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ORIGINAL PAPER

Application of molecular techniques in the study of natural infection of *Leishmania infantum* vectors and utility of sandfly blood meal digestion for epidemiological surveys of leishmaniasis

M. Magdalena Alcover · Marina Gramiccia · Trentina Di Muccio · Cristina Ballart · Soledad Castillejo · Albert Picado · Montserrat Portús · Montserrat Gállego

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Abstract Epidemiological studies on the distribution of leishmaniasis caused by *Leishmania infantum* Nicolle, 1908 (Kinetoplastida: Trypanosomatidae) have been based principally on serological surveys of the canine reservoir. This methodology is useful due to the facility of sampling, the rapidity in obtaining results, its consistency and because it allows the detection of heterogeneous foci of canine leishmaniasis (CanL) even in small areas. Other investigations have analysed *Leishmania* parasitism in sandflies (Diptera: Psychodidae: Phlebotominae) by using classical dissection techniques. These techniques allow the vector species to be incriminated in different foci, although they suffer from being very time consuming. Lately, studies in this field are increasingly using molecular techniques, which are faster and easier to perform. In the present work, we applied a nested-PCR in a study of natural infection of sandflies by *Leishmania* in three isolated farms where serological data on canine leishmaniasis of local dogs were also obtained. The analysis allowed the detection of 38.7% of females with positive nested-PCR (78%, 18% and 0%, respectively, in the different isolated farms). The positive *Leishmania* DNA samples were genotyped and identified as *L. infantum*. The results of this work provide new data for the vectorial capacity of *Phlebotomus ariasi* in a Pyrenean area, which can be considered at risk of becoming a new focus of CanL. The females with positive nested-PCR displayed blood in the midgut at different degrees of digestion, and/or were gravid. According to the multivariate logistic regression analysis, the risk of nested-PCR-positivity increased significantly with the degree of blood digestion (OR=1.3; *P* value=0.025). The *Phlebotomus* species and the presence of eggs were not statistically associated with nested-PCR positivity (*P* value of >0.05). The correlation of positive nested-PCR results with the presence of seropositive dogs in the farm confirms the utility of this technique in the study of the distribution and intensity of leishmaniasis foci. Also, the importance of sandfly blood-meal digestion for epidemiological surveys of leishmaniasis foci has been demonstrated.

M. M. Alcover · C. Ballart · S. Castillejo · M. Portús · M. Gállego (✉)
Laboratori de Parasitologia, Facultat de Farmàcia,
Universitat de Barcelona,
Av. Joan XXIII s/n,
08028 Barcelona, Spain
e-mail: mgallego@ub.edu

M. M. Alcover · C. Ballart · S. Castillejo · M. Gállego
Barcelona Centre for International Health Research
(CRESIB, Hospital Clinic-Universitat de Barcelona),
Barcelona, Spain

M. Gramiccia · T. Di Muccio
Section of Vector-Borne Diseases and International Health,
MIPI Department, Istituto Superiore di Sanità,
Rome, Italy

A. Picado
Department of Public Health, Institute of Tropical Medicine,
Antwerp, Belgium

Introduction

The leishmaniases are transmissible vector-borne diseases that affect humans and other reservoir hosts, mainly dogs in

Mediterranean areas, and are transmitted by the bite of infected female sandflies (Gállego 2004). The leishmania life cycle in the vector and vector competence have been reviewed by several authors (Sacks 2001; de Almeida et al. 2003; Volf et al. 2008). The amastigotes in the vertebrate hosts are ingested when the female sandfly takes an infected bloodmeal. After morphological and functional modifications in the insect's midgut, an infection is established at the stomodeal valve at the metacyclic promastigotes stage, which in some cases is accompanied by an invasion of the mouthparts. When the infected phlebotomine bites a new host, it regurgitates about 1 to 1,000 metacyclic promastigotes, closing the life cycle. Transmission may also occur by the transfer of parasites from heavily infected mouthparts into the skin during biting or by invasion of the salivary glands and deposition of metacyclic promastigotes into the skin with the saliva (Killick-Kendrick and Rioux 2002). The exact time from an infected bloodmeal until a female can transmit leishmaniasis is not known and varies according to the gonotrophic cycle and the ambient temperature. Other factors could be influential, such as the sugars taken by the females (WHO 2010).

