



Research paper

Geospatial analysis applied to seroepidemiological survey of canine leishmaniosis in east-central Portugal

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ABSTRACT

Despite the high prevalence of canine *Leishmania infantum* infection in Portugal, significant differences associated with different risk factors can be found between geographically contiguous areas. In this study, a geographical area within the central region of Portugal (municipalities of Proença-a-Nova, Mação and Vila de Rei) was investigated. An epidemiological survey involved the analysis by an indirect enzyme-linked immunosorbent assay of serum samples collected during the anti-rabies vaccination campaign from 282 dogs. Geospatial analysis showed the distribution of geospatial prevalence of leishmaniosis and has delimited two areas (clusters) with a statistically significant higher risk of seropositivity in dogs ($p = 0.003$ and $p = 0.027$, for clusters 1 and 2, respectively). The highest seroprevalence (56.0%; CI: 41.2–70.0) was found in Vila de Rei. Five land occupation types showed a possible influence on the geographic distribution of seropositivity, with statistically significant differences between seropositive and seronegative dogs. Land occupied by temporary irrigated crops ($p = 0.026$), olive groves ($p = 0.013$), complex cultural systems and parcelling ($p = 0.021$), open forests, logging and new plantations ($p = 0.043$) and watercourses ($p = 0.012$) influenced the geographical distribution of canine *Leishmania* infection. Seropositive dogs had a greater average area of occupied land (i.e. open forests, logging and new plantations) than the seronegative ones (3.1439 km² versus 2.5650 km², respectively; $p = 0.043$).

1. Introduction

The World Health Organization (WHO, 2019) considers leishmaniosis as one of the most important neglected zoonoses in the world. The circumstances of transmission are continually changing in relation to environmental, demographic and human behavioural factors, which all together contribute to a variable epidemiology of infection and disease (Antoniou et al., 2013).

In Portugal, there are differences in the prevalence of canine *Leishmania infantum* infection, even between contiguous areas, a circumstance that may be associated with different risk factors (Campino, 2002; Dujardin et al., 2008; Ready, 2010; Franco et al., 2011; Pereira et al., 2017). The municipalities of Proença-a-Nova, Mação and Vila de

Rei (which encompass the geodesic centre of Portugal) are characterized by being a mountainous region with fertile plateaus cut by valleys and with high humidity, conditions which potentiate the proliferation of phlebotomine sand flies, the vectors of *Leishmania* spp. (Silva, 2010). In Portugal the identified vectors of *L. infantum* are *Phlebotomus perniciosus* and *P. ariasi*, although there are three other phlebotomine sand fly species in the country (*P. sergenti*, *P. papatasi* and *Sergentomyia minuta*). *Leishmania infantum* DNA was detected by molecular techniques in a *S. minuta* specimen, but the vector role of this sand fly species has not been confirmed (Pereira et al., 2017).

This study used geospatial analysis to identify areas of high risk for *Leishmania* spp. infection in central Portugal, with a view to potentially optimizing the prediction of its impact on public health, the application

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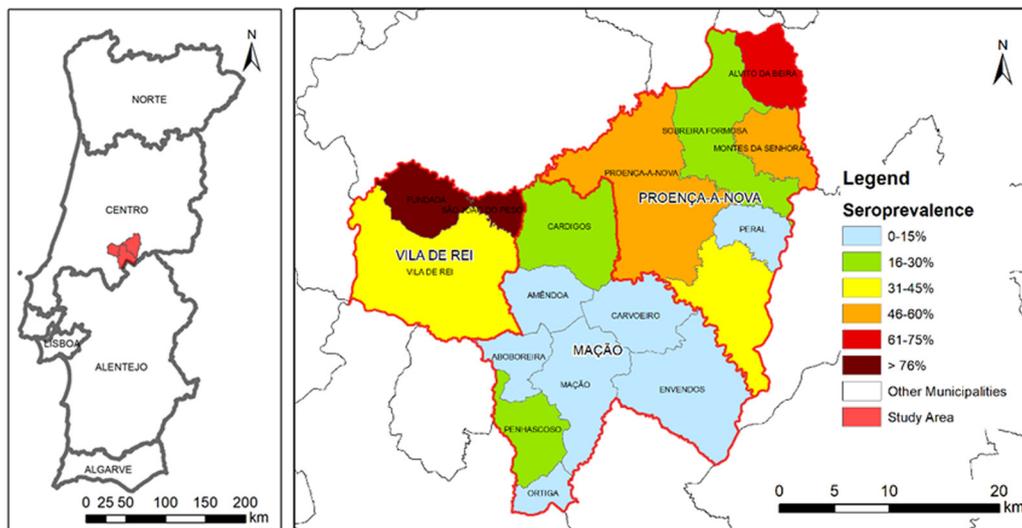


