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Original article

Spotted fever group rickettsiae detected in immature stages of ticks parasitizing on Iberian endemic lizard *Lacerta schreiberi* Bedriaga, 1878

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ABSTRACT

Spotted fever rickettsioses are tick-borne diseases of growing public health concern. The prevalence of rickettsia-infected ticks and their ability to parasitize humans significantly influence the risk of human infection. Altogether 466 *Ixodes ricinus* ticks (428 nymphs and 38 larvae) collected from 73 *Lacerta schreiberi* lizards were examined by PCR targeting the citrate synthetase gene *gltA* for the presence of *Rickettsia* spp. Rickettsial DNA was detected in 47% of nymphs and 31.6% of larvae. They were subsequently subjected to a second PCR reaction using primers derived from the outer membrane protein rOmpA encoding gene (*ompA*) to detect spotted fever group rickettsiae (SFG). This analysis shows that 41.4% of nymphs and 7.9% of larvae collected from the lizards contain DNA of SFG rickettsiae. Sequencing of 43 randomly selected samples revealed two different haplotypes, both closely related to *R. monacensis* (39 and 4 samples, respectively). The remaining *ompA* negative *Rickettsia* spp. samples were determined to be *R. helvetica* based on sequencing of *ompB* and *gltA* fragments. Our results indicate that the role of Iberian endemic lizard *L. schreiberi* and its ectoparasites in the ecology and epidemiology of zoonotic SFG rickettsioses may be appreciable.

1. Introduction

Rickettsial diseases are an emerging public health concern. Infections are caused by obligate intracellular Gram-negative bacteria of the genus *Rickettsia* occurring worldwide. Ixodid ticks play a key role in transmission of the spotted fever group (SFG) rickettsiae, which are maintained in tick populations through transovarial and transstadial passage (Azad and Beard, 1998). At least 19 validated species of SFG rickettsiae are associated with human infections (Parola et al., 2013), and new *Rickettsia* species with unknown pathogenicity are reported regularly. The most frequent symptoms of spotted fever rickettsioses are fever, rash, local lymphadenopathy and cutaneous eschar at the side of tick-bite (Brouqui et al., 2007). The *Ixodes ricinus* tick is a predominant vector of a large variety of pathogens in Europe (Heyman et al., 2010). This typical three-host tick species has been considered as the most anthropophilic and therefore the most potentially human health-threatening tick species in north-western Spain (Fernández-Soto et al., 2004). Moreover, *I. ricinus* was previously described as the dominant vector of two species of SFG rickettsiae – *Rickettsia monacensis* and *Rickettsia helvetica*, on the Iberian Peninsula (Márquez, 2008; Milhano et al., 2010). Both of these rickettsiae were considered as non-pathogenic, but recently they were identified as causative agents of human rickettsioses. *R. helvetica* commonly causes flu-like febrile illness (Fournier et al., 2000) but has also been associated with the death of two young tourists from Sweden (Nilsson et al., 1999). To date two cases of *R. monacensis*-associated human disease manifested as common acute SFG rickettsiosis have been reported from Spain (Jado et al., 2007). Our survey aimed to evaluate prevalence of SFG rickettsiae in immature stages of *I. ricinus* ticks collected from the endemic Iberian lizard, *Lacerta schreiberi*. Samples were obtained during a non-invasive field study

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(Stuart-Fox et al., 2009), and the results offer new data on ecology and epidemiology of zoonotic rickettsioses in Iberian Peninsula.

2. Material and methods

2.1. Collection of ticks

Iberian endemic lizards – *L. schreiberi* Bedriaga, 1878 were captured from a hybrid zone in the Central System Mountains of the Iberian Peninsula during April and May 2006 and May 2007. Lizards carried two types of ectoparasites – ticks (*I. ricinus*) and mites (*Ophionyssus schreibericolus*) (Moraza et al., 2009). The number of ticks was counted on each individual and a sub-sample collected to check their developmental stages. The majority were nymphs (73%) and the remaining ticks were at the larval stage (27%). The total number of ticks (irrespective of developmental stage) per individual was determined as a measure of relative ectoparasite load (mean = 12; range = 0–81) (for details see Stuart-Fox et al., 2009). Ticks were collected using tweezers, put immediately into plastic vials filled with 70% ethanol, and stored at room temperature until laboratory treatment. Tick species and life-stage determination was done using key by Estrada-Peña et al. (2004). Altogether 466 immature stages (38 larvae and 428 nymphs) of ticks *I. ricinus* sampled from 73 *L. schreiberi* were randomly selected for further PCR analysis.

