Veterinary Parasitology 202 (2014) 119–127

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

High rates of *Leishmania infantum* and *Trypanosoma nabiasi* infection in wild rabbits (*Oryctolagus cuniculus*) in sympatric and syntrophic conditions in an endemic canine leishmaniasis area: Epidemiological consequences

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ARTICLE INFO

Article history:
Received 28 December 2013
Received in revised form 17 March 2014
Accepted 26 March 2014

Keywords:
Leishmania infantum
Trypanosoma nabiasi
Wild rabbits
Reservoir host

ABSTRACT

Leishmania infantum infection has been reported in various host species, both domestic and wild, in some cases with high prevalence rates. However, until the recent discovery of infected hares, no studies had provided clear evidence of any significant reservoir other than domestic dogs. Our focus was on another lagomorph, *Oryctolagus cuniculus* or wild rabbit. This species is native to the Iberian Peninsula and its presence and abundance gave rise to the name of Spain. In an endemic area for canine leishmaniasis in the southeast of Spain, 150 rabbits were captured over a period of three years. Samples of blood, bone marrow, liver, spleen, heart and skin were taken and analysed through parasitological, serological and molecular techniques in order to detect *Leishmania* and *Trypanosoma*. 20.7% of the rabbits were infected with *L. infantum* and 82.4% with *Trypanosoma nabiasi*, and 14.8% of mixed infections were detected. Both parasites were found in all the animal organs analysed, a factor which, along with the presence of serological cross-reactions, must be taken into account in epidemiological studies on leishmaniasis. *O. cuniculus* is an abundant and gregarious species, with a long enough average lifespan to ensure *L. infantum* transmission. The presence of the parasite in the skin and blood of these rabbits with no acute manifestation of disease ensures its contact with the vector, which finds in their warrens a suitable biotope to inhabit. The rabbit therefore seems to meet the most of conditions for being considered a reservoir host of *L. infantum*.

1. Introduction

Leishmania infantum is the species responsible for zoonotic visceral leishmaniasis (ZVL) in Mediterranean countries (both Southern Europe and Northern Africa), Middle East and certain parts of Asia, and was brought into Latin America from the Iberian Peninsula, where it adapted to the local permissive sandfly *Lutzomyia longipalpis* (Volf and Myskova, 2007; Alvar et al., 2012). In southern Europe, the incidence of ZVL in humans is relatively low (0.02–0.49/100,000 in the general population) (Alvar et al., 2012; Antoniou et al., 2013). However, outbreaks or recrudescence may occur periodically in foci like the new one in Spain, where incidences reached 56 per

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http://dx.doi.org/10.1016/j.vetpar.2014.03.029
0304-4017/

100,000 (Molina et al., 2012; Arce et al., 2013). Dogs, which may suffer from severe disease (CanL), are the primary domestic reservoir hosts of ZVL. Furthermore, the infection has been described in various host species, both domestic and wild (Ashford, 1996; Quinnell and Courtenay, 2009). It has been found in marsupials, primates, rodents, carnivores, xenarthrans and bats (De Lima et al., 2008; Quinnell and Courtenay, 2009; Malta et al., 2010; Antoniou et al., 2013; De Araújo et al., 2013). The use of PCR in these studies confers great advantages such as higher sensitivity and the opportunity to identify the species involved. Several domestic species have now been shown to have a high prevalence of infection in some areas. In particular, a number of European studies have shown a high prevalence in domestic cats; for example, 26% of 183 cats tested in a Spanish study were PCR positive (Martín-Sánchez et al., 2007). Infections have also been reported in horses in Europe (Fernández-Bellón et al., 2006). In wild animals, infections have been reported in red foxes, wolves, genets, lynxes, Egyptian mongooses, black rats, etc. (Criado-Fornelio et al., 2000; Portús et al., 2002; Di Bella et al., 2003; Ranieri et al., 2006; Dipineto et al., 2007; Sobrino et al., 2008). In studies of foxes, for example, 14.1% and 74% tested PCR positive in Spain (Criado-Fornelio et al., 2000; Sobrino et al., 2008) and 40% in southern Italy (Ranieri et al., 2006). The absence or low prevalence of lesions is a characteristic common to these studies. Infections have also been described in animals kept in captivity such as non-human primates (Malta et al., 2010), Barbary lions (Libert et al., 2012) and a Bennett's wallaby (Ramírez et al., 2012).

