Borrelia burgdorferi sensu lato and spotted fever group rickettsiae in small rodents and attached ticks in the Northern Apennines, Italy

Full Title

Borrelia burgdorferi sensu lato and spotted fever group rickettsiae in small rodents and attached ticks in the Northern Apennines, Italy.

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Abstract.

Ticks and ear biopsies were collected from wild small rodents in 2011 and 2012 in the northern Apennines (Italy), up to 1650 m above sea level. *Apodemus* spp. (n=83) and *Myodes glareolus* (n=22) were infested by *Ixodes ricinus* (192 larvae and two nymphs), *Dermacentor marginatus* (179 larvae and 29 nymphs), and *Ixodes trianguliceps* (three larvae and two nymphs). We detected several *Borrelia burgdorferi* sensu lato (s.l.) genospecies (*B. afzelii*, *B. burgdorferi* sensu stricto, *B. garinii*, *B. lusitaniae*, *B. valaisiana*) in *I. ricinus* and skin biopsies. The most common genospecies found in *I. ricinus* was *B. valaisiana*, while it was *B. lusitaniae* in tissues. Spotted fever group (SFG) rickettsiae (*Rickettsia monacensis, R. slovaca* and *R. raoultii*) infected *I. ricinus, D. marginatus* and rodent tissues. *Rickettsia slovaca* was the *Rickettsia* species most frequently found in our samples. Coinfections by *B. burgdorferi* s.l. and SFG rickettsiae indicate an overlap of transmission cycles and potential risk for humans to be infected by multiple pathogens, resulting in more severe symptoms. The findings of *B. lusitaniae* and *R. slovaca* in bank voles, and of *B. valaisiana* in small rodents, open new questions about host-pathogen interactions. In addition, our results highlight the importance of small rodents as data sources for studying tick-borne pathogens.

Keywords.

Borrelia burgdorferi s.l., spotted fever group rickettsiae, rodents, altitude, coinfection, Italy.

1. Introduction.

Data collection from small rodents is a valid method for detecting presence and absence, as well as spatial and temporal trends of ticks and tick-borne pathogens (TBPs) at local scale (Kiffner et al., 2011; Mannelli et al., 2012; Paziewska et al., 2010; Silaghi et al., 2012). Moreover, these animals can be used in tick-borne zoonoses surveillance programs. For example, active data collection on wild small rodents has been used for the surveillance on tick-borne encephalitis virus (TBEV) in Germany: given the generally low prevalence of the agent in tick vectors, these animals were considered the most effective data source to detect the presence of the virus (Achazi et al., 2011).

In turn, associations between small rodents and ticks, and the role of rodents in the transmission cycles of related zoonoses, are very relevant with respect to the occurrence of multiple infections (Mihalca et al., 2012; Morand et al., 2006). This is a topic of great public health interest, because human patients coinfected by different TBPs may experience more severe and acute illness with atypical clinical symptoms (Swanson et al., 2006). So far, *Borrelia burgdorferi* sensu lato (s.l.) was found coexisting in patients with the agents of TBE (Cimperman et al., 2002) and anaplasmosis (Santos et al., 2006); moreover, patients affected by Lyme borreliosis were found co-infected by different *B. burgdorferi* s.l. genospecies (Da Franca, 2004). The evaluation of TBP co-occurrence is thus important for facilitating prognosis and drug treatment in humans (Andersson et al., 2013; Milhano et al., 2010).

Despite many years of research on *B. burgdorferi* s.l. genospecies specificity, the association among genospecies and rodent hosts is not clear yet and it may vary in different geographic areas (Mannelli et al., 2012). For example, in the case of *B. burgdorferi* sensu stricto (s.s.), spirochetes are considered less specialized compared to *B. afzelii* and they may thrive in both rodents and birds. With regards to spotted fever group (SFG) rickettsiae, the relative importance of rodents and other vertebrates in the transmission cycles is still under debate for several *Rickettsia* species (Tomassone et al., 2018).

During our investigations on tick geographical range expansion in the Northern Apennines, Italy, we have captured rodents to study the distribution of immature stages of hard ticks. Small rodents were found infested, up to the altitudinal limit of the tree vegetation (1650 m above sea level - asl), by

Ixodes ricinus and *Dermacentor marginatus* immatures (Martello et al., 2014). We identified *B. burgdorferi* s.l. in questing *I. ricinus* nymphs, collected in sites up to 1370 m asl (Ragagli et al., 2016), and SFG rickettsiae in *I. ricinus* nymphs collected from lizards up to 1450 m asl (Tomassone et al., 2017) and in *D. marginatus* collected from small rodents up to 1220 m asl (Martello et al., 2013). In this paper, we report on prevalence and characterization of *B. burgdorferi* s.l. and SFG rickettsiae in attached ixodid ticks and tissues from small rodents collected up to 1650 m asl in 2011-2012. These results complete the picture of our study area, integrating data on zoonotic agents from different sources (vectors and animals) (Martello et al., 2013; Ragagli et al., 2016; Tomassone et al., 2017), to provide better knowledge of the occurrence of a variety of TBPs of human relevance after the recent altitudinal range expansion of *I. ricinus* (Martello et al., 2014). Moreover, our findings contribute to the knowledge of associations between small rodents, ticks and different *B. burgdorferi* s.l. genospecies and SFG rickettsiae, including the occurrence of coinfections.

2. Materials and methods.

2.1. Study area and small rodent trapping

Data collection on small rodents was performed in the Tuscan side of the Tuscan-Emilian Apennine National Park, province of Lucca, Italy (44° 12’ N, 10° 22’ E). The study area is characterized by a favourable forested habitat for ticks and is inhabited by several sylvatic animal species (Mannelli et al., 1997; Ragagli et al., 2016).

