Epidemiological evaluation of Leishmania infantum zoonotic transmission risk in the recently established endemic area of Northwestern Italy

DOI:10.1111/zph.12477

(Article begins on next page)

Epidemiological evaluation of *Leishmania infantum* zoonotic transmission risk in the recently established endemic area of Northwestern Italy.

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Summary

Leishmania infantum infection had been expanding into new areas due to changes in vector and host biology. Zoonotic visceral leishmaniasis has become endemic in previously unsuitable areas as vectors find favorable climatic conditions and an increasing number of reservoir dogs are moved between traditionally and new endemic areas. Monitoring vector and disease expansion in areas of recent colonization is needed to understand transmission mechanisms and patterns of disease establishment. Here we studied the infection status of 815 human blood donors and of 803 sympatric dogs from 5, newly endemic, areas in Northwestern Italy. In autochthonous dogs, the seroprevalence of anti-*Leishmania infantum* antibodies, recorded by Western Blot, reached 42.22%, while in humans, the seroprevalence was of 16.81%. No significant correlation between the infection status of dogs and that of their human owners was found but *L. infantum* infection was recorded in the different study areas with significant levels of diversity. Restriction Fragment Length Polymorphism showed a high genetic variability of the circulating strains and gave useful insights on patterns of disease establishment into a naïve area.

Keywords

Leishmania infantum, WB, PCR, RFLP, Italy, zoonotic transmission risk

Impacts

- *Leishmania infantum* prevalence has increased in dogs and humans after less than a decade from becoming endemic in the area.
- PCR-RFLP pattern analysis showed high genetic diversity of circulating *L. infantum* strains
- Infection status of dogs is not directly related to *L. infantum* infection in human owners.

1. Introduction

Zoonotic visceral leishmaniasis (ZVL) is a vector-borne parasitic disease which is caused in the Mediterranean basin, by *Leishmania infantum*. *L. infantum* is transmitted in the Old World by sandflies of the genus Phlebotomus (Bates et al., 2015) and in the urban environment, has the dog as main reservoir of infection (Quinnell and Courtenay, 2009) together with various species of sylvatic hosts (Millan et al., 2011, Molina et al.,2012; Zanet et al., 2014; Diaz-Saez et al., 2014; Jimenez et al 2014). The geographic range of occurrence for *L. infantum* infection in dogs has changed in the recent past, as the endemic area of ZVL has broaden to include Northern Italy (Maroli et al.,2008; Dereure et al., 2009) and Northern European countries (i.e. Germany (Naucke et al., 2008), United Kingdom (Shaw et al., 2009), Hungary (Farkas et al., 2011; Tánczos et al., 2012) and France (Chamaillé et al., 2010). The geographical expansion of phlebotomine vectors toward higher latitudes and elevations (Ferroglio et al., 2005; Millan et al., 2016) has been related to climate change and in particular to the rise of winter temperatures (Stainforth et al., 2013) that allows for successful overwintering (Githeko et al., 2000) in previously unsuitable areas. In newly endemic areas, prevalence and incidence of *L. infantum* infection varies greatly. In Northwestern Italy *L. infantum* was recorded with a seroprevalence of 7.4% in asymptomatic healthy adults (Ferroglio et al., 2005; Biglino et al., 2010; Ferroglio et al., 2006) and PCR-RFLP pattern analysis from the same area, identified three domestic dogs as source of infection for their owners (Ferroglio et al., 2006). Within a context of a rapidly changing epidemiology of ZVL, we analyzed with the same diagnostic techniques used in the previous studies (to guarantee data comparability), sympatric human and dog samples from 5 geographically distinct and specific areas in Northwestern Italy recently become endemic for *L. infantum*. The main goals of the work were to assess presence and prevalence of infection in humans and dogs, to identify species-specific risk factors and common epidemiological traits. These data will serve as base-reference to assess how the epidemiology of *L. infantum* has changed over the past decade.

2. Materials and Methods

2.a Study area

Within a context of geographic expansion of ZVL, in a newly endemic area of Piedmont Region (Northwestern Italy) we identified 5 areas (which will be referred to as areas A, B, C, D and E) distinct for topographical, altitudinal, land cover and demographic characteristics. Details of each study site are reported in table 1.