A mammal host responsible for the long-term maintenance of a population of infectious agents is called a reservoir host. In addition to the reservoir host(s) essential to the maintenance of parasite populations, there may be numerous incidental hosts that are irrelevant to long-term persistence. Occasionally, these incidental hosts may be responsible for some transmission. They may even become secondary reservoir hosts (Ashford, 1996). In addition to domestic dogs, the ability to transmit infection has been confirmed by xenodiagnosis in ground squirrels, Syrian hamster, black rats, domestic cats and recently, in hares (Gradoni et al., 1983; Maroli et al., 2007; Quinnell and Courtenay, 2009; Molina et al., 2012; Antoniou et al., 2013). An understanding of the reservoir system is important in the design of rational control measures (Ashford, 1996; Quinnell and Courtenay, 2009).

The domestic dog is the main reservoir host for *L. infantum* and as such, it is able to maintain the parasite indefinitely as its only host (Ashford, 1996); however, it is important to consider the potential importance of other hosts and its implications in the control of ZVL. Until recently, no study had provided clear evidence of any important ZVL reservoir other that the domestic dog, which does not mean to say that they did not exist. Then Molina et al. (2012) demonstrated for the first time that apparently healthy naturally infected hares (*Lepus granatensis*) can be infectious to a competent *L. infantum* vector (*Phlebotomus perniciosus*), revealing that hares may be playing a role of active reservoirs in the recently reported leishmaniasis outbreak in the southwestern area of the Madrid autonomous community in Spain (Arce et al., 2013; Jiménez et al., 2013). The finding of positive hares in each of the six Spanish regions surveyed by Ruiz-Fons et al. (2013), suggests that the parasite is widespread in Spanish hare populations. These findings highlight the need for further research on this topic. Studies on the role of lagomorphs as reservoirs of leishmaniasis are very scarce worldwide. Our focus was on another lagomorph, the wild rabbit, which unlike the hare is a burrowing animal.

Moreover, when the distribution of *Trypanosoma* and *Leishmania* species overlaps in the same geographical area, mixed infections can appear within the same host. In the Amazon Basin, for example, De Araújo et al. (2013) describe a triple mixed infection of *T. cruzi*, *T. rangeli* and *L. infantum* in the anteater *Tamandua tetradactyla.*

Trypanosomes are haemoflagellate protozoans (Kinetoplastida) found worldwide that infect a wide range of animals and man. Some species that parasitize animals are nonpathogenic under normal conditions, although they can produce fatal infections in young or immunologically compromised hosts (Molyneux, 1970). Nonpathogenic trypanosomes have strong host specificity, usually being infective for only one host species. Although the nonpathogenic trypanosome group nowadays includes at least 45 morphologically indistinguishable species, it is accepted that *T. nabiasi* is the species that infects the domestic and wild rabbit (Molyneux, 1970; Hamilton et al., 2005; Reglero et al., 2007). In spite of being included in the same family and sharing some antigenic and molecular characteristics, *Trypanosoma* (*Herpetosoma*) and *L. infantum* selected different life strategies in the course of their evolution. The former is transmitted by fleas, whilst *L. infantum* is transmitted to several mammal species by parasite regurgitation during bloodmeals taken by infected sandflies, mainly belonging to the subgenera Larroussius and Adlerius in the Old World.

Our aim was to investigate natural *L. infantum* infection in wild rabbits (*Oryctolagus cuniculus*) and discuss their potential role as reservoirs in a recognized endemic area with ample epidemiological knowledge, as is the case of southeastern Spain. The sympatric and syntrophic presence of another kinetoplastid, *T. nabiasi*, was taken into consideration to avoid confusion.

2. Materials and methods

2.1. Specimens and capture area

From July 2009 to October 2011, on a private farm in the province of Granada in southeastern Spain (geographical coordinates: 37°17′18″ N, 3°52′47″ W), 150 wild rabbits (*O. cuniculus*) were captured. The farm has controlled hunting grounds and restricted access. Extending over 1000 ha, it is located in the Mesomediterranean bioclimatic level, at an altitude of 750–900 m above sea level. With an annual rainfall of approximately 600 mm, the summers are hot and dry. The terrain is undulating, with tree cover consisting mainly of olives in 45% of the estate, and 10% pine and Holm oak on scrubland; the remaining 45% is destined to dry land crops such as wheat and barley and irrigated crops such as alfalfa. A stream runs through the estate. There is livestock, mainly sheep which are put out to pasture during the day

and overnight in stables. There were 7 dogs, 3 of which were diagnosed with canine leishmaniasis by our group through serology and culturing. The ground is not too hard or stony, making burrowing easier. The density of the lagomorph population is high, at 11 rabbits per hectare, with an average life span of 2.5 years. The rabbits on the farm were genotyped as belonging to the subspecies *O. cuniculus algirus* and no repopulation measures have ever been taken; the rabbits from this farm are used to repopulate farms in other parts of Spain (Land agent of the state "La Torre", personal communication).