Approximately 800 species of sandflies have been described, 95 of which are proven or suspected vectors of more than 20 *Leishmania* species (Killick-Kendrick 1999; WHO 2010). Vector efficiency depends on several factors, including biting habits and ability to survive and the parasite's ability to multiply and survive during the bloodmeal digestion.

Epidemiological studies regarding the endemicity of leishmaniasis foci have been carried out principally by serological surveys of human (HL) and canine leishmaniasis (CanL) (Fisa et al. 1999; Iniesta et al. 2002; Riera et al. 2004). The classical method of vector incrimination involves the dissection of sandflies and culture of parasites found in the intestine (Rioux et al. 1986; Martín-Sánchez et al. 2006; Rossi et al. 2008). This method allows *Leishmania* strains to be isolated from sandflies but needs fresh or cryopreserved specimens, dissecting expertise, is time consuming and lacks sensitivity (Kato et al. 2005). Since the 1990s, several molecular techniques have been developed and applied in this specific field (Perez et al. 1994; Aransay et al. 2000; Kato et al. 2005; Velo et al. 2005; Martín-Sánchez et al. 2006; Pandey et al. 2008; Rossi et al. 2008). The advantages of PCR techniques include greater sensitivity, specificity, versatility and rapid processing of a large number of phlebotomine sandfly samples, as described by the above authors. For these reasons, molecular techniques have also been applied in the study of arthropods other than sandflies as potential vectors of *Leishmania infantum* (Dantas-Torres et al. 2010; Colombo et al. 2011).

In the present work, we applied a nested-PCR (n-PCR) method in the study of natural infection of sandflies by *L. infantum* Nicolle, 1908 at three isolated farms in three localities of Catalonia. Positive DNA specimens were identified by *Leishmania* sp. Internal-transcribed spacer-1 (ITS-1) molecular typing to confirm the role of sandfly species as *Leishmania* vectors. Serological data on canine leishmaniasis were also obtained for the dogs living in the farms at the time of sandfly capture. The degree of blood meal digestion was also evaluated to identify the vectorial capacity of the sandflies.

Materials and methods

Study area

The study was carried out in three isolated farms from three localities of Catalonia (North East Spain): Torroja del Priorat, Sant Just Desvern and Ainet de Besan, each one in a different county ("comarca") and province of Catalonia (Fig. 1). The characteristics of the sampling stations and the periods of sandfly captures are included in Table 1.

Torroja del Priorat is located in a known endemic focus of HL and CanL, with an overall CanL seroprevalence of 20% (Fisa et al. 1999), which is highest in suburban and rural areas (33%) (Castillejo 2008). No data concerning CanL seroprevalence are available for the other two villages. Official data on the annual incidence of HL in each county are included in Table 1 (Portús et al. 2007).

Serological analysis of the farm dogs

Samples of peripheral blood were obtained by cephalic vein puncture with the agreement of the dog owners at the time of sandfly capture. The dogs were analysed by an in-house ELISA assay performed as described elsewhere with some modifications (Riera et al. 1999). Sonicated promastigotes of an autochthonous *L. infantum* strain (MCAN/ES/92/BCN-83) belonging to zymodeme MON-1 were used at a protein concentration of 20 μg/ml in 0.05 M carbonate buffer, pH 9.6. The sera were diluted to 1:400 in phosphate-buffered saline-Tween 1% milk (Sigma, St. Louis, MO, USA) and protein A peroxidase (1:30,000; Sigma) was used as the second antibody. The reaction was quantified in units (U) by reference to a positive serum arbitrarily set at 100 U. The cut-off was established at 24 U.