2.b Collection and identification of sandflies

In order to confirm the local presence of competent *L. infantum* vectors, in each of the study sites, we carried out a preliminary entomological survey to assess the presence of phlebotomine sandflies. Sandflies were collected using sticky traps made from 20X20 cm castor oiled paper. Ten (sticky trap total area of 0.4 m^2) to 20 traps (sticky trap total area of 0.8 m^2) were placed for one night, from sunset to sunrise in 105 trapping points evenly distributed among areas (A to E). A single trapping session was performed at each of the 105 sites during the local peak of sandflies abundance at the beginning of July 2007 (Takken and Knols, 2007). Collected insects were kept in 70% alcohol for further analysis**.** Males and females were cleared in lactophenol and identified according to specific taxonomical keys (Corradetti et al., 1961; Biocca et al., 1977). The density and frequency of captured specimens in each study area are reported in table 2.

2.c Sample collection and processing

Peripheral blood samples were taken by venipuncture from 803 dogs from 4 areas (A, B, C and D) (Table 1). Dogs were randomly sampled by local veterinarians and for each dog a questionnaire was used to collect information on individual and environmental factors that might influence their exposure to *L. infantum*. The information included in the questionnaire were: breed, age, sex, municipality of origin, coat type, housing, sleeping sight and movements to classical endemic areas (vs. autochthonous dogs: subjects for which any movement outside the area of origin could be excluded). Symptoms related to Leishmaniasis were also annotated in the questionnaire.

Human serum samples were collected from 815 anonymous blood donors as part of routine surveillance screenings from all study areas (A, B, C, D and E). A questionnaire was administered to the sampled individuals to collect information on sex and age, living place, hobby, job and contact with dogs. A subset of 112 human subjects form area B were sampled together with their dogs (n=121) for direct comparative analysis of infection status.

All samples were stored at -20 °C until further use. Human and dog samples were collected over a period of three years (2007-2009) from December to March of each year to maintain a suitable time distance from sandfly active season.

Ethical approval was obtained by the Ethical committees of the Department of Veterinary Sciences, University of Turin and by the San Luigi Gonzaga Teaching Hospital, University of Turin for dog and human sampling respectively. All information was collected in compliance with privacy rules.

2d. Diagnosis of *L. infantum* infection in dogs and humans

L. infantum specific antibodies were detected in both dogs and human samples, by means of Western Blotting (WB) using as antigen source late-log phase promastigotes of *L. infantum* (IPT-1 Roma) (Ferroglio et al., 2007).

For the direct diagnosis of *L. infantum* by PCR, total genomic DNA was extracted from 200 µl of dog's whole blood samples using the commercial kit GenElute Mammalian Genomic MiniKit (Sigma–Aldrich) following manufacturer's instructions.

Human white-blood cells where pelleted from 2 ml of serum, and DNA was extracted using the same commercial kit used for dog's samples. A specific fragment of 145bp of *L. infantum* kDNA was amplified by end-point PCR with primers mRV1and mRV2 as published elsewhere (Zanet et al., 2014). The same WB and PCR protocols were used to test dogs (autochthonous and non) and humans. All positive amplicons were purified using (QIAQuick PCR purification kit, QIAGEN) and both DNA strands were directly sequenced (Macrogen, The Netherlands) to confirm *L. infantum* identification. The resulting sequences were compared with the ones available on Genbank using the Basic Local Alignment Search Tool (BLAST).

In an endemic focus of leishmaniasis the number of PCR positive individuals exceeds the number of seropositive subjects (Baneth et al., 2008). In order to obtain information on parasite prevalence in the study area, dogs (for whom whole blood was available) were at first, all tested by PCR and only autochthonous dogs were screened by WB. Humans (for whom blood serum was available) were instead all screened by WB at first (Baneth et al., 2008), and only WB-positive individuals were subsequently tested, for confirmation, by PCR.

2e. PCR-RFLP analysis

All PCR positive samples of both dogs and humans were double digested with restriction enzymes M1sI (MscI) and BseLI (Bs1I) (Fermentas, Milan, Italy) as previously described (Millan et al., 2011). To confirm digestion specificity, an in-silico Restiction Fregment Length Polymorphism (RFLP) was carried out, on all positive samples, using the online software tool NEBcutter (Vincze et al., 2003).

2.f Statistical and Geospatial analysis

To identify possible associations between *L. infantum* infection status and anamnestic variables, we used Pearson's Chi square implemented in the statistical environment R version 3.0.2 (R Development Core Team). Cohen's kappa coefficient (k) was used to assess test agreement between PCR and WB on autochthonous dogs, and between RFLP and in-silico RFLP (Landis and Koch, 1977). A Geographic Information System (GIS) environment (QGIS 2.8.0; Quantum GIS Development Team 2015) was used to calculate the Minimum Convex Polygon (MCP) area (for patterns shared between n≥3 individuals) or the linear distance (for patterns shared between n=2 individuals) of each RFLP pattern.

