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Rickettsia slovaca in immature *Dermacentor marginatus* and tissues from *Apodemus* spp. in the Northern Apennines, Italy.

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Abstract.

Immature *Dermacentor marginatus* ticks and tissues from small rodents were tested for infection by *Rickettsia slovaca* in the Northern Apennines, Lucca Province, where tick-borne lymphadenopathy (TIBOLA) was previously reported in people. Prevalence of infestation by *D. marginatus* was 30.5% (n=131, 95%CI: 22.8, 39.2) in *Apodemus* spp., and 26.5% (n=34, 95%CI: 12.9, 44.4) in *M. glareolus*, which were captured during 1980 trap nights in 2009 and 2010. *R. slovaca* was identified by polymerase chain reaction, targeting the gltA and OmpA genes, in ear biopsies from eight out of 37 tested *Apodemus* spp. (21.6%, 95%CI: 9.8, 38.2), but not from nine *M. glareolus*. The prevalence of *R. slovaca* in *D. marginatus* feeding on *Apodemus* spp. was 52.9% in larvae (n=51, 95%CI: 38.5, 67.1), and 47.5 % in nymphs (n=59, 95%CI: 34.3, 60.9). No larvae (0.0%, 95%CI: 0, 36.9), but one nymph from *M. glareolus* was positive (10.0%, 95%CI: 0.3, 44.5). Prevalence of *R. slovaca* in host-seeking *D. marginatus* larvae, collected in the same area, was 42.1% (n=38; 95%CI: 26.3, 59.2). Prevalence of *R. slovaca* was greater in larvae feeding on PCR-positive *Apodemus* spp. than in those feeding on negative mice (78.6% vs 37.1%). Furthermore, levels of infestation by *D. marginatus* larvae were greater for *R. slovaca*-positive mice. The infection of *Apodemus* spp. was probably the result of repeated bites by transovarially-infected larvae. On the other hand, the finding of *R. slovaca* in mice tissues would be compatible with transmission from these hosts to feeding *D. marginatus*. Based on such an hypothesis, the most heavily infested *Apodemus* spp. might play a role as amplifiers of the infection.

Keywords

Rickettsia slovaca, tick-borne lymphadenopathy, *Dermacentor marginatus*, *Apodemus* spp., *Myodes glareolus,* Italy

Introduction

Rickettsia slovaca is the causative agent of TIBOLA (tick-borne lymphadenopathy; Lakos, 2002) which is the most widespread rickettsiosis in Europe (Méchaï et al., 2009). It is transmitted by *Dermacentor* spp. ticks. Like other spotted fever group (SFG) rickettsiae, it multiplies in almost all organs of the vector, including ovaries, thus enabling transovarial transmission between subsequent generations of ticks. *Dermacentor* spp. are, therefore, both vectors and reservoirs of the pathogen (Brouqui et al., 2007; Samoylenko et al., 2009).

Vertebrates serve as sources of blood for tick vectors, small mammals being hosts for immature *Dermacentor* spp. (Pluta et al., 2010; Dörr and Gothe, 2001), and ungulates and carnivorafor adults (Cringoli et al., 2005). On the other hand, the role of vertebrates in the maintenance of SFG rickettsiae is not completely clarified. The percentage of successful transovarial transmission of SFG rickettsie gradually decreases with each tick generation (Rehácek, 1989), therefore, vertebrates might be important in the perpetuation of these agents. In fact, SFG rickettsiae might be transmitted to tick following systemic infection of hosts (Brouqui et al., 2007). For example, some vole, mouse, and rat species may develop sufficient levels of *Rickettsia rickettsii* in blood to infect vector ticks (Walker and Fishbein, 1991; Socolovschi et al., 2009). Accordingly, *R. slovaca* and specific antibodies were found in *Apodemus flavicollis* and *Myodes glareolus* blood, following the bite of infected *D. marginatus* (Rehácek et al., 1976, 1992). Furthermore, *R. slovaca* was detected in *D. marginatus* and tissues collected on wild boars (*Sus scrofa*) (Sanogo et al., 2003; Ortuño et al., 2007; Selmi et al., 2009; Masala et al., 2012), and these ungulates may develop specific antibodies (Ortuño et al., 2007).

