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Molecular detection of *Rickettsia*, *Borrelia*, and *Babesia* species in *Ixodes ricinus* sampled in northeastern, central, and insular areas of Italy

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Abstract The aim of the present study was to provide insight into the diversity of tick-borne pathogens circulating in Italy, carried/transmitted by *Ixodes ricinus*, one of the most abundant tick species in the country. A total of 447 specimens sampled in five areas of northeastern, central and insular Italy were analysed by polymerase chain reaction and sequencing for the presence of rickettsiae, borreliae and babesiae. Several rickettsial species of the spotted fever group of zoonotic concern and other zoonotic pathogens were found, such as *Borrelia burgdorferi* s.s., *Borrelia afzelii*, *Borrelia garinii*, and *Babesia venatorum*. These findings confirm a wide distribution of tick-borne bacterial and protozoan species in Italy, and highlight the sanitary importance of *I. ricinus*, often recorded as feeding on humans.

Keywords *Rickettsia* spp. · *Borrelia* spp. · *Babesia venatorum* · Italy · PCR

Introduction

Ticks (Acari: Ixodidae) are among the most important vectors of diseases in temperate climates (Heyman et al. 2010). In Europe, a wide range of tick-borne pathogens, including viruses, bacteria, and protozoa can cause diseases in both animals and humans. Moreover, some tick-borne pathogens are recognized as important zoonotic pathogens (Sparagano et al. 1999).

Rickettsiae, for example, are intracellular bacteria maintained in nature by Ixodid ticks through trans-stadial and trans-ovarial transmission; therefore their geographical distribution coincides to the distribution of the carrier ticks (Parola and Raoult 2001). Several

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species, like *Rickettsia conorii* in Europe, are well-known causative agents of diseases. However, molecular approaches had enabled recent identification of other rickettsiae of the spotted fever group of interest to animal and human health (Oteo and Portillo 2012). Of these, *Rickettsia helvetica*, *Rickettsia monacensis*, *Rickettsia raoultii*, *Rickettsia aeschlimannii* and *Rickettsia massiliae* have been reported in *Ixodes ricinus* (Parola et al. 2013).

Also, spirochetes of the complex *Borrelia burgdorferi* s.l., etiological agents of Lyme borreliosis (LB), are transmitted mainly by *I. ricinus* (Hengge et al. 2003), and are reported as endemic in Italy (Lindgren and Jaenson 2006). Several studies, especially in northern regions, have confirmed the presence of the disease (Cinco et al. 2004; Pajoro et al. 2010; Pistone et al. 2010; Ravagnan et al. 2010).

Concerning protozoa, many species of the genus *Babesia*, which can cause malaria-like syndrome in animals and humans, are tick-transmitted and, in Europe, most of them have *I. ricinus* as the main vector (Duh et al. 2001; Hilpertshauser et al. 2006; Bonnet et al. 2007). In Italy, babesioses affects from 2.5 to 30.4 % of livestock, pets and wild animals (Cringoli et al. 2002; Cancrini et al. 2007; Pietrobelli et al. 2007; Torina and Caracappa 2007; Cassini et al. 2009; Moretti et al. 2009), and recent studies revealed evidence of these protozoa in about 2.5 % of ticks sampled from central and northern Italy (Iori et al. 2010). As a confirmation of their zoonotic importance, about 19 % of people living in the same areas showed reactivity to different babesial antigens (Gabrielli et al. 2014).

The aim of this study was to add new knowledge to the complex puzzle of pathogens transmitted by *I. ricinus* through the analysis of specimens collected in areas of Italy that had been scarcely or not at all studied previously.

Materials and methods

Samples

Host-seeking ticks were collected during 2007–2010 from the environment in selected sites of northeastern (Belluno), central (Perugia, Rieti) and insular (Pianosa and Capri) areas of Italy (Fig. 1). Sampling was carried out using the dragged woollen blanket technique (Mannelli et al. 1999), in a restricted area of 1 ha, over six sessions. Sites were chosen on the basis of suitable biotope structures and on the previous descriptions of tick occurrence, with the exception of Capri for which no previous data were available. Morphological identification was performed by microscopy, recording the developing stage and using morphological keys (Manilla 1998; Iori et al. 2005).

