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(Article begins on next page)

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

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20 **Summary**

21 *Leishmania infantum* infection had been expanding into new areas due to changes in vector and
22 host biology. Zoonotic visceral leishmaniasis has become endemic in previously unsuitable areas
23 as vectors find favorable climatic conditions and an increasing number of reservoir dogs are moved
24 between traditionally and new endemic areas. Monitoring vector and disease expansion in areas of
25 recent colonization is needed to understand transmission mechanisms and patterns of disease
26 establishment. Here we studied the infection status of 815 human blood donors and of 803
27 sympatric dogs from 5, newly endemic, areas in Northwestern Italy. In autochthonous dogs, the
28 seroprevalence of anti-*Leishmania infantum* antibodies, recorded by Western Blot, reached
29 42.22%, while in humans, the seroprevalence was of 16.81%. No significant correlation between
30 the infection status of dogs and that of their human owners was found but *L. infantum* infection
31 was recorded in the different study areas with significant levels of diversity. Restriction Fragment
32 Length Polymorphism showed a high genetic variability of the circulating strains and gave useful
33 insights on patterns of disease establishment into a naïve area.

34

35 **Keywords**

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

37 **Impacts**

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

43

44 **1. Introduction**

45 Zoonotic visceral leishmaniasis (ZVL) is a vector-borne parasitic disease which is caused in the
46 Mediterranean basin, by *Leishmania infantum*. *L. infantum* is transmitted in the Old World by
47 sandflies of the genus *Phlebotomus* (Bates et al., 2015) and in the urban environment, has the dog
48 as main reservoir of infection (Quinnell and Courtenay, 2009) together with various species of
49 sylvatic hosts (Millan et al., 2011, Molina et al., 2012; Zanet et al., 2014; Diaz-Saez et al., 2014;
50 Jimenez et al 2014). The geographic range of occurrence for *L. infantum* infection in dogs has
51 changed in the recent past, as the endemic area of ZVL has broaden to include Northern Italy
52 (Maroli et al., 2008; Dereure et al., 2009) and Northern European countries (i.e. Germany (Naucke
53 et al., 2008), United Kingdom (Shaw et al., 2009), Hungary (Farkas et al., 2011; Tánčzos et al.,
54 2012) and France (Chamaillé et al., 2010). The geographical expansion of phlebotomine vectors
55 toward higher latitudes and elevations (Ferroglio et al., 2005; Millan et al., 2016) has been related
56 to climate change and in particular to the rise of winter temperatures (Stainforth et al., 2013) that
57 allows for successful overwintering (Githeko et al., 2000) in previously unsuitable areas. In newly
58 endemic areas, prevalence and incidence of *L. infantum* infection varies greatly. In Northwestern
59 Italy *L. infantum* was recorded with a seroprevalence of 7.4% in asymptomatic healthy adults
60 (Ferroglio et al., 2005; Biglino et al., 2010; Ferroglio et al., 2006) and PCR-RFLP pattern analysis
61 from the same area, identified three domestic dogs as source of infection for their owners
62 (Ferroglio et al., 2006). Within a context of a rapidly changing epidemiology of ZVL, we analyzed
63 with the same diagnostic techniques used in the previous studies (to guarantee data comparability),
64 sympatric human and dog samples from 5 geographically distinct and specific areas in
65 Northwestern Italy recently become endemic for *L. infantum*. The main goals of the work were to
66 assess presence and prevalence of infection in humans and dogs, to identify species-specific risk

67 factors and common epidemiological traits. These data will serve as base-reference to assess how
68 the epidemiology of *L. infantum* has changed over the past decade.

69 **2. Materials and Methods**

70 2.a Study area

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

75 2.b Collection and identification of sandflies

76 In order to confirm the local presence of competent *L. infantum* vectors, in each of the study sites,
77 we carried out a preliminary entomological survey to assess the presence of phlebotomine
78 sandflies. Sandflies were collected using sticky traps made from 20X20 cm castor oiled paper. Ten
79 (sticky trap total area of 0.4 m²) to 20 traps (sticky trap total area of 0.8 m²) were placed for one
80 night, from sunset to sunrise in 105 trapping points evenly distributed among areas (A to E). A
81 single trapping session was performed at each of the 105 sites during the local peak of sandflies
82 abundance at the beginning of July 2007 (Takken and Knols, 2007). Collected insects were kept
83 in 70% alcohol for further analysis. Males and females were cleared in lactophenol and identified
84 according to specific taxonomical keys (Corradetti et al., 1961; Biocca et al., 1977). The density
85 and frequency of captured specimens in each study area are reported in table 2.

86 2.c Sample collection and processing

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

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

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

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

106 2d. Diagnosis of *L. infantum* infection in dogs and humans

107 *L. infantum* specific antibodies were detected in both dogs and human samples, by means of
108 Western Blotting (WB) using as antigen source late-log phase promastigotes of *L. infantum* (IPT-
109 1 Roma) (Ferroglia et al., 2007).