Small mammal trapping locations were selected in order to collect data at different altitudinal levels, as described by Martello and colleagues (2014). In 2011, from June to August, traps were set at an altitude ranging from 1140 - 1200 m asl, in three 3 x 10 grids (30 traps, 10 m apart) within 2 km from the park premises, for a total of 540 trap nights. In 2012, from June to September, traps were set between 1200 m and 1650 m asl, 20 m apart, in two parallel lines in three transects, for a total of 764 trap nights.

Small rodents were trapped using Sherman live traps (230x80x90 mm, Sherman Live Traps Co., Tallahassee, FL, USA) and Ugglan live traps (240×60x90 mm, Grahnab, Hillerstorp, Sweden), baited with cereals and apples. Cotton was provided to protect against cold temperature during the night. Animals were anesthetized as described by Amore and colleagues (2007). A microchip (transponder AEG ID162 ISO, AEG, Ulm, Germany) was injected in the interscapular region of each individual for identification. After recording trapping site, species, gender, and age class (sub-adult, adult), rodents were screened for ticks on the entire body. Then, all ticks and an ear biopsy were stored in 70% ethanol. Animals were released at their capture site after reversing anaesthesia. Animal capture and sampling procedures were approved by the Bioethics Commission of the University of Turin.

2.2. Laboratory analyses

DNA was extracted from all small rodent ear biopsies regardless of the tick infestation status of the rodents. Skin biopsies are commonly tested for the detection of *B. burgdorferi* s.l. (Pospisilova et al., 2019; Cutler et al., 2017) and SFG rickettsiae in vertebrate hosts (Schex et al., 2011; Levin et al., 2016).

Ticks were identified to species using a taxonomic key (Manilla, 1998). From each infested rodent, a sample of *I. ricinus* and *D. marginatus* ticks was selected for DNA extraction. We measured the tick engorgement rate (TEI) of all fed ticks (index 2 of Yeh et al., 1995) and selected, from each rodent, the two most engorged larvae per each tick species and all attached nymphs, if present.

DNA of ticks and tissues was extracted using the DNeasy blood and tissue kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instruction. We used negative controls (distilled water) to verify possible cross-contaminations during this phase. Quality and quantity of extracted DNA were evaluated with a spectrophotometer (NanoDrop ND-1000, Peqlab, Erlangen, Germany). Success of DNA extraction was verified by using a PCR assay for tick mitochondrial 16S rDNA (d'Oliveira et al., 1997), and an assay targeting the cytB gene of vertebrates (Lee et al., 2002).

Infection by *B. burgdorferi* s.l. in *I. ricinus* ticks and ear biopsies was investigated by a PCR assay targeting the intergenic spacer (IGS) region (Rijpkema et al., 1995). We used DNA of *B. afzelii* (Nancy strain) as positive control.

For the specific detection of *Rickettsia slovaca* in ear tissues and *D. marginatus* ticks, we used the quantitative PCR (qPCR) protocol described by Tomassone and colleagues (2016). Briefly, we used the primers RafrS and RafrA, and the specific TaqMan probe (CCTGCTGGCAGTCCTCAA) marked 5'-FAM e 3'-TAMRA, targeting *R. slovaca OmpA* gene. The qPCR was run in an Applied Biosystems 7300 thermal cycler; protocol: 50°C for 2 min, 95°C for 3 min, [40x] 95°C for 3 s, 60 °C for 30 s. In each qPCR run, we included negative controls (RNase and DNase-free water), and DNA from *R. slovaca* as positive control. Experiments were conducted in duplicate, in a 10-µl volume. *Dermacentor marginatus* ticks and ear biopsies which were negative for the *R. slovaca* qPCR, and all DNA extracts from *I. ricinus*, were tested for the presence of other rickettsial species by conventional PCR assays targeting fragments of the *gltA* and *OmpA* genes (Martello et al., 2013). DNA from a *Rickettsia conorii* isolate was used as positive control.

Amplicons were purified using ExoSAP-IT PCR Clean-up Kit (GE Healthcare Limited, Chalfont, UK) and sent to an external service (Macrogen Inc., Amsterdam, The Netherlands) for automatic sequencing. The sequences were analyzed by Chromas 2.0 software (Technelysium, Helensvale, Australia) and submitted to BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi) to identify similarities to known sequences. We performed an *in silico* RFLP analysis and a 'virtual hybridization' to confirm the genospecies identification of *B. burgdorferi* s.l., as described by Rudenko and colleagues (2009).

2.3.Statistical analysis

Prevalence and 95% exact binomial confidence intervals (CIs) of tick infestation on rodents and tick and tissue infection were calculated using the FREQ procedure in SAS System 9.4 (SAS, 2013). Mean numbers of ticks on rodents and 95% confidence intervals (CIs) were obtained using intercept-only, generalized linear models (GLM) using PROC GENMOD, with negative binomial error (log

link) (Littell et al., 2006; Martello et al 2014). We used the Fisher exact test to compare the proportions of infection in larvae and nymphs, by tick species, in tissues from infested and not infested rodents, and in ticks and tissues collected from different rodent species. Based on the low number of *I. ricinus* nymphs collected from small rodent hosts, some statistical analysis was only performed on larvae, or considering all immatures together. A two-tailed significance level of α=0.05 was adopted. The degree of concurrent infection by *B. burgdorferi* s.l. and SFG rickettsiae in the same tick and in the same individual rodent tissue was tested by Kappa statistics. Kappa is commonly used as a measure of agreement between categorical classification criteria, and a value not significantly different from zero indicates no agreement beyond chance. For most purposes, values > 0.75 indicate excellent agreement beyond chance, whereas values between 0.40 and 0.75 indicate fair to good agreement (Fleiss, 1981). Median (Q1, Q3) TEI were calculated for *B. burgdorferi* s.l. and *Rickettsia*-positive and negative host-attached larvae. The two sample Wilcoxon rank sum test was used to compare medians.

