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Leishmaniases and the Cyprus Paradox

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Abstract. In Cyprus, leishmaniasis has been considered exclusively a veterinary problem. It was prevalent before 1945, and until its recent reemergence, it was nearly eradicated by 1996 as a consequence of the destruction of reservoir hosts and vectors. A survey carried out to provide an unbiased estimate of current transmission rates in dogs and humans showed a 9-fold increase in dog seroprevalence (reaching 14.9%) compared with 10 years ago. However, no human cases caused by *Leishmania infantum* were detected, although *L. donovani* cases were reported recently. The 62 strains isolated from dogs were typed as *L. infantum* MON-1 (98.4%), which is the predominating zymodeme in the Mediterranean region, and MON-98 (1.6%). The *Phlebotomus* species *P. tobbi* (vector of *L. infantum* in Cyprus), *P. galilaeus*, and *P. papatasi* were the predominant species captured. Two transmission cycles seem to run in parallel in Cyprus: in dogs with *L. infantum* and in humans with *L. donovani*.

INTRODUCTION

Leishmaniases are vector-borne diseases endemic in 88 countries with a wide range of clinical symptoms; visceral leishmaniasis (VL) is the most aggressive form of the disease and is life-threatening, if untreated. VL caused by *Leishmania infantum* is endemic in all countries of southern Europe with up to 25% seroprevalence in domestic dogs (the reservoir host) and 700 autochthonous human cases per year.[1]

Before 1945, canine VL (CanL) was widespread in Cyprus,[2] and *Phlebotomus tobbi* was incriminated as the vector of *L. infantum*.[3,4] The malaria-eradication campaign, 1940–1950, greatly reduced the sand-fly fauna in Cyprus,[5] whereas dog numbers fell dramatically (from 46,000 to 6,000) as a consequence of the successful anti-echinococcosis campaign of 1970–1975.[6] These actions resulted in the almost complete eradication of CanL in the government-controlled part of the island, which stayed clear of the disease for over 20 years.[4,7,8] However, sand-fly populations increased and the number of dogs (according to the number of dog vaccines sold in the island) recovered to an estimated 100,000 (17 dogs/km^2) after the end of the two control programs. As a consequence, CanL reemerged, and dog cases were again recorded in coastal areas in 1996, implicating *L. infantum* MON-1 as the causative agent.[4,7] In contrast to the relatively high infection rates observed in domestic dogs,[2–4] no passive or active surveys of human leishmaniasis have been conducted in the past in Cyprus, and only two human cases (infantile VL) are known to have occurred since 1935.[2,7] Because the possibility of human infection typically increases with the number of infected dogs in an area and human VL cases caused by *L. infantum* are known in all areas in the Mediterranean basin with high prevalence in CanL,[9] the assumption that there are no anthropophilic vectors on the island was reached.[4] Yet, in 2006, six autochthonous human cases were diagnosed as caused by *L. donovani*,[10] indicating that indigenous competent vectors are present on the island. Thus, the question remains: why are there no leishmaniasis cases in Cyprus caused by *L. infantum*? The survey conducted to resolve this was based on the assumption that if there were to be VL cases, they had to be in the areas with the highest dog seroprevalence. To locate these areas, an epidemiological study was carried out on the dog population. A map of Cyprus was divided into 82 equal squares of which 30 were chosen randomly as the study area. Dog seroprevalence revealed the areas with the highest risk for humans. In two such areas, as well as one area with zero dog seroprevalence, a seroepidemiological study was conducted on the human population, and sand-fly collections were done to compare species in these three areas.

MATERIALS AND METHODS

Study location. The government-controlled part of the island, southern Cyprus (Figure 1), which covers 5,896 km^2 and is comprised of five prefectures, was divided into 82 equal but arbitrary squares on a map. The squares were given numbers, and 30 numbers were drawn from a ballot to select the squares to be included in the study. One or more villages falling in each of the 30 squares, according to dog population size, were chosen so as to have at least 200 dogs per square. The size of the 30 areas, representing the 30 squares in our sampling protocol, depended on each village's geographic boundaries, which is different for each village; this area is the official reference to a village in Cyprus, and it includes the villagers' fields and farms, the areas where the dogs of the village mostly move.

Dog samples. A total of 900 dogs (379 males and 521 females; 719 hunting, 87 guard, and 94 companion) living in the 30 areas were sampled (30 dogs per area) during 2005 and 2006. Owned dogs, living permanently in the area irrespective of race, age, color, or health status, were included in the study. To select the dogs, the houses in each village in the study area were given numbers and drawn from a ballot. The dog owners visited with a government veterinarian; they were informed about leishmaniasis and asked to participate in the study by providing a written consent. The dogs were examined clinically,

