Exp Appl Acarol (2008) 45:185–194
DOI 10.1007/s10493-008-9181-7

Spotted fever group *Rickettsia* in ticks from southeastern Spain natural parks

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Received: 18 February 2008 / Accepted: 17 July 2008 / Published online: 2 August 2008

Abstract During an 8-years study, we collected from vegetation or domestic and wild mammals 1246 ticks (624 males, 511 females and 111 nymphs) belonging to 13 species in Jaen province (Andalusia) and we analyzed these ticks by PCR and sequencing for the presence of rickettsiae. Specific rickettsiae DNA was detected in 243 (19.5%) of the ticks tested. Sequence analysis of amplicons of *gltA*, *ompA* and *ompB* genes revealed that *Ixodes ricinus* were infected with *R. monacensis,* including strain IRS3, and *R. helvetica* (prevalences of 27.0% and 2.7%, respectively), while in *I. ventalloi* we found only this last species (12.5%). Moreover, *Dermacentor marginatus* presents *R. slovaca* (24.7%) and *R. raoultii* (59.9%). In *Rhipicephalus sanguineus* group ticks (*Rh. sanguineus, Rh. turanicus* and *Rh. pusillus*) only *R. massiliae* (15.2%) was found. *Haemaphysalis punctata* and *Ha. sulcata* were infected with a *Rickettsia* sp. near *R. hoogstraalii* (prevalence of 3.1% and 16.1%, respectively). In addition, *Ha. punctata* appeared infected *with R. monacensis*—like *Rickettsia* (1.0%) and *R. raoultii* (9.3%). None of *I. hexagonus, Hyalomma lusitanicum, Hyalomma* sp., *Ha. hispanica* or *Rh. bursa* studied ticks contained rickettsiae.

Keywords Spotted fever group *Rickettsia* · Ixodidae · Jaen · Andalusia · Spain

Introduction

Rickettsiae are gram-negative, obligate intracellular bacteria that are associated with arthropods and are able to grow only within the cytoplasm or, occasionally in the nucleus, of a variety of eukaryotic host cells. Based on the differences in etiology, serology, epidemiology, and intracellular growth characteristics, the genus *Rickettsia* has traditionally been divided into three different groups and clusters based on their molecular and antigenic

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similarities (Raoult and Roux 1997), namely, the typhus group (TG), the scrub typhus group (that includes only one species, *Orientia tsutsugamushi*), and the spotted fever group (SFG). Recently, a revision of the classification of *Rickettsia* was proposed by erecting the transitional group (TRG) as a distinct lineage that shares immediate ancestry with the members of the spotted fever group (SFG) rickettsiae, coupled with the TG and ancestral group (AG) rickettsiae (Gillespie et al. 2007, 2008). The TG comprises only two species, *R. prowazekii* and *R. typhi.* The SFG, the largest rickettsial group, contains a multitude of pathogenic and nonpathogenic antigenically related species (Sekeyova et al. 2001), whereas TRG includes *R. felis* and *R. akari*. The ancestral group also includes marginal species, such as *R. belli*, the most divergent species within the rickettsiae (Stothard et al. 1994), and *R. canadensis*. The transovarial and transtadial passage of SFG rickettsiae within tick vectors in nature ensures rickettsial survival with distribution limited to that of their tick vectors (Azad and Beard 1998; Raoult and Roux 1997; Walker and Fishbein 1991).

In the Mediterranean area, some SFG rickettsiae have been implicated in human disease and are therefore defined as the pathogenic species (Brouqui et al. 2007; Parola et al. 2005). These rickettsiae include *R. conorii,* formerly the causal agent of Mediterranean spotted fever, comprising a variety of genospecies of *R. conorii* (Zhu et al. 2005), *R. slovaca* (Sekeyova et al. 1998), implicated in development of TIBOLA-DEBONEL in humans (Lakos and Raoult 1999; Oteo et al. 2004; Raoult et al. 1997). In addition, several other species of SFG have been found initially in ticks and nowadays it is thought that they have low pathogenicity toward humans, as *R. massiliae* (Beati and Raoult 1993; Vitale et al. 2006), *R. aeschlimanni* (Beati et al. 1997; Raoult et al. 2002), *R. helvetica* (Beati et al. 1994; Fournier et al. 2000), *R. monacensis* (Jado et al. 2007; Simser et al. 2002) or *R. akari* (Radulovic et al. 1996).