Sandfly collection, blood digestion, and morphological identification

Captures were made with CDC-like traps constructed at the *Serveis Cientifico-tècnics* at the *Universitat de Barcelona*. The traps were set at sunset during the sandfly transmission

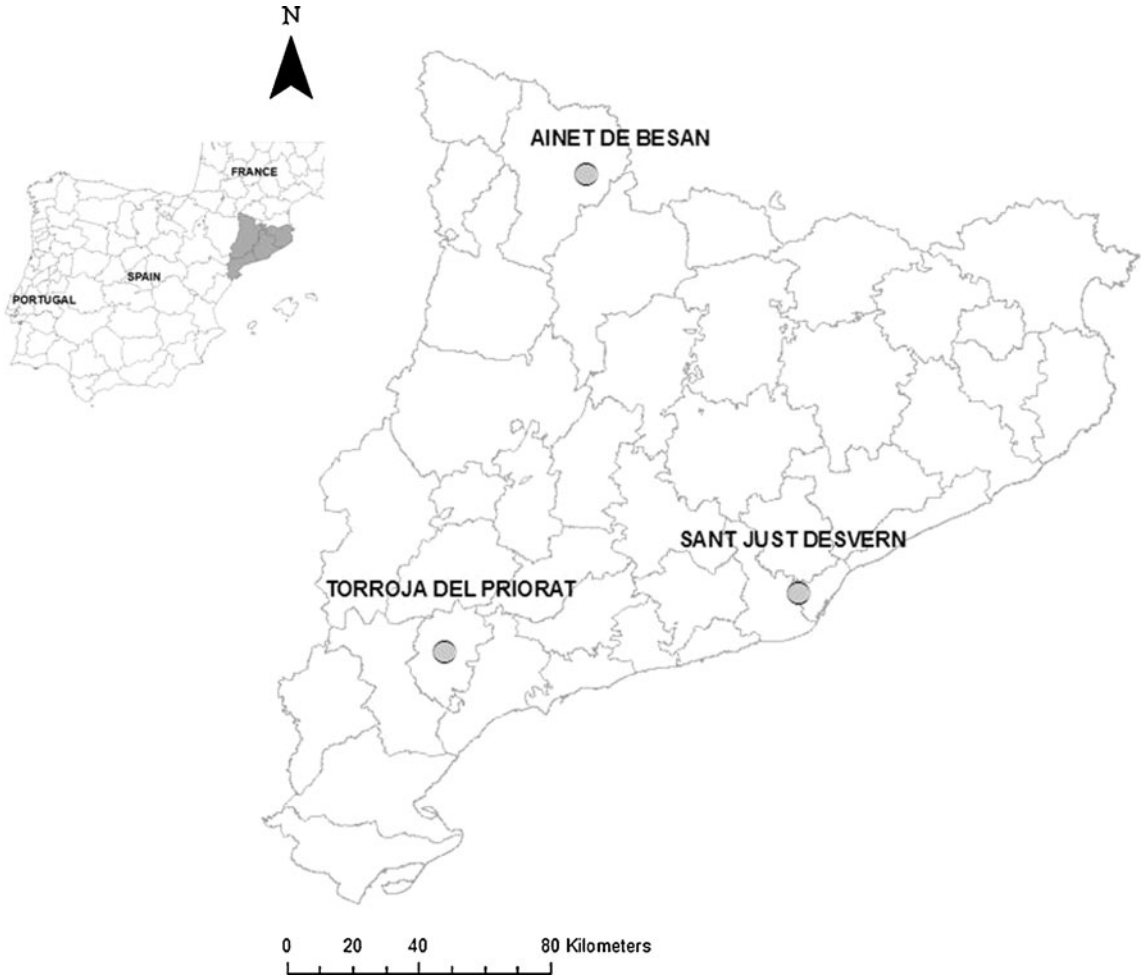

Fig. 1 Sampling sites of sandflies in Catalonia (NE Spain)

season and left in operation all night (from 8 p.m. to 8 a.m.) in the proximity of the dogs and other animals present in the farms (Table 1). Sandflies were removed from the traps with an electrical aspirator and then placed in a freezer to reduce their activity. Finally, about 30–50 specimens were put in single cryotubes and conserved in liquid nitrogen until their analysis.