Fig. 1. The municipalities of Proença-a-Nova, Mação and Vila de Rei (study area, 677.1 km²), in central Portugal and seroprevalence of canine *Leishmania* infection by parish.

of preventive measures and other action plans aiming at controlling this zoonosis.

2. Materials and methods

Blood samples were collected from 282 dogs in the municipalities of Proença-a-Nova, Mação and Vila de Rei (Fig. 1) during the annual anti-rabies vaccination campaign of 2011. The number of samples was determined for an estimated population of 2000 dogs and an expected prevalence of 30%, with a 95% confidence interval and a 5% error (WinEpi, 2006). A systematic sampling was based on the collection of one blood sample for every seventh dog present at the vaccination campaign. The methodology was adopted in order to avoid sampling errors and to obtain a representative sample of the canine population.

Serum samples were analysed by an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of serum immunoglobulins specific to *Leishmania* spp. Microtitre plates (MaxiSorp Surface, Nunc, USA) were coated with 50 µL of soluble antigen obtained from sonicated promastigotes of a *L. infantum* strain and adjusted to a protein concentration of 20 µg/ml in 0.05 M sodium carbonate buffer (pH 9.6). Plates were left at 4 °C for 16 h, washed four times with 0.3% (v/v) phosphate-buffered saline-Tween 20 (PBS-T, pH 7.2), blocked with 200 µL of a 5% (w/v) bovine haemoglobin (Bhb) solution in PBS-T at 37 °C for 20 min, and then washed one time with PBS-T. Canine sera were diluted at 1:100 in a solution of PBS-T with 1% Bhb. One hundred µL of each serum dilution was added per well and incubated at 37 °C for 30 min. All plates included a blank, a positive and four negative control sera. After three washings with PBS-T, phosphatase-conjugated anti-dog IgG (Sigma-Aldrich, USA) was used as secondary antibody, at the dilution of 1:6000 in PBS-T. A volume of 100 µL per well of diluted conjugate was incubated at 37 °C for 1 h, and the plates were then re-washed four times with PBS-T. The color reaction was developed with 100 µL of a substrate solution of 0.01% (w/v) 4-nitrophenylphosphate (Sigma-Aldrich, USA) incubated at 37 °C for 10 min in the absence of light. Optical densities (OD) were immediately read at 405 nm by using an automatic spectrophotometer. The cut-off value was calculated as the average plus 3 standard deviations of OD from seronegative dogs.

A geospatial data analysis of the seroepidemiological data was performed. For geo-referencing, each blood sample corresponded to the geographical location where the owner of the dog lived and was coded accordingly (Open Office 3). The maps with georeferenced points were constructed using Quantum GIS, version 1.6.0, and cartographic projections were WGS 84, EPSG 4326 and Goolge Mercator, EPSG 900913.

Spatial clusters of seropositivity and the relative risk were evaluated by SaTScan v9. 1.