The purpose of this study was to investigate, identify and characterize SFG rickettsiae in ticks (Acarina, Ixodidae) collected from environmental samples and wild and domestic host in Jaen province. Prevalence studies of infection in the vectors can be used as an indicator of a change in the intensity of *Rickettsia* transmission. However, these studies are difficult to carry out as prevalence in the vector is usually low and its estimation requires a large number of ticks to be dissected.

Material and methods

Sampling

The fieldwork was conducted in two ecologically different areas of medium altitude mountainous system surrounded by olive tree cultures from Jaen Province (Andalusia, Spain) between January 2000 to December 2007. First, Sierra of Andujar and Despeñaperros natural parks (38°16′ N, 4°6′ W and 38°23′ N, 3°32′ W), a siliceous inland formed by sandstone. Secondly, Sierra of Cazorla and Sierra Magina natural parks (38°5′ N, 2°45′ W and 37°44′ N, 3°28′ W), a calcareous inland area constituted by calcite and dolomite rocks. Both areas have a Mediterranean climate with average air temperature between 4.0–6.6°C in January and 23.3–27.7°C in July, and an altitude between 500 and 2,167 m. Rainfall varies with altitude, between 505 mm to 793 mm per year.

Ticks were collected on vegetation by dragging or removed from domestic (dogs, sheeps and goats) and wild mammals (greater white toothed shrew *Crocidura russula,* west European hedgehog *Erinaceus europaeus,* wood mouse *Apodemus sylvaticus,* wild rabbit *Oryctolagus cuniculus,* red deer *Cervus elaphus,* fallow deer *Dama dama,* mouflon *Ovis aries musimon,*

wild boar *Sus scrofa* and stone marten *Martes foina*). After collection, the ticks were immediately placed in vials with 70% ethanol, properly labeled, and were later identified in the laboratory by species, gender and stage using existing taxonomic keys (Gil-Collado et al. 1979; Iori et al. 2005; Manilla 1998; Márquez et al. 1992; Walker et al. 2000).

Molecular methods

Ticks were rinsed with distilled water, dried on sterile filter paper and then crushed in sterile Eppendorf tubes. DNA was extracted individually using the Macherey-Nagel DNA tissue Kit (Düren, Germany) according to the manufacturer's instructions. The efficiency of DNA extraction was verified in all samples by PCR assay, which amplifies 12S rRNA of tick origin as well in the cases that doubt persisted in discrimination of closely related species (i.e. between *R. sanguineus* and *R. turanicus*) using the oligonucleotides T1B and T2A (Beati and Keirans 2001) and amplification conditions described elsewhere (Bernasconi et al. 2002). Negative controls consisted of distilled water extracted in the same laboratory. Specific rickettsial sequences were detected by using PCR primers that amplify a portion of *gltA*, *ompA* and *ompB* genes, respectively (Márquez et al. 1998). Subsequent direct sequencing of amplified products was performed on selected samples in order to provide an objective and precise identification.

Positive PCR products were sequenced using PCR primers and the GenomeLab DTCS—Quick Start kit (Beckman Coulter) and a CEQ 2000XL capillary DNA sequencer (Beckman Coulter) according to the manufacturer's instructions. The resulting sequences were manually aligned and analyzed with Bioedit vers. 7.0.1. sequence analysis software (Hall 1999) to obtain consensus sequences and to align and compare other rickettsiae sequences found on GenBank, including previously sequenced and identified species of *Rickettsia*, with homologous sequences obtained directly from ticks. Sequences were identified using the BLAST feature of GenBank (http://ncbi.nlm.nih.gov/blastn) (Altschul et al. 1990). Phylogenetic relationships among sequences from tick associated rickettsiae, representatives of the established *Rickettsia* taxa, and other partially characterized *Rickettsia* isolates were analyzed using the resulting alignment into the PAUP (Phylogenetic Analysis Using Parsimony) vers. 4.0 beta 10 win software package for parsimony analysis (Center for Biodiversity, IL Natural History Survey, Champaign, IL).

Results

A total of 1,246 ixodid ticks (624 males, 511 females and 111 nymphs), representing 13 species (Table 1) were sampled in both natural and nearby urbanized areas. *Hyalomma lusitanicum* was the predominant tick species in siliceous inland, while *R. bursa* was the main species in Sierra of Cazorla and Magina. *Rh. sanguineus* was the unique species collected in urban and suburban areas.