One cryovial was randomly chosen from each farm among those conserved in liquid nitrogen. Females were transferred to a glass slide and dissected by removing the terminal segment of the abdomen in order to carry out their identification. The dissected females were examined under a microscope in order to identify the degree of blood digestion, following a previously established protocol (Dolmatova and Demina 1971) that allows the identification of seven categories of digestion. Sandflies were identified on the basis of their morphological features following the keys of Gállego et al. (1992). Accordingly, genitalia were clarified with chloralactofenol, mounted in Canada balsam, and identified under an optical microscope.

Leishmania infection in sandflies: ssu rDNA n-PCR

The presence of *Leishmania* DNA was assayed individually in randomly selected females. Genomic DNA was extracted from each specimen identified as *Phlebotomus* spp. with phenol–chloroform, precipitated with 100% ethanol, and then centrifuged for 30 min at 13,000 rpm. The DNA pellet was resuspended in 50 μl of sterile water and stored at −20°C until the n-PCR assay (Di Muccio et al. 2000).

In the n-PCR assay, the first amplification was carried out in a 50 μl volume of 10 μl DNA plus 40 μl PCR Master Mix (Promega) containing 50 pmol of the kinetoplastid-specific primers R221 and R332 of the small subunit rRNA gene (ssu rDNA) (van Eys et al. 1992). For the second amplification, 3 μl of the first PCR product was added to 47 μl of PCR Master Mix (Promega) containing 50 pmol of the *Leishmania*-specific primers R223 and R333 of the same gene (van Eys et al. 1992). The cycling conditions were denaturation at 94°C for 30 s, annealing at 60°C for 30 s (65°C for 30 s for the second PCR) and extension at 72°C

Table 1 Characteristics of sandfly capture sites and data on the incidence of HL in counties of Catalonia

County (province)	Nearest locality (distance)	Year of capture	Site relative to settlement	Latitude	Longitude	Altitude (m)	Adjacent flora	Presence of dogs (number)	Presence of other animals	Incidence of HL in the counties (Portús et al. 2007)
Priorat (Tarragona)	Torroja del Priorat (0.8 km)	July 2003	Between localities	41°13′ 10.89″ N	0°48′ 12.76″ E	289	Mixed oak	5	Rabbits	>5 cases/100,000 inhabitants
Pallars Sobirà (Lleida)	Ainet de Besan (1.37 km)	July 2007	Between localities	42°32′ 11.77″ N	1°16′ 51.23″ E	953	Mixed oak	3	Cows and rabbits	0.5–0.9 cases/100,000 inhabitants
Baix Llobregat (Barcelona)	Sant Just Desvern (0.82 km)	July 2002	Between localities	41°24′ 0.01″ N	2°55.05″ E	177	Holm oak	3	Sheep and goats	0.25–0.49 cases/100,000 inhabitants

for 30 s (Velo et al. 2005). Two negative controls (no DNA and DNA from male *Phlebotomus perniciosus* Newstead, 1911 or *Phlebotomus ariasi* Tonnoir, 1921 samples) and one positive control (a mixture of DNAs from *L. infantum* cultured promastigotes and colonized *P. perniciosus* samples) were used. A sample of 20 μl of the amplification products was analysed on 1.5% agarose gel and visualized under UV light. Positive samples yielded a predicted n-PCR product of 358 bp. This technique can detect virtually 5×10^{-4} parasite DNA.

Contamination by amplicons was avoided, as previously described, by using physical separation (rooms and materials) as well as decontamination procedures (UV exposure and bleaching of materials and surfaces). Cross contamination was monitored with negative controls for sample extraction and PCR solutions.

