Molecular detection of Hemoprotozoa and Rickettsia sp in arthropods collected from wild animals in the Burgos province, Spain

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Complete List of Authors: Lledó, Lourdes; Alcalá de Henares University, Microbiology and Parasitology
Consuelo, Gimenez; Alcalá de Henares University, Microbiology and Parasitology
Domínguez, Gerardo; Consejería de Sanidad y Bienestar Social de la Junta de Castilla y León
Sousa, Rita; Centro de Estudos de Vetores e Doenças Infecciosas, Instituto Nacional de Saúde
Isabel, Gegundez; Alcalá de Henares University, Microbiology and Parasitology
Nieves, Casado; Alcalá de Henares University, Microbiology and Parasitology
Criado, Angel; Alcalá de Henares University, Microbiology and Parasitology

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Molecular detection of Hemoprotozoa and *Rickettsia* sp in arthropods collected from wild animals in the Burgos province, Spain

L. LLEDÓ¹, C. GIMÉNEZ-PARDO¹, G. DOMÍNGUEZ-PEÑAFIEL², R. SOUSA³, MI. GEGÚNDEZ¹, N. CASADO¹, A. CRIADO¹

¹Departamento de Microbiología y Parasitología, Universidad de Alcalá, Spain

²Consejería de Sanidad y Bienestar Social de la Junta de Castilla y León, Spain

³Centro de Estudios de Vetores e Doenças Infecciosas, Instituto Nacional de Saúde, Portugal

Corresponding author/correspondence address and reprint to:

Lourdes Lledó  MD, PhD

- Departamento de Microbiología y Parasitología, Universidad de Alcalá,
- Ctra. Madrid-Barcelona, Km. 33,6- 28871- Alcalá de Henares, Madrid (España)
- phone: 34 91 8854794
- fax: 34 91 8854663
- e-mail: lourdes.lledo@uah.es

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Abstract

Limited information on presence of bacterial and hematozoan infections in parasitic arthropods from Spain is available. In an attempt to address this issue, prevalence of Theileria, Babesia, Hepatozoon and Rickettsia species was investigated by PCR plus sequencing. In a survey for zoonotic pathogens in ectoparasites, 42 wild animals (which included rodents, carnivores, Sciuridae and Cervidae) were captured in 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 (2 Ctenocephalides felis), ii) Hepatozoon sp infected some fleas (2 Ctenophtalmus sp and a DNA pool of Ceratophyllus sciurorum) and Acari (1 Neotrombicula 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 hemoproteozoa with proven significance as human pathogens were found in the survey. However, diagnostic of Theileria annae in ticks recovered from wild canids suggests possible connection links of sylvatic and domestic cycles for some piroplasmida.
INTRODUCTION

During the past few years, there has been in Spain an increase of the incidence of some zoonoses, especially those transmitted by arthropod vectors (Blanco and Oteo 2006). Climate change and global warming are inducing some ecological changes in living conditions of animal reservoirs. This may lead to increased contact to humans, which in turn contributes to more disease cases. After mosquitoes, ticks are the most important vectors of pathogens that can cause disease to man. 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 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).

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 with a study on pathogens found in arthropods from wildlife in Burgos (Spain). Organisms such as Rickettsia spp and hemoproteoza (piroplasmida and Hepatozoon spp) were identified by PCR and sequencing. These pathogens are important because they may cause disease either in animal and man. 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), that is located in North West of Spain (42° 55’ 52” N, 3° 29’ 2” W). Summer temperature ranges between 16-20°C and winter temperatures range between 2-5°C. The rainfall is usually high in winter and ranges from 900-1,100 mm/year. It is a mainly rural area, but recreational activities attracting non-residents 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 (2004) and for mites were used those of 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 that amplify a 381-bp fragment, 190-kDa protein (ompA) gene, using Rr 190.70p/Rr 190.602n primers pair which amplifying a 532-bp fragment (Regnery et al. 1991) and ompB gene using 120-M59'/120-807 primers that amplifying 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 amplifies 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, GmbH, Germany) and sequenced using an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were edited using the software Lasergene (DNASTAR, inc., Madison, USA), the homology searches of amplicons were aligned with corresponding sequences of other Rickettsia, Babesia, Hepatozoon species available in GenBank/EMBL database, using the BLASTN software (Altschul et al 1990, Burland 2000).

RESULTS

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%], 78 larvae [73.89%]), 76 fleas and 73 mites (48 trombiculids, 25 not trombiculids).

The most prevalent flea species was Paraceras melis (32%), followed by Ctenophthalmus sp. (22.6%), Ctenocephalides felis (17.3%) and Ceratophyllum 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 (non-trombicula) 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 diagnostic, 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 and *ompB* - fragment of 825 bp) were 100% identical to *R. felis* (AF540555). Both isolates were obtained in *Ctenocephalides felis* (1 individual and 1 pooled sample). These fleas had been recovered from two different *Martes foina* (beech marten).