3. Results.

3.1. Rodent trapping and tick collection

In 2011, we trapped a total of 30 *Apodemus* spp. mice (19 males and 11 females), and 18 bank voles (*Myodes glareolus;* 13 males and 5 females), while in the following year we collected 53 *Apodemus* mice (44 males and 9 females) and only 4 *M. glareolus* males. All *Apodemus* spp. mice were classified as belonging to *A. sylvaticus* (wood mouse) and/or *A. flavicollis* (yellow-necked mouse), but it was not possible to distinguish with certainty between the two species (Martello et al., 2013 and 2014).

Immature ticks, but no adults, were found feeding on small rodent hosts. The two tick species we collected most frequently over the two year study period were *I. ricinus* (184 larvae and one nymph from *Apodemus* mice; 8 larvae and one nymph from *M. glareolus*) and *D. marginatus* (177 larvae and 20 nymphs from *Apodemus* mice; two larvae and 9 nymphs from *M. glareolus*). In addition, we

identified a few *I. trianguliceps* - one larva on an *Apodemus* mouse and one on a *M. glareolus*, whereas two nymphs were found on one *M. glareolus* and one on one *Apodemus* mouse.

The prevalence (95% CI) of infestation by *I. ricinus* larvae was 55% (44, 66) on *Apodemus* mice, with a mean (95% CI) number of 2.2 (1.5, 3.2). For *M. glareolus*, 18% (5, 40) were infested by *I. ricinus* larvae, with a mean number of 0.4 (0.1, 1.1). The prevalence (95% CI) of infestation by *D. marginatus* larvae was 23% (14, 33) on *Apodemus* mice with a mean (95% CI) number of 2.1 (0.9, 4.8). Only two *M. glareolus* (9%; 95% CI: 1, 29) were infested by one *D. marginatus* larva each, while seven (32%; 95% CI: 14, 55) carried nymphs. The mean (95% CI) number of nymphs on *M. glareolus* was 0.4 (0.4, 0.8).

The median engorgement status of larvae, as evaluated by TEI, was 2.4 (Q1 = 1.8, Q3 = 2.8) in *I. ricinus* and 2.1 (Q1 = 1.7, Q3 = 2.6) in *D. marginatus*.

3.2. Pathogen detection

Detection of B. burgdorferi s.l.

The overall infection prevalence by *B. burgdorferi* s.l. in rodent-feeding *I. ricinus* larvae (n=67) in the study period was 21% (n=14 positives). The only two *I. ricinus* nymphs collected from rodents tested negative. No significant difference in infection prevalence of larvae (OR=1.3, 95% CI: 0.13-68.9) was detected between the ones collected from mice (21%, 95% CI: 12-34) and from voles (17%, 95% CI: 1-61). *Borrelia*-positive amplicons were identified as *B. afzelii*, *B. burgdorferi* s.s., *B. garinii*, *B. lusitaniae*, and *B. valaisiana* (Table 1). Sequences were identical to those previously identified in the study area (Ragagli et al., 2016).

Overall, 11% (n=11 positives) of the tested ear biopsies collected from animals (n=102, infested and non-infested by *I. ricinus* ticks) were infected with Lyme borreliosis spirochetes, namely *B. lusitaniae*, *B. afzelii* and *B. valaisiana* (Table 1). Some of the amplicons could not be identified by genospecies and were classified as *B. burgdorferi* s.l. The difference in infection prevalence between the small rodents infested (10%, 95% CI 3-22) and the non-infested (13%, 95% CI 5-25) by *I. ricinus* ticks,

was not significant (OR=0.8, 95% CI 0.2-3.1). *Borrelia burgdorferi* s.l. infection prevalence was higher in bank voles (24%, 95% CI: 8-47) than in mice (7%, 95% CI: 3-15), but the difference was not significant (OR=3.8, 95% CI: 0.8-17.3). The TEI was not significantly different (p = 0.7) between infected and non-infected larvae.

Details on infection rates and genospecies classification by tick stage and rodent group are further summarized in Table 1.

Detection of SFG rickettsiae

Results from the *R. slovaca*-specific qPCR assay revealed a 46% (n=26 positives; 95% CI: 33-60) infection prevalence in *D. marginatus* ticks collected from the two rodent groups. We did not detect differences in infection prevalence between larvae (15 positives; 479%, 95% CI: 29-65) and nymphs (11; 46%, 95% CI: 26-67) (OR=1.04, 95% CI: 0.3-3.4). The proportion of infected *D. marginatus* carried by *M. glareolus* (55%, 95% CI 23-83) and *Apodemus* spp. (44%, 95% CI: 30-60) was not significantly different (OR=1.5, 95% CI: 0.3-7.2).

