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ORIGINAL ARTICLE

Molecular Detection of Hemoprotozoa and *Rickettsia* Species in Arthropods Collected from Wild Animals in the Burgos Province, Spain

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Abstract

Limited information on the presence of bacterial and hematozoan infections in parasitic arthropods from Spain is available. In an attempt to address this issue, the prevalence of *Theileria*, *Babesia*, *Hepatozoon*, and *Rickettsia* species was investigated by polymerase chain reaction plus sequencing. In a survey for zoonotic pathogens in ectoparasites, 42 wild animals (which included rodents, carnivores, Sciuridae, and Cervidae) were captured in AU3 ▶ Burgos (Spain). A total of 258 arthropods (including 107 ticks, 76 fleas, and 73 mites) were collected from these mammals. Molecular diagnostic results showed that (i) *Rickettsia felis* was found in fleas (two *Ctenocephalides felis*), (ii) *Hepatozoon* sp. infected some fleas (two *Ctenophtalmus* sp. and a DNA pool of *Ceratophyllus sciurorum*) and Acari (one *Neotrombicul*a sp.), and (iii) *Theileria annae* was found in *Ixodes ricinus* and *I. hexagonus* (each a single infected specimen). All microorganisms and parasites were genetically identical to pathogens already described in Spain or elsewhere. Infected arthropods were recovered from beech marten, bank vole, squirrel, wood mouse, and red fox. Our findings emphasize the potential risk for transmission of rickettsias to humans (namely, *R. felis*) in Burgos, since *C. felis* is capable to seek out humans for feeding. No hemoprotozoa with proven significance as human pathogens were found in the survey. However, finding *T. annae* in ticks recovered from wild canids suggests possible links of sylvatic and domestic cycles for some Piroplasmida.

Key Words: *Babesia*—Epidemiology—*Hepatozoon*—*Rickettsiae*—*Theileria*—Vector-borne.

Introduction

During the past few years, there has been an increase in the incidence of some zoonoses, especially those transmitted by arthropod vectors, in Spain (Blanco and Oteo 2006). Climate change and global warming induce some ecological changes in living conditions of animal reservoirs. This may lead to increased contact with humans, which in turn contributes to more disease cases. After mosquitoes, ticks are the most important vectors of pathogens that can cause disease to humans. Although most of *Rickettsiae* are transmitted by ticks, other vectors such as fleas, lice, and mites can also be important vectors of these bacteria. Fleas are vectors of *Rickettsia typhi* and *R. felis*. *R. prowazekii* and *R. akari* are transmitted by lice and mites, respectively. Emerging zoonoses caused by *Babesia* spp. and *Theileria* spp. are diseases that mainly affect domestic animals and, to a lesser extent, humans. Both genera are transmitted by ixodid tick bites (Blaschitz et al. 2008). In other respects, *Hepatozoon* sp. has a life cycle that includes two hosts: the invertebrate (definitive) host, which is a tick, louse, flea, or mosquito, and the vertebrate (intermediate) host, which is in some instances a mammalian species (Watkins et al. 2006). *Hepatozoon* sp. is usually transmitted by ingestion of the invertebrate host, but in the last years some studies have shown the experimental transmission of this protozoa in reptiles by mosquitoes (Adham et al. 2007, Sloboda et al. 2007) or in mammals by injection of the sporozoites recovered from ticks like *Amblyomma ovale* (Forlano et al. 2005). ◀ AU4

In a previous study performed by Giménez et al. (2009), some zoonotic agents (Piroplasmida and *Hepatozoon* sp.) were characterized in domestic and wild mammals from Northern Spain. In the present work such information is completed

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with a study of pathogens found in arthropods from wildlife in Burgos (Spain). Organisms such as *Rickettsia* spp. and hemoprotozoa (Piroplasmida and *Hepatozoon* spp.) were identified by polymerase chain reaction (PCR) and sequencing. These pathogens are important because they may cause disease in animals and humans. In addition, detailed information concerning vectors and reservoirs is essential to implement appropriate control measures (Torina et al. 2007).