Overall, rickettsial DNA was detected in 243 (19.5%) of the examined ticks by PCR amplifying specific fragments of *glt*a, as well as *omp*A and *omp*B genes (Table 2). The presence of rickettsiae was demonstrated in 29.73% of *I. ricinus* studied (33/111), and in 12.5% of *I. ventalloi* (2/16). Furthermore, 84.57% of *D. marginatus* (137/162), 13.4% of *Ha. punctata* (13/97) and 16.13% of *Ha. sulcata* (15/93) ticks examined were positive. In *Rhipicephalus sanguineus* group (*Rh. sanguineus, Rh. turanicus* and *Rh. pusillus*) ticks 15.25% were positive (43/282). None of *I. hexagonus, Ha. hispanica, Hy. lusitanicum,*

Table 1 Distribution by gender, stage and origin of ticks included in this study

Tick species	No. of ticks analyzed	Gender stage	Vegetation	Shrew	Wood mouse	West European hedgehog	Wild rabbit	Sheep	Goat	Red Deer	Fallow deer	Mouflon	Wild boar	Dog	Beech marten	Total per gender/ stage	Fallow deer
I. ricinus	111	M	31														31
		F	55														55
		N	18	2	5												25
I. ventalloi	16	M					2										2
		F					14										14
I. hexagonus	3	M	1														1
		F	2														2
D. marginatus	162	M	61					4					9				74
		F	80					1					7				88
Ha. punctata	97	M	31														31
		F	46														46
		N	20														20
Ha. sulcata	93	M	38														38
		F	36														36
		N	19														19
Ha. hispanica	14	M					8										8
		F					6										6
Rh. Sanguineus	228	M	20											105	2		127
		F	8											48			56
		N	3											42			45
Rh. turanicus	29	M				5		11	5								21
		F				3		3	2								8
Rh. pusillus	25	M					14										14
		F					11										11
Rh. bursa	235	M	97						8	17	2	2					126
		F	80						9	15	1	2					107
		N								2							2
Hy lusitanicum	230	M	25						11	71		4	37				148
		F	46						5	10		6	15				82
Hyalomma sp.	3	M	3														3
Total	1,246																

M = male, F = female, N = nymph

Table 2 Distribution among ticks (by gender and stage) of SFG rickettsiae identified

Tick species	Number of ticks analyzed	Number of ticks infected	No. of ticks infected/analyzed (infection rate)	Identified SFG rickettsiae	No. of positive Rickettsiae amplicons per gender							Infection rate in positive ticks	Infection rate in anlyzed ticks
					Total	M	M (%)	F	F (%)	N	N (%)		
I. ricinus	111	33	29.73	IRS3	19	2	6.45	16	29.09	1	4.00	57.58	17.12
				R. monacensis	11	1	3.23	8	14.55	*2*	8.00	33.33	9.91
				R. helvetica	3			3	5.45			9.09	2.70
I. ventalloi	16	2	12.50	IRS3	2			2	14.29			100.00	12.50
I. hexagonus	3	0	0.00										
D. marginatus	162	137	84.57	*R. slovaca*	40	18	24.32	22	25.00			29.20	24.69
				R. raoultii	97	35	47.30	62	70.45			70.80	59.88
Ha. punctata	97	13	13.40	*R. monacensis*	1			1	2.17			7.69	1.03
				R. raoultii	9	3	9.68	6	13.04			69.23	9.28
				Rickettsia sp.	3			3	6.52			23.08	3.09
Ha. sulcata	93	15	16.13	*Rickettsia* sp.	15	3	7.89	11	30.56	1	5.26	100.00	16.13
Ha. hispanica	14	0	0.00										
Rh. sanguineus	228	32	14.04	*R. massiliae*	32	17	13.39	12	21.43	3	6.67	100.00	14.04
Rh. turanicus	29	6	20.69	*R. massiliae*	6	2	9.52	4	50.00			100.00	20.69
Rh. pusillus	25	5	20.00	*R. massiliae*	5	1	7.14	4	36.36			100.00	20.00
Rh. bursa	235	0	0.00										
Hy. lusitanicum	230	0	0.00										
Hyalomma sp.	3	0	0.00										
Total	1246	243	19.50										

M = male, F = female, N = nymph

Hyalomma sp, or *Rh. bursa* studied ticks contained rickettsiae. PCR water controls and DNA extraction control were negative.