A risk factors analysis (Quantum GIS, version 1.6.0) was carried out to detect land occupation types, in order to explain the geospatial distribution of seroprevalence, with an average radius of 1.5 km of territorial area for each dog (buffers). The average area of a given land occupation type as a function of the seropositive or seronegative groups was calculated from the formula: $(\pi r^2) \times f$, where π is the value of Pi, r the radius value and f corresponds to value of the fraction (%) of the buffer's area. The buffers were defined based not only on the area of dispersion of sand flies, but also attending to the dynamics of the movement that occurs in the population of dogs of the region, which is fundamental for the characterization of the land occupation type according to the dog's habitat.

For the individualization of each land occupation type, software GVSIG 1.10 OADE 2010 (Oxford Archaeology Digital Edition) was used, with GRASS 6 extension and the Sextant toolkit, version 0.6. The data treatment to determine the risk factors was done through the function "sample t-test" using the program R version 2.12.2.

To obtain the Land Use Cover in Portugal for 2010 (COS2010), maps were used with a scale of 1:25000, a minimum cartographic unit of 1 ha, a five-level hierarchical classification with 193 classes in the most detailed level and which are available through Web Feature Services (WFS) (Direção-Geral do Território, 2016, 2017), allowing a highly detailed analysis for the land use cover. The administrative boundaries were aggregated from the information in CAOP (2012).

A spatial analysis was carried out on landscape diversity of the studied area to evaluate its importance in the distribution of *Leishmania* cases using a toolbox Patch Analyst 5.2 (Rempel et al., 2012).

3. Results

The seroprevalence values were lower in the parishes of Peral, Envedos, Carvoeiro, Mação, Amêndoa, Ortiga e Aboboreira (0–15%), and higher in the parishes of São João do Peso and Fundada (76–100%), both in the municipality of Vila de Rei, and in Alvito da Beira (61–75%), in Proença-a-Nova (Fig. 1). This geospatial distribution was influenced by the localities or parishes where the dogs came from.

Eighty-nine centroids (Fig. 2) and two clusters were identified (Fig. 3). The coordinates of the centroids related to the clusters allowed to project circular areas with a defined radius: cluster 1 was located in the northern region of the municipality of Vila de Rei, and covered the parishes of São João do Peso and Fundada, with a radius of 10.809 m.

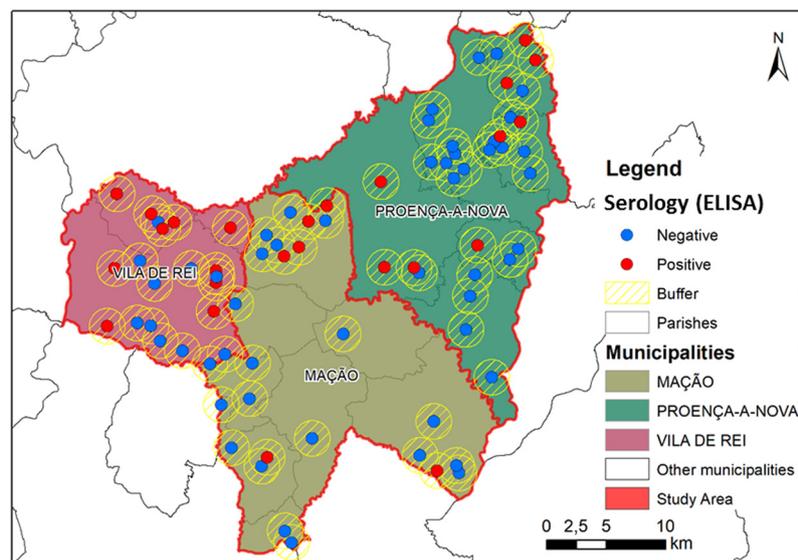


Fig. 2. The buffer distribution for dogs seropositive and seronegative to *Leishmania infantum* in the study area (municipalities of Proença-a-Nova, Mação and Vila de Rei, central Portugal).

Cluster 2's centroid, located in the southern region of the parish of Proença-a-Nova, presented a radius of 3.051 m. The relative risk (RR) of *Leishmania* spp. infection was 2.45 and 2.89, respectively (Table 1).