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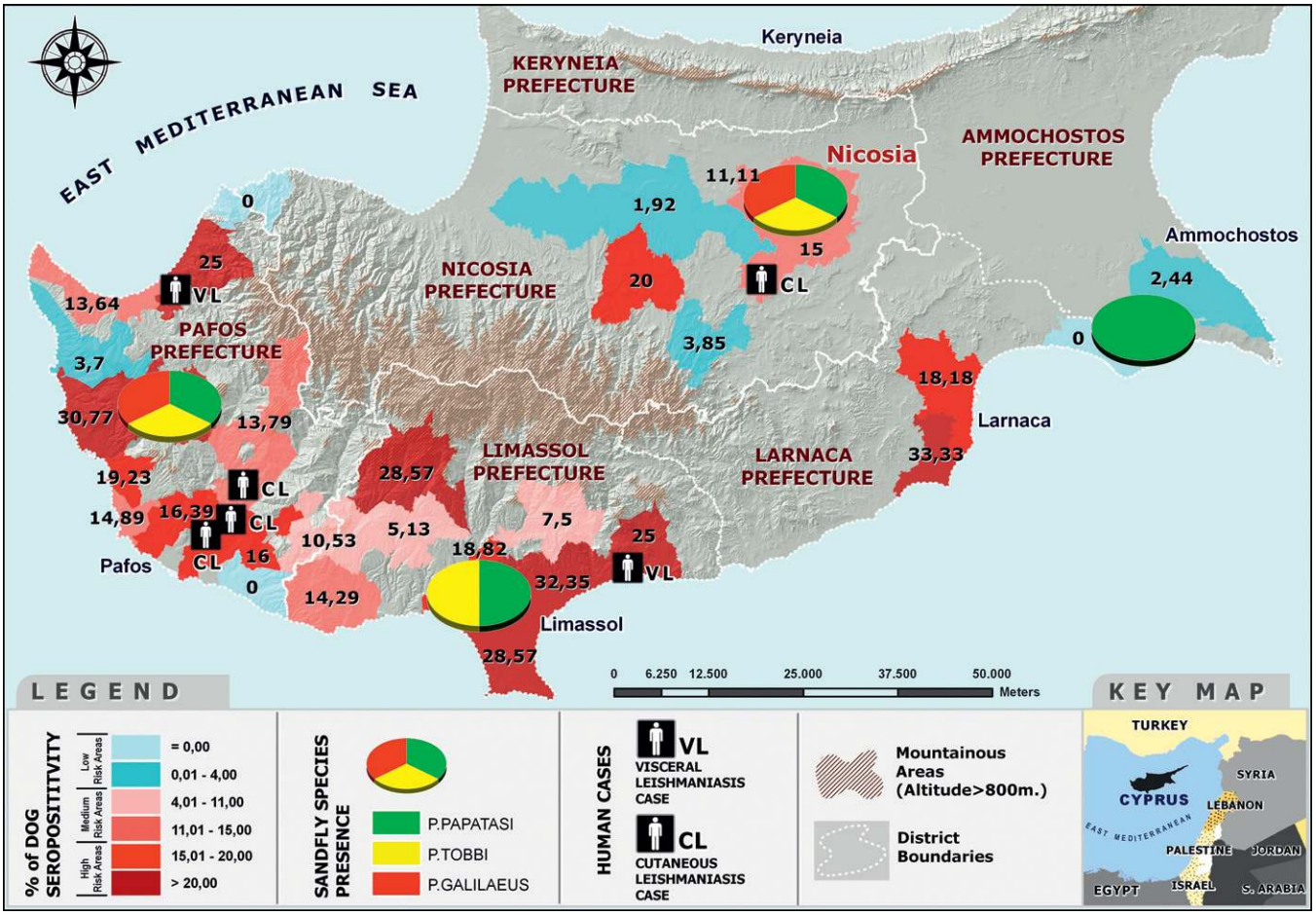

FIGURE 1. Map of Cyprus showing dog seropositivity (%) caused by *L. infantum* in the 30 squares studied (the boundaries of the selected villages in each square are shown). CL and VL, both caused by *L. donovani* MON-37, and the presence of the main *Phlebotomus* spp. caught in the four prefectures are shown.

and peripheral blood (900 samples) and lymph-node aspirates, when enlarged lymph nodes were observed, (22 samples) were collected. Personal, epidemiological, and clinical data were registered in questionnaires for each dog. Severe leishmaniasis symptoms were diagnosed in 18 dogs, and the disease was confirmed by serology and polymerase chain reaction (PCR). These dogs were euthanized with the consent of the owner, and the spleen was provided for examination. In addition, 2,056 dog sera were provided by veterinarians throughout the island for routine testing for leishmaniasis. Both the study and the protocols used were approved by the Ethical Scientific Committee of the University of Crete, Medical School.