Leishmania sp. molecular typing: n-PCR—restriction fragment length polymorphism analysis

DNAs from sandfly specimens positive to ssu rDNA n-PCR were genotyped by ribosomal internal-transcribed spacer-1 (ITS-1) n-PCR, followed by restriction fragment length polymorphism (RFLP) analysis (ITS-1 n-PCR-RFLP) for *Leishmania* species molecular identification. Primers LITSR and L5.8 S were used to amplify a specific ITS-1 region in the ribosomal operon. Nested ITS-1 PCR was performed on 2 μl of the previous ITS-1 PCR products using the same primer combination and PCR conditions as for the first round of amplification (Schönian et al. 2003). Negative (no DNA, DNA from colonized *P. perniciosus*) and two different positive *Leishmania* spp. (*L. infantum*, MHOM/TN/80/IPT1; *Leishmania tropica* MHOM/SU/74/K27) DNA controls were used in amplifications. Molecular genotyping of *Leishmania* species was achieved by RFLP analysis of the 300–350 bp amplified sequence. Ten microliters of the PCR product were added to 2 μl of the enzyme buffer and 0.5 μl (10 U) of the restriction enzyme *Hae*III; this mixture was incubated at 37°C for 3 h, as recommended by the manufacturer (Promega). All PCR-RFLP products were analyzed by 4% MetaPhor agarose gel.

Statistical analyses

The number of n-PCR-positive samples of female sandflies was tabulated per location and species. The proportion of n-PCR-positive vectors (*P. ariasi* and *P. perniciosus*) per location was compared using Fisher's exact test.

To evaluate the association between n-PCR-positivity and the degree of blood digestion, only the blood-fed females of *P. ariasi* and *P. perniciosus* of farms from Torroja del Priorat and Ainet de Besan were used. A logistic regression model was used to assess the association between PCR-

positivity and the degree of blood digestion. The origin of the sandflies, the *Phlebotomus* species and the presence of eggs (i.e., yes or no) were included in the model as covariates but were removed from the final model if not statistically significant (P value of <0.05). The predicted values of the logistic regression model (i.e., probability of obtaining a n-PCR-positive in function of the degree of blood digestion) were plotted.

Results

Canine leishmaniasis in the farms

A total of 11 dogs were examined from the three farms: five from Torroja del Priorat, three from Ainet de Besan, and three from Sant Just Desvern. Six of the examined dogs were serologically positive: the five dogs from the rabbit farm of Torroja del Priorat and one of the three dogs from the farm in Ainet de Besan. None of the three dogs from the Sant Just Desvern farm was serologically positive (Table 1).

Leishmania positivity infection in sandflies by n-PCR

Only females of the genus *Phlebotomus* were included in the analysis (70 *P. ariasi*, 31 *P. perniciosus*, and 10 *Phlebotomus sergenti*) and the three captured males, one of *P. ariasi* (Ainet de Besan), and two of *P. perniciosus* (Torroja del Priorat and Sant Just Desvern) were used as negative controls. The proportions of n-PCR-positive samples in the different farms were 76.6% in Torroja del Priorat, 18.9% in Ainet de Besan, and 0% in Sant Just Desvern (Table 2). The differences were statistically significant (P value of <0.001). In addition, the single specimen of *P. sergenti* collected in Torroja del Priorat was n-PCR positive (Table 2).

A total of 68 blood-fed female sandfly vectors of *L. infantum* (56 *P. ariasi* and 12 *P. perniciosus*) captured in Torroja del Priorat and Ainet de Besan were included in the statistical model to study the association between blood digestion and n-PCR results. According to the multivariate logistic regression model, the risk of n-PCR positivity increased significantly with the degree of blood digestion (OR=1.3; P value=0.025). The level of risk differed between the two farms (OR=0.1, P value of <0.001), which was clearly visualized when the probability of n-PCR positive results per degree of digestion was plotted (Fig. 2). The sandfly species and the presence of eggs in female sandflies were not statistically associated with n-PCR positivity (P value of >0.05) and were not kept in the final model.

Leishmania sp. molecular typing

Leishmania molecular typing by ITS-1 n-PCR-RFLP analysis was carried out on the 43 sandfly specimens that were *Leishmania* ssu rDNA n-PCR positive: 30 *P. ariasi* (23 from Torroja del Priorat and 7 from Ainet de Besan), 12 *P. perniciosus* (from Torroja del Priorat) and 1 *P. sergenti* (from Torroja del Priorat). Twenty-six *P. ariasi* (20 from Torroja del Priorat and 6 from Ainet de Besan), nine *P. perniciosus* from Torroja del Priorat, and one *P. sergenti* from Torroja del Priorat were positive to ITS-1 n-PCR-RFLP showing the specific *L. infantum* pattern (184–72–55 bp bands) (Fig. 3).