Materials and Methods

Study area

The study was carried out in the region of the Merindades (Burgos), which is located in northwest of Spain (42°55′52″ N, 3°29′2″ W). The summer temperature ranges between 16°C and 20°C and the winter temperature ranges between 2°C and 5°C. The rainfall is usually high in winter and ranges from 900 to 1100 mm/year. It is a rural area, but recreational activities attracting nonresidents have increased over the last few years.

Animal samples

Forty-two wild animals (belonging to 13 species; see Table 1 for details on species and number of animals studied) were collected during the period between June 2006 and September 2007.

Wild animals were live-trapped, captured, or in some instances found dead (in the latter case death was due to road accidents). In all cases mammals were combed for ectoparasites such as ticks, fleas, lice, or mites. All these invertebrates were kept in 70% ethanol in sterile tubes until further processing. Arthropods were identified on the basis of morphometric characteristics. The keys used to identify fleas were those of Beacornu and Launay (1990); for ticks, Estrada-Peña et al. (2004); and for mites, Baker (1999).

DNA extraction, PCR, and sequencing

Samples were taken from 70% ethanol and were rinsed in distilled water before being dried on sterile filter paper. DNA was extracted from arthropods using alkaline hydrolysis, as described previously by Shouls et al. (1999) and Sousa et al. (2006). Whenever possible, DNA was extracted from pooled samples of 12 specimens of the same arthropod species (all recovered from a single host). DNA of *Rickettsia* genera was detected by amplification of citrate synthase (*gltA*) gene using the primers RpCs 1258/RpCs 877, which amplify a 381-bp fragment; 190-kDa protein (*ompA*) gene using Rr 190.70p/Rr 190.602n primers, which amplify a 532-bp fragment (Regnery et al. 1991); and *ompB* gene using 120-M59′/120–807 primers, which amplify a 833-bp fragment (Roux and Raoult 2000).

Piroplasmids (*Babesia* sp. and *Theileria* sp.) were detected using the Universal *Babesia–Theileria* primers BT1-F/BT1-R, which amplify a fragment of approximately 400 bp of the 18s rRNA gene (Criado-Fornelio et al. 2006). For the detection of *Hepatozoon*, primers HEP1/HEP 4 were employed. These amplify a fragment of 660 bp of the 18s rRNA gene (Criado-Fornelio et al. 2006). Negative and positive controls were included in all experiments. Positive amplicons were purified with QIAquick Spin PCR purification kit (Qiagen, Hilden, Germany) and sequenced using an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA). The sequences were edited using the software Lasergene (Dnastar,

Table 1. Ectoparasites Identified in Wild Mammals

Mammal species (no. of animals studied)	*Tick species (no., stage[a])*	*Mite species (no.)*	*Flea species (no.)*	*Louse species (no.)*
Arvicola terrestric (2)	None	None	*Ctenophtalmus* sp. (1 pool)	None
Apodemus flavicollis (3)	*Ixodes ricinus* (1, L)	*Laelaps agilis* (1 + 1 pool)	*Ctenophtalmus* sp. (2)	None
Apodemus sylvaticus (11)	*Ixodes trianguliceps* (1, A)	*L. agilis* (1 pool)	*Ctenophtalmus* sp. (1)	None
	I. ricinus (16 L +4 pool, L)	*Neotrombicula* sp. (12)		
Capreolus capreolus (5)	*I. ricinus* (1 A +1 pool, A)	None	None	None
Myodes glareolus (4)	None	*Neotrombicula* sp. (12 + 2 pool)	*Ctenocephalides felis* (1 + 1 pool)	None
Martes foina (2)	*Ixodes hexagonus* (5, N)	None	*Pulex irritans* (1)	*Trichodectes melis* (1)
			Paraceras melis (1 pool)	
Martes martes (2)	*I. hexagonus* (2, N)	None	*Ceratophyllus sciurorum* (1)	None
Meles meles (1)	None	None	*P. melis* (1 pool)	*T. melis* (1)
Putorius putorius (1)	*I. hexagonus* (1 pool, N)	None	None	None
Sciurus vulgaris (2)	*I. ricinus* (1, N)	None	*C. sciurorum* (1 pool)	None
Strix aluco (1)	*I. ricinus* (2, N)	None	None	None
Talpa occidentalis (3)	None	None	*Palaeopsylla minor* (3)	None
Vulpes vulpes (5)	*I. hexagonus* (2, N; 3, A)	None	*P. irritans* (3)	None
	I. ricinus (1, N)		*Ctenocephalides canis* (2)	

[a] Pool, 12 specimens of the same arthropod species.
L, larvae; N, nymph; A, adult.