Human samples. The two areas (squares) with high dog seropositivity, 25% and 29% (high risk for humans), and one with no seropositive dogs (low risk) were considered for the human survey. The areas were chosen such as to be located near a local hospital. In collaboration with the Ministry of Health, doctors from local hospitals provided samples from a total of 600 people that lived permanently in these three areas (200 people per area), irrespective of nationality, age, or health status, who visited the local hospital for any reason. The people were informed of the study, and if they agreed to participate, they provided a written consent and were examined clinically for symptoms of VL and cutaneous leishmaniasis (CL). They completed a personal questionnaire with epidemiological and clinical data. The ages of the people providing blood samples ranged from 10 to 88 years with an average age of 52 years (standard deviation [SD]). In addition, blood (35), skin tissue (4), and bone-marrow (4) samples from 35 patients with suspicious symptoms of leishmaniasis (high persisting fever or skin lesions difficult to cure) were provided from hospitals from different cities of the island, irrespective of the sampled areas.

Both the study and the protocols used were approved by the Ethical Scientific Committee of the University of Crete, Medical School.

Sand flies. Sampling of sand flies was carried out from May to October 2006. Live sand flies were collected by Centers for Disease Control and Prevention (CDC) light traps (Hausherr's Machine Works, Tom's River, NJ) from 20 villages with and without human and/or dog cases; 10 of 20 villages belonged to the three sampling areas for human leishmaniasis in the prefectures of Pafos, Limassol, and Ammochostos (with 25%, 28.6%, and 0% dog seropoprevalence, respectively). The other 10 were villages with a human case near Nicosia or with or without seropositive dogs, mainly from the Nicosia prefecture (Table 1) The traps were battery-operated and placed near animal shelters from dusk until early morning; they were placed near houses with human and/or dog cases (if any in the village) for one or more nights. Species identification was carried out according to morphology-based keys.

TABLE 1
The *Phlebotomus* species found in the 20 villages studied

Village	Prefecture	*P. papatasi*	*P. tobbi*	*P. galilaeus*	*P. sergenti*	*P. alexandri*	*P. mascittii*	*P. economidesi*
Chrysochous*	Pafos	†	†	†	‡	‡	‡	‡
Polis Chrysoch*	Pafos	†	†	†	†	‡	‡	‡
Pelathousa*	Pafos	†	†	‡	†	‡	‡	‡
Makounta*§	Pafos	†	†	†	‡	‡	‡	‡
Ag. Georgios*§	Pafos	†	†	†	†	‡	‡	‡
Anarita*	Pafos	†	†	†	‡	‡	‡	‡
Dora*	Limassol	†	†	‡	‡	‡	‡	†
Ipsonas*	Limassol	†	†	‡	‡	‡	‡	‡
Pissouri*	Limassol	†	†	‡	‡	†	‡	‡
Kantou*	Limassol	†	†	‡	†	‡	‡	‡
Kolossi*	Limassol	†	†	‡	‡	‡	‡	‡
Agioi Trimithias	Nicosia	†	‡	‡	‡	‡	‡	‡
Akaki	Nicosia	‡	‡	†	‡	‡	‡	‡
Kokkinotrimithia	Nicosia	†	†	‡	‡	‡	‡	‡
Nicosia*	Nicosia	†	†	†	‡	‡	†	‡
Paliometocho	Nicosia	†	†	‡	‡	‡	‡	‡
Sotira	Ammochostos	†	‡	‡	‡	‡	‡	‡
Xylofagou	Ammochostos	†	‡	‡	‡	‡	‡	‡
Paralimni	Ammochostos	†	‡	‡	‡	‡	‡	‡
Aradippou	Larnaca	‡	‡	‡	‡	‡	‡	‡

* Presence of CanL cases in the area.
† Presence of species.
‡ The species was not captured during this study.
§ Presence of a human case (see also Figure 2).

Serology. The 900 dog and 635 human sera were screened for the presence of *Leishmania* IgG antibodies by enzyme-linked immunosorbent assay (ELISA) (*L. infantum* promastigote soluble antigens; Bordier Affinity Products SA, Crissier, Swizerland). Seroprevalence measures were derived solely from the ELISA result to have comparable results with previous publications on Cyprus.[7] Cut off was 0.318 optical density (OD), which was the average cut off of the weak-positive serum used in all tests performed according to the manufacturer's directions; Lot 503L and Lot 513L were used. All sera were further tested by the Indirect Immunofluorescence Test (IFAT) using anti-human or anti-dog anti-IgG antibodies accordingly (VMRD, Inc., Pullman, WA). A series of 2-fold serum dilutions starting from 1/50 were performed, and cut-off titers ≥ 1/200 for dogs were considered. The 2,056 extra dog sera were tested only by ELISA. Positive-control sera (from dogs with parasitologically proven leishmaniasis) and negative-control sera (from *Leishmania*-free dogs living in areas with no leishmaniasis) were included in each batch of tests for both techniques.