Transmission of rickettsiae might also occur among infected and susceptible ticks feeding, simultaneously, in close proximity on the same host's skin (transmission via co-feeding). This mechanism may enable the transmission of a pathogen even between ticks feeding on a non-competent host (Zemtsova et al., 2010). The transmission of *R. slovaca* from infected male ticks to non infected females has also been demonstrated (Socolovschi et al., 2009).

In this study, we carried out PCR analysis for the detection of *R. slovaca* in small rodents and ticks, in an area of the province of Lucca (Tuscany, Italy), where *D. marginatus* was previously shown as the dominant tick species on *Apodemus* spp. and *M. glareolus* (Mannelli et al., 1997). TIBOLA cases were reported in the same province, and *R. slovaca* was detected in *D. marginatus* removed from humans (Selmi et al., 2008), as well as in *D. marginatus* and tissues from wild boars (Selmi et al., 2009). The objectives of the present study included the identification of a focus of *R. slovaca* transmission involving immature *D. marginatus* and wild rodents, and gathering information on the role of these animals in the agent's maintenance.

Materials and methods

Small rodents capture and tick collection.

Small rodents trapping was carried out from June to September 2009 and 2010, on the Tuscan side of the Tuscan-Emilian Apennine National Park, province of Lucca, Italy (44° 12' N, 10° 22' E). Three trapping sites were chosen, within 2 km from the park premises, based upon convenience of access, and at locations where rodents were previously captured and found infested by *D. marginatus* (Mannelli et al., 1997). Site A, at 1220 m above the sea level (a.s.l.), was characterized by a mixed wood with common hazels (*Corylus avellana*), Turkey oaks (*Quercus cerris*), wild apples (*Malus sylvestris*), and shrubs (*Spartium junceum*, *Rosa canina*). Site B (1140 m a.s.l.) was dominated by oaks, whereas site C (1185 m a.s.l.) was characterized by mixed wood with a predominance of black alders (*Alnus glutinosa*), and including sycamore maples (*Acer pseudoplatanus*), silver firs (*Abies alba*) and European ashes (*Fraxinus excelsior*). Sherman live traps (230x80x90 mm, Sherman Live Traps Co., Tallahassee, FL) and Ugglan live traps (240×60x90 mm, Grahnab, Sweden) were baited with cereals and apples, provided with cotton during cold weather, and set 10 m apart in a 3x10 grid in each capture site (30 traps per site), for a total of 1980 trap nights.

Captured rodents were anesthetized with a mixture of medetomidine and ketamine, as described in Amore et al. (2007). They were individually identified in 2009 by ear punch, and in 2010 by a microchip (transponder AEG ID162 ISO, AEG, Germany) which was subcutaneously injected. Biometric data were also recorded: species, sex, and age class (sub-adult, adult). Biopsies were taken by cutting a little triangular

fragment from the outer margin of a ear and stored in 70% ethanol. Each processed animal was carefully screened for the presence of ticks on the entire body. Any attached tick was removed and stored in 70% ethanol. After examination, anesthetized mice were injected with 5 μg atipamezole HCl (Antisedan®, Pfizer Animal Health, Rome, Italy) to reverse the effects of medetomidine, and were released at capture site after they had completely recovered from the anaesthesia. Animal capture and tissue sampling were approved by the Bioethics Commission of the University of Turin. Ticks were identified by species by using keys from Manilla (1998). The tick engorgement index (TEI) was evaluated as the ratio of body length to scutum width (Yeh et al., 1995).

Molecular analyses.

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), from ear biopsies from small rodents that were infested by *D. marginatus*, and from a sample of the ticks found attached. Ticks were selected for molecular analysis based on the following criteria: two larvae, if present, from each rodent, and all of the attached nymphs. When more than two larvae were found from an individual rodent, the less, and the more engorged ones (as evaluated by TEI) were analysed, with the objective of estimating the effect of engorgement on *R. slovaca* infection for larvae collected on the same animals. Moreover, to estimate prevalence of *R. slovaca* resulting from transovarial transmission, DNA was extracted from questing *D. marginatus* larvae, which were collected by dragging in the study area in 2010 and 2011 (Ragagli et al., in preparation). A negative control every five samples (distilled water) was added to verify the potential contamination of samples during this phase.

