Leishmania infection and host-blood feeding preferences of phlebotomine sandflies and canine leishmaniasis in an endemic European area, the Algarve Region in Portugal

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The Algarve Region (AR) in southern Portugal, which is an international tourist destination, has been considered an endemic region of zoonotic leishmaniasis caused by Leishmania infantum *since the 1980s. In the present study, phlebotomine and canine surveys were conducted to identify sandfly blood meal sources and to update the occurrence of* Leishmania *infection in vectors and dogs. Four sandfly species were captured:* Phlebotomus perniciosus, Phlebotomus ariasi, Phlebotomus sergenti *and* Sergentomyia minuta. *In one* P. perniciosus *female,* L. infantum *DNA was detected. Blood meal tests showed that this species had no host preferences and was an opportunistic feeder. An overall canine leishmaniasis (CanL) seroprevalence of 16.06% was found; the seroprevalence was 3.88% in dogs housed in kennels and 40.63% in dogs that attended veterinary clinics. The simultaneous occurrence of dogs and* P. perniciosus *infected with* L. infantum *in the AR indicates that the region continues to be an endemic area for CanL. Our results reinforce the need for the systematic spatial distribution of phlebotomine populations and their* Leishmania *infection rates and the need to simultaneously perform pathogen monitoring in both invertebrate and vertebrate hosts to investigate the transmission, distribution and spreading of* Leishmania *infection.*

Key words: phlebotomine sandflies - vectors - *Leishmania infantum* - dog - blood meal preferences - Portugal

Zoonotic leishmaniasis, which is caused by *Leishmania infantum*, is endemic in the Mediterranean basin. Dogs are considered the major host for these parasites and the main reservoir for human infection. In nature, transmission among dogs and to humans occurs through the bite of infected phlebotomine sandflies.

The Algarve Region (AR) in southern Portugal is the most popular tourist destination in the country and one of the most popular in Europe. AR was considered an endemic region of human leishmaniasis in the 1980s. Forty-three infantile cases of visceral leishmaniasis were diagnosed in the Paediatric Service at the Faro District Hospital between 1980-1988, most of which were from the municipality of Loulé (Vicente 1990). However, in the last decade, human leishmaniasis in this region was only notified in four adult cases: two cases in 2006, one in 2007 and one in 2008 (dgs.pt). This frequency suggests that humans only develop this disease under special conditions, such as immunosuppression (Campino & Maia 2010). Five phlebotomine species have been reported in the AR (*Phlebotomus perniciosus, Phlebotomus ariasi, Phlebotomus papatasi, Phlebotomus sergenti* and *Sergentomyia minuta*) and *L. infantum* zymodeme MON-1 has been isolated from *P. perniciosus*, dogs and humans (Campino et al. 2006).

The purpose of this work was to record an updated *Leishmania* infection rate in phlebotomine sandflies and determine their potential vertebrate hosts. In parallel, a serological survey of domestic and kennelled dogs was conducted.

MATERIALS AND METHODS

Study area - The AR is the southern region of mainland Portugal (Figure). It has an area of 5,412 Km^2 with 451,005 permanent inhabitants and incorporates 16 counties (INC 2011). The human population triples in the summer season. Tourism, fisheries and agricultural activities are important for the local economy. Figs (*Ficus carica*), almonds (*Prunus amygdalus*), oranges (*Citrus sinensis*), carobs (*Ceratonia siliqua*), strawberries trees (*Arbutus unedo*) and cork oak (*Quercus suber*) are the most common crops in the region (Franco 1994).

The AR has a Mediterranean climate with warm weather (annual average temperature of 18ºC) and low rainfall almost the entire year (annual average of 500 mm). Summer (June-September) is the driest and warmest season, with average monthly temperatures between 16-28/30ºC (-meteo.pt).