With regard to hemoproteozoa, *Hepatozoon* DNA was found both in fleas and mites. In fleas, *Hepatozoon* sp (100% identity to *Hepatozoon* sp BV2 - AY600625) was diagnosed 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 1 DNA pool of *Ceratophyllus 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 - *Myodes glareolus*). *

*Theileria annae* was found in ticks. Two isolates (100% identity to AY150069) were diagnosed in *Ixodes ricinus* larvae (from wood mouse - *Apodemus sylvaticus*) and also in adult *Ixodes 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 (either in Spain or elsewhere: Marquez *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 finding of *R. felis* in fleas (*Ctenocephalides felis*) from wild animals in Spain. In our study the prevalence of Rickettsiae in *Ctenocephalides felis* from wildlife animals represented at least a 15%, whilst 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 it is interesting to mention that these wild mammals may approach human settlements in search of food (Villoria *et al.*, 2008), and it is possible 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 (Sousa *et al.*, 2006; Bitam *et al.*, 2006). By this reason the possibility of transmission to man by flea bite should not be disregarded. The interferences between sylvatic and domestic cycles might influence prevalence infection in peridomestic animals, thus increasing the risk of man exposure. Ticks such as *I. ricinus* and *I. hexagonus* have been found to be 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 transmisssion, but data on their vectorial ability are scarce in Spain. However, Choi *et al.* (2007) reported rickettsias belonging to spotted fever group (SFG) and typhus group (TG) 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 *Laelaps agilis* (mite). Molecular procedures showed the existence of hepatozoons in Spanish bank voles by 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 (*Ceratophyllus sciurom* 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, so that 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 chances 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 *Theileria 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 findings in the present study, where one specimen of *I. hexagonus* was infected by *Theileria 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,
and Giménez et al, 2009). Since foxes 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 and 2003).

Our results emphasize the potential risk of arthropods-transmitted infections in this study area. Further studies are 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.
REFERENCES


Table 1- Ectoparasites identified in wild mammals.

<table>
<thead>
<tr>
<th>Mammal species (animals studied)</th>
<th>Tick species (no., stage*)</th>
<th>Mite species (no.)</th>
<th>Flea species (no.)</th>
<th>Louse species (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arvicola terrestris (2)</td>
<td>None</td>
<td>None</td>
<td><em>Ctenocephalides felis</em> (1 pool)</td>
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</tr>
<tr>
<td>Apodemus flavicollis (3)</td>
<td><em>Ixodes ricinus</em> (1 L)</td>
<td><em>Laelaps agilis</em> (1+ 1 pool)</td>
<td><em>Ctenocephalides felis</em> sp (2)</td>
<td>None</td>
</tr>
<tr>
<td>A. sylvaticus (11)</td>
<td><em>Ixodes trianguliceps</em> (1 A)</td>
<td><em>Neotrombicula sp</em> (12)</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Capreolus capreolus (5)</td>
<td><em>Ixodes ricinus</em> (1 A+1 pool A)</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Myodes glareolus (4)</td>
<td>None</td>
<td><em>Neotrombicula sp</em> (12+2 pool)</td>
<td><em>Ctenocephalides felis</em> sp (2)</td>
<td>None</td>
</tr>
<tr>
<td>Martes foina (2)</td>
<td><em>Ixodes hexagonus</em> (5 N)</td>
<td>None</td>
<td><em>Ctenocephalides felis</em> (1 pool)</td>
<td><em>Pulex irritans</em> (1)</td>
</tr>
</tbody>
</table>

* For Peer Review
<table>
<thead>
<tr>
<th>Species</th>
<th>Arthropod</th>
<th>Infestations</th>
<th>Vectors</th>
<th>Pests</th>
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<tbody>
<tr>
<td>Martes martes (2)</td>
<td>Ixodes hexagonus</td>
<td>None</td>
<td>Ceratophyllum sciuorum (1)</td>
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<tr>
<td>Meles meles (1)</td>
<td>None</td>
<td>None</td>
<td>Paraceras melis (1 pool)</td>
<td>Trichodectes melis (1)</td>
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<tr>
<td>Putorius putorius (1)</td>
<td>I. hexagonus</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Sciurus vulgaris (2)</td>
<td>Ixodes ricinus</td>
<td>None</td>
<td>Ceratophyllum sciuorum (1 pool)</td>
<td>None</td>
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<tr>
<td>Strix aluco(1)</td>
<td>Ixodes ricinus</td>
<td>None</td>
<td>None</td>
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<tr>
<td>Talpa occidentalis (3)</td>
<td>None</td>
<td>None</td>
<td>Palaeopsylla minor (3)</td>
<td>None</td>
</tr>
<tr>
<td>Vulpes vulpes (5)</td>
<td>Ixodes hexagonus</td>
<td>None</td>
<td>Pulex irritans (3)</td>
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</tr>
<tr>
<td></td>
<td>(2 N; 3 A)</td>
<td></td>
<td>Ctenocephalides canis (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>I. ricinus</td>
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- Abbreviations: L= larvae; N=nymph; A=Adult
- Pool= 12 specimens of the same arthropod species