Of the 150 rabbits, 107 were shot by hunters and 43 were captured live with ferrets. 52% (78) were male. Weight varied from 400 to 1200 g: 8.7% weighing ≤500 g, classified as small; 75.3% weighing > 880 g, classified as large and 16.0% classified as medium weight.

2.2. Obtaining and processing samples

Blood samples were collected from the recently shot rabbits (107) by cardiac puncture; this was the only type of sample analysed in this case. The rabbits captured live (43) were sacrificed by concussion in the laboratory and samples of blood, bone marrow, liver, spleen, heart and skin were taken. Some of the blood was reserved for separation of the serum with a view to detecting antibodies and the rest was mixed with EDTA as an anticoagulant to preserve the whole blood. As well as the whole blood, all the other types of samples were also divided into three parts for microscopy, culture and PCR. The PCR part was processed in a room exclusively destined to DNA extraction. Bone marrow was extracted by passing RPMI medium supplemented with 20% Foetal Bovine Serum through the clean femur or tibia of the animal, and placed on a Petri dish. Due to the small amount of collected sample, the bone marrow was destined exclusively for culturing.

2.3. Laboratory techniques

Parasitological, serological and molecular techniques were all used to detect *Leishmania* and *Trypanosoma* infection.

2.3.1. Microscopic techniques

For detection of trypomastigotes both direct observation of a drop of blood placed between slide and coverslip and microhematocrit technique were performed.

Thin blood smears and liver and spleen imprints were made, and then fixed in pure methanol and Giemsa-stained in order to detect amastigotes.

Cultures were made with peripheral blood, bone marrow and macerates of liver, spleen and heart, using a combination of EMTM solid phase made with rabbit blood and RPMI supplemented with 20% Foetal Bovine Serum and 5% human urine as the liquid phase. The cultures were kept for 6 months before being rejected as negative. In vitro subinoculations were performed weekly during the first two months and then every 15 days.

2.3.2. Serum antibody testing by indirect fluorescent antibody test (IFAT)

A suspension of 2 million/ml *L. infantum* zymodeme MON-1 = GR-1 promastigotes from strain MCAN/ES/91/DP204 or *T. nabiasi* epimastigotes from strain MLAG/ES/2010/DP543 were used as antigen in the IFAT. The antibody titre against *Leishmania* or *Trypanosoma* was determined in geometric dilutions from the serum obtained from each blood sample as previously reported for dogs; a starting dilution of 1/20 was used (Acedo Sánchez et al., 1996). Rabbit anti-gamma globulin (ICN Biomedicals) was used as a conjugate at a concentration of 1/100 in Evans blue previously diluted at $1/10^4$ with phosphate-buffered saline. A standard cut-off dilution for positivity in rabbits was not available for any of the parasites.

2.3.3. DNA extraction

DNA was obtained from the different samples using the REAL DNA SSS Extraction Kit (RBME01). Each extract was rehydrated in a final volume of 20 μL of sterile water. To make sure there was no contamination at this stage, extraction controls were carried out. These consisted of tubes of sterile water to which the whole extraction process was applied simultaneously with the biological samples. One control was used for every group of 7 biological samples. The extracted DNA was kept at −20 °C until its amplification by PCR.

As a totally independent procedure to the DNA extraction from biological samples taken from wild rabbits, DNA was also extracted from *L. infantum* promastigotes (MCAN/ES/91/DP204) and *T. nabiasi* epimastigotes (MLAG/ES/2010/DP543) taken from cultures. The parasites were washed and counted with a hemocytometer and adjusted to a final concentration of 1000 parasites/μl to be used as positive controls in the PCR. Various negative controls were used: (i) tube of PCR reagents without DNA, (ii) extraction controls, (iii) DNA from an uninfected domestic rabbit.

2.3.4. PCR-ELISA

This technique is specific to the *L. infantum* species. It was performed following the protocol of Martín Sánchez et al. (2001) using kits PCR-ELISA DIG Labelling and PCR-ELISA DIG Detection (Roche Diagnostics GmbH. Mannheim, Germany). Every sample was analysed in duplicate, adding 2 and 3 μL of DNA in a final reaction volume of 25 μL. The results were read in a spectrophotometer at a λ of 405 nm. Samples returning absorbance values of ≥1 were considered positive, in accordance with the results obtained previously by Martín-Sánchez et al. (2001). When optical density was ≥0.5 and <1, amplification was repeated using 4 and 5 μL of DNA.