Regarding the 49 land occupation types, 16 classes were detected in the study area (Fig. 3) and only five showed a possible influence on the geographic distribution of seropositivity, with statistically significant differences between seropositive and seronegative dogs, and land areas occupied by temporary irrigated crops ($p = 0.026$), olive groves ($p = 0.013$), complex cultural systems and parcelling ($p = 0.021$) and watercourses ($p = 0.012$) (Table 2).

Likewise, there were significant differences ($p = 0.043$) between seropositive and seronegative dogs in the areas occupied by open forests, logging and new plantations (total of 1050.5 ha in the three municipalities; Fig. 4 and Table 2), but the seropositive group had an average area larger than the seronegative group. The areas occupied by open forests, logging and new plantations in cluster 1 and cluster 2 were 47.36% and 33.63%, respectively.

In relation to other land occupation types, no statistical relationship was established, but there was a lower Shannon's Diversity Index (SDI) in the municipalities where the two clusters were marked.

4. Discussion

This study reveals the geospatial distribution of *Leishmania* spp. infection seroprevalence in the three municipalities assessed in the central region of Portugal, highlighting the endemic character of infection and disease as described by other studies (Brançal et al., 2009; Silva, 2010). Additional studies involving more dogs in each parish are needed to validate current results, as no other study had been performed on dogs in nine out of the 17 parishes currently evaluated.

The analysis of spatial aggregates, aimed at identifying areas with higher relative risk of canine infection, delimited two geographic areas (clusters). Cluster 1 encompassed the northern zone of the municipality of Vila de Rei municipality (RR = 2.45; $p = 0.003$), and cluster 2 the southern zone of Proença-a-Nova parish (RR = 2.89; $p = 0.027$).

Biotope with hollowed tree trunks and organic matter in the soil can serve as shelter or breeding sites for dipterans. And factors such as the use of insecticide-impregnated protector collars against vector bites can influence the results of the geospatial distribution of canine infection (Saraiva, 2008).

Geospatial analysis seems to be a useful tool to identify areas of high risk for *Leishmania* infection, contributing to optimize the application of

preventive measures and action plans. When the buffers were characterized, open forests, logging and new plantations represented a land occupation type among the 49 under study, with influence on the geographical distribution of infection, i.e. there was an association between seropositive dogs and this type of land occupation ($p = 0.043$). This type of land occupation is characterized in detail by areas of grass or natural pastures with dispersed trees (whose lower limit of existence is 10%), as well as degraded or regenerated forest (Caetano et al., 2007).

Statistically significant differences were observed between the average of the areas, comparing the group of seropositive and seronegative cases, a fact which indicates that this type of land occupation can be associated with the highest number of seropositive cases, probably due to the higher density of vectors. This result is in agreement with other studies that have also associated this type of land occupation with the highest seroprevalence found (Aparicio and Bitencourt, 2004). In relation to land occupation by watercourses, this other study has found that there was no association between open watercourses and the occurrence of *Leishmania* spp. infection. For the watercourses, as in land areas occupied by temporary irrigated crops, olive groves, and complex cultural systems and parcelling, it is necessary to complement the present study for a more correct interpretation. It is possible that vectors of *Leishmania* spp. do not find optimal survival conditions in these environments, including for the survival of larvae in the soil. Shannon's Diversity Index pointed out a lower diversity of the landscape in the cluster areas, which may be related to the vectorial activity.

In the present study, in relation to the risk analysis of factors for land occupation types, urban landscape (discontinuous areas of industry and commerce) and agricultural areas (permanent or temporary crops, and heterogeneous agricultural areas) were not associated with seropositive or seronegative cases. Likewise, some other studies have reported that there is apparently no association between seropositive cases and these types of land occupation (Saraiva, 2008).

Whether on a regional or local scale, the spatial and temporal patterns of the distributions of the sand fly populations are influenced by humidity, temperature, lightness and altitude (Elnaïem et al., 2003; Signorini et al., 2014; Shirzadi et al., 2015; Mokhtari et al., 2016; Ramezankhani et al., 2017).

