

Absence of *Leishmania infantum* in cave bats in an endemic area in Spain

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Abstract Though dogs have been historically considered the main reservoir of *Leishmania infantum*, the role of wildlife in its epidemiology is attracting increasing attention. Rodents, wild carnivores and, recently, hares (*Lepus* spp.) have been proposed as sylvatic reservoirs for this parasite. Bats have never been tested for *L. infantum* infection in Europe. Nevertheless, bats have a widespread distribution, they live in abundant colonies, and some species inhabit caves, where constant temperatures and humidity provide ideal habitat for the sand fly vector. We tested blood samples from 35 Schreibers' bats (*Miniopterus schreibersii*), abundant cave bats in NE Spain, which is an enzootic area of leishmaniasis. A PCR-amplifying fragment of the high copy of *Leishmania donovani* group kDNA minicircles was used. None of the analyzed samples were positive (maximum possible prevalence=8.20 %). Though the susceptibility of this bat to parasitization by *L. infantum* cannot be ruled out, our survey indicates that this species may not be a relevant sylvatic reservoir of *L. infantum* in the Mediterranean area. Nevertheless, even if the prevalence of infection in bats is low, such an abundant taxonomic group would still provide a significant maintenance population for the parasite.

Keywords Canine leishmaniasis · Catalonia · *Leishmania infantum* · Zoonotic visceral leishmaniasis

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Parasites of the genus *Leishmania* cause a disease complex known as leishmaniasis. According to the World Health Organization, leishmaniasis is endemic in 98 countries or territories, with more than 350 million people at risk (WHO 2010). This vector-borne disease is endemic in Mediterranean Europe (Cabezón et al. 2010; Trotta et al. 2012). There, *Leishmania (Leishmania) infantum* causes the potentially fatal zoonotic visceral leishmaniasis (ZVL) in humans as well as canine leishmaniasis in dogs, a disease of significant veterinary importance. ZVL is re-emerging in southern Europe (Baneth 2006). For example, a recent outbreak took place in central Spain, where there was a fivefold increase in the number of human cases during the last quarter of 2010 compared to annual numbers in the previous years (Arce et al. 2013). Though dogs have been historically considered the main reservoir of the disease, the role of wildlife in its epidemiology is attracting increasing attention (Sobrino et al. 2008; Millán et al. 2011). For example, hares (*Lepus granatensis*) were proposed as the origin of the abovementioned outbreak in Spain (Molina et al. 2012). Bats, however, have received little attention thus far as potential reservoirs for *Leishmania* in endemic areas. In fact, as far as we know, bats have never been tested for *L. infantum* infection in Europe. Nevertheless, bats do have some characteristics that make them potentially good reservoirs: they have a widespread distribution, they live in abundant colonies, and some species inhabit caves, where constant temperatures and humidity provide ideal habitat for the sand fly vector (Feliciangeli 2004). Evidence of infection of bats with parasites of the *Leishmania donovani* group has only been found in South America. In Venezuela, *Leishmania chagasi* (syn. *L. infantum*) DNA was detected in one out of eight Seba's short-tailed bats (*Carollia perspicillata*), a common species that dwells in caves and tunnels (De Lima et al. 2008). In Brazil, infection was also confirmed in three individuals belonging to two different species out of 683 bats analyzed (Savani et al. 2010). In this latter study, 18 bats

infected by *Leishmania amazonensis* were also detected. In the Old World, Mutinga (1975) reported the detection of *L. donovani* bodies in 3 out of 104 bats in Kenya, and antibodies against *Leishmania* sp. were detected in Egypt (Morsy et al. 1987). The aim of the present study was to determine whether bats in a hyperendemic area in the Western Mediterranean are parasitized by *L. infantum* and as a first step to determine whether bats should be considered when describing the epidemiology of this parasite and in implementing control strategies.