2.3.5. PCR amplification assay ITS 1 fragment and restriction fragment length polymorphism (RFLP)

ITS1 was amplified with primer pair LITSR/L5.8S and PCR conditions described elsewhere (El Tai et al., 2000, 2001; Schönian et al., 2003). The amplification products were tested by electrophoresis in 1.5% agarose gels in 1× TAE-buffer and visualized by staining with ethidium

bromide. This technique permits differentiation between the *Trypanosoma* and *Leishmania* genera according to the size of amplified fragment. Digestion was carried out with restriction enzyme Hae III (BsuRI) (Thermo Scientific) for 3 h at 37 °C and the digestion products were controlled in 3% agarose gels.

2.3.6. Isoenzymatic identification

Starch gel electrophoresis was performed according to the method described by Rioux et al. (1990), using 15 enzyme systems. The studied strain was analysed along with diverse zymodeme reference strains and MHOM/FR/78/LEM75 *L. infantum* as the basic reference zymodeme.

2.3.7. Statistical treatment of data

The statistical analysis of data was conducted using the IBM SPSS Statistics 20 package. Significance level was set at 5%. Concordance between techniques was determined with the Kappa coefficient, interpreted in the following way: almost perfect (1.00–0.81), substantial (0.80–0.61), moderate (0.60–0.41), fair (0.40–0.21) and slight (0.20–0.0).

The existence of associations between *Leishmania* or *Trypanosoma* infection (dependent variable) and the characteristics of the rabbits (sex, weight or size) or their capture (capture period) and antibody response, was determined by logistic regression analysis.

3. Results

3.1. L. infantum infection

3.1.1. General results

We were able to analyze all 150 of the rabbits captured by at least one of the techniques used to check for *L. infantum* infection. Using the whole array of techniques, the parasite was detected in peripheral blood, liver, spleen, heart, bone marrow and skin, returning a result of 31 infected rabbits (20.7%). An external visual inspection of the apparently healthy rabbits shot by hunters did not reveal the presence of clinical symptoms in any of the specimens. The 43 live rabbits which were transferred to the laboratory were more closely inspected and cutaneous lesions were found on 10 specimens (23.3%). The number of lesions varied from 1 to 4, located on hind legs, ears or nose. Amastigotes in the lesions of two specimens were detected under microscope, both of which tested PCR positive for *L. infantum* and *Trypanosoma*; amastigotes were not detected in any other organ with the exception of the liver of one these two rabbits. A strain of *Leishmania* taken from the bone marrow of another rabbit was isolated in culture (MLAG72011/ES/DP568).

Leishmania infection in rabbits is not associated with the sex of the specimen ($p = 0.723$). It is however associated with size – large rabbits are more parasitized than small/medium ones ($p = 0.018$) – and with capture period ($p < 0.001$). The detection of the parasite is positively associated with the antibody titre determined using promastigotes as the antigen ($p = 0.003$; OR = 1.017), so as the antibody titre increases, the probability of detecting *Leishmania* by molecular techniques increases. In the multivariate model created with the three variables which turned out to be significant in the previous univariate models, the antibody titre lost significance and was therefore removed from the model, resulting in the structure displayed in Table 1. Thus when capture period is equal, the risk of *Leishmania* infection in large rabbits is 15 times higher than in smaller rabbits. Similarly, when size is equal, with respect to the figures for 2009, the risk of infection is 54 times higher in 2010 and 793 times higher in 2011. To rule out the possibility that capture method and the additional use of diagnostic methods was determining the influence of capture period, a parallel logistic regression analysis was conducted using the results of the blood diagnosis with PCR techniques as the dependent variable, confirming that the independent variable "capture period" continued to maintain its association and that its behaviour was similar.

Table 1
Factors associated with *Leishmania infantum* infection in rabbits. Multivariate model obtained by logistic regression. *N* is the number of rabbits, *P* is the prevalence of infection in each category, *p* shows the level of significance, OR shows the risk of infection with respect to the reference category, ref is the reference category.

Variable	*N*	*P*	*p*	OR
Size	150	20.7%	0.05	–
Small/médium rabbits (ref)	37	5.4%	–	–
Large rabbits	113	25.7%	–	15,220
Capture period	150	20.7%	<0.001	–
July 2009 (ref)	86	1.1%	–	–
October 2009	21	9.5	0.165	
October 2010	24	50.0%	<0.001	54.205
October 2011	19	84.2%	<0.001	792.711

3.1.2. Detection of L. infantum infection by PCR

20.7% (31/150) of the rabbits were found to be infected by *Leishmania* spp. on applying one of the two PCR techniques to the samples obtained.

The PCR-ELISA indicated the presence of *L. infantum* in 13.4% (20/149). The PCR of the ITS-1 fragment amplified a 300 bp fragment characteristic of the *Leishmania* genus in 10.9% of specimens (15/138); only two of the 15 positives were apt for digestion with restriction enzyme Hae III, giving unclear results for differentiation between *L. infantum* and *L. tropica* (in all other cases the band obtained was too weak to permit digestion).