Sequence analysis based on the portion of the *glt*A, *omp*A and *omp*B genes revealed that *D. marginatus* ticks are infected with two rickettsial species. In 40 *D. marginatus* (29.2% of rickettsiae positive *Dermacentor*) a rickettsia indistinguishable from *R. slovaca* was found. Moreover, in 97 *D. marginatus* (70.8% of rickettsiae positive *Dermacentor*) and in 9 *Ha. punctata* a rickettsiae similar to *R. raoultii* (Mediannikov et al. 2008), including RpA4, candidatus *R. rioja* and others, was found. In the case of species of the genus *Ixodes,* 2 *I. ventalloi* and 19 *I. ricinus* were infected with IRS3 strain. Moreover, in 11 *I. ricinus* and one *Ha. punctata*, the sequences from a portion of the *gltA* and *omp*A genes were closely related to that of *R. monancensis.* In only three *I. ricinus* we detected *R. helvetica* (*gltA* and *ompB*). In Jaen province both *Haemaphysalis (Herpetobia) sulcata* and *Ha. punctata* appeared infected with an exclusive SFG rickettsia. The *gltA* sequence found in all positive *Ha. sulcata* and 43% of positive *Ha. punctata* (GenBank accession code EU863190) agrees (98% of similarity, 376/381 bp) with the endosymbiont of *Ha. sulcata* described in southern Croatia (Duh et al. 2006) (GenBank accession code DQ081187) and with candidatus *R. hoogstraalii* (Mattila et al. 2007) co-isolated along with cellular line CCE2 (98% similarity, 358/363 bp) (GenBank accession code EF629539). We detected *gltaA, ompA* and *ompB* sequences of *R. massiliae* (Beati and Raoult 1993) in 43 of 282 *Rhipicephalus sanguineus* group examined ticks (32/228 *Rh. sanguineus*, 6/29 *Rh. turanicus* and 5/25 *Rh. pusillus*). No double infection was detected in any case.

Discussion

The Iberian Peninsula (Bartolomé et al. 2005; Cardeñosa et al. 2003, 2006; Guerrero et al. 2006; Herrero-Herrero et al. 1989; Jado et al. 2007; Lledó et al. 2006; Oteo et al. 2006) and particularly Andalusia (Bernabeu-Wittel et al. 2005, 2006) are known to be endemic regions of Mediterranean spotted fever (MSF) for humans. The epidemiology of rickettsiae and rickettsial diseases in Andalusia (south of Iberian Peninsula) is not well known, and a limited number of previous studies affecting *Rickettsia* sp. prevalence in ticks from domestic or feral animals have been made (Márquez et al. 1998, 2006, 2008).

In our area, both *I. ricinus* and *I. ventalloi* were infected mainly with *R. monacensis sensu lato* (formerly "Cadiz agent", IRS3, IRS4, etc.), and in the case of *I. ricinus* a lower proportion with *R. helvetica*. In previous studies in Castilla-Leon (northern Spain) *I. ricinus* was infected also with rickettsiae IRS3 (2.44%) and *R. helvetica* (0.61%) and lesser percentages for rickettsiae RpA4 (Fernández-Soto et al. 2004). No rickettsia was detected previously in *I. hexagonus.* In northern Portugal, a 5.9% of *I. ventalloi* parasitizing the short-eared owl *Asio flammeus* was infected with *R. helvetica* (Santos-Silva et al. 2006).

The prevalence of *R. slovaca* infection in *D. marginatus* ticks (29.19%) in this study is similar to that obtained by other investigators: 30.3–45.4% in Switzerland (Beati et al. 1994), 36.8% in Croatia (Punda-Polić et al. 2002). However, that value was higher than 21.1% observed in Hungary (Lakos and Raoult 1999), 5.5% in Portugal (Bacellar 1999) or 1.8–3.3% obtained in Russia (Shpynov et al. 2001). In Castilla-Leon (northern Spain) Fernández-Soto et al. (2003, 2004, 2006a, b) detected by PCR the presence in *D. marginatus* and *D. reticulatus* of *R. slovaca* (11.43% and 4.93% respectively), *R. raoultii* (Marne strain formerly RpA4, and Khabarovsk strain initially referred as DnS14/DnS28) (4.93% and 10.0%). Moreover, the frequency values for other rickettsiae genospecies like DnS1 and *R. monacensis* (formerly "Cadiz agent", IRS3 and IRS4) were very low (Fernández-Soto et al. 2006a). Immature

D. marginatus ticks usually feed on small mammals and birds, whereas adult ticks mainly feed on large mammals but frequently also on humans (Rehácek and Tarasevich 1991).

