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Rickettsia slovaca and Rickettsia raoultii in Dermacentor marginatus Ticks Collected on Wild Boars in Tuscany, Italy

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ABSTRACT
During the hunting season 2007-2008, 494 *Dermacentor marginatus* (Sulzer) ticks were collected from 109 hunter-killed wild boars, Sus scrofa, in Lucca's province, Tuscany, Italy. *Rickettsia slovaca*, the causative agent of tick-borne lymphadenopathy (TIBOLA), was detected in 32.1% of ticks tested (n = 112) by using polymerase chain reaction primers targeting gltA, ompA, and ompB rickettsial genes. Moreover, *Rickettsia raoultii* was found for the first time in Italy, with 1.8% infection prevalence. This study confirms the risk posed to humans by ticks and tick-borne pathogens in the study area, where cases of spotted fever rickettsiosis (TIBOLA) are reported.

KEY WORDS
Rickettsia slovaca, *Rickettsia raoultii*, *Dermacentor marginatus*, wild boars

INTRODUCTION

Spotted fever group (SFG) rickettsiae are gram-negative obligate intracellular bacteria responsible for emerging tick-borne diseases (Parola et al. 2005). They exist in endemic and enzootic foci and can occasionally cause sporadic or seasonal outbreaks (Azad and Beard 1998). The rickettsial life cycle involves vertebrate and invertebrate hosts. Ixodid ticks maintain rickettsiae in nature through transstadial and transovarial transmission, and the pathogens' distributions correspond with those of the tick vectors (Parola and Raoult 2001). In Italy, rickettsial agents have been detected in different tick species, but human rickettsioses were traditionally considered to be caused by *Rickettsia conorii*, the agent of Mediterranean spotted fever (MSF), and transmitted by the brown dog tick, Rhipicephalus sanguineus (Latreille). Recently, the tickborne zoonoses surveillance system established at the Lucca local health unit (ASL 2) indicated the emerging role of *Rickettsia slovaca* as a human pathogen in Tuscany. In fact, a low incidence of MSF cases has been reported in the study area, whereas an increasing number of patients showed symptoms of tick-borne lymphadenopathy (TIBOLA), and *Rickettsia slovaca* was detected in *Dermacentor marginatus* (Sulzer) ticks collected from the same patients (Selmi et al. 2008).
D. marginatus and *D. reticulatus* have now been implicated as both vectors and reservoirs of *R. slovaca* by detection or isolation in many European countries (Sekeyova et al. 1998, Ortuno et al. 2007). *D. marginatus* normally feeds on ungulates, wild boars in particular, and is considered to be an anthropophilic tick (Cringoli et al. 2005). Studies on *D. marginatus* removed from wild boars in southern Europe revealed an infection prevalence by *R. slovaca* ranging from 15.7% in France (Sanogo et al. 2003) to 30.5% in northeastern Spain (Ortuno et

al. 2007). In Tuscany, this tick species is widespread in hardwood forests, where wild boars are abundantly distributed. *D. marginatus* can occasionally bite humans frequenting these habitats for agricultural and recreational purposes; however, their infection prevalence by SFG rickettsiae in the area is unknown.
To better understand the distribution of tick-borne pathogens in Tuscany, we collected *D. marginatus* ticks from hunter-killed wild boars, *Sus scrofa*, during the 2007-2008 hunting season and tested them by polymerase chain reaction (PCR) for the presence of Rickettsia spp.

Materials and Methods

Sample Collection

Sixteen hunting districts located in the province of Lucca, northwestern Tuscany, were included in the investigation. The study area limits are 44° 17.210' N, 10° 18.024' E northward, 43° 58.548' N, 10° 8.762' E westward, 44° 4.966' N, 10° 43.215' E eastward, and 43° 45.461' N, 10° 37.032' E southward. Altitude of the study sites ranges from 10 to 1,707 m above sea level. Vegetation is mainly hardwood forests, with a predominance of sweet chestnut, Castanea sativa Mill., trees; oak (Quercus spp.) trees; and maritime pine, Pinus pinaster Aiton, trees.
European wild boars hunted from November 2007 to January 2008 were rapidly examined when brought from hunters to hunting lodges at the end of each daily expedition. A sample of ticks was collected from each animal at different body sites and stored in 70% ethanol. When possible, skin biopsies at the tick attachment site were taken from the killed animals. Stereoscopic microscope and taxonomic keys (Manilla 1998, Cringoli et al. 2005) were used for tick identification.

Molecular Analyses

The tick sample was chosen to test at least one tick per infested wild boar. Ticks were randomly selected and individually homogenized with a pestle in microcentrifuge tubes. DNA was extracted from ticks and from skin biopsies using the DNeasy blood and tissue kit (QIAGEN GmbH, Hilden, Germany). Negative controls (distilled water) were used to check for sample contamination during this phase. Success of DNA extraction was verified using PCR targeting the 16s ribosomal RNA coding region of tick mitochondrial DNA (d'Oliveira et al. 1997). To identify SFG rickettsiae, a fragment of the citrate synthase gene (gltA, 380-bp product), which is common to the whole Rickettsia genus (Regnery et al. 1991), was amplified. Subsequently, ticks that were positive with this first PCR were tested by a second assay with primers targeting the outer membrane protein gene (ompA, 530-bp product), allowing the differentiation of closely related strains of SFG rickettsiae (Weller et al. 1998). To better characterize the bacterial strains, a 856-bp fragment of the ompB gene (Roux and Raoult 2000) also was amplified from positive samples. *Rickettsia conorii* DNA was used as a positive control and water as a negative control in all PCR assays. PCR products were purified and DNA sequencing was performed as described previously (Bertolotti et al. 2006). Direct nucleotide sequences of positive ompA and ompB samples were obtained and compared with reference sequences from GenBank.