Sandfly collection and identification - From March-November 2007, CDC miniature light traps were set out from sunset to sunrise in 175 biotopes located at alti-

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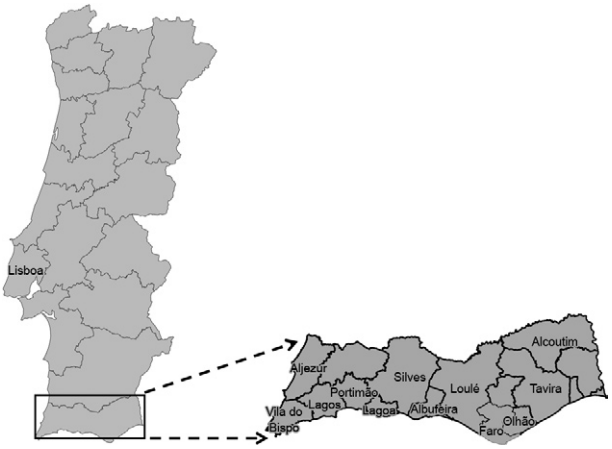

Map of Portugal with the location where the entomological survey was made: Algarve Region.

tudes between 2-209 m above sea level and monitored for sandfly presence. Captures were made in the counties of Albufeira, Aljezur, Lagoa, Lagos, Portimão, Silves and Vila do Bispo in the west area of the AR (Barlavento), as well as in Alcoutim, Faro, Olhão, Loulé and Tavira in the east area (Sotavento) (Table I). Collections were made in farms and cottage houses with yards and gardens. In most of the studied biotopes, in addition to humans and dogs, the major vertebrates available within a 50 m radius were cats, cows, sheep, goats, pigs, donkeys, rabbits and birds (poultry and exotic). In 41 of the 175 biotopes, insecticides (collars, spot-on or spray) were used on dogs.

A total of 1,663 sandfly adults were collected. The specimens were preserved in 70% ethanol and maintained at room temperature (22-26ºC) for further analysis. Phlebotomine specimens of both genders were identified by their morphological characteristics at the species level according to Pires (1979). In addition, for each female, it was determined if it was gravid (with eggs in the abdomen), engorged (blood in the abdomen, total or partial) or unfed (no visible blood in the abdomen).

Leishmania spp detection in female sandflies - After microscopic species identification, the body of each female (minus genitalia) was used for molecular analysis. The individual female's body was homogenised in lysis buffer (200 µL) from a polymerase chain reaction (PCR) kit (PCR-Template Preparation kit, Roche Diagnostics GmbH, Germany) and incubated with the buffer and proteinase K (40 µL). The DNA was then extracted according to the kit's instructions. PCR amplification for the identification of *Leishmania* spp in females was performed using specific primers for two molecular markers: the internal transcribed spacer 1 of the ribosomal operon of *Leishmania* and the kinetoplastid minicircle DNA (kDNA) sequence of the *Leishmania donovani* complex (Schonian et al. 2003, Cortes et al. 2004). Amplification of 314 base pair (bp) and 447 bp PCR products was analysed on a 1.5% agarose gel stained with ethidium bromide. Positive (*L. infantum* and *Leishmania tropica* DNA) and negative controls were included.

Sandfly blood meal identification - Blood meals were identified using the modified vertebrate-universal specific primers cytB1-F and cytB2-R to amplify a 350 bp segment of the host mitochondrial cytochrome *b* gene (Svobodova et al. 2008). PCR-amplified products were sequenced on both strands using a BigDyeTerminator v1.1 Applied Biosystem ABI PRISM 3700 DNA Analyzer (Stabvida® Sequence Service, Portugal). Sequences were compared with sequences deposited in the GenBank database using vertebrate standard nucleotide BLAST searches.

Canine leishmaniasis (CanL) serosurvey - In parallel with the phlebotomine survey, a total of 193 adult dogs of both genders were analysed, including both mongrel and pedigree animals; 129 animals were from official and private kennels and 64 attended veterinarian clinics.

TABLE I

Characteristics of the sandfly collecting sites

	County	Altitude (m)	Hosts available within a 50 m radius
West area (Barlavento)	Albufeira	195	Humans, dogs
	Aljezur	2	Humans, dogs, cats
	Lagoa	2	Humans, dogs
	Lagos	2	Humans, dogs, cats
	Portimão	2	Humans, dogs, cats, cows, chickens
	Silves	15	Humans, dogs, sheep, chickens
	Vila do Bispo	2	Humans, dogs
East area (Sotavento)	Alcoutim	2	Humans, dogs, donkeys, cows, goats, sheep, pigs, chickens
	Faro	209	Humans, dogs, cats, domestic and wild rabbits, chickens, geese, ducks, exotic birds
	Loulé	181	Humans, dogs, cats, sheep, chickens
	Olhão	35	Humans, dogs, cats, chickens
	Tavira	2	Humans, dogs, cats