Discussion

The estimation of transmission intensity in a leishmaniasis focus is based on different indicators. In Spain, as in other countries worldwide, these studies have included serological

Table 2 Distribution of sandfly species and results of n-PCR-positive samples per location

Location	Species	Number of specimens	n-PCR positive (number; percentage %±95% confidence interval)
Torroja del Priorat	*Phlebotomus ariasi*	32	23 (71.9±6.3)
	Phlebotomus perniciosus	14	12 (85.7±5.7)
	Phlebotomus sergenti	1	1 (100)
	Total	47	36 (76.6±12.1)
Ainet de Besan	*P. ariasi*	36	7 (19.4±12.9)
	P. perniciosus	1	0 (0)
	Total	37	7 (18.9±12.05)
Sant Just Desvern	*P. ariasi*	2	0 (0)
	P. perniciosus	16	0 (0)
	P. sergenti	9	0 (0)
	Total	27	0 (0)

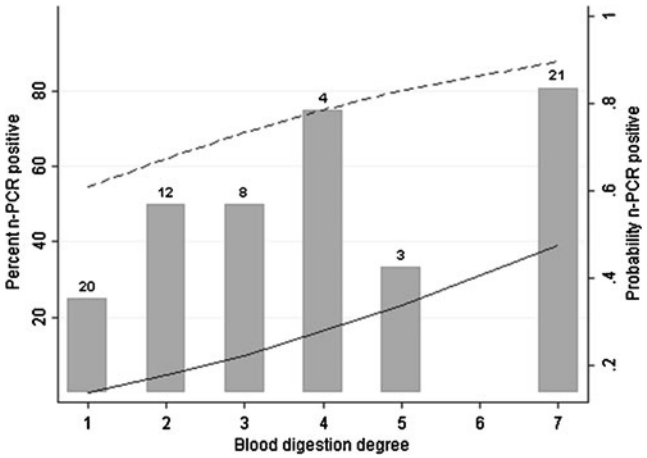

Fig. 2 Histogram representing the percentage of n-PCR-positive sandlfies (*P. ariasi* and *P. perniciosus*) degree of blood digestion. The total number of sandflies per degree of digestion is also presented on the top of each bar. The logistic regression predictive values representing the probability of a n-PCR-positive sample in function of the degree of blood digestion in Torroja del Priorat (*dashed line*, sandflies analyzed—*P. ariasi*, 26 and *P. perniciosus*, 11) and Ainet de Besan (*solid line*, sandflies analyzed—*P. ariasi*, 30 and *P. perniciosus*, 1) are also plotted

surveys mainly of the canine reservoir but also of the human population (Riera et al. 2004; Portús et al. 2007). Serological surveys are useful due to the facility of sampling, rapidity in obtaining results, consistency and because they allow the detection of a heterogeneous distribution of CanL even in small foci. Nevertheless, some inconveniences have been reported, including the limited sensitivity of the serological techniques or the detection of false-positive results (Alvar et al. 2004). Other invasive techniques, like Montenegro's intradermal reaction test, have been used in humans and more recently in dogs (Iniesta et al. 2002, Riera et al. 2004), but false-positive results can occur due to the phenol-containing diluent (Pineda et al. 2001). Other investigations in leishmaniasis endemicity have analysed *Leishmania*