PCR. All 35 patient samples and all 900 random dog samples were tested by PCR on peripheral blood, skin, and/or bone marrow from patients and blood, lymph node, or spleen from dogs, according to availability. QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany) and DNeasy Tissue kit (QIAGEN) were used for DNA extraction from blood and tissue, respectively, and the primers T2 and B4 were used for the reaction.[11]

Parasite isolation and typing. An attempt to culture the parasite was made from biological samples from all of the 900 dogs and 35 patient samples. Two culture media were used: the NNN[12] medium and the RPMI 1640 (Invitrogen, Paisley, UK).[12] The isolates were typed by starch gel electrophoresis using 15 enzymatic systems[13] and the *K26* PCR assay.[14]

Statistical analysis and mapping of the results. Dog seroprevalence was estimated for each of the 30 areas and 5 prefectures, and it was mapped using the geographical-information system software (GIS, Redlands, CA; ArcGIS 9.2). Possible associations between dog seropositivity and PCR positivity and 10 possible risk factors were initially assessed using the χ^2 test and univariate logistic regression models. The risk factors considered were geographical origin, sampling season, dog use (hunting, companion, or guard dog), sex, age, weight, coat color, length of dog hair, ectoparasite presence, and lastly, presence of at least three of any of the following CanL symptoms (lymph-node swelling, alopecia, onychogryphosis, epistaxis, ocular lesions, or splenomegaly). Subsequently, a multivariable logistic regression model was fitted to include all nine factors, and backwards stepwise-selection procedures were used to obtain a final predictive model.[15] A 5% significance level was chosen throughout the study; and SPSS version 15 was used.

RESULTS

All 600 individuals from the three areas under investigation were found to be seronegative. Of the 900 dogs, 14.9% were positive by ELISA, 11.8% by IFAT at the 1/200 cut-off titer, 12% by IFAT at the 1/100 cut-off titer, and 25% by PCR. In some squares, seropositivity by ELISA reached 33.3% (Figure 1). Of the additional 2,056 dogs tested, 402 (19.6%) were seropositive by ELISA. Parasites were isolated from the blood (25 isolates), lymph node (19), and spleen (18) of 62 dogs, 9 of which were healthy-looking, seronegative animals. All but three dogs with a positive culture gave a positive PCR. Typing showed 61 of 62 isolates to be *L. infantum* MON-1 and one isolate to be *L. infantum* MON-98.[13,14]

Using univariate statistical analyses, the risk of a dog being seropositive was associated with the following variables: (1) the season of sampling with an increased risk in the spring (OR = 2.8; 95% CI = 1.4–5.3), summer (OR = 2.2; 95% CI = 1.1–4.3), and autumn months (OR = 2.6; 95% CI = 1.2–5.8) compared with the winter months; (2) the geographic origin of the dog ($P = 0.009$) with Pafos and Limassol prefectures having the greater risk; (3) the age with an increased risk of seropositivity in older dogs ($P < 0.0001$), and the risk in 2–3-year-old dogs is 3.2 times that of < 2-year-old dogs (95% CI = 1.6–6.2), the risk

in 4–5-year-old dogs is 5.4 times that of < 2-year-old dogs (95% CI = 2.7–11.0), and the risk in ≥ 6-year-old dogs is 2.9 times that of < 2-year-old dogs (95% CI = 1.3–6.6); and (4) the presence of at least three symptoms increased the risk by 4.3 times (OR = 4.3; 95% CI = 2.7–6.8), although 67% of the seropositive dogs had no symptoms. Dog use, weight, color, hair length, gender, and presence of ectoparasites were found not to be significantly associated with seropositivity at univariate analysis at the 5% level. Further details are provided in Table 2.

When all variables were included in a multivariable logistic regression model, the final model selected the following four variables: (1) geographic location ($P = 0.003$; the risk of seropositivity for dogs living in Limassol being 3.1 with 95% CI = 1.4–6.5 and for dogs living in Pafos being 2.6 with 95% CI = 1.0–5.1 times that of dogs living in Nicosia); (2) age ($P < 0.0001$; OR = 3.0, 4.8, and 2.7 for age groups 2–3, 4–5 and ≥ 6 years, respectively, compared with < 2 years); (3) presence of ectoparasites ($P = 0.016$; OR = 0.22; 95% CI = 0.06–0.72); and (4) presence of symptoms ($P < 0.0001$; OR = 4.3; 95% CI = 2.7–6.8).

Using univariate statistical analyses, the risk of a dog being positive by PCR was found to differ significantly according to the following variables: (1) the season of sampling ($P < 0.0001$) with an increased risk in the spring (OR = 10.8; 95% CI = 5.9–19.9), summer (OR = 5.3; 95% CI = 2.9–9.9), and autumn months (OR = 4.2; 95% CI = 2.03–8.8) compared with winter months; (2) the geographic origin of the dogs ($P < 0.0001$) with Pafos prefecture having the greater number of PCR-positive animals (OR = 2.1; 95% CI = 1.4–3.3); (3) the symptoms of leishmaniasis observed in 119 dogs, because the risk of a symptomatic dog to be PCR positive was 1.5 times that of a dog without symptoms (OR = 1.5; 95% CI = 1.0–2.3). Age, dog use (hunting, guard, or companion), gender, weight, coat shade, hair length, and presence of ectoparasites were found not to be significantly associated with PCR positivity in univariate analysis at the 5% level.