Peripheral blood samples were collected by cephalic or jugular venipuncture. Serum samples for serological testing were separated by centrifugation and preserved at -20ºC until use. Determination of the total anti-*Leishmania* antibodies was performed by an indirect immunofluorescence antibody test (IFAT), counterimmunoelectrophoresis (CIE) and an immunochromatographic dipstick test rK39 (InBios®, USA). IFAT and CIE were performed as previously described (Maia et al. 2010). Briefly, for CIE, all reactions with at least one precipitation were considered positive. The IFAT cut-off value was established at a serum dilution of 1/64. In both tests, negative and positive sera were used. The rK39 test was performed according to the manufacturer's guidelines. The appearance of two pink lines indicated a positive result, while the appearance of only one line indicated a negative result. Dogs were considered seropositive if at least two of the techniques were positive (Maia & Campino 2008). A bone marrow biopsy was performed in 13 animals (seropositive and/or with clinical signs compatible with CanL) for in vitro culture in Novy-MacNeal-Nicolle medium.

The study was approved by the Ethical Committee of the Institute of Hygiene and Tropical Medicine, Nova de Lisboa University, and followed Portuguese legislation guidelines (Lei 92/95, 12.9).

RESULTS

In this work, four sandfly species were captured: *P. perniciosus, P. ariasi, P. sergenti* and *S. minuta*. *L. infantum* DNA was detected in one *P. perniciosus* female. Cats, rodents, chickens and lizards were identified as blood sources. An overall CanL seroprevalence of 16.06% was found.

Sandflies survey - A total of 1,663 sandflies (888 females and 775 males) were collected in 55 of the 175 monitored biotopes and identified morphologically. The prevalence was 86.59% for *P. perniciosus* (1,440 specimens), 10.35% for *S. minuta* (172 specimens) and 2.4% for *P. ariasi* (40 specimens). A few (11) of *P. sergenti* specimens (0.66%) were also collected.

Summer was the season with the highest phlebotomine density (Table II). *P. perniciosus* was the dominant species and was collected in all biotopes from western AR-eastern AR, except in Lagoa (Table III).

Leishmania spp detection in sandflies - One *P. perniciosus* female was found to be infected with *Leishmania* DNA; thus, there was a 0.13% (1/773) overall infection rate for this species. The infected specimen was detected in September in Olhão County (Sotavento). Gene sequences of the kDNA products were compared with *Leishmania* sequences available in GenBank and the specimen was found to have a 98% homology with *L. infantum*. Although humans, poultry and dogs (1 of which had clinical and laboratory-confirmed leishmaniasis) were present in the biotope where the infected sandfly female was captured, no blood (fresh or digested) was visible in its midgut.

Sandfly blood meal identification - Sixty-four (full or partial) blood-fed females were tested for host blood identification. After sequencing the amplified part of the cytochrome *b* gene, 37 (57.81%) blood meals were identified. One cat, 27 rodents, six chickens and three lizards were identified as blood sources (Table IV). It was not possible to identify the blood source in 27 samples.

CanL survey - A simultaneous CanL survey revealed that 31 of the 193 (16.06%) screened dogs were seropositive for *Leishmania* infection (Table V). A prevalence of 3.88% (5/129) was found in kennelled dogs and 40.69% (26/64) in dogs that were taken to veterinarian clinics. Parasites were isolated from one dog and identified as *L. infantum* MON-1 by iso-enzymatic typing at the National Reference Center for *Leishmania*, Montpellier University (Rioux et al. 1990).

DISCUSSION

Phlebotomine sandflies are present in periurban, rural and sylvatic environments and distributed in all countries around the Mediterranean basin. Therefore, human populations and domestic animals living in these areas are potential targets of sandfly-borne diseases, such as

TABLE II

Phlebotomine sandfly densities in Algarve Region

	Month[a]								
Phlebotomine species	March	April	May	June	July	August	September	October	November
Phlebotomus perniciosus	0.75	0.00	0.00	19.90	16.84	0.20	11.21	3.89	0.00
Phlebotomus ariasi	0.00	0.00	0.00	0.20	0.88	0.00	0.19	0.17	0.00
Phlebotomus sergenti	0.00	0.00	0.00	0.30	0.20	0.00	0.00	0.00	0.00
Phlebotomus papatasi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sergentomyia minuta	0.33	0.00	0.00	0.25	4.64	0.20	0.90	0.22	0.00
Total[a]	1.08	0.00	0.00	20.65	22.56	0.40	12.30	4.28	0.00

a: sandfly density = total number of sand flies/CDC light trap (month).