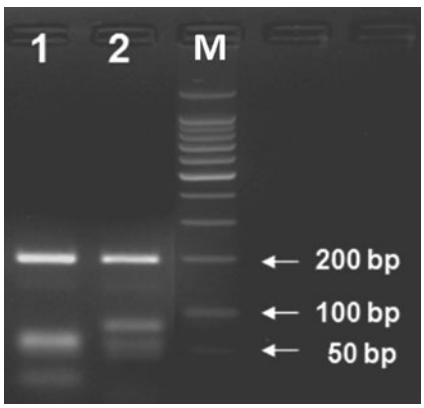

Fig. 3 RFLP analysis (ITS-1 region). *Line 1*, *L. tropica* (185, 57, 53, and 24 bp), *line 2*, *L. infantum* (184, 72, and 55 bp), and *M*, 50-bp DNA ladder marker

parasitism in sandflies by classical dissection methods. Although these are time-consuming and need a large number of specimens (Rossi et al. 2008), they allow vector species to be incriminated in different foci, as has been done with *P. ariasi* and *P. perniciosus* in Spain (Rioux et al. 1986). The increasing application of molecular techniques in this field has considerably reduced the time involved in obtaining results, although their scarce use in Spain has resulted in only one publication to date (Martín-Sánchez et al. 2006).

In the present work, we applied a n-PCR technique in the study of three sandfly populations captured in isolated farms located in different counties of Catalonia, each with a different degree of HL and CanL prevalence. The overall n-PCR positivity in sandflies was high (38.7%) and the results obtained from the three sandfly populations were related with the number of infected dogs found in each farm as well as the level of HL endemicity in the respective counties (Portús et al. 2007) (Tables 1 and 2). Torroja del Priorat is a well-known endemic focus of CanL (Fisa et al. 1999) and HL, but none of the dogs studied in this location showed clinical signs of the disease, despite the high percentage of sandflies detected with leishmanial DNA (76.6%). This supports the postulation that asymptomatically infected dogs may play an important role in the transmission of the disease (Molina et al. 1994). In the other two locations, despite the low number of dogs studied, the n-PCR results correlated with the serological results. In Sant Just Desvern the negative serological and n-PCR results obtained in the dogs and sandflies could be explained by the limited size of the sampling area and the low dispersal capacity of sandflies since the HL data for the county and the opinion of local veterinarian practitioners confirm the presence of CanL in this location. The results demonstrate the utility of this technique for the study of the distribution and intensity of leishmaniasis foci.

The detection of *Leishmania* DNA was similar in the *P. ariasi* and *P. perniciosus* specimens captured in the farm of Torroja del Priorat. These results match those previously obtained in this locality by classical dissection, confirming that both species act as *L. infantum* vectors in sympatric conditions (Rioux et al. 1986). Nevertheless, it is difficult to compare the proportion of infected sandflies detected in the two studies because of the different times of sandfly capture, locations and size of sampling sites. In the farm of Ainet de Besan only *P. ariasi* was n-PCR-positive (the single specimen of *P. perniciosus* captured did not allow vectorial comparison).

Susceptibility and resistance of sandflies to the complete development of *Leishmania* appears to be under genetic control. It has been shown experimentally that differences exist in the ability of the parasite to survive within the early blood-fed midgut and/or to persist in the gut after excretion of the digested bloodmeal (Sacks 2001). The mere detection

of *Leishmania* DNA in a sandfly specimen does not prove that it is acting as a competent vector (Rossi et al. 2008). Thus, the finding of *L. infantum* DNA in one specimen of *P. sergenti* does not implicate this sandfly as a vector of this *Leishmania* species in the area. *P. sergenti* is a vector of *L. tropica*, which causes cutaneous leishmaniasis in humans, and can also feed on and transmit *L. tropica* to dogs (Dereure et al. 1991). Nevertheless, despite the presence of *P. sergenti* populations in Spain, genetically identical to those found in a new focus of cutaneous leishmaniasis in Morocco (Barón et al. 2008), until now *L. tropica* has not been identified in the country.

Opportunistic behaviour of sandflies regarding feeding tropism has been observed for some species (Guy et al. 1984; de Colmenares et al. 1995). As mentioned, *P. sergenti* can bite dogs and it has been demonstrated in experimental conditions that this species can initially became infected with *L. infantum* to a similar extent as *P. perniciosus*, although after 3-4 days the parasites are lost (Killick-Kendrick 1985), reducing or voiding their transmission capacity. Due to their great sensitivity, PCR techniques are able to detect parasites in infected sandflies irrespective of their quantity, stage, location in the gut or proboscis and transmissibility (Perez et al. 1994). *Leishmania*-DNA-positive sandflies can include not only those carrying live promastigotes, but also those carrying DNA fragments of *Leishmania* resulting from non-established infections or blood meals containing *Leishmania* fragments (Gradoni 2002).