Overall, 6% of rodent tissues were infected by *R. slovaca*. Among the six positive ear biopsies, only one was sampled from *M. glareolus* (21 tissues tested, prevalence: 5%, 95% CI: 0-24) and the other five from *Apodemus* spp. (81; 6%, 95% CI: 2-14). Based on the *gltA/OmpA* gene sequences, we identified *R. raoultii* in a few *D. marginatus*: one larva from an *Apodemus* mouse and one larva and one nymph from two *M. glareolus*. Sequences of *R. slovaca* and *R. raoultii* were identical to the ones previously identified in the study area (Martello et al., 2013). Moreover, we identified *R. monacensis* in attached *I. ricinus* immatures: this agent infected 18% of the tested larvae (12 positives), and one of the two nymphs. The OmpA sequence (GenBank Accession No. MK543506) was 99% identical to *R. monacensis* from *I. ricinus* in Emilia Romagna, central Italy (HM161785). Further details on *Rickettsia* spp. detection are reported in Table 2.

The TEI was not significantly different between larvae infected and non-infected by *Rickettsia* spp., either for *I. ricinus* (p = 0.4) or *D. marginatus* (p = 0.5).

Borrelia burgdorferi s.l. and SFG rickettsiae coinfection

We observed coinfection by *B. burgdorferi* s.l. and SFG rickettsiae in two *Apodemus* spp. ear biopsies (2%, 95% CI: 0-7), both positive for *R. slovaca*, and coinfected with *B. lusitaniae* and *B. valaisiana*, respectively. Kappa, as a measure of concurrent infection by the two pathogens in the same individual tissue, indicated a low level of coinfection (Kappa= 0.17, 95% CI: -0.01-0.4).

Dual infections were also detected in three *I. ricinus* larvae from different *Apodemus* individuals (Kappa=0.06, 95% CI: -0.2-0.3), two with a combination of *R. monacensis* and *B. garinii* and one with *R. monacensis* and *B. afzelii*. None of the dually infected mice carried infected ticks, and none of the animals carrying dually infected ticks was infected by any of the pathogens under study. No *M. glareolus* sample was found coinfected.

The detection of the same disease agent in an ear tissue and in at least one attached tick from the same rodent was a rare finding in our study. As for *B. burgdorferi* s.l., all tested *I. ricinus* collected from infected rodents were negative, while all positive ticks were feeding on negative individuals. Regarding SFG rickettsiae, one *Apodemus* ear sample tested positive for *R. slovaca*, and two *D. marginatus* larvae and two nymphs attached on this animal were infected by the same agent. In another four cases, infection in tissues and in attached ticks was not caused by the same disease agent: two mice infected by *R. slovaca* carried *I. ricinus* larvae infected by *R. monacensis* and *B. burgdorferi* s.l. respectively; one *B. afzelii* positive animal carried two *D. marginatus* larvae, one infected by *R. slovaca* and one by *R. raoultii*. Finally, one *M. glareolus* infected by *B. burgdorferi* s.l. carried a *D. marginatus* larva infected by *R. raoultii*.

4. Discussion.

In the present study, *B. burgdorferi* s.l. and SFG rickettsiae were detected in ear tissues and ticks collected from small rodents in all study sites, including the highest altitude site. Our results demonstrate the altitudinal range expansion of these agents in the study area, as previous studies at

the same location in 1994, showed that *I. ricinus* was very rare, and *B. burgdorferi* s.l. was not found in small rodent samples (Mannelli et al., 1997).

Both rodent groups were found infected by *B. burgdorferi* s.l.; in literature, it was shown that the contribution of bank voles to the infection of ticks appears to be higher, despite their acquired resistance to *I. ricinus* (Dizij and Kurtenbach, 1995) and lower infestation levels by this tick species, compared to mice (Biernat et al., 2016; Martello et al., 2014).

We detected several *B. burgdorferi* s.l. genospecies, which we had previously reported from ticks collected on the vegetation and from lizards in the same study area (Ragagli et al., 2016; Tomassone et al., 2017). Based on the low probability to have transovarially infected larvae feeding on hosts (Bellet-Edimo et al., 2005; Karbowiak et al., 2018), our *Borrelia*-infected ticks may have acquired the infection by feeding on infected rodents, or by co-feeding with infected nymphs. The first hypothesis is well supported by the role of rodents as reservoirs, or potential reservoirs, for some genospecies, even if the number of spirochetes circulating in the host may not always be enough to be detected in tissues (Kurtenbach et al., 1998). The hypothesis of transmission via co-feeding is countered by the fact that few nymphs were found feeding on hosts during the collection period.

The identification of *B. afzelii* in our samples confirms its association with rodents (Kurtenbach et al., 2006). However, the finding of *B. burgdorferi* s.l. genospecies other than *B. afzelii* may indicate a generally great level of *B. burgdorferi* s.l. transmission in the area.

As regards *B. burgdorferi* s.s., detection in a single larva may reflect its low transmission efficiency in the *I. ricinus* vector (Richter et al., 2004), although rodents were identified as reservoir for this genospecies (Michalik et al., 2005). *Borrelia garinii* was also detected in feeding larvae; it is a very genetically and immunologically heterogeneous genospecies and, even though it is mainly associated with birds, some strains have been detected in rodents (Radzijevskaja et al., 2011). *Borrelia lusitaniae* infected tissues from both *Apodemus* spp. and *M. glareolus*; it is normally associated with lizards (Amore et al., 2007; Dsouli et al., 2006; Richter and Matuschka, 2006; Tomassone et al., 2017) and birds (Marie-Angele et al., 2006), but also mice were indicated as

possible reservoir hosts (de Carvalho et al., 2010). Our results confirm that mice may be involved in the *B. lusitaniae* transmission cycle and suggest that also *M. glareolus* might be involved.

Finally, ear samples and attached larvae were found infected by *B. valaisiana*, which we had already detected in our study area both in questing ticks and lizard ticks (Ragagli et al., 2016; Tomassone et al., 2017). This finding was unexpected because *B. valaisiana* is considered to be bird-associated (Amore et al., 2007), so further studies are needed to assess the role of small rodents in its transmission.