Other studies of geospatial analysis have shown that vegetation constitutes a land occupation type with a strong association with the distribution of seropositive cases due to *Leishmania* spp. infection

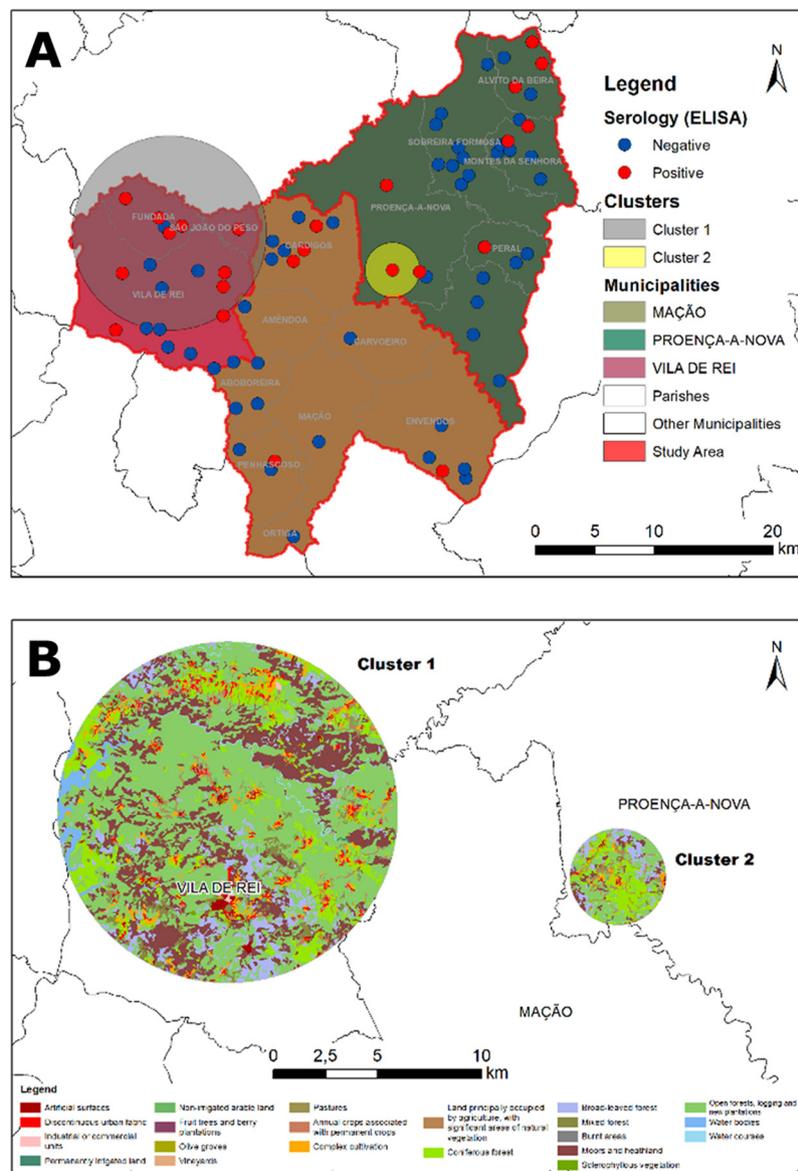


Fig. 3. A. Geospatial clusters of higher risk of seropositivity to *Leishmania infantum* in dogs in central Portugal. B. Land occupation types in geospatial clusters with cluster characterization.

Table 1

Characterization of two delimited areas (clusters) with a statistically significant higher risk for seropositivity to *Leishmania infantum* in dogs in central Portugal.

	Cluster 1	Cluster 2
ID code	131014; 12201; 124012; 121013; 120017; 114132; 110109; 32153; 127011; 30158; 123201; 129023; 26113; 107126; 132055; 134124; 136148,111111	157506; 152353
Coordinates (cartesian)	-905802,4825	-881816,482
Radius (m)	10809	3061
Population	36	12
Number of observed cases	23	10
Number of expected cases	11.3	3.8
Observed/expected cases	2.04	2.66
Relative risk	2.45	2.89
Likelihood log	9.52	7.33
p value	0.003	0.027

(Aparicio and Bitencourt, 2004; Saraiva, 2008; Mollalo et al., 2014; Golpayegani et al., 2018). In the present study, no area was identified with this type of land occupation.