2.2. DNA isolation

DNA was extracted from ticks using alkaline hydrolysis (Rijpkema et al., 1996; following the modified protocol as described in Kubelová et al., 2011). The concentration of isolated dsDNA was checked using Micro-Volume Spectrophotometer ASP-3700 (Avans Biotechnology Corp., Taipei City, Taiwan). Samples were stored at −20 °C.

2.3. PCR detection and identification of SFG rickettsiae

Rickettsial DNA was detected by PCR reaction with universal primers RpCS.877p (5′-GGG GGC CTG CTC ACG GCG G-3′) and RpCS.1258n (5′-AAT GCA AAA AGT ACA GTG AAC A-3′) amplifying the citrate synthetase gene *gltA* (Regnery et al., 1991). Reaction mixture was incubated at 95 °C for 7 min, followed by 35 cycles at 95 °C for 30 s, 48 °C for 30 s and 65 °C for 2 min. The final extension step lasted 7 min at 72 °C. Positive samples were visualised on 1.2% agarose gel with ethidium bromide under UV light as 382 bp long bands. All 213 positive samples were screened with a further PCR reaction amplifying a 489 bp fragment of the *ompA* gene to detect SFG rickettsiae using the SLO1F (5′-CAC CAC CTC AAC CGC AG-3′) and SLO1R (5′-GCC GGG GCT GCA GAT TG-3′) primer set (Raoult et al., 2002). This reaction was carried out under the following conditions: an initial denaturation step at 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 5 min (Stańczak, 2006). For both PCR reactions, the mixture in 25 μl total volume contained: 2 μl of DNA template, 10 pmol of each primer (Integrated DNA Technologies, Belgium), 0.625 Unit Taq Purple DNA Polymerase in 12.5 μl of PCR master mix (Combi PPP Master Mix, Top-Bio s.r.o. Prague, Czech Republic) and 8.5 μl of PCR water (Top-Bio s.r.o. Prague, Czech Republic). To confirm our results, randomly selected positive samples (n = 43, all nymphs) were purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taipei, Taiwan) and sequenced (Macrogen, Amsterdam, Netherlands). The two obtained *ompA* haplotypes were deposited in GenBank database under the accession numbers KF768801 and KF768802.

Furthermore, in two representatives of each of the two *ompA* haplotypes, a 475 bp fragment of *ompB* gene was amplified and sequenced using Rc.rompB.4362p (5′-GTC AGC GTT ACT TCT TCG ATG C-3′) and Rc.rompB4836n (5′-CCG TAC TCC ATC TTA GCA TCA G-3′) primer set under the conditions in Choi et al. (2005). In the same four samples, the *gltA* fragment was also sequenced. Obtained sequences were deposited to the NCBI GenBank database under the accession numbers KP283015 and KP283016, respectively, and compared with sequences from GenBank database using BLAST tool (http://blast.ncbi.nlm.nih.gov/).

Table 1
The GenBank accession numbers of the *ompA* sequences included in the phylogenetic analyses.

Organism	Acc. number	References
Rickettsia aeschlimannii	HQ335159	Abdel-Shafy et al. (2012)
Rickettsia africae	U43790	Roux et al. (1996)
Rickettsia amblyommi	JQ690647	Mukherjee et al. (unpublished)
Rickettsia australis	AF149108	Stenos and Walker (2000)
Rickettsia canadensis	CP000409	Madan et al. (unpublished)
Rickettsia conorii	U43791	Roux et al. (1996)
Rickettsia heilongjiangensis	AY280711	Mediannikov et al. (2004)
Rickettsia honei	AF018075	Stenos et al. (1998)
Rickettsia IRS3	AF141909	Sekeyová et al. (2000)
Rickettsia IRS4	AF141911	Sekeyová et al. (2000)
Rickettsia japonica	U43795	Roux et al. (1996)
Rickettsia massiliae	U43793	Roux et al. (1996)
Rickettsia monacensis	FJ919640	Corrain et al. (2012)
Rickettsia monacensis	KF768801	This study
Rickettsia monacensis	KF768802	This study
Rickettsia montana	U43801	Roux et al. (1996)
Rickettsia parkeri	U43802	Roux et al. (1996)
Rickettsia raoultii	JQ792153	Wang et al. (2012)
Rickettsia rhipicephali	U43803	Roux et al. (1996)
Rickettsia rickettsii	U43804	Roux et al. (1996)
Rickettsia sibirica	U43807	Roux et al. (1996)
Rickettsia slovaca	U43808	Roux et al. (1996)
Rickettsia tamurae	DQ103259	Fournier et al. (2006)