We observed a total absence of matching results on *B. burgdorferi* s.l. infection in tissue and in ticks collected from the same rodent. This could be explained first by the low number of *Borrelia* spirochetes present in a vertebrate host, therefore the detection of DNA in skin biopsy specimens may not always be possible, as shown by Kurtenbach and colleagues (1998). Secondly, ticks feeding on infected rodents are not always able to acquire the infection (Mather et al., 1989). Thirdly, ticks may have been removed prior to completing their feeding, thereby reducing the chance of pathogen acquisition. Finally, the results could be due to the limited number of fed ticks, collected on infected rodents, that we tested.

The only SFG rickettsia found in our *I. ricinus* was *R. monacensis*, the agent of Mediterranean spotted fever–like cases in humans. This was also the dominant rickettsial species in *I. ricinus* larvae and nymphs that we collected from lizards (Tomassone et al., 2017), indicating that this pathogen is widespread in the area. In contrast with previous findings on lizards, we did not detect *R. helvetica*, the etiological agent of uneruptive tick bite fever in humans, either in rodent tissues or ticks collected from the rodents. This could be due to the limited number of samples we tested. Conversely, studies in Germany (Fischer et al., 2018; Obiegala et al., 2017) and Lithuania (Mardosaite-Busaitiene et al., 2018) reported *R. helvetica* infection in small rodents, including *A. flavicollis* and *M. glareolus*.

We identified other SFG rickettsiae transmitted by *D. marginatus, R. slovaca* and *R. raoultii*, in ticks and ear tissues, demonstrating their occurrence also at high altitudes. These rickettsiae cause TIBOLA/DEBONEL, a disease that affects the human population in the study area (Selmi et al.,

2008), and we had already detected them in rodents (Martello et al., 2013) and wild boar (Selmi et al., 2009). Infection of bank voles by *R. raoultii* – but not by *R. slovaca* - had already been reported in Germany (Obiegala et al., 2017).

The coinfection by *B. burgdorferi* s.l. and SFG rickettsiae in our rodents and ticks was low. This may be due to the distinct transmission cycles and also associated with different monthly infestation patterns of the two tick species on rodents (Martello et al., 2014). In addition, according to Raulf and colleagues (2018), the associations of *Borrelia* and *Rickettsia* spp. are probably influenced by environmental factors. Rodent tissues were found infected by *R. slovaca* in association with *B. burgdorferi* s.l. At our knowledge, this is the first report of a rodent infected by *R. slovaca* and *B. lusitaniae*, while this same association was reported in *D. marginatus* ticks collected from vegetation in Portugal (Milhano et al., 2010). In addition to dual infections in tissues, we observed *R. monacensis* in association with *B. garinii* and *B. afzelii* in feeding *I. ricinus* larvae. This, once again, could be due to rodent infection (even if not detected by our analysis), co-feeding transmission, and to transovarial transmission in the case of *R. monacensis.*

In conclusion, our study provides new insights on tick and TBP occurrence at high altitude in the Northern Appennines; since this work was conducted in a park frequented by tourists and workers, our results are of importance for public health. Historical tick and pathogen distribution data were useful for making comparisons with more recent data in the same area, giving the most valuable evidence on how ticks and TBPs are spreading. In general, hosts exposed to multiple microbial species as well as the presence of co-infections indicate that there is an overlap of transmission cycles. In particular, our results suggest that small rodents have a crucial role in co-infections, since they are the natural hosts of several TBPs and they can be infected with more than one of these agents at the same time. Investigations carried out on the same samples for detecting multiple pathogens and on different data sources from the same area represent a valuable approach in order to have a comprehensive picture of the risk posed by ticks to public health.

List of Tables.

Table 1. Infection rates and genospecies classification of *Borrelia burgdorferi* s.l. in ticks and ear biopsies collected from small rodents (*Apodemus* spp. and *Myodes glareolus*) in 2011-2012, in the Northern Apennines, Italy.

Table 2. Infection rates and species classification by SFG rickettsiae in ticks and ear biopsies collected from small rodents (*Apodemus* spp. and *Myodes glareolus*) in 2011-2012, in the Northern Apennines, Italy.

Compliance with Ethical Standards.

Ethical approval: All applicable institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

Conflict of Interest.

The authors declare no conflicts of interest.

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References

Achazi, K., Ruzek, D., Donoso-Mantke, O., Schlegel, M., Ali, H.S., Wenk, M., Schmidt-Chanasit, J., Ohlmeyer, L., Ruhe, F., Vor, T., Kiffner, C., Kallies, R., Ulrich, R.G., Niedrig, M., 2011. Rodents as sentinels for the prevalence of tick-borne encephalitis virus. Vector Borne Zoonotic Dis. 11, 641-647.

Amore, G., Tomassone, L., Grego, E., Ragagli, C., Bertolotti, L., Nebbia, P., Rosati, S., Mannelli, A., 2007. *Borrelia lusitaniae* in immature *Ixodes ricinus* (Acari: Ixodidae) feeding on common wall lizards in Tuscany, central Italy. J. Med. Entomol. 44, 303-307.

Andersson, M., Bartkova, S., Lindestad, O., Raberg, L., 2013. Co-infection with '*Candidatus* Neoehrlichia mikurensis' and *Borrelia afzelii* in *Ixodes ricinus* ticks in southern Sweden. Vector Borne Zoonotic Dis. 13, 438-442.

Bellet-Edimo, R., Betschart, B., Gern, L., 2005. Frequency and efficiency of transovarial and subsequent transstadial transmission of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks. Bull. Soc. Neuchl. Sci. Nat. 128, 117-125.