Of the 10 rabbits with cutaneous lesions, for 2 of these no skin samples were reserved for molecular diagnosis, whilst 6 of the remaining 8 tested positive with these techniques on the skin lesions (6 positives with PCR-ELISA, of which 3 were also positive for *Leishmania* with PCR-ITS); all 6 specimens had *L. infantum* in other organs simultaneously. Of the two skin samples testing positive under microscope, one was positive with PCR-ELISA and the other could not be studied by molecular methods.

There are statistically significant differences between the results of the two PCR techniques ($p = 0.002$), with the PCR-ELISA returning a higher percentage of positive results. Its concordance expressed in terms of the kappa index is 31.4% ($k = 0.314$): the two PCR assays were positive for 7 animals and negative for 109.

Table 2
Factors associated with *Trypanosoma* infection in rabbits. Univariate models obtained by logistic regression. *N* is the number of rabbits, *P* is the prevalence of infection in each category, *p* shows the level of significance, OR shows the risk of infection with respect to the reference category, ref is the reference category.

Variable	*N*	*P*	*p*	OR
Size	142	85.2%	0.049	–
Large rabbits (ref)	107	81.3%	–	–
Small/médium rabbits	35	97.1%	–	7816
Antibody titres for the antigen made up of *Trypanosoma* epimastigotes	134	85.2%	0.05	0.994
Capture period	142	85.2%	0.009	–
July 2009 (ref)	80	95.0%	–	–
October 2009	19	75.0%	0.009	0.147
October 2010	24	68.4%	0.008	0.158
October 2011	19	73.7%	0.002	0.114

3.1.3. Serology for L. infantum promastigotes

Antibody titres for the antigen made up of *L. infantum* promastigotes varied from 0 to 320: 71.4% of specimens had zero titres; 8.2% had titres of 20; 10.9% titres of 40; 6.8% titres of 80, 2.0% titres of 160 and 0.7% titres of 320. Thus 28.6% of the rabbits had antibody titres ≥20, and 20.4% had titres of ≥40. On comparing separately the positivity results obtained from these two different cut-off titres with those obtained with direct techniques, the resulting kappa index values were 0.563 ($p < 0.001$) and 0.372 ($p < 0.001$) respectively.

As stated previously, the detection of the parasite was positively associated with antibody titre ($p = 0.003$; OR = 1.017): the likelihood that rabbits with an antibody titre of 20 are infected with *Leishmania* is 1.7% higher than that of rabbits with a titre of 0; in the case of rabbits with a titre of 40 is 1.7^2% higher, and 1.7^3% for animals with an antibody titre of 80, and so on.

3.1.4. Specific identification of L. infantum

Thirteen blood samples, 6 of spleen, 8 of liver, 3 of heart and 7 of skin, taken from a total of 20 rabbits, tested positive by PCR-ELISA, a technique which is specific to *L. infantum*. A strain taken from the bone marrow of one of the rabbits was also isolated in culture (MLAG72011/ES/DP568). DNA was extracted from the cultured parasite and submitted to PCR-ELISA with positive results indicative of *L. infantum*, and to PCR-RFLP of the ITS-1, giving a band pattern characteristic of *L. infantum*. Following mass culturing, it was submitted to isoenzyme electrophoresis for characterization and identified as *L. infantum* zymodeme MON-1. The specimen did not have skin lesions and amastigotes were not observed by direct techniques in any of the samples. The PCR of the ITS was negative in all organs analysed, whilst the PCR-ELISA returned positive results in liver, spleen, heart and blood. As with the rest of the specimens, no bone marrow was reserved for PCR.

3.2. Trypanosoma spp. infection

3.2.1. General results

Of the 150 rabbits studied, 142 were apt for use to determine the presence of *Trypanosoma* spp.; of these, 85.2% (121/142) were infected. The parasite was detected with the different techniques in peripheral blood, liver, spleen, heart, bone marrow and skin. In the peripheral blood, trypomastigotes were observed under microscope in 31.6% of cases (6/19); performing the microhematocrit technique did not increase diagnostic sensitivity. Through culturing we were able to visualize, and in most cases also isolate, epimastigotes from all the organ types except skin, as these cultures were contaminated with fungi. The positivity values obtained by culture were: 21.2% in spleen (7/33), 27.3% in liver (9/33), 21.1% in heart (4/19), 10.5% in blood (2/19), 30.3% in bone marrow (10/33). The overall percentage of rabbits testing positive with this technique was 45.5% (15/33).