TABLE III

Sandfly specimens collected, number of fed, gravid and infected females detected per county

		Sandfly species											F		
		Phlebotomus perniciosus		*Phlebotomus ariasi*		*Phlebotomus sergenti*		*Sergentomyia minuta*		Total					
	County	F	M	F	M	F	M	F	M	F	M	F + M	Blood fed	Gravid	Infected
West area (Barlavento)	Albufeira	188	122	7	5	0	0	21	19	216	146	362	25	11	0
	Aljezur	28	33	4	4	0	0	5	31	37	68	105	2	0	0
	Lagoa	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Lagos	5	1	0	0	0	0	0	0	5	1	6	2	1	0
	Portimão	1	1	0	0	0	0	0	0	1	1	2	0	3	0
	Silves	130	113	2	0	0	0	0	0	132	113	245	4	3	0
	Vila do Bispo	30	44	0	0	0	0	3	2	33	46	79	0	2	0
East area (Sotavento)	Alcoutim	62	75	3	3	0	0	5	4	70	82	152	10	3	0
	Faro	1	2	0	0	0	0	0	1	1	3	4	0	4	0
	Loulé	18	6	1	0	0	0	3	1	22	7	29	1	2	0
	Olhão	229	214	4	2	7	1	11	8	251	225	476	12	21	1
	Tavira	81	56	3	2	2	1	34	24	120	83	203	7	22	0
Total		773	667	24	16	9	2	82	90	888	775	1,663	63	72	1

F: female; M: male.

leishmaniasis. An updated analysis of the distribution, relative abundance, *Leishmania* infection rate and blood feeding preferences of phlebotomine sandflies was performed in the AR, which is the most popular summertime tourist destination in Portugal and one of the most popular tourist destinations in Europe. Simultaneously, a CanL seroprevalence survey was also performed because dogs are considered the main host of *Leishmania*.

Four sandfly species previously known to be present in AR were captured: *P. perniciosus, P. ariasi, P. sergenti* and *S. minuta*. Summer had the highest sandfly density and activity. *P. perniciosus* was the most abundant species, followed by *S. minuta*. *L. infantum* was detected in one *P. perniciosus* female, resulting in a 0.13% (1/773) infection rate of this species. The presence of *L. infantum* demonstrates that *P. perniciosus* remains a vector of *Leishmania* in this region. This result, which is similar to the 0.35% and 0.52% infection rates observed previously (Alves-Pires et al. 2001, Maia et al. 2009), was expected because no phlebotomine control strategies have been developed. In the present study, the infected sandfly was detected in September, confirming that the probability of *Leishmania* vectorial transmission is higher during summertime. We observed by microscopy that the infected sandfly did not contain either fresh or digested blood in its midgut.

Knowledge of the host preferences of phlebotomine sandflies under natural conditions is essential to understand their role as vectors and detect other potential reservoir hosts. The lack of identification of the blood sources in 27 of the 64 blood-fed sandfly females was likely related to the inherent requirement of PCR for relatively fresh blood. In fact, it was observed microscopically that blood was partially or almost completely digested in some specimens.

The results of the blood meal analyses suggest that *P. perniciosus* appears to be an opportunistic feeder, as females were found to be engorged with chicken, rodent and cat blood. This result is partially in agreement with previous reports from Spain (Colmenares et al. 1995) and Italy (Bongiorno et al. 2003). The high number of rodents found to be blood sources for *P. perniciosus* was likely because this work was primarily conducted in rural areas, including several sylvatic biotopes. *P. sergenti* and *S. minuta* exhibited blood meal preferences for chickens and lizards, respectively. This finding is in agreement with what is known about the *Sergentomyia* genus. These sandflies are considered vectors of *Sauroleishmania* and they frequently feed on reptiles, such as *Tarentola mauritanica* and *Hemydactilus turcicus*, which are abundant in southern Portugal and widely distributed around the Mediterranean area (Bates 2007, Alves-Pires et al. 2008). Surprisingly, in our study, no dog or human blood was found in engorged sandfly females. The absence of blood from dogs could have been due to the use of insecticides and repellents in approximately 23%