The difference of results in comparison with other studies, regarding the relation of land occupation type by vegetation and seropositive

cases due to the presence of antibodies to *Leishmania* spp., might be explained by the different adjustments of the nomenclature of the types of land occupation or by the soil and climatic characteristics of the Pinhal Interior Sul sub-region. The types of land occupation nomenclature adapted in Portugal indicate that areas of natural grassland with

Table 2
Analysis of land occupation types as risk factors for seropositivity dogs to *Leishmania infantum* in dogs in central Portugal.

Risk factor	Average value of the fraction (%)	Average area (km ²)	Standard deviation	Number of cases	p value
Temporary irrigated crops					
Seropositive group	0.0012	0.0184	0.0078	82	0.026
Seronegative group	0.0046	0.1195	0.0163	182	
Olive grove					
Seropositive group	0.0026	0.0085	0.0141	82	0.013
Seronegative group	0.0169	0.0368	0.0736	182	
Cultural systems and parcelling					
Seropositive group	0,1044	0.7379	0.1387	82	0.021
Seronegative group	0.1469	1.0383	0.1326	182	
Open forests, logging and new plantations					
Seropositive group	0.4448	3.1439	0.3114	82	0.043
Seronegative group	0.3629	2.5650	0.2762	182	
Watercourses					
Seropositive group	0.0012	0.0085	0.0078	82	0.012
Seronegative group	0.0052	0.0325	0.0174	182	

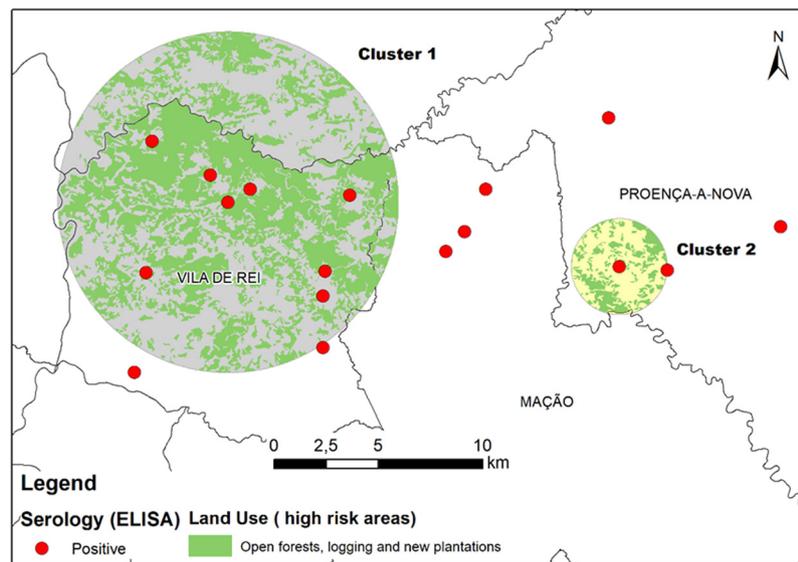


Fig. 4. High risk areas for seropositivity to *Leishmania infantum* in dogs in geospatial clusters.

herbaceous vegetation, essentially grass, without human intervention and areas of natural vegetation with shrub species, were mistaken and are somewhat controversial from the satellite image, raising problems in their strict identification (Caetano et al., 2007).

5. Conclusion

Modelling environmental factors affecting ecological disease need attention to better clarify their importance in the distribution of the infection by *L. infantum* in the central region of Portugal. Studies to be further developed should also comprise the use of other diagnostic methodology to confirm the presence of the disease among the seropositive dogs.

Declaration of Competing Interest

The authors declare no conflict of interest.

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