Trypanosoma infection in rabbits is not associated with the sex of the specimen ($p = 0.667$). It is however associated with weight ($p = 0.049$): the risk of infection in small/medium rabbits is 7.8 times higher than in larger ones. It is also associated with capture period ($p = 0.009$), with a higher risk in July 2009 than in subsequent periods (Table 2).

3.2.2. PCR of the ITS

The PCR of the ITS-1 fragment amplified a 500 bp band which could not be digested with restriction enzyme Hae III in 82.4% of the rabbits (117/142), and another of 480 bp in 2.8% of cases (4/142). The two fragments were sequenced and their sequences compared with those of other species of *Trypanosoma* recorded in GenBank. This allowed us to confirm the identification of *T. nabiasi* in the rabbits of southern Spain.

3.2.3. Serology for T. nabiasi

The antibody titres for the antigen made up of *T. nabiasi* epimastigotes varied from 0 to 640: 28.9% of specimens had antibody titres equal to 0; 38.7% had titres of 20; 18.3% titres of 40; 9.2% titres of 80, 2.8% titres of 160; 1.4% titres of 320 and 0.7% titres of 640. Thus 71.1% of the rabbits had antibody titres of ≥20, and 32.4% a titre of ≥40. On comparing separately the positivity results obtained from these two different cut-off titres with those obtained by direct techniques, the kappa index values returned were −0.011 ($p = 0.557$) and −0.003 ($p = 0.958$) respectively.

The detection of the parasite is negatively associated with the antibody titre determined using both epimastigotes ($p = 0.05$, OR = 0.994) and promastigotes ($p = 0.007$; OR = 0.985) as the antigen, meaning that as the antibody titre increases, the probability of detecting the parasite decreases.

3.3. Mixed infections

Mixed *Leishmania*/*Trypanosoma* infections were detected in 14.8% of specimens (21/142). Joint infection by the two *Trypanosoma* spp. haplotypes was found in 2.1% of the rabbits (3/142).

The correlation (Pearson correlation coefficient) between the antibody titres determined using the two types of antigen is 10.4% ($p = 0.216$).

4. Discussion

The wild rabbits taken from the endemic ZVL area that is southeastern Spain, with a CanL seroprevalence of 20.1% at the Mesomediterranean bioclimatic level (Martín-Sánchez et al., 2009) and PCR positivities of 54.5% (Morales-Yuste et al., 2012), were found to be infected by *L. infantum* in 20.7% of cases when direct techniques were used. The identity of the species was confirmed using a specific PCR (PCR-ELISA, Martín-Sánchez et al., 2001) and isoenzymatic characterization of a strain isolated in culture. It has become almost automatic that on detecting the parasite by techniques that do not permit specific identification, including PCR techniques which are only specific to genus, it is classified as *L. infantum* due simply to the fact that the area in question is endemic for ZVL (Criado-Fornelio et al., 2000; Di Bella et al., 2003; Dipineto et al., 2007; Helhazar et al., 2013). This lack of rigour in the expression of results can lead to errors, especially if there is a possibility that other species are present, not only in sympatry but also in syntrophy. The prevalence of infection of these rabbits by another kinetoplastid parasite, *T. nabiasi*, was very high, with values of up to 82.4%. Both parasites were detected in all the rabbit organs analysed, peripheral blood, liver, spleen, heart, bone marrow and skin. Finding these parasites in the heart probably reflects their presence in the blood. The amastigotes from both parasites are indistinguishable and culture forms of flagellated *Trypanosoma* initially were mistaken by these authors, despite their great experience with *Leishmania* culture. The PCR of the ITS1 proved useful for distinguishing between the two genera, although it was not sensitive enough to allow specific identification of *L. infantum* through RFLP, and its diagnostic sensitivity for leishmaniasis was lower than that of the PCR-ELISA ($p = 0.002$). *Trypanosoma* acts as protection factor against *Leishmania* infection: the presence of *Leishmania* was 4.3 times greater in rabbits non infected with *Trypanosoma* compared with the infected animals ($p < 0.05$).

The correlation between the antibody titres detected in the wild rabbits using the two types of antigen, *L. infantum* promastigotes and *T. nabiasi* epimastigotes, was low (10.4%), but we must take into account that this is not an indication of the reactivity for both antigens but it shows whether the two antibody titres have the same value. In general terms, the results reflect the existence of cross-reactions due to the presence of antigens shared by members of the same family, so caution must be exercised in the interpretation of serological results for lagomorphs when the studies do not include procedures that allow us to distinguish between the two kinetoplastids, such as that conducted by Moreno et al. (2013). In any case, on comparing the results obtained with direct and indirect diagnostic methods, taking titres 20 and 40 as threshold values for the latter, we find a moderate correlation in the diagnosis of leishmaniasis (56.3% and 37.2% respectively) and a very poor one in the case of trypanosomiasis (negative kappa values). In fact, as the antibody titre for *Leishmania* increases, the probability of the rabbit being infected by this parasite increases (1.7% with every increase in antibody titre, $p = 0.003$), whilst the association is negative in the case of *Trypanosoma*, whose likelihood of being present falls by 0.6% with every increase in antibody titre. A positive association between antibody titre and the presence of *L. infantum* is a characteristic found in dogs (Morales-Yuste et al., 2012), whilst in cats the association is negative (Martín-Sánchez et al., 2007); such negative association may suggest a possible protective role for the acquired immunity in infected animals.