When all variables were initially included in a multivariable logistic regression model, the final model selected, following the stepwise selection procedures, consisted only of the following variable for $P < 0.0001$: season (the risk being 5.5 and 3.8 for spring and summer, respectively, compared with winter; 95% CI = 2.6–11.5 and 95% CI = 1.8–7.7 respectively). Therefore, dogs sampled during spring and summer had the highest risk of being PCR positive for *Leishmania* (Table 2).

Of the 35 patients with suspicious symptoms of leishmaniasis, two with high persisting fever developed VL, and four with cutaneous lesions developed CL. The VL patients were positive by ELISA and IFAT (both titers 1/200), and only one of four CL patients had antibodies (by ELISA and IFAT; titer 1/400). PCR was positive in all six patients (two VL from blood sample and four CL from skin biopsy), and parasite isolation was made from five patients.

A total of 1,716 sand flies (649 males and 1,067 females), comprising 10 species, were collected from the 20 villages (altitude range = 37–176 m; 1,512 *Phlebotomus* and 204 *Sergentomyia*). *P. papatasi* was found in almost all areas studied (18/20). *P. tobbi* was found in 14 of 20 areas, *P. galilaeus* in 7 of 20, *P. sergenti* in 4 of 20, *P. alexandri* in 1 of 20, *P. mascittii* in 1 of 20, and *P. economidesi* in 1 of 20 (Table 1; Figures 1 and 2). Because sampling was not done for the same number of nights and in the same month for each village, the results are reported as presence of species found.

TABLE 2

The prevalence of leishmaniasis seropositivity (ELISA) and PCR positivity in dogs in southern Cyprus according to 10 possible risk factors ($N = 900$)

Variable	Number of dogs positive by ELISA (%)	Number of dogs positive by PCR (%)	Total no. of dogs sampled
Geographical origin*			
Nicosia	12 (7.7%)	34 (21.9%)	155
Ammochostos	1 (1.0%)	1 (1.0%)	101
Larnaca	3 (15.0%)	3 (15.0%)	20
Limassol	43 (16.3%)	56 (21.2%)	264
Pafos	49 (13.6%)	135 (37.5%)	360
Sampling season†			
Winter	13 (6.0%)	13 (6.0%)	215
Spring	44 (15.1%)	120 (41.1%)	292
Summer	36 (12.4%)	74 (25.5%)	290
Autumn	15 (14.6%)	22 (21.4%)	103
Age‡ (years)			
< 2	11 (4.5%)	61 (25%)	244
2–3	46 (12.9%)	91 (25.6%)	356
4–5	36 (20.3%)	51 (28.8%)	177
> 6	15 (12.2%)	26 (21.1%)	123
No. of symptoms (≥ 3)§			
Absent	72 (9.2%)	190 (24.3%)	781
Present	36 (30.3%)	39 (32.8%)	119
Dog use¶			
Hunting	86 (12.0%)	185 (25.8%)	718
Guard dog	13 (14.9%)	22 (25.3%)	87
Companion	9 (9.5%)	22 (23.2%)	95
Sex			
Male	53 (14.0%)	104 (27.4%)	379
Female	55 (10.6%)	125 (24.0%)	521
Shade of coat††			
Light	10 (10%)	26 (26.0%)	100
Dark	50 (12.4%)	113 (28.1%)	402
Mixed	48 (12.1%)	90 (22.6%)	398
Hair length‡‡			
Short	86 (11.3%)	198 (26.0%)	763
Medium	17 (15.5%)	27 (24.5%)	110
Long	5 (18.5%)	4 (14.8%)	27
Weight§§			
< 11 kg	6 (7.7%)	13 (16.7%)	78
11–20 kg	67 (13.1%)	129 (25.3%)	510
> 20 kg	35 (11.2%)	87 (27.9%)	312
Ectoparasites¶¶			
Absent	105 (12.4%)	214 (25.3%)	846
Present	3 (5.6%)	15 (27.8%)	54

* ELISA X^2 statistic 19.9 on 4 df; $P = 0.001$; PCR X^2 statistic 64.07 on 4 df; $P < 0.0001$.
† ELISA X^2 statistic 10.50 on 3 df; $P = 0.015$; PCR X^2 statistic 81.26 on 3 df; $P < 0.0001$.
‡ ELISA X^2 statistic 24.91 on 3 df; $P < 0.0001$; PCR X^2 statistic 2.29 on 3 df; $P = 0.515$.
§ ELISA X^2 statistic 43.26 on 1 df; $P < 0.0001$; PCR X^2 statistic 3.88 on 1 df; $P = 0.049$.
¶ ELISA X^2 statistic 1.24 on 2 df; $P = 0.539$; PCR X^2 statistic 0.30 on 2 df; $P = 0.860$.
ELISA X^2 statistic 2.44 on 1 df; $P = 0.118$; PCR X^2 statistic 1.38 on 1 df; $P = 0.241$.
†† ELISA X^2 statistic 0.48 on 2 df; $P = 0.798$; PCR X^2 statistic 3.20 on 2 df; $P = 0.202$.
‡‡ ELISA X^2 statistic 2.64 on 2 df; $P = 0.267$; PCR X^2 statistic 1.76 on 2 df; $P = 0.415$.
§§ ELISA X^2 statistic 2.20 on 2 df; $P = 0.332$; PCR X^2 statistic 4.15 on 2 df; $P = 0.125$.
¶¶ ELISA X^2 statistic 2.04 on 1 df; $P = 0.153$; PCR X^2 statistic 2.24 on 1 df; $P = 0.134$.