We investigated the presence of *Rickettsia* spp. DNA in our samples by a PCR assay targeting the citrate synthase gene (gltA, 401-bp product; Labruna et al., 2004). Positive samples were further tested to detect the ompA gene, specific for SFG rickettsiae, using Rr190.70F and Rr190.602R primers (530-bp product; Regnery et al., 1991). In all the PCR reactions, 2.5 μl of DNA sample was tested. Distilled water was used as negative control to determine any possible cross-reactivity or contamination; one negative control every five field samples was put into each PCR run. DNA from a *Rickettsia conorii* isolate was used as positive control. The efficiency of the extraction protocol was verified: for PCR-negative ticks, a 16S rDNA PCR

specific for mitochondrial sequences of hard and soft ticks was carried out (d'Oliveira et al., 1997); while a 383-bp fragment of the cytB gene was amplified (Lee et al., 2002) to verify the presence of vertebrate DNA from each ear biopsy extract.

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 and therefore to classify pathogens at species level.

Statistical analysis.

Mean rodent weight (grams) and standard deviation (sd) were calculated. Prevalence of infection by *R. slovaca*, and 95% exact binomial confidence interval (95%CI) were calculated in mouse ear biopsies, and in *D. marginatus* immatures, by using the *binom.test* function in the R software (R Development Core Team 2012). Prevalence of infestation of rodents by *D. marginatus* was also calculated. Data from recapture of the same rodents during one trapping session were excluded from the analysis. Frequency distributions, median, first (Q_1) and third (Q_3) quartiles of numbers of attached immature *D. marginatus* were separately obtained for mice which resulted positive or negative to PCR for the detection of *R. slovaca* in ear tissues. Median (Q_1, Q_3) TEI were calculated for *R. slovaca*-positive and negative host-attached larvae. The two-sample Wilcoxon rank sum test (*wilcox.test* function) was used to compare medians. Prevalence of infection by *R. slovaca* in *D. marginatus* larvae which were collected from *R. slovaca*-positive and negative *Apodemus* spp. was compared by a generalised estimating equation (GEE) taking into account non independence among outcomes of laboratory test carried out on ticks collected from the same mouse (*gee* function) (Diggle et al., 2002).

Results.

Rodent trapping, during 1980 trap-nights, yielded 82 *Apodemus* mice plus 49 recaptures in different trapping sessions, and 24 bank voles (*Myodes glareolus*) plus 9 recaptures, which were infested by a total of 297 *D. marginatus* larvae and 69 nymphs. It was not possible to distinguish between the wood mouse *Apodemus sylvaticus* and the yellow-necked mouse *Apodemus flavicollis* (Barciova and Macholán 2009) due to morphological similarities. Captured *Apodemus* spp. included 76 males (32.9% sub-adults), and 55 females (36.4 % sub-adults); *M. glareolus* included 21 males (19.0% sub-adults), and 12 females (16.7% sub-adults). The age class of five *Apodemus* spp. and one *M. glareolus* individuals could not be determined. The mean (sd) weight for *Apodemus* spp. was 24.4 grams (5.4) in males and 23. 7g (5.6) in females. *M. glareolus* males weighted 27.1g (4.7), while females 26.1g (5.7).