Madison, WI), and the homology searches of amplicons were aligned with corresponding sequences of other *Rickettsia*, *Babesia*, and *Hepatozoon* species available in GenBank/EMBL database, using the BLASTN software (Altschul et al. 1990, Burland 2000).

Results

AU3 ▶ A total of 258 arthropods were collected from 42 wild animals (details on invertebrate species found are shown in Table 1). These included 107 ticks (16 adults [14.95%], 13 nymphs [12.15%)], and 78 larvae [73.89%]), 76 fleas, and 73 mites (48 trombiculids and 25 not trombiculids).

The most prevalent flea species was *Paraceras melis* (32%), followed by *Ctenophthalmus* sp. (22.6%), *Ctenocephalides felis* (17.3%), and *Ceratophyllus sciurorum* (17.3%). Other less frequent flea species accounted for the remaining 10.8%. All of the trombiculids found belonged to the genus *Neotrombicula* (34.2%), and the other mites (nontrombicula) were classified as *Laelaps agilis* (65.8%). In ticks, *Ixodes ricinus* was the most frequent species (76.6%), followed by *Ixodes hexagonus* (22.4%) and *Ixodes trianguliceps* (0.93%).

Concerning microbiological and parasitological diagnosis, two fleas were infected by identical rickettsia isolates. BLASTN sequence comparison showed that in both cases the two studied genes (*gltA*, fragment of 381 bp; *ompB*, fragment of 825 bp) were 100% identical to *R. felis* (AF540555). Both isolates were obtained in *C. felis* (one individual and one pooled sample). These fleas had been recovered from two different *Martes foina* (beech marten).

With regard to hemoprotozoa, *Hepatozoon* DNA was found in both fleas and mites. In fleas, *Hepatozoon* sp. (100% identity to *Hepatozoon* sp. BV2-AY600625) was found in two specimens of *Ctenophtalmus* sp. (recovered from bank vole—*Myodes glareolus*). A different *Hepatozoon* sp. isolate (100% identity to *Hepatozoon* sp. red squirrel EF222259) was found in one DNA pool of *C. sciurorum* (recovered from red squirrel—*Sciurus vulgaris*). In mites, a *Hepatozoon* isolate (100% identity to *Hepatozoon* sp. BV2 AY600625) was found in a DNA pool from *Neotrombicula* sp. mites (recovered from bank vole—*M. glareolus*). *Theileria annae* was found in ticks. Two isolates (100% identity to AY150069) were found in *I. ricinus* larvae (from wood mouse—*Apodemus sylvaticus*) and also in adult *I. hexagonus* (from red fox—*Vulpes vulpes*).