TABLE IV

Identification of sandfly blood meal sources

Species	Blood meal identification/ fed females	Blood meal source			
		Chicken	Rodents	Cat	Lizard
Phlebotomus perniciosus	31/56	3	27	1	0
Phlebotomus ariasi	3/3	2	0	0	1
Phlebotomus sergenti	1/3	1	0	0	0
Sergentomyia minuta	2/2	0	0	0	2
Total	37/64	6	27	1	3

TABLE V

Anti-*Leishmania* antibodies analysed by rK39, CIE and IFAT in dogs from Algarve Region

Source	Dogs (n)	Negative (n)	Positive				
			rK39 + CIE	rK39 + IFAT	CIE + IFAT	rK39 + CIE + IFAT	Total
Veterinarian clinics	64	38	2	10	7	7	26
Kennels	129	124	2	1	0	2	5
Total	193	162	4	11	7	9	31

CIE: contraimmunoelectrophoresis test; IFAT: indirect immunofluorescence antibody test; rK39: immunocromatographic test.

of the animals present in the studied biotopes. Alternatively, this result could be related to the fact that in rural areas, humans and dogs might not be the major blood source target for sandflies due to the presence of a high number of other vertebrates, such as the great predominance of rodents. Our results clearly showed that females preferred to take their blood meals from small animals, such as rodents, lizards and chickens, rather than from bigger/larger vertebrates. To confirm this conclusion, further work should be carried out to specifically determine such a preference and to determine if rodents play any role in the maintenance of *Leishmania* in the Algarve focus, which has observed in other endemic areas (Quinnell & Courtenay 2009).

In the CanL survey, each sample was screened by three tests and to improve diagnosis, only dogs with specific anti-*Leishmania* antibodies detected by at least two techniques were considered seropositive (Maia & Campino 2008).

An overall seroprevalence of 16.06% was obtained. The high prevalence of 40.63% (26/64) in dogs that attended veterinarian clinics likely reflects the fact that all animals were clinically suspected of having *Leishmania* infection. This CanL seroprevalence was higher than the 5.02% obtained in 2009 in 206 randomly screened animals from the AR that attended veterinarian clinics (Cortes et al. 2012) and the 25.7% obtained in 105 dogs with clinical signs compatible with vector borne diseases in 2010-2011 (Cardoso et al. 2012). This discrepancy could be related to the small number of the animals screened in our study and the fluctuation of the prevalence of infection between transmission seasons. Our results indicate the maintenance of leishmaniasis in the AR and reinforce the importance of *P. perniciosus* as a vector of *L. infantum* in southern Portugal. These results are in contrast with what has been observed in the central and northern regions, where *P. ariasi* specimens were also found to be infected (Campino et al. 2006, Branco et al. 2013). This geographical distribution of known vectors may be related to the high density of *P. perniciosus* in the AR and/or due to bioecological aspects that should be determined in future work. The gap between the number of human and CanL cases observed in this region emphasises the fact that *Leishmania* can be considered an accidental or opportunistic infection in humans (3 adult cases notified in 2006-2007). Nevertheless, it is also important to note that global climate changes associated with a higher density and activity of sandfly species over a longer period might (i) enhance the number of days favourable for the transmission of parasites and therefore (ii) increase leishmaniasis incidence not only in animals, but also in humans. Moreover, it is important to alert tourists from non-endemic countries who are spending their summer holidays in the AR with their dogs that they should implement prophylactic measures prior to travelling to ensure their pets are not infected (Shaw et al. 2009, Menn et al. 2010).

The *P. sergenti* vector of *L. tropica* was identified in the AR; however, no *L. tropica* DNA was amplified from the *P. sergenti* specimens collected in this study. Although human cases of *L. tropica* have not been recorded in Portugal, the risk of the potential introduction of new *Leishmania* species from travellers or immigrants from North Africa should not be neglected given that its vector is already present in the country (Afonso et al. 2005).

Programmes and/or monitoring projects should be developed to detect areas at risk for vector transmission to enable the implementation of specific and effective control measures. To do so, it is necessary that the local population, technicians, public and private institutions and institutions of local and regional management be aware of these problems and to actively participate in the control of sandflies, particularly in facilitating the placement of material to capture sandflies (i.e., the maintenance of collecting stations and participation in local CanL surveys). According to the illustrious parasitologist Killick-Kendrick (2010), education is key for controlling visceral leishmaniasis.

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