DISCUSSION

Although it is clear that in Cyprus, there are competent vectors for the transmission of *Leishmania* to dogs and humans (CanL cases caused by *L. infantum* MON-1[4,7] and VL or CL cases in humans caused by *L. donovani* MON-37[10]), the question of why there are no VL or CL cases caused by *L. infantum* remains unanswered. CanL is widespread in the island, and seroprevalence had an almost 9-fold increase in the last 10 years (overall average increased from 1.7%[7] to 14.9%). This shows that the parasite is circulating actively, transmitted by autochthonous sand-fly species found in most parts of the Republic,[16] and that conditions favor its geographic

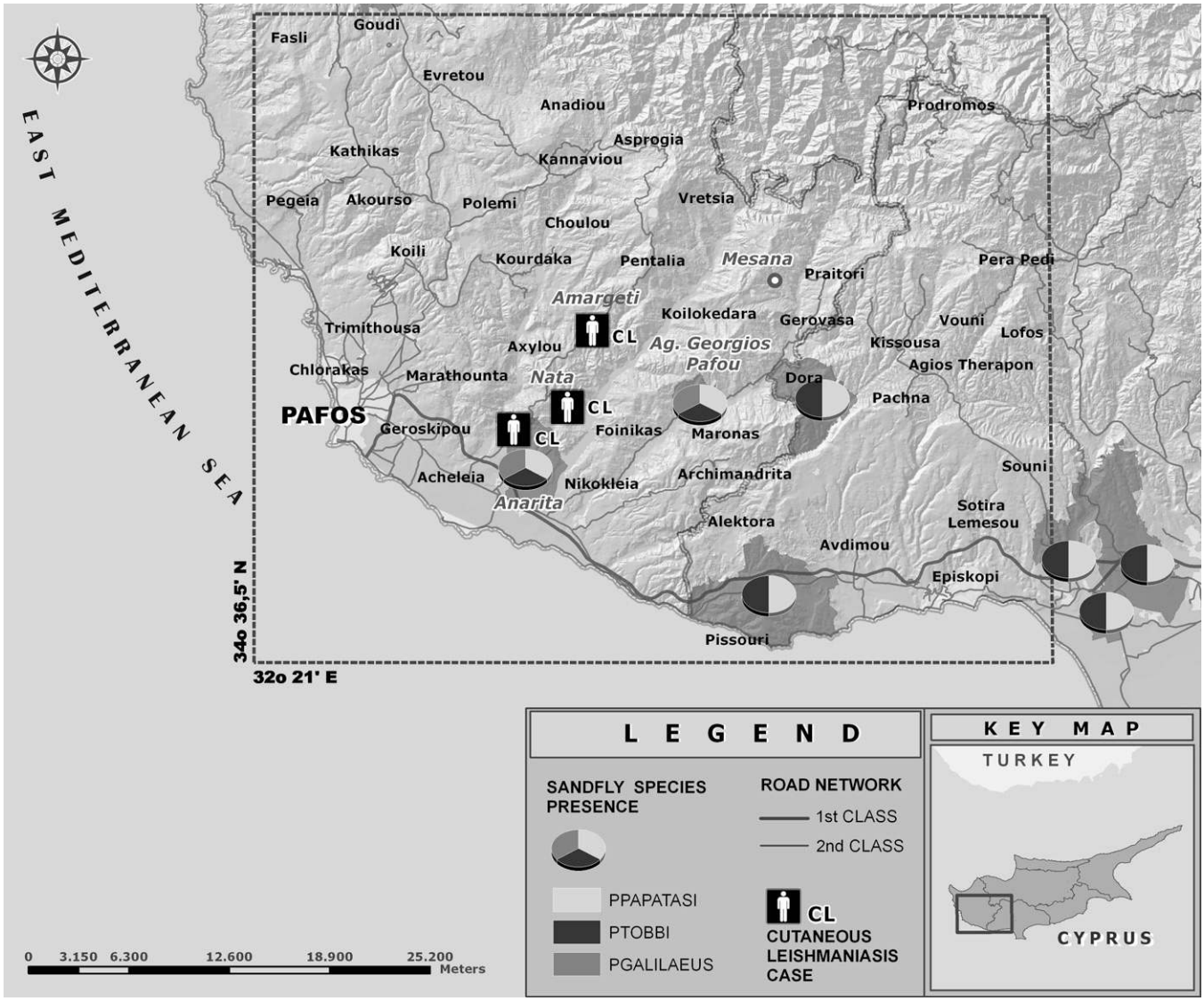

FIGURE 2. Map of Southwestern Cyprus showing the location of the three villages where the three human CL cases occurred as well as the village of Mesana, where the fourth CL individual was probably infected; the presence of the main *Phlebotomus* spp. is also shown.