Discussion

Changes in human habits or in the ecology of some reservoir hosts have contributed to a closer contact of humans and arthropods vectors. This may have facilitated the spreading of some emerging zoonoses. Defining vector species in a particular area is of the foremost importance for disease control. In the present work, some putative vector species have been found in a population of parasitic arthropods in Burgos. Fleas (from genera *Archeopsylla*, *Ctenophtalmus*, and *Ctenocephalides*) have been found to be likely rickettsia carriers for domestic animals, as previously pointed out by other authors (in Spain or elsewhere: Márquez et al. 2002, Rolain et al. 2003, Bitam et al. 2006, Sousa et al. 2006). However, we must underline that the present study is the first one that has found *R. felis* in fleas (*C. felis*) from wild animals in Spain. In our study the prevalence of Rickettsiae in *C. felis* from wildlife animals represented at least a 15%, whereas the prevalence in fleas of domestic mammals ranged from 26.4% (Blanco et al. 2006) to 54.17% (Márquez et al. 2006). Positive fleas were obtained from beech martens; this fact probably should be considered anecdotic, but these wild mammals may approach human settlements in search of food (Villoria et al. 2008), and it is possible that the flea infection transferred from domestic animals (cat or dog to beech marten). To our knowledge, this is the second report of molecular detection of *R. felis* from fleas obtained from wild animals, other than wild rodents, in Europe. In Portugal and Algeria, *R. felis* was found in the pulicid flea *Archeopsylla erinacei* from hedgehogs (Bitam et al. 2006, Sousa et al. 2006); for this reason, the possibility of transmission to humans by flea bite should not be disregarded. The interference between sylvatic and domestic cycles might influence the prevalence infection in peridomestic animals, thus increasing the risk of human exposure. Ticks such as *I. ricinus* and *I. hexagonus* have been found to be the transmitters for different species of rickettsiae (Schouls et al. 1999). In contrast, we failed to detect any rickettsiae in the tick specimens analyzed. Other Acari-like *Trombiculidae* may be responsible of rickettsial transmission, but data on their vectorial ability are scarce in Spain. However, Choi et al. (2007) reported rickettsias belonging to spotted fever group and typhus group in these mites.

Hemoprotozoa present in arthropods have been scarcely studied in Spain by molecular methods. Thus, the present study is the first report of *Hepatozoon* sp. in trombiculids or fleas of wild mammals. Since no analysis of the vectorial capacity of these arthropods has been done in the present study, the definitive hosts for *Hepatozoon* sp. BV2/red squirrel remain uncertain. Smith (1996), in his review of *Hepatozoon* species of mammals, mentioned the presence of *H. sylvatici* in bank voles and *L. agilis* (mite). Molecular procedures showed the existence of hepatozoons in Spanish bank voles (Criado-Fornelio et al. 2006) and in trombiculid mites (present work; prevalence 2%). Thus, the latter are likely definitive hosts. The fact that fleas (in our study *Ctenophtalmus* sp. with a prevalence of 11.7%) from bank voles harbored the same parasite is not surprising, since these arthropods may easily feed on several hosts (Service 1996), thus increasing the chances of finding infected specimens. Concerning *Hepatozoon* sp. red squirrel, it has been found in a flea (*C. sciurorum* with a prevalence of 7.6%), but this does not demonstrate vectorial capacity. Smith (1996) pointed out that *H. griseisciuri* was found in squirrels and mites as well; therefore, previous findings do not point out to fleas as the likely definitive hosts. Finally, it seems that *Hepatozoon* species from arthropod species parasitizing Sciuridae or rodents have little chance to infect domestic animals (particularly, cats and dogs), and their only potential risk as pathogens remains only for wildlife, in agreement with data published by Criado-Fornelio et al. (2006).

Molecular methods revealed the existence of *T. annae* infections in *Ixodid* ticks. This is in agreement with the hypothesis of Camacho et al. (2003), who suggested that *Ixodidae* (particularly *I. hexagonus*) was a good candidate vector for the protozoa (Camacho et al. 2003). This is in agreement with the findings of the present study, where one specimen of *I. hexagonus* was found to be infected by *T. annae* (prevalence 4.16%). The tick was recovered from fox, which has been found to be frequently infected by Piroplasmida in Spain (Criado-Fornelio et al. 2003, Giménez et al. 2009). Since foxes ◀ AU7

have been seen many times visiting human settlements, they may carry infected ticks close to domestic canids. Although there had been no reports of human infections caused by these protozoa, this possibility cannot be totally disregarded (Camacho et al. 2001, 2003).

Our results emphasize the potential risk of arthropod-transmitted infections in this study area. Further studies must be performed in the same area to determine the vectorial capacity of arthropod species. These data are essential for the development of future control campaigns in Spain or elsewhere.

AU8 ▶ **Disclosure Statement**

No competing financial interests exist.

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