DNA extraction

Genomic DNA was extracted from single adults, single nymphs, and pools of larvae ($n = 5$) by the phenol–chloroform method (Favia et al. 2001). Briefly, DNA was extracted by grinding the ticks in TES buffer/Tris–HCl 50 mM, EDTA 1 mM, sucrose 15 %, pH 8, after abdomen incision. Proteinase K was added at a final concentration of 1 mg/mL, and samples were incubated overnight at 42 °C. Phenol–chloroform was used for further DNA isolation and DNA was ethanol-precipitated. The pellet was resuspended in 50 µL of double-distilled water. Finally, DNA quality and concentration was verified by electrophoresis using 1 % agarose gels.

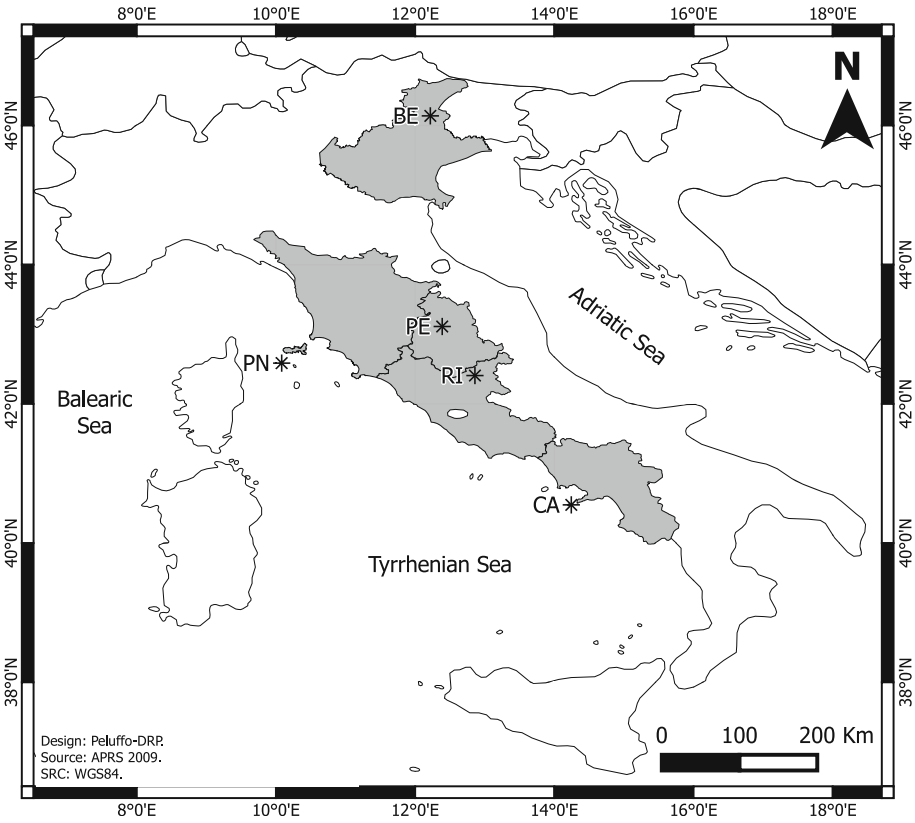

Fig. 1 Map of Italy showing the regions and areas where ticks were collected: BE (Belluno), PE (Perugia), RI (Rieti), PI (Pianosa), CA (Capri)

PCR, and DNA sequence analysis

DNA samples were screened for the *gltA* gene of *Rickettsia* spp., the *18S* rRNA gene of *Babesia* spp. and the flagellin gene (*Fla*) of *Borrelia* spp. using primers and conditions previously described (Table 1). *Borrelia* sp. positive samples were further amplified with species-specific *16S* identification primers (Marconi and Garon 1992). Amplifications were performed in a volume of 25 μL containing: 0.2 μL BIO-X-ACT Short DNA Polymerase (Bioline, Italy), 0.5 μL of each primer (50 mM), 3 μL of template (25 ng/μL), 2.5 μL of dNTP (10 mM), 2 μL of $MgCl_2$ (50 mM), 2.5 μL PCR Buffer (10×) and 13.8 μL of ddH_2O. All the PCR reactions included positive controls that had been identified by our group in previous works (Cassini et al. 2009; Iori et al. 2002; Veronesi et al. 2006), and negative (sterile deionized water) controls.

Amplifications were followed by 2 % agarose-gel electrophoresis and were visualized with GelRed® (Biotium, Hayward, USA) under UV-light. PCR products were purified with SureClean purification Kit (Bioline, Italy) and sequenced by Eurofins MWG Operon (Ebersberg, Germany). Sequence comparisons were performed with the BLAST tool provided by the National Center for Biotechnology (http://blast.ncbi.nlm.nih.gov/).