Prevalence of infestation by *D. marginatus* was 30.5% (n=131, 95%CI: 22.8, 39.2) in *Apodemus* spp., and 27.3% (n=33, 95%CI: 13.3, 45.5) n *M. glareolus*. As regards rodent tissues, ear biopsies from three infested *Apodemus* spp. were not available. Two of the tested ear biopsies were from one *M. glareolus* which was found infested by *D. marginatus* in two different trapping sessions. *R. slovaca* was detected in *Apodemus* mice ear biopsies (n tested=37; prevalence=21.6%; 95%CI: 9.8, 38.2), but not from bank voles (n=9; 0.0%; 95%CI: 0.0, 33.6). All of the positive biopsies were from adult male *Apodemus* spp., which were also characterized by relatively great levels of infestation by *D. marginatus* larvae. Up to 102 larvae were collected from a *R. slovaca*-positive mouse in July 2009 (Figure 1, A). Accordingly, the median number of *D. marginatus* larvae, on *Apodemus* spp. infested by at least one immature *D. marginatus*, was significantly greater for positive (median=8.5; Q_1=1.75, Q_3=11.5), than for negative mice (median=1.0; Q_1=0.0, Q_3=4.0) ($P<0.05$). On the other hand, certain *R. slovaca*-negative *Apodemus* spp. were infested by numerous *D. marginatus*, and up to 51 larvae were found on one of these individuals (Figure 1, A). Counts of *D. marginatus* nymphs were greater on *R. slovaca*-negative, than on positive mice (Figure 1, B).

R. slovaca was detected in 52.9% of larvae (n=51, 95%CI: 38.5, 67.1), and in 47.5 % of nymphs (n=59, 95% CI: 34.3, 60.9) collected from *Apodemus* spp. Prevalence of *R. slovaca* in host-seeking *D. marginatus* larvae was 42.1% (n=38; 95%CI: 26.3, 59.2).

Eleven out of 14 (78.6%) larvae feeding on PCR-positive *Apodemus* spp. were positive for *R. slovaca*, whereas 13 out 35 (37.1%) larvae from PCR-negative mice were positive, and such a difference was statistically significant (GEE, P<0.05). Engorgement status, as evaluated by TEI, was greater in *R. slovaca*-positive (median=2.9; Q1=2.5, Q3=3.3) than in negative (median= 2.5; Q1= 1.8, Q3=3.1) larvae, but this difference was not statistically significant (P= 0.24).

R. slovaca was not detected in eight larvae collected from *M. glareolus* (prevalence=0.0%, 95%CI:0, 36.9), whereas it was found in one out of ten nymphs from the same host species (10.0%, 95%CI: 0.3, 44.5).

Sequence analysis of the *R. slovaca* ompA gene showed the identity of our specimens, derived from mice tissues (GenBank accession no.: KC700048), and from *D. marginatus* ticks collected on rodents (KC700049, KC700050, KC700051) and vegetation (KC700052). Sequences were 100% similar to GenBank *R. slovaca* ompA sequences originating from Emilia Romagna region in Italy, Slovakia and Turkey (JQ691724, CP002428, HM161798).

Two nymphs from *Apodemus* spp. were infected by *R. raoultii*. The two ompA sequences were identical (GenBank accession no. KC700054) and 100% similar to reference sequences from Emilia Romagna, Hungary, Slovakia and Turkey (HM161794, JQ798907, JN398480, JQ691731).

Discussion.

At our study location, *R. slovaca* was found, by PCR, in immature *D. marginatus* from small rodents and ear tissues collected from *Apodemus* spp. The infection was not found testing a limited number of tissues from *M. glareolus*.

D. marginatus larvae were most likely to be *R. slovaca*-positive when collected from *Apodemus* spp. which were positive for the same agent. The infection of mice was probably the result of repeated bites by transovarially-infected larvae. However, some of the tested mice were PCR-negative in spite of relatively high levels of infestation. Given the prevalence of infection detected in host-seeking larvae and the infestation of mice, larvae might play a central role in the transmission dynamics of *R. slovaca* in our study area.

Based on our study, no conclusion can be drawn on the transmission of *R. slovaca* from small rodents to feeding *D. marginatus*. Nevertheless, the finding of *R. slovaca* in mice tissues would be compatible with such a process (Rehácek et al., 1976; Rehácek et al., 1992). Accordingly, *Apodemus* spp. might play a role as amplifiers of the infection, and contribute to the perpetuation of *R. slovaca*.