Results

3.a Sand fly:

Phlebotomine sandflies were monitored in the 5 areas of interest for the current study. *Phlebotomus perniciosus* was identified in all study areas (A to E) with an overall frequency of positive trapping sites of 42.86% (IC95% 15.82 – 74.95%; 45/105 positive trapping stations). No statistical difference was recorded among capture frequency nor density values of *P. perniciosus* in the different areas (Tab. 2). *P. perniciosus* was trapped with an average density of 15.2 individuals/m^2. *Sergentomya minuta* was reported from all trapping sites in all areas (A to E) (capture frequency of 100%, IC95% 96,47 – 100%). The average density of *S. minuta* was 18 individuals/m^2 (Tab.2).

3b. Dogs

An overall prevalence of 40.35% (CI95% 37.01% - 43.78%) was recorded in dogs with 324/803 animals testing positive to *L. infantum* by PCR (Table 3). PCR prevalence on autochthonous dogs was 48.52% (CI 95% 42.62% - 54.46%) with 131/270 dogs testing positive. Autochthonous dogs, tested by WB likewise human samples, showed an overall seroprevalence of 42.22% (CI95%

36.48%-48.18%) with 114/270 subjects testing positive (Table 3). K coefficient of agreement between PCR and WB on autochthonous dogs showed substantial (area D) to almost perfect agreement (areas A and B). No exclusively autochthonous dogs were available from area C.

On autochthonous dogs, statistically significant differences existed between study areas: sites B and D, which are agricultural and urbanized hilly areas, have prevalence and seroprevalence values higher than those recorded in area A, a low urbanized and natural mountain area (Table 4). No other risk factors showed p values ≤ 0.05 at Pearson's Chi square (data not shown).

3c. Humans

The overall seroprevalence recorded by WB on human samples is P=16.81% (CI95% 14.40 - 19.53%) with 137 positives on 815 tested individuals. Of these, 100 were also positive by PCR (P=72.99%; IC95% 65.01% - 79.73%). Individuals resident in the two mainly agricultural areas, B (X^2= -0.59163; p= 0.0124) and C (X^2= -0.5043; p= 0.0396) had significantly lower seroprevalence than those from the other study areas (Table 5). No other factors showed p values ≤ 0.05 at Pearson's Chi square analysis. Notably no significant association was found with periodic stays in traditionally endemic areas (p>0.05) nor with owing a dog (p>0.05) even if the dog itself is *L. infantum* infected (p>0.05 in the subset of owners/dogs from area B; P=54.40% - CI95% 36.6-71.5 in owners with *L. infantum* positive dog *vs.* P=44.45% - CI95% 28.5-63.4 in owners with dogs negative to *L. infantum*).

3d. RFLP pattern analysis:

A total of 116 different patterns were detected in the 424 dogs and humans' samples positive by PCR. Only 17 patterns were detected in 2 or more individuals in an overall number of 66 dogs and/or humans. Geographical distribution of the most frequently occurring patterns is pictured in Figure 1.

The three most common patterns were numbers 7, 11 and 14 (Figure 2). Pattern 7 was reported from 4 municipalities of area B (reported from 2 men and 2 dogs) and from one dog in a municipality of area D. The MCP area of pattern 7 is 435 km^2. Pattern 11 was reported from areas A (n=1 dog), B (n= 6 dogs) and C (n=1 human) and the overall area of presence is of 1691 km^2. Pattern 14 was reported from area A (n=5 dogs), area B (n= 6 dogs and n=1 human; notably the human subject is resident in the same municipality as one of the dogs infected with this same *L. infantum* strain), area C (n=1 human) and area D (n=7 dogs). The MCP area for pattern 14 is the biggest recorded within the study (3154 km^2, Table 6). Patterns 2, 8, 17 (Figure 2) were shown to be localized to specific areas as they were shared among 2 subjects each, and present exclusively in municipalities of the B area. Likewise, pattern 16 was found only in 2 dogs in 2 municipalities of area D at a distance of 4.8 km. The other 10 patterns were shared among two individuals (dogs and/or humans) thus resident in different study areas. Among the analyzed patterns, only 4 were shared among individuals resident in the same municipality (patterns 6,11,13 and 14). A perfect agreement (k=1) between RFLP and in-silico RFLP was recorded, as the same pattern resulted for all tested samples with both techniques.