Table 1 Primers used in the performed molecular studies

Detected pathogen	Amplified gene	Amplicon size (bp)	Primer name	Primer sequence (5′–3′)	Annealing temp. (°C)	Reference
Rickettsia spp.	*gltA*	300–380	RpCS.877p RpCS.1258n	GGGGGCCTGCTCACGGCGG ATTGCAAAAAGTACAGTGAACA	52	Regnery et al. (1991)
Borrelia spp.	*Fla*	482	FLA1	AGAGCAACTTACAGACGAAATTAAT CAAGTCTATTTTGGAAAGCACCTAA	50	Skotarczak et al. (2002)
Borrelia valaisiana	*16S*	591	FLA2	GCATGCAAGTCAAACGGA ATATAGTTTCCAACATAGT	50	Marconi and Garon (1992)
Borrelia burgdorferi s.s.	*16S*	574	V5′	GGGATGTAGCAATACATTC ATATAGTTTCCAACATAGG	46	Marconi and Garon (1992)
Borrelia garinii	*16S*	574	V3′	GGGATGTGTAGCAATACATCT ATATAGTTTCCAACATAGT	50	Marconi and Garon (1992)
Borrelia afzelii	*16S*	600	BUR5′	GCATGCAAGTCAAACGGA ATATGTTTCCAACATAGC	47	Marconi and Garon (1992)
Babesia spp.	*18S*	411–452	BUR3′	GTCTTGTAATTGGAATGATGG TAGTTTATGGTTAGGACTACG	54	Casati et al. (2006)

Phylogenetic analysis

In order to support BLAST amplifications phylogenetic trees were constructed. Nucleotide alignments were performed using MUSCLE (Edgar 2004) as implemented in MEGA V.5 (Tamura et al. 2011). The model of molecular evolution was calculated using jModelTest 2 (Darriba et al. 2012), and Bayesian analyses were performed using MrBayes (Ronquist and Huelsenbeck 2003). Two independent runs of 2,000,000 generations were performed, with trees sampled every 100 generations, 25 % of the trees were discarded as burnin. Convergence was validated by the standard deviation of split frequencies (<0.01), by plotting the likelihood values over time, and by using the sump command in MrBayes. The percentage of trees recovering a particular clade was used as a measure of that clade's posterior probability (Huelsenbeck and Ronquist 2001).

Results

Among the ticks collected a total of 447 (341 adults, 77 nymphs and 29 larvae) were morphologically identified as *I. ricinus.* Altogether 86 ticks (82 adults, 4 nymphs) (19.23 %) showed products of the expected length in the PCR for *Rickettsia* spp. (Table 2). Sequencing of rickettsial *gltA* products and BLAST comparisons with the GenBank database revealed 99–100 % identity with the following rickettsial species: *R. monacensis* in 5 isolates from Rieti (e.g. KC996728.1, JX04639.1, KJ663735.1), *R. helvetica* in 2 isolates from Rieti and 1 from Belluno (KF447530.1, JX040636.1), *R. massiliae* in 2 isolates from Perugia (KC428030.1). Samples from Pianosa (6 isolates) matched to both *R. raoultii* (e.g. KF003009.1, KC428020.1), and *R. aeschlimanni* (e.g. AY259084.1, HQ335153.1). There were no positive samples in Capri Island. The phylogenetic tree constructed with the *Rickettsia* sequences confirmed the BLAST results, and the different sequences amplified from *I. ricinus* in this study clustered with expected species of the spotted fever group of the genus *Rickettsia* (Fig. 2).

Concerning *Borrelia,* 17/447 specimens (3.8 %), all adults, were infected with *B. burgdorferi* DNA (Table 2). The sequences obtained showed a nucleotide identity of 99 % to members of the *B. burgdorferi* s.l. Unfortunately, the sequenced *Fla* region did not allow a more detailed identification, because, for the same product, we obtained 99 % identity with more than one species of the complex. Figure 3a shows the phylogenetic tree

Table 2 Prevalence of investigated microorganisms in *I. ricinus* ticks collected from different regions of Italy