spread, because it is no longer found restricted in coastal areas (Figure 1).[3,7] The seroprevalence found in the two groups of dogs tested (14.9% in the 900 randomly selected dogs and 19.6% in the 2,056 extra dog sera sent by veterinarians from all over the country) is comparable. Univariate statistical analysis on the data from the 900 dogs showed that the geographic origin of the dog was a significant risk for seropositivity, and the Pafos prefecture (the prefecture with four of six human cases) had the greatest risk for PCR positivity; however, the season of sampling was also statistically significant for seropositivity and PCR positivity with an increased risk in spring, summer, and autumn (Table 2). The high percentage of PCR positivity observed compared with ELISA positivity in the prefectures of Nicosia (21.9% and 7.7%, respectively) and Pafos (37.5% and 13.6%, respectively) is explained by the fact that the majority of the animals were sampled during spring and summer, the time when the sand flies are active and transmitting the parasite.

This is the first survey of human leishmaniasis conducted in Cyprus. Until the time of the study, only two infantile VL and no CL cases were known since 1935.[2,7] In Cyprus, leishmaniasis in humans and dogs is notifiable. Since VL is lethal if untreated all VL patients come to the government hospitals where they are registered for the disease. In the Mediterranean basin, clinical and subclinical human infections are being reported from endemic regions in healthy and human immunodeficiency virus (HIV)-infected individuals,[9,17] where *L. infantum* MON-1 comprises up to 90% of the typed *L. infantum* strains and is responsible for the majority of CanL and human VL cases.[18] Although the incidence of VL is relatively low in CanL-endemic European countries, asymptomatic infections are common. In endemic areas in southern France and Greece, for example, 3.4% and 15%, respectively, seropositivity was reported.[1]

Serological methods for detecting active VL present large amounts of specific antibodies; however, in subclinical infections, antibody levels are low, and serological tests show low sensitivity.[19] The lack of a gold-standard technique to detect cryptic infections by *L. infantum* is an important drawback

in the epidemiology of leishmaniasis in the Mediterranean region.[20] IFAT, ELISA, direct agglutination test (DAT), or delayed-type hypersensitivity reaction test (DTH) are traditionally used. A positive result indicates previous exposure to *Leishmania*, which is expected in an endemic area, but a negative result does not necessarily mean no contact with the parasite.[20] The leishmanin skin test (LST) has been used on a large scale for epidemiological studies and presents a good sensitivity and specificity in cured VL, but it is difficult to define its value in asymptomatic infections.[21,22] Higher sensitivity is obtained with nested PCR, and a significant statistical association was reported between nested PCR and ELISA, indicating that serology could be used to detect infection in asymptomatic subjects but with lower sensitivity.[17,20]

In a similar study, Riera and others[20] reported cryptic leishmaniasis in 2.4% of 656 blood donors from the endemic Eivissa Island of Spain by using ELISA and 7.6% by using Western blot, but different ELISA tests, using different promastigote antigens, seem to produce different results with different sensitivity.[23] In the two endemic areas sampled with 25% and 28.6% dog seropositivity, the 400 persons tested (of the 21,967 registered permanent residents of this area according to Census 2001; www.mof.gov.cy/mof/cystat/statistics.nsf) were all permanent residents of the study areas with a mean age of 52.7 years, factors which favor them coming into conduct with the parasite.[24] Yet, the ELISA test performed on the human sera did not produce any positives. Nevertheless, the two VL and the one of four CL cases caused by *L. donovani* MON-37 were seropositive with the same ELISA test, which used *L. infantum* promastigote-soluble antigens. Hence, although the planning of the study expected a result in a small percentage of seropositives, the negative result needs to be considered with caution, and more tests are required before excluding the possibility of missing seropositive individuals.

The absence of human cases caused by *L. infantum* is intriguing, suggesting a paradox of a minimal risk of *L. infantum* infections in humans compared with a high risk observed in dogs in southern Cyprus; this situation is not found in the surrounding countries where CanL and VL caused by *L. infantum* coexist. On the contrary, reports from the northern part of Cyprus show CL cases to be increasing from 2 cases in 1985 to 36 in 1990 in the Turkish Cypriot population.[25] Additionally, one VL case[2] implicating *L. infantum* has been reported.[26] This could possibly be explained by the presence of *P. neglectus*, the usual vector of *L. infantum* in the eastern Mediterranean, in Northern but not in Southern Cyprus.[4,27] This species is reported north of the Keryneia mountains (Figure 1). Taking into account that sand flies are weak fliers and do not usually disperse more than a few hundred meters from their breeding places, *P. neglectus* spreading toward the south is in part prevented by the mountainous terrain of the island (Figure 1).