2.4. Phylogenetic analyses of the ompA gene in SFG rickettsiae

Obtained *ompA* haplotypes together with additional sequences of SFG rickettsiae species from GenBank (see Table 1) were aligned in MEGA version 5.05 (Tamura et al., 2011). Bayesian inference analysis (BI) was carried out in the MrBayes 3.1.2. program with a GTR + Γ + I model for 10 million iterations (Ronquist and Huelsenbeck, 2003). Chain convergence and burn-in were estimated according to the indices implemented in the MrBayes program (deviation of split frequencies, potential scale reduction factor – PSRF) and using the Tracer program (Rambaut and Drummond, 2007). The trees were summarized after removing burn-in (700 trees). Maximum likelihood analysis (ML) was performed in PHYML 2.4.4. (Guindon and Gascuel, 2003), with the GTR + Γ + I model and parameters estimated from the data; bootstrap values were calculated for 1000 replicates. Resulting trees including *Rickettsia canadensis* as an outgroup were visualized using TreeGraph 2.0.56 (Stöver and Müller, 2010).

2.5. Identification of ompA-negative rickettsiae

In seven representatives of the *gltA*-positive, but *ompA*-negative samples, fragments of *ompB* and *gltA* genes were amplified and sequenced, as above. Obtained sequences were deposited in GenBank database under the accession numbers KP283017 and KP283018, respectively, and compared with sequences from GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/).

3. Results and discussion

From a total sample of 466 *I. ricinus* ticks, 47% of nymphs (201/428) and 31.6% of larvae (12/38) were found *Rickettsia* (*gltA*)

