and unknown wild animals. In contrast, we did not detect any LB spirochetes in ticks removed from ungulates ($n = 113$). This statistically significant difference is consistent with previous studies (Díaz-Cao et al., 2022; Kazimírová et al., 2018; Michalski et al., 2021) that demonstrated the borreliacidal effect of serum from wild ruminants and thus resulting in the loss of *B. burgdorferi* sensu lato in ticks during feeding (Pacilly et al., 2014; Pearson et al., 2023). On the other hand, we found *B. miyamotoi* both in females taken from ungulates (2 %) and from humans (3 %). This pathogen has rarely been detected in northern Italy, with a prevalence of 0.6 % and 0.9 % in Piedmont and Lombardy regions, respectively (Garcia-Vozmediano et al., 2020; Ravagnan et al., 2018; Rigamonti et al., 2021). All the findings above indicate that this pathogen is circulating at a local level, which seems to require further attention. Differently from *Borrelia* causing LB, the *B. miyamotoi* genospecies is not eliminated by ungulates during tick feeding, likely due to different host-pathogen interaction mechanisms (Richter and Matuschka, 2010).

With regard to *A. phagocytophilum*, our screening showed a statistically higher occurrence in ticks taken from ungulates. This confirms

previous studies from Europe on both feeding ticks and ungulate sera, and is in accordance with the fact that wild cervids are the primary and most significant reservoirs (Fabri et al., 2021; Kazimírová et al., 2018; Stuen et al., 2013). We also observed that infection rates in adult females was significantly higher compared to those of nymphs. This could be attributed to the pathogen's preferential transstadial maintenance rather than transovarial transmission, and the increased likelihood for females, with their additional blood meal, to acquire the infection from the vertebrate hosts (Stuen et al., 2013). In ticks collected from humans, *A. phagocytophilum* occurrence was lower than those collected from ungulates (~4 %), but higher with respect to other reports in Italy, that registered percentages with a prevalence ranging from 0 to 2 % (Baráková et al., 2018; Battisti et al., 2019; Capelli et al., 2012; Corrain et al., 2012; Otranto et al., 2014).

In our study, the overall proportion of ticks positive to *Rickettsia* spp. (~13 %) was in line with recent studies in Italy, where prevalence varied between 1 and 23 % (Beltrame et al., 2018; Bertola et al., 2021; Cafisoet al., 2021; Capelli et al., 2012; Corrain et al., 2012; Garcia-Vozmediano et al., 2021; Otranto et al., 2014; Pistone et al., 2017). The two detected species, *R. helvetica* and *R. monacensis*, are the most common circulating *Rickettsia* species in *I. ricinus* populations in northern Italy (Guccione et al., 2021), both causing a Mediterranean spotted fever-like illness (Ciceroni et al., 2006; Madeddu et al., 2012). Many *Rickettsia* spp. are vertically transmitted in ticks, which thus can play a major role both as vectors and as reservoirs (Parola et al., 2013). While it has been suggested that lizards can act as a reservoir for *R. monacensis*, the role of big mammals in the eco-epidemiology of *Rickettsia* spp., like ungulates investigated herein, is still to be defined (Remesar et al., 2023).

Interestingly, we detected a tick-borne apicomplexan parasite, *H. martis*, in every female collected from the stone marten. Indeed, *H. martis* is predominantly present in mustelids all over Europe with very high infection rate (Hodžić et al., 2018). *Hepatozoon* spp. can be acquired by ticks during their blood meal, but transmission back to the vertebrate host only occurs when they ingest the tick containing parasite oocysts (Uiterwijk et al., 2023).

Regarding other pathogenic agents investigated, the overall infection rates of *N. mikurensis* and *B. venatorum* are consistent with previous reports, both in Italy and Europe (Cafisoet al., 2021; Castro et al., 2015; Lejal et al., 2019; Otranto et al., 2014; Wennerås, 2015). It is also worth noting that no pathogens of the genera *Coxiella*, *Francisella* and *Bartonella* were detected in this study. The two pathogenic bacteria *C. burnetii* and *F. tularensis* were quite rarely reported in the same area (Mancini et al., 2014; Pajoro et al., 2018; Pistone et al., 2017), while *Bartonella* has been detected in many reports in Italy (Corrain et al., 2012; Ebani et al., 2015; Sanogo et al., 2003).

We detected a small number of multiple infections in the same tick individual ($n = 24$), with the most frequent dual infection observed being *A. phagocytophilum* in combination with *Rickettsia* spp. ($n = 15$), consistently with other studies on engorged *I. ricinus* collected from wild animals (Michalski et al., 2021; Rocha et al., 2022). A complex and still not well understood interplay between various factors, related to ticks and reservoir hosts, determines the occurrence and prevalence of co-infections. In our dataset, we did not observe statistically significant differences in frequency of co-infections among the main pathogens investigated. The only significant exception pertains to the pairing of *B. burgdorferi* sensu lato and *A. phagocytophilum*, which is significantly less common than expected. However, this seems likely linked to their different vertebrate host tropism, in particular clearance of *Borrelia* by ungulates, rather than to direct interactions between these bacteria. In any case, we underline that co-infections are relevant for their potential consequences on public health, e.g. for enhancing the disease severity, and should be monitored accordingly (Diuk-Wasser et al., 2016).

Since the incidence of TBDS in humans is underestimated, the goal of this study was to increase the knowledge of TBPs circulating, adopting a time-saving and cost-effective approach. Our work was performed using

a high-throughput real-time PCR system, which allowed us to easily and rapidly detect multiple pathogens in ticks from the study area. Our results were mainly consistent with previous studies and moreover confirm the presence of the pathogenic agent *B. miyamotoi* in this area. Future studies should expand to include questing ticks and to investigate the presence of TBE virus, taking advantage of this high-throughput approach in order to continuously monitor the presence of pathogenic microorganisms in very populated and urbanised areas, such as the Lombardy region in northern Italy.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ttbdis.2023.102285](https://doi.org/10.1016/j.ttbdis.2023.102285).

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