Biernat, B., Stanczak, J., Michalik, J., Sikora, B., Wierzbicka, A., 2016. Prevalence of infection with *Rickettsia helvetica* in *Ixodes ricinus* ticks feeding on non-rickettsiemic rodent hosts in sylvatic habitats of west-central Poland. Ticks Tick Borne Dis. 7, 135-141.

Cimperman, J., Maraspin, V., Lotric-Furlan, S., Ruzic-Sabljic, E., Avsic-Zupanc, T., Strle, F., 2002. Double infection with tick borne encephalitis virus and *Borrelia burgdorferi* sensu lato. Wien. Klin. Wochenschr. 114, 620-622.

Cutler, S.J., Rudenko, N., Golovchenko, M., Cramaro, W.J., Kirpach, J., Savic, S., Christova, I., Amaro, A.,2017. Diagnosing Borreliosis. Vector Borne Zoonotic Dis. 17(1):2-11.

d'Oliveira, C., van der Weide, M., Jacquiet, P., Jongejan, F., 1997. Detection of *Theileria annulata* by the PCR in ticks (Acari:Ixodidae) collected from cattle in Mauritania. Exp. Appl. Acarol. 21, 279-291.

Da Franca, I. 2004. Infeccao por espiroquetideos do complexo *Borrelia burgdorferi* em Portugal: perspectiva dermatologica e implicacao da especie *Borrelia lusitaniae*. Public Health, Ph.D Thesis, Universida de da Beira Interior.

de Carvalho, I.L., Zeidner, N., Ullmann, A., Hojgaard, A., Amaro, F., Ze-Ze, L., Alves, M.J., de Sousa, R., Piesman, J., Nuncio, M.S., 2010. Molecular characterization of a new isolate of *Borrelia lusitaniae* derived from *Apodemus sylvaticus* in Portugal. Vector Borne Zoonot. Dis. 10, 531-534.

Dizij, A., Kurtenbach, K., 1995. *Clethrionomys glareolus*, but not *Apodemus flavicollis*, acquires resistance to *Ixodes ricinus* L., the main European vector of *Borrelia burgdorferi*. Parasite Immunol. 17, 177-183.

Dsouli, N., Younsi-Kabachii, H., Postic, D., Nouira, S., Gern, L., Bouattour, A., 2006. Reservoir role of lizard *Psammodromus algirus* in transmission cycle of *Borrelia burgdorferi* sensu lato (Spirochaetaceae) in Tunisia. J. Med Entomol. 43, 737-742.

Fischer, S., Spierling, N.G., Heuser, E., Kling, C., Schmidt, S., Rosenfeld, U.M., Reil, D., Imholt, C., Jacob, J., Ulrich, R.G., Essbauer, S., 2018. High prevalence of *Rickettsia helvetica* in wild small mammal populations in Germany. Ticks Tick Borne Dis. 9, 500-505.

Fleiss, J.L., 1981. Statistical methods for rates and proportions. 3rd Edn., J. Wiley, New York.

Kiffner, C., Vor, T., Hagedorn, P., Niedrig, M., Ruhe, F., 2011. Factors affecting patterns of tick parasitism on forest rodents in tick-borne encephalitis risk areas, Germany. Parasitol. Res. 108, 323-335.

Karbowiak, G., Biernat, B., Stańczak, J., Werszko, J., Szewczyk, T., Sytykiewicz, H., 2018.

The role of particular ticks developmental stages in the circulation of tick-borne pathogens in Central Europe. 5. Borreliaceae. Ann Parasitol. 64(3):151-171.

Kurtenbach, K., Peacey, M., Rijpkema, S.G., Hoodless, A.N., Nuttall, P.A., Randolph, S.E., 1998. Differential transmission of the genospecies of Borrelia burgdorferi sensu lato by game birds and small rodents in England. Appl Environ Microbiol. Apr;64(4):1169-74.

Kurtenbach, K., Hanincova, K., Tsao, J.I., Margos, G., Fish, D., Ogden, N.H., 2006. Fundamental processes in the evolutionary ecology of Lyme borreliosis. Nat. Rev. Microbiol. 4, 660-669.

Lee, J.H., Hassan, H., Hill, G., Cupp, E.W., Higazi, T.B., Mitchell, C.J., Godsey, M.S., Jr., Unnasch, T.R., 2002. Identification of mosquito avian-derived blood meals by polymerase chain reaction-heteroduplex analysis. Am. J. Trop. Med. Hyg. 66, 599-604.

Levin, M.L., Snellgrove, A.N., Zemtsova, G.E., 2016. Comparative value of blood and skin samples for diagnosis of spotted fever group rickettsial infection in model animals. Ticks Tick Borne Dis. 7(5):1029-1034.

Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D., Schabenberger, O., 2006.SAS for Mixed Models, 2nd ed. SAS®Institute, Cary, USA.

Manilla, G., 1998. Acari: Ixodida. (Fauna d'Italia 36). Ed. Calderoni, Bologna, Italy.

Mannelli, A., Martello, E., Tomassone, L., Calzolari, M., Casalone, C., De Meneghi, D., Dottori, M., Estrada-Peña, A., Fabbi, M., Ferreri, L., Ferroglio, E., Luini, M., Nicolau Solano, S., Ortega, C., Pautasso, A., Prati, P., Vesco, U., 2012. Inventory of available data and data sources and proposal for data collection on vector-borne zoonoses in animals. EFSA Supporting Publications 9, 234E.

Mannelli, A., Tolari, F., Pedri, P., Stefanelli, S., 1997. Spatial distribution and seasonality of ticks (Acarina: Ixodidae) in a protected area in the northern Apennines. Parassitologia 39, 41-45.