3. Discussion

The epidemiology of Leishmaniasis has been changing rapidly over the past decades. In Europe and in the Mediterranean basin, *L. infantum* has spread to higher latitudes and elevations (Ballart et al.,2012; Guernaoui et al., 2006) and has become endemic in areas with continental climate with active foci registered both in humans and dogs (Biglino et al., 2009; Ferroglio et al., 2005). The entomological survey confirmed the local presence of competent phlebotomine sandflies vectors in all 5 study areas, with trapping density values within the range of those obtained in the same area in 2000-2001 (Ferroglio et al., 2005) while capture frequency of *P. perniciosus* increased from 21.8% in 2000-2001 (Ferroglio et al., 2005) to 42.86% of positive trapping stations. The survey was conceived as a single sampling during seasonal abundance peak to maximize capture sensitivity. No inference can be made on eventual differences in seasonal dynamics nor abundance among the study areas. When the first stable foci of infection were identified in the study area (Northwestern Italy) in the years 2000-2001, seroprevalence by indirect fluorescent antibody test (IFAT) in dogs ranged from 3.9% to 5.8% (Ferroglio et al., 2005) and reached 7.41% by WB in asymptomatic healthy humans (Biglino et al., 2009). Data collected within this study, refer to the period 2007-2009 and recorded a marked increase in prevalence in both canine and human population. The overall prevalence of *L. infantum* by PCR reached 40% in dogs and exceeded 48% in autochthonous dogs, with animals living in areas B and D being significantly more infected by *L. infantum*. Even the difference is not statistically significant, sandfly capture frequency in areas B and D was higher than in the other study sites. We hypothesized that a more frequent presence of competent vectors within the study area, increased the risk of host-vector contact and thus the risk of *L. infantum* infection (Ferroglio et al., 2005; Ferroglio et al., 2006). The same risk factors did not apply in regards of human infection. Antibodies against *L. infantum* were detected by WB in more than 16% of the tested individuals, and the DNA of the parasite was detected in 73% of the seropositive subjects. The only significant association with infection was found, as for dogs, with the area of origin of the tested subjects. Humans from the two agricultural areas considered (B and C), were significantly less infected than those from the other study sites. No direct relation was indeed found between prevalence in dogs and humans from the same areas, and further research is needed to assess the reasons behind the contemporary high prevalence recorded in dogs and the low infection rate recorded in humans from the same area B. Notably, also owning a dog positive to *L. infantum* was not a risk factor for human infection as shown by the subset of dogs and human owners tested in parallel. This might suggest that at least in the first years after the disease becoming endemic, the infection status of dogs in the immediate surroundings is not a critical point for human infection. This hypothesis is also corroborated by RFLP pattern analysis. The high number of circulating strains confirms the multiple-introduction origin of *L. infantum* in the study area since as previously demonstrated, in classically endemic areas, the same high variability of RFLP patterns was recorded (Millan et al., 2011)Especially those RFLP pattern strains localized to limited areas like patterns 2,8,16 and 17 are suggestive of patterns that have been somehow recently introduced into the territory or have been weakly spread among a small number of subjects. Patterns that are found in a higher number of subjects (i.e. pattern 7, 11 and 14) are strains that have been circulating in the Piedmont territory for a longer time; Thanks to the frequent travels and journeys to which the population is subject and to an expanding presence of the vectors in terms of capture frequency, these are now circulating in much of the Piedmont territory, probably in a stable manner considering the size of their presence area. The complete agreement between RFLP and in-silico RFLP confirms the possibility of using either technique with the same degree of accuracy in pattern determination.

Vector territorial expansion and the consequent establishment of vector-borne diseases into naive areas are an issue of concern for both public health and veterinary authorities (Githeko et al., 2000), especially for diseases with a high social impact like ZVL. Monitoring vector and disease expansion in areas of recent colonization is of paramount importance for understanding transmission mechanisms and patterns of disease establishment (Bern et al., 2008).

Acknowledgments

The authors would like to thank the veterinarians and medical doctors who participated to this study as well as all the people that volunteered to participate as anonymous blood donors.

Conflict of Interest

None

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Caption to figures

Figure 1 Geographical distribution of RFLP patterns identified in dogs and humans in study areas A, B, C and D. The pattern identification number is reported close to each identifier on the map.

Figure 2 RFLP digestion with M1sI (lanes a1-a9) and BseLI (lanes b1-b9) separated on 2% agarose high-resolution gel. Fragment size was estimated using the molecular weight standard pBR 322 HaeIII Digest (lanes mrk). Patter 2 (lanes a1, b1), pattern 7 (lanes a2, b2, a6, b6), pattern 11 (lanes a3, b3, a7, b7), pattern 14 (lanes a4, b4, a8, b8), pattern 8 (lanes a5, b5) and pattern 17 (lanes a9, b9).