The aggregation of *D. marginatus* larvae on certain *Apodemus* spp. individuals, which were also characterised by the highest prevalence of *R. slovaca*, might favour the transmission of the agent. This is in agreement with the hypothesis that a small fraction of individuals in the population of hosts are responsible for the majority of transmission events and are, therefore, critical for the maintenance of an infectious agent (Woolhouse et al., 1997). Even in the absence of the hosts' systemic infection, tick aggregation might favour the transmission of *R. slovaca* between transovarially infected, and uninfected larvae co-feeding on the same individuals.

The transmission of the infection to feeding ticks, either via the host's systemic infection, or via co-feeding, might be particularly important in case *R. slovaca* had a negative effect on the vector's survival and reproduction (as it was demonstrated for *R. rickettsii*) and it might compensate for the loss of infected tick lines (Walker and Fishbein, 1991).

The finding of *R.raoultii* confirms the presence of this agent, responsible of a less severe form of TIBOLA, in Lucca province, as reported in a previous study (Selmi et al., 2009).

The simultaneous presence of *Apodemus* spp. and abundant *D. marginatus*, together with abundant wild boars serving as hosts for adult ticks, may create favourable conditions for the occurrence of *R. slovaca* foci. Factors associated with rodent infestation by ticks and infection by *R. slovaca* at our study location should be further investigated.

Conflict of interest

No competing financial interests exist.

Acknowledgements

We thank all personnel of the Corpo Forestale dello Stato, Ufficio Territoriale per la Biodiversità, Lucca, for providing accommodation, animal handling facilities, and valuable help. Michela Adami, Donal Bisanzio, Leonardo A. Ceballos, and Maurizio Pieroni, helped with fieldwork. This study was funded by the Fondazione Cassa di Risparmio di Lucca.

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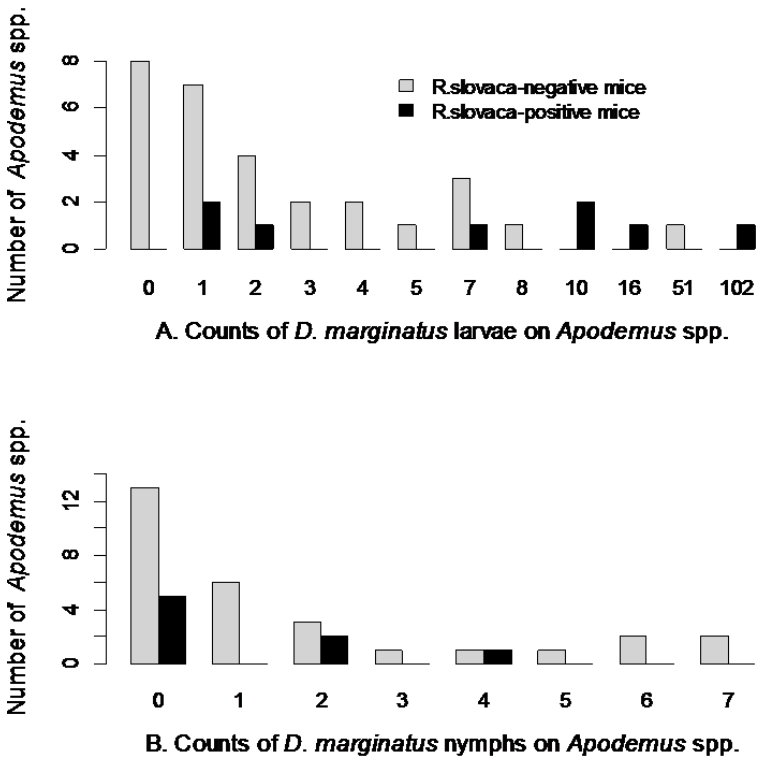

Fig. 1. Frequency distribution of immature *Dermacentor marginatus* (A: larvae; B: nymphs) on *Apodemus* spp., in the Northern Apennines, Italy, from June through September, in 2009 and 2010. The included *Apodemus* spp. were found infested by at least one *D. marginatus*, and were tested for *R. slovaca* infection by PCR on ear biopsies.