Mardosaite-Busaitiene, D., Radzijevskaja, J., Balciauskas, L., Paulauskas, A., 2018. First detection of *Rickettsia helvetica* in small mammals in Lithuania. New Microbes New Infect. 22, 19-23.

Marie-Angele, P., Lommano, E., Humair, P.F., Douet, V., Rais, O., Schaad, M., Jenni, L., Gern, L., 2006. Prevalence of *Borrelia burgdorferi* sensu lato in ticks collected from migratory birds in Switzerland. Appl. Environ. Microbiol. 72, 976-979.

Martello, E., Mannelli, A., Ragagli, C., Ambrogi, C., Selmi, M., Ceballos, L.A., Tomassone, L., 2014. Range expansion of *Ixodes ricinus* to higher altitude, and co-infestation of small rodents with *Dermacentor marginatus* in the Northern Apennines, Italy. Ticks Tick Borne Dis. 5, 970-974.

Martello, E., Selmi, M., Ragagli, C., Ambrogi, C., Stella, M.C., Mannelli, A., Tomassone, L., 2013. *Rickettsia slovaca* in immature *Dermacentor marginatus* and tissues from *Apodemus* spp. in the northern Apennines, Italy. Ticks Tick Borne Dis. 4, 518-521.

Mather, T.N., Wilson, M.L., Moore, S.I., Ribeiro, J.M., Spielman, A., 1989. Comparing the relative potential of rodents as reservoirs of the Lyme disease spirochete (*Borrelia burgdorferi*). Am. J. Epidemiol. 130, 143-150.

Michalik, J., Skotarczak, B., Skoracki, M., Wodecka, B., Sikora, B., Hofman, T., Rymaszewska, A., Sawczuk, M., 2005. *Borrelia burgdorferi* sensu stricto in yellow-necked mice and feeding *Ixodes ricinus* ticks in a forest habitat of west central Poland. J. Med. Entomol. 42, 850-856.

Mihalca, A.D., Dumitrache, M.O., Sandor, A.D., Magdas, C., Oltean, M., Gyorke, A., Matei, I.A., Ionica, A., D'Amico, G., Cozma, V., Gherman, C.M., 2012. Tick parasites of rodents in Romania: host preferences, community structure and geographical distribution. Parasit. Vectors 5, 266.

Milhano, N., de Carvalho, I.L., Alves, A.S., Arroube, S., Soares, J., Rodriguez, P., Carolino, M., Nuncio, M.S., Piesman, J., de Sousa, R., 2010. Coinfections of *Rickettsia slovaca* and *Rickettsia helvetica* with *Borrelia lusitaniae* in ticks collected in a Safari Park, Portugal. Ticks Tick Borne Dis. 1, 172-177.

Morand, S., Krasnov, B., Poulin, R., Degen, A., 2006. Micromammals and macroparasites: Who is who and how do they interact? Conference: Symposium on Parasites and Mammals. DOI: 10.1007/978-4-431-36025-398 4_1

Obiegala, A., Krol, N., Oltersdorf, C., Nader, J., Pfeffer, M., 2017. The enzootic life-cycle of *Borrelia burgdorferi* (sensu lato) and tick-borne rickettsiae: an epidemiological study on wild-living small mammals and their ticks from Saxony, Germany. Parasit. Vectors 10, 115.

Paziewska, A., Zwolinska, L., Harris, P.D., Bajer, A., Sinski, E., 2010. Utilisation of rodent species by larvae and nymphs of hard ticks (Ixodidae) in two habitats in NE Poland. Exp. Appl. Acarol. 50, 79-91.

Pospisilova, T., Urbanova, V., Hes, O., Kopacek, P., Hajdusek, O., Sima, R., 2019. Tracking of *Borrelia afzelii* transmission from infected *Ixodes ricinus* nymphs to mice. Infect. Immun. Mar 25 [Epub ahead of print].

Radzijevskaja, J., Paulauskas, A., Rosef, O., 2011. Molecular detection and characterization of *Borrelia burgdorferi* sensu lato in small rodents. Vet. Med. Zoot. 55, 40-46.

Ragagli, C., Mannelli, A., Ambrogi, C., Bisanzio, D., Ceballos, L.A., Grego, E., Martello, E., Selmi, M., Tomassone, L., 2016. Presence of host-seeking Ixodes ricinus and their infection with *Borrelia burgdorferi* sensu lato in the Northern Apennines, Italy. Exp. Appl. Acarol. 69, 167-178.

Raulf, M.K., Jordan, D., Fingerle, V., Strube, C., 2018. Association of *Borrelia* and *Rickettsia* spp. and bacterial loads in Ixodes ricinus ticks. Ticks Tick Borne Dis. 9, 18-24.

Richter, D., Klug, B., Spielman, A., Matuschka, F.R., 2004. Adaptation of diverse Lyme disease spirochetes in a natural rodent reservoir host. Infect. Immun. 72, 2442-2444.

Richter, D., Matuschka, F.R., 2006. Perpetuation of the Lyme disease spirochete *Borrelia lusitaniae* by lizards. Appl. Environ. Microbiol. 72, 4627-4632.

Rijpkema, S.G., Molkenboer, M.J., Schouls, L.M., Jongejan, F., Schellekens, J.F., 1995. Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. J. Clin. Microbiol. 33, 3091-3095.

Rudenko, N., Golovchenko, M., Grubhoffer, L., Oliver, J.H., Jr., 2009. *Borrelia carolinensis* sp. nov., a new (14th) member of the *Borrelia burgdorferi* sensu lato complex from the southeastern region of the United States. J. Clin. Microbiol. 47, 134-141.