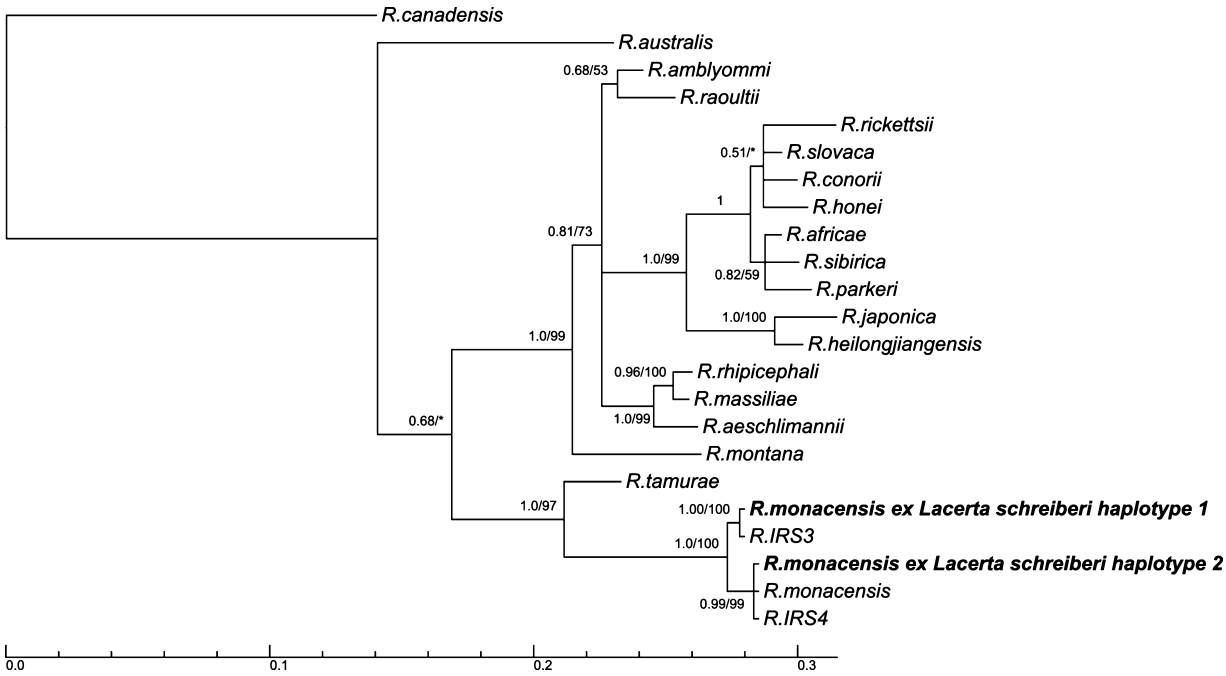

Fig. 1. Phylogenetic tree of SFG rickettsiae as revealed by Bayesian analysis based on 455 bp long alignment of *ompA* gene. Branch lengths indicate expected numbers of substitutions per nucleotide site. Numbers along branches indicate Bayesian posterior probabilities/percent bootstrap values as obtained by ML analysis. * denotes bootstrap support lower than 50 percent. Haplotypes identified in this study are highlighted in bold.

positive. From those *Rickettsia* positive samples, 177 nymphs were also positive for the *ompA* gene (88.1% of the *Rickettsia* positive nymphs; 41.4% of all tested nymphs). From the 12 *Rickettsia* positive larvae, 3 were also positive for the *ompA* gene (7.9% of all tested larvae). We obtained two different *ompA* haplotypes (differing only in four nucleotides) from the 43 sequenced samples. The two haplotypes have been identified in 39 and 4 samples, respectively. BI and ML provided phylogenetic trees with identical topologies. In the phylogenetic analysis, haplotype 1 shows 100% identity to "*R. IRS3*", and haplotype 2 shows 100% identity to *R. monacensis* and "*R. IRS4*" (Fig. 1). However, further sequencing of *ompB* and *gltA* fragments in representatives of both haplotypes provided us with identical sequences for both haplotypes, which were in the case of both genes identical with sequences of *R. monacensis* from GenBank (JX625150 and JX040639, respectively). Thus, we consider both our haplotypes to be identified as *R. monacensis*.

Furthermore, we also tried to ascertain identity of the *ompA*-negative rickettsiae. Sequencing of *ompB* and *gltA* fragments resulted in identical sequences in all tested samples, which were, in the case of both genes, identical with available sequences of *R. helvetica* in GenBank (HQ232245 and KF447530, respectively).

Although SFG rickettsioses have been reported from Iberian Peninsula, none of those studies have considered the potential for reptiles and their ticks to play a role in transmission of rickettsial bacteria. High infection rates of *Rickettsia* spp. in adult *I. ricinus* ticks collected by flagging were described in south-eastern Spain (S) and southern Portugal (P) (29.73% and 55.1% respectively). Sequencing revealed that *R. monacensis* (S, P prevalences 27.0% and 51.7%) and *R. helvetica* (S, P 2.7% and 48.3%) are the dominant *Rickettsia* species on the Iberian Peninsula (Márquez, 2008; Milhano et al., 2010). A long-term study performed in north-western Spain showed that altogether 31.2% of *I. ricinus* ticks found on humans carried pathogenic rickettsiae (Fernández-Soto et al., 2004). Nevertheless, rickettsial DNA in ticks collected from lizards has been previously described from some European countries. Tijsse-Klasen et al. (2010) found 19% *R. helvetica*-positive ticks from *Lacerta agilis*, Václav et al. (2011) found 4.5% rickettsia-positive *I. ricinus* nymphs, 2.7% of larvae and 7% of *Dermacentor marginatus* nymphs from *Lacerta viridis*. In comparison, we detected significantly higher prevalence of rickettsiae in *I. ricinus* from *L. schreiberi* (47% of nymphs and 31.6% of larvae). Thus, our results suggest the complexity of tick-borne disease occurrence, which depends on many factors – such as diversity of hosts in different biotopes, vegetation, climate and human influence (Silaghi et al., 2012).

A previous study demonstrated that the lizard *L. agilis* might be a reservoir host for *R. helvetica* (Tijsse-Klasen et al., 2010). According to molecular screening of ticks parasitized on *L. schreiberi*, we suggest that this Iberian endemic lizard could also play a role in the natural cycle of this *Rickettsia*. Moreover, *L. schreiberi* and its ticks may contribute to maintenance of another pathogenic rickettsia – *R. monacensis*. It should be borne in mind, however, that further research would be necessary to demonstrate that *L. schreiberi* is a competent reservoir. The importance of our findings is highlighted by the growing incidence of clinically detected cases of human rickettsioses, as we find high prevalence of rickettsial DNA in immature *I. ricinus* ticks (47% of nymphs and 31.6% of larvae), a tick with wide host range including humans (Parola et al., 2013; Vennestrøm and Jensen, 2007).

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