Leishmania DNA has also been found in ticks and fleas parasitizing dogs, including in experimental conditions, but these findings do not conclusively demonstrate that these arthropods can act as leishmaniasis vectors (Dantas-Torres et al. 2010; Paz et al. 2010; Colombo et al. 2011).

Despite these inconveniences, detection and identification of *Leishmania* species within naturally infected sandflies is important for predicting the risk and expansion of leishmaniasis in endemic areas. In our study, n-PCR has been useful in detecting differences between farms in areas with varying levels of CanL and HL incidence. Furthermore, the specificity of PCR techniques avoids the common mistake of considering motile flagellates (not only *Leishmania* spp.) in sandfly guts as necessarily an indication of *Leishmania* infection (Perez et al. 1994).

Most female sandflies require a bloodmeal for egg development, which allows contact with both infected and non-infected hosts and the transmission of leishmanias. *P. ariasi* and *P. perniciosus* are gonotrophically concordant and do not feed again until the bloodmeal is digested and the eggs are laid (Killick-Kendrick and Rioux 2002; WHO 2010). When the parasites arrive in a susceptible sandfly gut, to be infective they should multiply at the same time as the blood is digested, and the promastigotes should remain in the gut at the moment of defecation before finally reaching the stomodeal valve and mouth parts. Presumably, an increasing degree of blood digestion would result in the presence of more parasites and thus a higher possibility of detecting leishmania DNA. The intensity of infection by *L. infantum* in *P. ariasi* in natural conditions increases progressively during the second and third ovarian cycles (Killick-Kendrick and Rioux 2002).

A literature search (PubMed and ISI Web of Knowledge) did not yield any studies on the correlation between positivity in dissecting and/or molecular methods and the degree of blood digestion in sandflies in natural conditions. When different techniques (classical dissection and estimation in situ, direct counting with a hemocytometer and quantitative PCR) for calculating the degree of infestation were compared in experimental conditions, the results differed depending on whether the sandflies were examined before or after defecation. The greater sensitivity of PCR has also been demonstrated (Myskova et al. 2008).

The results obtained in the present study show a higher percentage of n-PCR positivity with increased blood digestion. In previous studies, it has been difficult to calculate the degree of infestation by non-molecular techniques when a semidigested bloodmeal contains erytrocytes or when promastigotes remain attached to the midgut tissue (Myskova et al. 2008).

In sandflies with a low degree of digestion, there is a strong possibility that the detected DNA belongs to the recently ingested amastigotes or to initially formed non infective promastigotes. As the bloodmeal digestion increases, so does the multiplication of the promastigotes and the probability in finding metacyclic forms.

No sandfly without a blood meal gave positive n-PCR results, possibly due to the loss of parasites during defecation (Myskova et al. 2008). The possibility that most females were nulliparous must also be taken into account, even though the captures were performed during July when several gonotrophic cycles had probably taken place.

The only specimen of *P. sergenti* found positive for *L. infantum* DNA showed blood digestion level 2, but, as mentioned before, it is likely that this specimen would lose the parasites after ingestion (Killick-Kendrick 1985). The vectorial role of this species for *L. tropica* has also been commented on.

The results of this work confirm the vectorial capacity of *P. ariasi* and *P. perniciosus* in the transmission of *L. infantum* in the locality of Torroja del Priorat and also provide new data for *P. ariasi* in Ainet de Besan, which can be considered at risk of becoming a new focus of CanL (Ballart et al. 2012). The utility of molecular techniques and importance of sandfly blood meal digestion for epidemiological surveys of leishmaniasis foci have been demonstrated.

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Ethical standards The experiments of the manuscripts submitted for publication comply with the current laws of Spain and Italy.

Conflict of interest The authors have no conflicts of interest concerning the work reported in this paper.

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