Santos, A.S., Bacellar, F., Dumler, J.S., 2006. Human exposure to *Anaplasma phagocytophilum* in Portugal. Ann. N. Y. Acad. Sci. 1078, 100-105.

SAS Institute Inc. 2013. SAS® 9.4 Statements: Reference. Cary, NC.

Schex, S., Dobler, G., Riehm, J., Müller, J., Essbauer S.,2011. *Rickettsia* spp. in wild small mammals in Lower Bavaria, South-Eastern Germany. Vector Borne Zoonotic Dis. 11, 493-502.

Selmi, M., Bertolotti, L., Tomassone, L., Mannelli, A., 2008. *Rickettsia slovaca* in *Dermacentor marginatus* and tick-borne lymphadenopathy, Tuscany, Italy. Emerg. Infect. Dis. 14, 817-820.

Selmi, M., Martello, E., Bertolotti, L., Bisanzio, D., Tomassone, L., 2009. *Rickettsia slovaca* and *Rickettsia raoultii* in *Dermacentor marginatus* ticks collected on wild boars in Tuscany, Italy. J. Med. Entomol. 46, 1490-1493.

Silaghi, C., Woll, D., Hamel, D., Pfister, K., Mahling, M., Pfeffer, M., 2012. *Babesia* spp. and *Anaplasma phagocytophilum* in questing ticks, ticks parasitizing rodents and the parasitized rodents--analyzing the host-pathogen-vector interface in a metropolitan area. Parasit. Vectors 5, 191.

Swanson, S.J., Neitzel, D., Reed, K.D., Belongia, E.A., 2006. Coinfections acquired from *Ixodes* ticks. Clin. Microbiol. Rev. 19, 708-727.

Tomassone, L., Ceballos, L.A., Ragagli, C., Martello, E., De Sousa, R., Stella, M.C., Mannelli, A., 2017. Importance of common wall lizards in the transmission dynamics of tick-borne pathogens in the Northern Apennine Mountains, Italy. Microb. Ecol. 74, 961-968.

Tomassone, L., De Meneghi, D., Adakal, H., Rodighiero, P., Pressi, G., Grego, E., 2016. Detection of *Rickettsia aeschlimannii* and *Rickettsia africae* in ixodid ticks from Burkina Faso and Somali Region of Ethiopia by new real-time PCR assays. Ticks Tick Borne Dis. 7, 1082-1088.

Tomassone, L., Portillo, A., Novakova, M., de Sousa, R., Oteo, J.A., 2018. Neglected aspects of tick-borne rickettsioses. Parasit. Vectors 11, 263.

Yeh, M.T., Bak, J.M., Hu, R., Nicholson, M.C., Kelly, C., Mather, T.N., 1995. Determining the duration of Ixodes scapularis (Acari: Ixodidae) attachment to tick-bite victims. J. Med. Entomol. 32, 853–858.

List of tables.

Table 1. Infection rates and genospecies classification of *Borrelia burgdorferi* s.l. in ticks and ear biopsies collected from small rodents (*Apodemus* spp. and *Myodes glareolus*) in 2011-2012, in the Northern Apennines, Italy.

Borrelia burgdorferi s.l.								
Source [no.]		% prevalence (95% CI)	no. infected					
			Bafz	Bgar	Blus	Bb ss	Bval	NI[c]
I. ricinus	Larvae [67]	21 (12-33)	1[a]	1[a]	1[a]	1[a]	5[a]	4[a]+1[b]
	Nymphs [2]	0 (0-84)	-	-	-	-	-	-
	total [69]	20 (12-32)						
Ear biopsy [102]		11 (6-19)	1[a]	-	2[a]+3[b]	-	2[a]	1[a]+2[b]

Bafz*: B. afzelii*; Bgar: *B. garinii*; Blus: *B. lusitaniae*; Bb ss: *B. burgdorferi* sensu stricto; Bval: *B. valaisiana*
[a] Data refers to *Apodemus* spp.
[b] Data refers to *Myodes glareolus*
[c] NI: genospecies not identified

Table 2. Infection rates and species classification of SFG rickettsiae in ticks and ear biopsies collected from small rodents (*Apodemus* spp. and *Myodes glareolus*) in 2011-2012, in the Northern Apennines, Italy.

		SFG rickettsiae		
	Source [no.]	**Prevalence (%[95% CI])**		
		R. slovaca	*R. raoultii*	*R. monacensis*
I. ricinus	Larvae [67]	0	0	18 (10-29)
	Apodemus sp. [61]	0	0	16 (8-28)
	M. glareolus [6]	0	0	33 (4-78)
	Nymphs [2]	0	0	50 (1-99)
	Apodemus sp. [1]	0	0	0 (0-98)
	M. glareolus [1]	0	0	100 (3-100)
	total [69]	0	0	19 (11-31)
D. marginatus	Larvae [32]	47 (29-65)	6 (1-21)	0
	Apodemus sp. [30]	47 (28-66)	3 (0-17)	0
	M. glareolus [2]	50 (1, 99)	50 (1, 99)	0
	Nymphs [24]	46 (26-67)	4 (0-21)	0
	Apodemus sp. [15]	40 (16-68)	0 (0-22)	0
	M. glareolus [9]	56 (21-86)	11 (0-48)	0
	total [56]	46 (33-60)	5 (1-15)	0
Ear biopsy [102]	*Apodemus* sp. [81]	6 (0-14)	0	0
	M. glareolus [21]	5 (0-2)	0	0
	total [102]	6 (2-12)	0	0