4.1. L. infantum infection

Similarly to other countries in southwestern Europe, in Spain leishmaniasis is an endemic zoonosis and the parasite responsible in all forms, visceral, cutaneous and mucosal, is *L. infantum*, with the main reservoir encountered so far being the dog (Morillas et al., 1996; Fisa et al., 1999; Aliaga et al., 2003; Martín-Sánchez et al., 2004, 2009; Miró et al., 2008; Gálvez et al., 2010; Ballart et al., 2012a; Morales-Yuste et al., 2012).

Studies on the infection of *O. cuniculus* by *Leishmania* are very scarce worldwide. A study carried out in southeastern Spain suggests that wild rabbits have a very low risk of becoming infected with *L. infantum* (Chitimia et al., 2011).

The absence or low prevalence of lesions is another characteristic to highlight in studies on hosts other than dogs, such as cats or wild animals (Martín-Sánchez et al., 2007; Maia et al., 2008; Quinnell and Courtenay, 2009; Millán et al., 2011; Sherry et al., 2011). In our case, when we were able to inspect the animals more closely, we found that 23.3% (10/43) had skin lesions. In six of these specimens we have confirmed the presence of *L. infantum* in the skin; one of them was only infected by this kinetoplastid and no trace of *T. nabiasi* was detected in any of its tissues; the rest had mixed infections.

The specimen from which the *L. infantum* strain was isolated was only infected by this trypanosomatid and although the parasite was detected by PCR in other organs, we were only able to isolate it in the bone marrow. We cannot rule out the possibility that, in cultures with few passages taken from specimens infected with both trypanosomatids, mixed populations of *L. infantum* and *T. nabiasi* may be present, and that over time the former may disappear leaving behind only *T. nabiasi*, a parasite for which 23 isolations were achieved.

Certain ecological parameters of reservoir hosts are particularly important in the understanding of their role in reservoir systems. It has been shown that distribution at the levels of geography, biotope, habitat, and home is the first essential, and that the other most important parameters are those of age structure of populations, their density, dispersion and movements, and social structure. Moreover, behavioural details may determine specific relationships

with sandflies. A convincing model would depend on a close specific interaction between the putative reservoir host and the sandfly vector (Ashford, 1996).

O. cuniculus is native to the Iberian Peninsula and is widely distributed across the whole of southwestern, central and northern Europe. It has also been introduced into Australia, New Zealand, the Unites States, Chile, etc. It was the Phoenicians who, on reaching the Iberian Peninsula, named this country "Sphania", derived from the Greek *Sphan* meaning rabbit. This animal was considered to be a plague throughout Europe, until 1954–1955, when its population was decimated by myxomatosis. Since then, its numbers have gradually increased in areas where they find food and protection, and there is a rational control of hunting. The rabbit is found throughout Spanish territory, with relative density values of between 1.6845 (Community of Madrid, central Spain) and 0.0026 (Community of La Rioja, northeastern Spain); in the province of Granada (southeastern Spain) the relative density is 0.94. In any case, the absolute rabbit densities increase tenfold over the course of the year between the season of minimum and maximum abundance. The main biotope for the protection and reproduction of these lagomorphs is the warren, dug by the rabbits themselves in fertile soil. It is a network of branching tunnels and galleries, in which they build their nests before giving birth. This is a gregarious, territorial and nocturnal species. It is polygamous (one male for various females). The roaming area of the male is larger than that of the female, reaching up to 7 ha. They have litters of 2 –8 pups, with higher numbers in the hot months, at one month intervals from January to June. Sexual maturity is reached at 3–4 months. The average lifespan of the rabbit is long enough to ensure its survival during the non-transmission period of leishmaniasis; on the farm where the specimens were collected, the average lifespan was 2.5 years. The distribution of rabbits is very irregular, and it is common to find one farm with a highly dense population while on a neighbouring farm there are very low numbers (Burton, 1978; Ministry of the Environment, 1996). On the farm surveyed, the density was much higher than the average for the Granada province and other areas of Spain.