In *Rh. sanguineus* group only *R. massiliae* were detected. *Rickettsia massiliae* has been detected previously in *Rh. sanguineus* and/or *Rh. turanicus* from France (Beati and Raoult 1993), Portugal (Bacellar 1999), Spain (Beati et al. 1996; Merino et al. 2005; Márquez et al. 2008), Switzerland (Bernasconi et al. 2002), Greece (Psaroulaki et al. 2006) and Algeria (Bitam et al. 2006), and recently was signalled in United States (Eremeeva et al. 2006). Our result agrees with that obtained by Fernández-Soto et al. (2006a, b) in Castilla-León (northwestern Spain). In Portugal, Bacellar (1999) found a prevalence of SFG rickettsiae of 1.8% (38 positives over 2207 *Rh. sanguineus* tested for the presence of rickettsiae, collected from vegetation, dogs and other wild mammals). Over 25 *Rickettsia* isolates 22 were *R. massiliae* and 3 correspond to *R. conorii*. In addition, *Rh. pusillus* parasitizing Egyptian mongoose (*Herpestes ichneumon*) was signaled as reservoir of *R. sibirica mongolotimonae* in south Portugal (de Sousa et al. 2006a, b)

In north Spain, *Ha. punctata* has been noted infected with IRS3 and *R. aechlimannii* (Fernández-Soto et al. 2003). In this investigation, *Ha. punctata* was infected with *R. monacensis,* candidatus *R. raoultii* and *Rickettsia* sp. *R. aechlimannii* appeared highly associated with *Hy. marginatum* (Matsumoto et al. 2004), showing higher prevalences (5.86%) for this ticks species than for other co-feeding species such as *Rh. sanguineus, Rh. bursa, Rh. turanicus, Ha. punctata* and *I. ricinus,* with observed prevalences values lower than 2% (Fernández-Soto et al. 2003). *Rickettsia aeschlimannii* was originally detected in Morocco in *Hy. marginatum*, which is the only tick species found to be associated with this rickettsia (Beati et al. 1997). The first human case of *R. aeschlimannii* infection was documented in a man who traveled to Morocco (Raoult et al. 2002).

The presence of an undescribed rickettsiae, closer to the endosymbiont described in Croatia and to candidatus *R. hoogtraalii*, is documented for first time in Iberian Peninsula. The adults of *Ha. sulcata* were found feeding habitually on wild and domestic mountain ungulates (Iberian ibex, mouflon, domestic sheep and goat) in high Mediterranean mountain situations. Larvae and nymphs of *Ha. sulcata* were found in several species of reptiles during feeding, mainly in the lizard *Psammodromus algirus* attached to skin pocket (Salvador et al. 1999; personal observations), sometimes co-feeding with larvae and nymphs of *Ha. punctata* and *I. ricinus*. This exposure may explain the presence of this rickettsial organism in both *Haemaphysalis* species that share some of the hosts and the middle-high calcareous mountains range.

Based on molecular methods, we have demonstrated the presence of several SFG rickettsiae in the southern part of Iberian Peninsula with still uncertain pathogenicity for humans. When a microorganism is found in an arthropod capable of biting humans, it could be considered a potential human pathogen (Gilot et al. 1990; Raoult et al. 1997; Walker and Fishbein 1991). The data presented in this article extend the knowledge of the geographic distribution of SFG rickettsiae in the southwestern Mediterranean part of Europe. This area will be severely affected by climate change and by the emergence and potential increase of tick species that may play an important role as vector of microorganisms pathogenic for both humans and animals (de Sousa et al. 2006a, b). In a near future, with unpredictable climate conditions, ticks might be considered as epidemiological markers for a number of infectious agents transmitted by them.

Acknowledgments We thank Javier Millán for his suggestions and comments on preparing the manuscript. We are grateful to Antonio Hidalgo, Damián J. Galán, Samer Alasaad, Pilar Simón and José Luis Rodríguez for their help collecting ticks. Financial support was provided by the Fondo de Investigación Sanitaria program, Ministerio de Sanidad y Consumo, Spain (grant 04–1521).

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