Sampling area	*Ixodes ricinus*			
	Examined no.	Infected by		
		Rickettsia no. (%)	*Borrelia* no. (%)	*Babesia* no. (%)
Belluno	148	6 (4)	2 (1.35)	1 (0.68)
Perugia	76	25 (32.89)	15 (19.73)	0 (0)
Rieti	98	22 (22.44)	0 (0)	0 (0)
Pianosa	35	28 (80)	0 (0)	0 (0)
Capri	90	0 (0)	0 (0)	0 (0)
Total	447	86 (19.23)	17 (3.8)	1 (0.22)

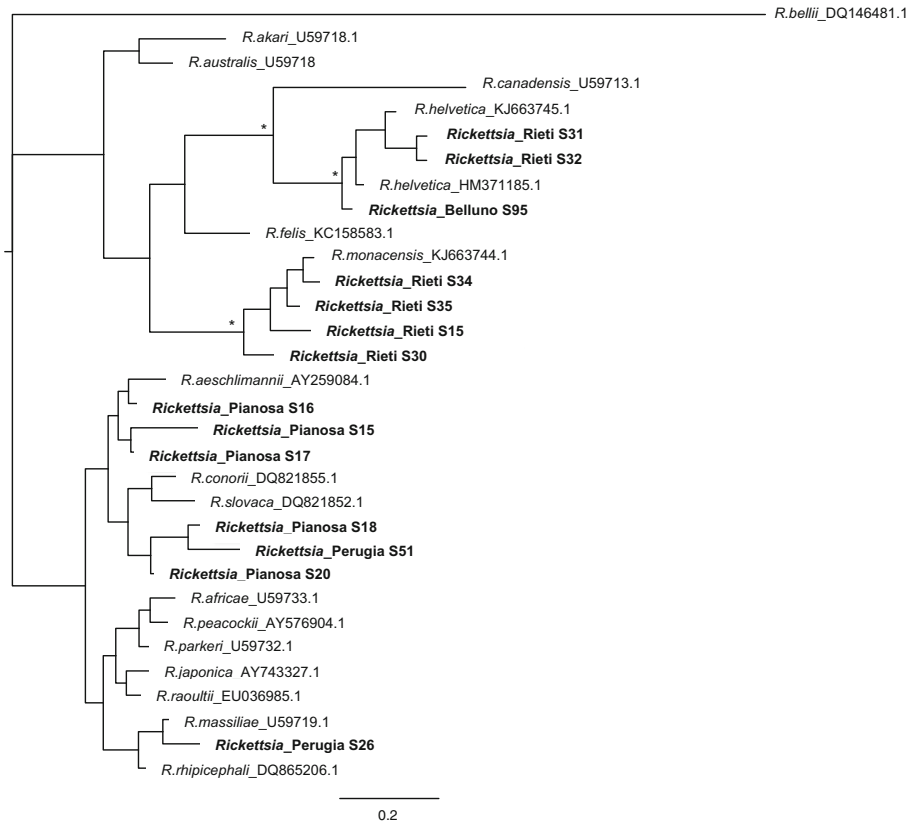

Fig. 2 Topology of the Bayesian phylogenetic analysis including *Rickettsia* spp. sequences from this study (*highlighted*), and others downloaded from GenBank (coded with each accession number). *Asterisks* represent posterior probabilities that were >0.95

reconstructed including *Borrelia Fla* sequences. Some of our sequences group with strong posterior probability with *Borrelia afzelii*, however, most of the groupings are not well supported. Instead, further PCR amplifications with *16S* species-specific primers allowed us to identify, in the two positive ticks from Belluno, *B. afzelii* and *B. burgdorferi* s.s., and, in the 15 specimens from Perugia, *B. burgdorferi* s.s. (n = 1), *B. afzelii* (n = 6), *Borrelia valaisiana* (n = 3), and *Borrelia garinii* (n = 5).

Babesial DNA was detected only in 1 adult specimen (0.22 %) (Table 2). Unidirectional sequencing results of the *18S* rRNA gene product revealed 100 % identity with *Babesia venatorum* (formerly *Babesia* sp. EU1) (accession no. KJ663730.1). The phylogenetic analysis also groups the species from this study with *B. venatorum* with high percent posterior probability (Fig. 3b).

No specimens were found simultaneously infected by more than one pathogen. All the sequences were deposited in GenBank under accession numbers: KM198331–KM198346.