Statistical Analysis

Prevalence of tick infestation on wild boars and prevalence of Rickettsia spp. in ticks were calculated, with 95% exact binomial confidence intervals (95% CI). Analyses were performed by R software (R Development Core Team 2008).

Results

In total, 163 hunter-killed wild boars from hunting districts in Lucca's province were examined for tick presence, and 66.9% were found infested with *D. marginatus* ticks (n = 109; 95% CI, 59.1-74.0). Overall, 494 *D. marginatus* were collected from the animals, 112 of which (88 females, 24 males) were tested for the presence of Rickettsia spp.
Thirty-eight *D. marginatus* resulted positive to gltA, ompA, and ompB PCRs, showing a SFG rickettsiae infection prevalence of 33.9% (95% CI, 25.2-43.5). Positive ticks were collected from 38 wild boars (34.8% of tested animals). Sequence analysis identified *R. slovaca* in 36 samples (32.1%; 95% CI, 23.6 41.6), whereas *R. raoultii* was detected in two ticks (1.8%; 95% CI, 0.2-6.3). Partial gene sequences showed a similarity of 100% compared with reference sequences in GenBank (R. slovaca ompA U43808.1, ompB AF123723; *R. raoultii* ompA: DQ365799.1, ompB: DQ365797) and to *R. slovaca* ompA gene sequences obtained by *D. marginatus* feeding on humans in Lucca province (Selmi et al. 2008).
Nine skin biopsies were removed from wild boars. One of the tissues was positive to SFG rickettsiae (11.1% prevalence; 95% CI, 0.3-48.2) and sequence analyses identified the *R. slovaca* ompA gene (GenBank reference sequence U43808.1, 100% nucleotide similarity). A *D. marginatus* positive to *R. slovaca* was collected from the site of the biopsy.

Discussion

The epidemiology of most tick-borne rickettsiae is not completely known, especially their sylvatic cycles, and the role of vertebrate hosts in the bacteria maintenance is unclear. Vertebrates can serve as hosts for the tick vector, supporting its life cycle and also may play a role in infecting new tick generations with SFG rickettsiae. Few animals were shown to have a rickettsemia long enough to allow the infection of feeding arthropods. Studies on *R. rickettsii* revealed sufficient blood levels of the bacteria in ground-living small mammals, particularly rodents, which are thus suspected to be the principal vertebrate reservoir of SFG rickettsiae (Rehacek 1989).
A possible role of wild boars in the eco-epidemiology of *R. slovaca* was suggested by Ortuno et al. (2007), who reported a 52.2% infection seroprevalence in wild boars. In our study area, 33.0% of sampled wild boars were feeding ticks infected by *R. slovaca*, and the finding of a positive skin biopsy shows their possible infection with the pathogen. However, whether wild boars are able to develop a sufficient rickettsemia to infect feeding ticks or simply act as hosts for infected vectors remains unclear. The *R. slovaca* infection prevalence in wild boars ticks found in this study is very close to the one detected in *D. marginatus* adults removed from patients admitted to emergency units in Lucca province (35.5%; Selmi et al. 2008). Moreover, preliminary results show similar *R. slovaca* infection prevalence in host-seeking *D. marginatus* adults collected by dragging in the same study area (36.4%; data not shown). These data suggest that *D. marginatus* become infected by *R. slovaca* before feeding at the adult stage, either by transovarial route or by feeding, at an immature stage, on a reservoir host. The fact that *D. marginatus* immature stages are nidicolous and feed on small rodents (Dorr and Gothe 2001) indicates the possible implication of small mammals in *R. slovaca* transmission.
R. raoultii was detected in 1.8% of the sampled ticks; this is a first report in Italy and extends its known geographic distribution in Europe. This species was previously detected in *D. marginatus* removed from wild boars in northeastern Spain (Ortuno et al. 2006, 2007) and in *D. marginatus* and *D. reticulatus* ticks collected from vegetation, domestic animals and deer (Shpynov et al. 2001, 2004; Dautel et al. 2006; Stanczak 2006; Nijhof et al. 2007, Vitorino et al. 2007; Sarih et al. 2008). *R. raoultii* was recently described as a new species; in fact, its isolates were identified with different names (DnS14, DnS28, and RpA4) from Dermacentor spp. in several European countries (Mediannikov et al. 2008). Its pathogenicity is unknown, but it was identified in 2002 from a *D. marginatus* specimen obtained from a patient with symptoms

of tick-borne lymphadenopathy in France (Mediannikov et al. 2008) and, in 2006, Ibarra and colleagues reported it in a serum sample and in two *D. marginatus* ticks removed from TIBOLA patients in Spain. Although the risk of developing the illness was estimated as greater in persons bitten by ticks infected by *R. slovaca* (Ibarra et al. 2006), these findings suggest a role for *R. raoultii* in the pathogenesis of TIBOLA.

In conclusion, tick-borne rickettsiae seem to be maintained in forested areas of Lucca province, which are inhabited by *D. marginatus* and wild ungulates. Although the role of wild boars in SFG rickettsiae life cycle is not clear, these animals are important in supporting the *D. marginatus* life cycle by feeding adult ticks and enable the transovarial transmission of rickettsiae to the next tick generations. Our findings confirm the presence of potentially pathogenic tick-borne rickettsiae in the study area and should be considered by physicians treating patients showing symptoms suggestive of TIBOLA and reporting a history of tick exposure.

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