L. infantum infection was detected in the rabbits throughout the entire capture period, from July 2009 to October 2011, with a sharp increase in infection prevalence which would be more indicative of an epidemic outbreak than an endemic steady state. The 1.1% of infection detected in the month of July probably indicates infections acquired in the previous transmission period, given the phenology of the main vector in the area, *P. perniciosus* (Morillas-Márquez et al., 1983). Furthermore, infection is more frequent in larger rabbits, which have been exposed to the vector's bites for longer. All these points appear to indicate that the rabbits can be infected for a long time without displaying acute symptoms of disease, given that the only clinical manifestation detected was skin lesions in 23.3% of the specimens. The presence of the parasite in the skin and blood allows it to be presented to the vector sandfly. CDC traps were placed next to the warrens, hanging from trees, when available, or from a stick driven into the ground for this express purpose, allowing us to capture these dipterans in considerable densities (10.6 sandflies/trap, collecting a total of 1812 sandflies). The most abundant species was *P. perniciosus* (72.3%), which is precisely the main vector of leishmaniasis in the area (Barón et al., 2011; Martín-Sánchez et al., 1994), followed by *S. minuta* (20.1%), *P. papatasi* (6%), *P. ariasi* (0.94%), which also acts as a vector (Ballart et al., 2012b; Morillas et al., 1996), and *P. sergenti* (0.71%) (data not shown).

4.2. *T. nabiasi* infection

T. nabiasi has been reported in *O. cuniculus* from various European countries (UK, France, Italy, Portugal and Spain) and in domestic *O. cuniculus* outside Europe. Little is known about the identification and distribution of *T. nabiasi* in Spanish rabbit populations. In a study conducted in southwestern Spain, Reglero et al. (2007) found that *Trypanosoma* was prevalent in the blood of rabbits, but this was the only type of sample analysed. Prevalence was 23.81% in young rabbits, 84.85% in juveniles and 47.37% in adults. The prevalence and abundance of parasites in the blood was higher in females than in males, and higher in juvenile rabbits, followed by adults and lastly, young rabbits. The figures were also higher in fenced nuclei than in open nuclei. In our case, no differences were found between males and females, whilst we were able to confirm the lower parasitization levels in larger rabbits, i.e. adults; young rabbits have an eight times higher risk of infection than adults. Effective self-cure of infection and a role for acquired immunity has been suggested as an explanation for this, which would be in keeping with the negative association between antibody titres and parasite presence reported in this study. The prevalence figures we found are clearly higher than those given by Reglero et al. (2007), and they remained high throughout the duration of the study. These differences may be due to these authors using only blood and direct observation methods to detect the parasites, as opposed to the variety of samples and diagnostic techniques which we used. Moreover we cannot rule out the potential influence of our survey site being a fenced farm, i.e. a closed nucleus.

5. Conclusion

In order to determine the role of a given host in a reservoir system, it is far from sufficient to simply discover infected individuals. Despite the fact that in southwestern Europe *L. infantum* antibodies and DNA have been found in a wide array of domestic and wild animals, and that in some cases it has been possible to isolate the parasite and submit it to isoenzymatic characterization, no clear evidence has ever been found of their involvement as reservoirs, with the possible exception of the recent study on hares. Without a doubt, the impossibility of finding an area where there are no dogs further hinders the process of evaluating the possible contribution of these other hosts to the epidemiology of leishmaniasis. In the systems that have been adequately described, the reservoir host is abundant, forming a large proportion of the mammalian biomass and it is often a gregarious species, all characteristics which are true of the wild rabbit to an even greater extent than the dog. Furthermore, an effective reservoir host can be expected to be

long-lived, at least surviving through any non-transmission season. Although the average lifespan of rabbits is shorter than that of dogs, it is long enough to ensure the transmission of *L. infantum*. If a rabbit reaches adulthood it may live for 4–5 years, clearly a much lower figure than the estimated average 14-year lifespan of dogs. The presence of the parasite in high proportions in the skin and peripheral blood of these rabbits with no apparent signs of acute disease ensures its contact with the vector, which finds in their warrens a suitable biotope to inhabit. Therefore, the rabbit appears to fulfil the most of conditions which would justify it being considered a reservoir host of *L. infantum* and it would be interesting to conduct xenodiagnostic experiments using the local phlebotomine vectors. The sympatric and syntrophic presence of *T. nabiasi* must be taken into account in order to avoid any confusion.

Acknowledgements

We would like to thank Dr. Ashford for making the suggestion of studying rabbits which led to this research. The work reported here was supported by the Regional Government of Andalusia [Junta de Andalucía] and the Spanish Ministry of Economía y Competitividad (project CGL2007-66943-CO2-O2/BOS). We are very grateful to the land agents of the state "La Torre" from Illora to provided us with all the sampling rabbits and the information we needed.

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