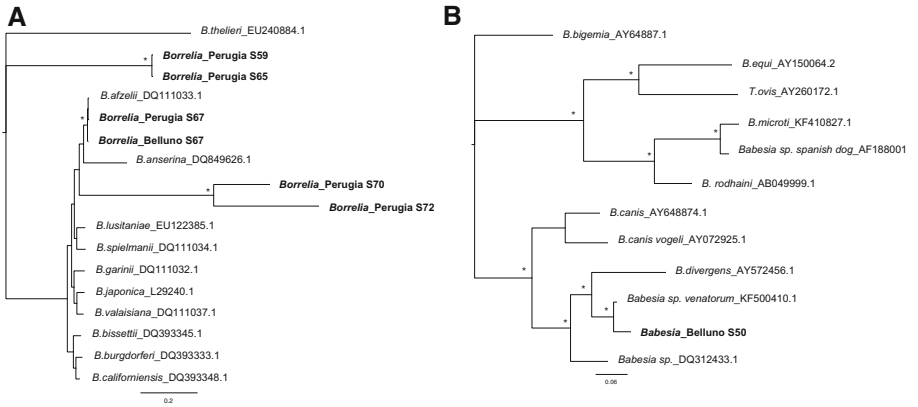

Fig. 3 Topology of the Bayesian phylogenetic analyses including *Borrelia* spp. (**a**) and *Babesia* spp. (**b**) sequences from this study (*highlighted*), and others downloaded from GenBank (coded with each accession number). *Asterisks* represent posterior probabilities that were >0.95

Discussion

Our results confirmed the presence of several *Rickettsia* species, and other zoonotic pathogens such as: *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, and *B. venatorum*, in *I. ricinus* sampled in different regions of Italy.

Rickettsiae were the most frequently found pathogenic agents, with six species found (*R. monacensis*, *R. helvetica*, *R. massiliae*, *R. raoultii* and *R. aeschlimanni*). It has been reported that clinical and epidemiological features vary depending on the rickettsial species involved in the disease (Raoult 2004; Parola et al. 2005). Thus, physicians should be aware of the different *Ricketsia* species that can be transmitted, and should be well trained on the clinical features of the corresponding infections in order to intervene, when needed, with the appropriate treatment. In Africa, for example, *R. conorii* and *Rickettsia africae* are prevalent, but clinical findings regarding patients infected with these agents are different. Patients infected with *R. africae* exhibit multiple eschars more commonly and exhibit a generalized rash less commonly than do patients infected by *R. conorii*. Moreover, vesicular eruption can be caused by *R. africae* but not by *R. conorii* (Raoult et al. 2001).

Concerning borreliae, the number of positive ticks was low (only 3.8 %), without positive samples from Rieti or the two islands. Our study confirms the presence of *B. burgdorferi* complex in *I. ricinus* from central and northeastern areas of Italy (Perugia and Belluno), with an infection rate of 20 % for the area of Perugia, which is similar to that reported in the Po River Valley (18 %) (Pistone et al. 2010), and in northern Italian provinces (17.6 %) (Ravagnan et al. 2010). In addition, the *16S* species-specific primers allowed us to identify, in the Perugia area, four genomic groups of the *B. burdorferi* s.l. complex: *B. valaisiana*, *B. garinii*, *B. afzelii* and *B. burgorferi* s.s., from which the last 3 species have been confirmed to be pathogenic to humans (Rauter and Hartung 2005), and are associated with different clinical symptoms of LB. Indeed, *B. afzelii* mainly match with skin manifestations and *B. burgdorferi* s.s with Lyme arthritis, whereas *B. garinii* is the only species correlated to neuroborreliosis (Balmelli and Piffaretti 1995; Van Dam et al. 1993). Our findings stress the poor benefits of the *Fla* gene to identify *Borrelia* species, and add new knowledge about the geographic distribution of the genospecies of the *B.*

burgdorferi complex that is not only of ecological and epidemiological interest, but also of clinical relevance.

Only one tick from Belluno was infected with *B. venatorum*, the species firstly identified in splenectomised patients living in Italy and in Austria (Herwaldt et al. 2003), and previously found in *I. ricinus* collected in northern-northeastern Italy with and infection rate of 0.85 %, similar to what we found (Cassini et al. 2010).

In conclusion, the present study evidenced a wide distribution of several bacterial and protozoan tick-borne pathogens. Therefore *I. ricinus*, one of the most abundant tick species in Italy, confirms to be a fearful possible source of infection.

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Conflict of interest The authors declare that they have no conflict of interest.

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