Until now, the absence of human leishmaniasis cases in southern Cyprus was explained by the assumption that the local populations of *P. tobbi,* the incriminated vector of *L. infantum* in Cyprus, may not bite humans.[8] *P. tobbi* was collected in 14 of 20 villages sampled. All but two of these villages had dog cases, whereas the remaining 6 of 20 villages with no *P. tobbi* had no CanL cases (Table 1). *P. galilaeus* feeding preferences, however, are not well-known, but because it is very close to the *P. perfiliewi galilaeus* vector of *L. infantum* in the Mediterranean basin, it is likely to be an opportunistic feeder, taking blood meals from dogs and humans. *P. galilaeus* was encountered in 7 of 20 villages studied, six of which had dog cases (Table 1). Both *P. tobbi* and *P. galilaeus* were encountered in the two villages studied with human (and dog) cases, and *P. tobbi* was found in all villages with *P. galilaeus*, except one in Nicosia prefecture (Table 1; Figures 1 and 2). *P. galilaeus* was found in the village of Makounta, where a VL case occurred, but not in Limassol, the origin of the other VL case; it was absent in 6 of 12 villages with dog cases (Table 1; Figures 1 and 2). *P. papatasi*, although encountered in 18 of 20 villages, is not considered a potential vector of either *L. infantum* or *L. donovani.*[8]

The three CL patients live in the neighboring villages of Amargeti, Nata, and Agios Georgios at about 7 km distance from each other, and the fourth lives in Nicosia but visits his birth village Mesana every summer and during most weekends, which is about 9 km from the other three villages (Figure 2). These four villages are situated in a valley near three rivers at 350–600 m altitude with 650 mm rainfall where people cultivate mainly vines, almond, and olive trees and rear sheep and goats. Interestingly, all villages studied near these rivers had a high number of dog infections compared with the villages not so close to the rivers, a factor indicating that the area is favorable for sand-fly development.

Because *L. donovani* MON-3 was isolated in Syria from *P. tobbi*,[28] this species is a putative vector of both *L. infantum* and *L. donovani.* The detection of both parasite species in one dog[10] (of the 25 studied; unpublished data) suggests that a sand fly biting both humans and dogs may be involved, but whether this is *P. tobbi* or *P. galilaeus* needs to be proved by further studies, although findings so far point to *P. tobbi*.[16]

Because a man-biting sand fly exists in Cyprus, which was confirmed by a number of tourists who developed VL after visiting Cyprus and other endemic countries,[29,30] the absence of human VL or CL cases caused by *L. infantum* may suggest that there is a mechanism protecting the Greek Cypriots from *L. infantum* but not from *L. donovani*, which caused CL and not VL in healthy individuals in Sri Lanka.[31] It has been shown that host-related factors may determine the evolution of the host-parasite interactions and that ethnic and familial factors can play an important role in the distribution of VL.[32] The two human VL cases caused by *L. donovani* (a 9-month-old female, gypsy in origin, with Epstein–Barr virus co-infection,[33] and a 73-year-old British male with no other health complication), both permanent residents of Cyprus[10] in different geographical areas without any connection, developed severe disease. The other four cases, however, were all healthy Greek Cypriots 44–55 years old;[10] they developed CL in the less severe form of leishmaniasis. In line with the above information, microsatellite analysis showed that the genetic makeup of the strains did not correlate to the clinical manifestation of leishmaniasis.[34] Further work is planned to address this important question and to clarify the epidemiology of leishmaniasis on the island by conducting studies on vector infectivity to prove the ability of the vectors present to transmit *L. donovani*, which was a limitation in this study.

Thus, the Republic of Cyprus presents a unique situation where two distinct leishmaniasis transmission cycles run in parallel: in dogs with *L. infantum* MON-1 and in humans with *L. donovani* MON-37. Recent data using microsatellites[34] showed that there are substantial differences between the MON-37 Cypriot strains and the MON-37 strains from India,[35] Israel,[36] Sri Lanka,[37] and Kenya.[38] Thus, one cannot assume that

a very recent introduction by immigrants or infected sand-fly vectors in Cyprus occurred, at least from the above-mentioned countries.[34] However, the scenario that Cypriots were infected in Cyprus by parasites imported by infected people from endemic areas not yet studied cannot be excluded.

The results of the dog seroepidemiological study led the Cyprus government to take measures to prevent the dispersal of leishmaniasis (a decree for monitoring the disease in the dog). These measures, however, will protect the dog but not the human population, if indeed *L. donovani* patients play the role of parasite reservoir. Measures must be taken to protect the population in this emerging disease hot spot, or otherwise, *L. donovani* may spread fast as it has done in Sri Lanka.[31] At a time when VL caused by *L. infantum* is spreading northwards in Europe,[39] the emergence of *L. donovani* MON-37 in a Mediterranean country may result not only in a dramatic change in the epidemiology of leishmaniasis, but it could also give the opportunity to the circulating species to generate hybrids.[40] To avoid the spread of this species, it is crucial that the disease is placed under public-health surveillance at the European level, because putative vectors occur in many European countries.[41]

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