The Schreibers' bat (*Miniopterus schreibersii* Kuhl, 1817), a member of the Miniopteridae family, is distributed from the Iberian Peninsula to the Caucasus, with the largest populations found in the warmer Mediterranean area. It has an estimated population size in the Iberian Peninsula of about 250,000 individuals. It is a colonial species that roosts mostly in caves and mines, often in large mixed colonies with other cave-dwelling bat species, where it shows highly gregarious behavior, especially during its breeding and hibernation periods (Serra-Cobo 1989). The roosting requirements of this species vary seasonally throughout its life cycle making movements of up to 300 km between different roosts (Serra-Cobo et al. 1998). Large warm caves are preferred during the nursing season. In winter, it hibernates in underground sites (usually large caves with a constant microclimate). Consequently, it is considered a regional migratory species (Hutterer et al. 2005). Many caves provide shelter for more than one species. Bats may benefit energetically by roosting in direct contact with other species or in close proximity to large aggregations. Schreibers' bats often form mixed colonies, especially during summer period, with greater mouse-eared bat (*Myotis myotis*, Borkhausen, 1797) and long-fingered bat (*Myotis capaccinii*, Bonaparte, 1837) and sometimes with species of *Rhinolophus* spp.

Studied bats were captured with nets between July and August 2013 in three caves in Catalonia (NE Spain), two in the province of Barcelona (41° 38' N 2° 44' E and 41° 17' N 1° 47' E), and a third one in the province of Lleida (42° 1' N 0° 57' E). The minimum sample size needed to detect *L. infantum* infection in this species of bat was calculated with WinEpi (de Blas et al. 2000) as $n=35$ for an unknown population size, with a 99 % confidence level and an expected prevalence of 12.5 %, which was the prevalence previously reported by De Lima et al. (2008). Blood was obtained by venipuncture of the cephalic vein and stored in EDTA. Polymerase chain reaction was used to amplify fragments of the high copy of *L. donovani* group kDNA minicircles. Briefly, DNA was extracted from blood samples using a commercial DNA extraction kit (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. Five microliters of the purified DNA was used for PCR with the BIOTAQ™ DNA Polymerase kit (Bioline Reagents Ltd., UK). Primers used were RV1 (5' CTTTCTGGTCCCGCGGGTAGG 3') and

RV2 (5' CACCTGGCCTATTTTACACCA 3') (Lachaud et al 2002). Water was used as a negative PCR control, and the positive control was obtained from a dog infected with *L. infantum*. Parasite DNA was not detected in any of the 35 bats analyzed (95 % confidence intervals=0.00–0.096). Maximum possible prevalence (calculated using WinEpi) was 8.20 %.

Though the susceptibility of European bat species to parasitization by *L. infantum* cannot be ruled out, as evidence of contact exists both in South America (De Lima et al. 2008; Savani et al. 2010) and Africa (Mutinga 1975; Morsy et al. 1987), this pilot survey, aimed at detecting at least one infected bat, showed no positive cases. Moreover, the PCR protocol used in the present study is highly sensitive (up to 1 parasite/15 ml of blood) and thus capable of detecting the presence of parasites in very low numbers (Millán et al. 2011). Our results agree with those reported for French Guiana, an area endemic for five species of *Leishmania*, where Rotureau et al. (2006) found no infected individuals among 216 bats belonging to 29 different species, including 54 *C. perspicillata*, using a genus-specific PCR. On the other hand, the prevalence observed in the present study contrasts with the usually high prevalences reported for other mammalian species in endemic areas of Spain (e.g., Millán et al. 2011, Molina et al. 2012). The absence of *L. infantum* infection in the sample of Schreibers' bat indicates that this species may not be a relevant sylvatic reservoir of *L. infantum* in the Mediterranean area. Nevertheless, the results of the present cross-sectional survey must be carefully interpreted. In the first place, even if prevalence of infection is very low, such an abundant species still provides a significant maintenance population, as observed by Rotureau et al. (2006). Also, since differences in the susceptibility to the infection among species can exist, other bat species must be studied in the future. The identification of the sandfly species inhabiting Mediterranean caves for which information is almost nonexistent (Felicangeli 2004), together with studies of blood meal preferences of sandflies in the caves inhabited by bats—as those carried out by Jiménez et al. (2013) in the focus of human leishmaniasis in Spain—will provide additional information to determine the role of bats in the epidemiology of *L. infantum* in the Mediterranean basin.

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