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

113 Human white-blood cells were pelleted from 2 ml of serum, and DNA was extracted using the
114 same commercial kit used for dog's samples. A specific fragment of 145bp of *L. infantum* kDNA
115 was amplified by end-point PCR with primers mRV1 and mRV2 as published elsewhere (Zanet et
116 al., 2014). The same WB and PCR protocols were used to test dogs (autochthonous and non) and
117 humans. All positive amplicons were purified using (QIAQuick PCR purification kit, QIAGEN)
118 and both DNA strands were directly sequenced (Macrogen, The Netherlands) to confirm *L.*
119 *infantum* identification. The resulting sequences were compared with the ones available on
120 Genbank using the Basic Local Alignment Search Tool (BLAST).

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

127 2e. PCR-RFLP analysis

128 All PCR positive samples of both dogs and humans were double digested with restriction enzymes
129 M1sI (MscI) and BseLI (Bs1I) (Fermentas, Milan, Italy) as previously described (Millan et al.,
130 2011). To confirm digestion specificity, an in-silico Restriction Fragment Length Polymorphism
131 (RFLP) was carried out, on all positive samples, using the online software tool NEBcutter (Vincze
132 et al., 2003).

133 2.f Statistical and Geospatial analysis

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

142 **Results**

143 3.a Sand fly:

144 Phlebotomine sandflies were monitored in the 5 areas of interest for the current study. *Phlebotomus*
145 *perniciosus* was identified in all study areas (A to E) with an overall frequency of positive trapping
146 sites of 42.86% (IC95% 15.82 – 74.95%; 45/105 positive trapping stations). No statistical
147 difference was recorded among capture frequency nor density values of *P. perniciosus* in the
148 different areas (Tab. 2). *P. perniciosus* was trapped with an average density of 15.2 individuals/m².
149 *Sergentomya minuta* was reported from all trapping sites in all areas (A to E) (capture frequency
150 of 100%, IC95% 96,47 – 100%). The average density of *S. minuta* was 18 individuals/m² (Tab.2).

151 3b. Dogs

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

156 36.48%-48.18%) with 114/270 subjects testing positive (Table 3). K coefficient of agreement
157 between PCR and WB on autochthonous dogs showed substantial (area D) to almost perfect
158 agreement (areas A and B). No exclusively autochthonous dogs were available from area C.
159 On autochthonous dogs, statistically significant differences existed between study areas: sites B
160 and D, which are agricultural and urbanized hilly areas, have prevalence and seroprevalence values
161 higher than those recorded in area A, a low urbanized and natural mountain area (Table 4). No
162 other risk factors showed p values ≤ 0.05 at Pearson's Chi square (data not shown).

163 3c. Humans

164 The overall seroprevalence recorded by WB on human samples is $P=16.81\%$ (CI95% 14.40 -
165 19.53%) with 137 positives on 815 tested individuals. Of these, 100 were also positive by PCR
166 ($P=72.99\%$; IC95% 65.01% - 79.73%). Individuals resident in the two mainly agricultural areas,
167 B ($\chi^2= -0.59163$; $p= 0.0124$) and C ($\chi^2= -0.5043$; $p= 0.0396$) had significantly lower seroprevalence
168 than those from the other study areas (Table 5). No other factors showed p values ≤ 0.05 at
169 Pearson's Chi square analysis. Notably no significant association was found with periodic stays in
170 traditionally endemic areas ($p>0.05$) nor with owing a dog ($p>0.05$) even if the dog itself is *L.*
171 *infantum* infected ($p>0.05$ in the subset of owners/dogs from area B; $P=54.40\%$ - CI95% 36.6-71.5
172 in owners with *L. infantum* positive dog vs. $P=44.45\%$ - CI95% 28.5-63.4 in owners with dogs
173 negative to *L. infantum*).

174 3d. RFLP pattern analysis:

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

179 The three most common patterns were numbers 7, 11 and 14 (Figure 2). Pattern 7 was reported
180 from 4 municipalities of area B (reported from 2 men and 2 dogs) and from one dog in a
181 municipality of area D. The MCP area of pattern 7 is 435 km². Pattern 11 was reported from areas
182 A (n=1 dog), B (n= 6 dogs) and C (n=1 human) and the overall area of presence is of 1691 km².
183 Pattern 14 was reported from area A (n=5 dogs), area B (n= 6 dogs and n=1 human; notably the
184 human subject is resident in the same municipality as one of the dogs infected with this same *L.*
185 *infantum* strain), area C (n=1 human) and area D (n=7 dogs). The MCP area for pattern 14 is the
186 biggest recorded within the study (3154 km², Table 6). Patterns 2, 8, 17 (Figure 2) were shown to
187 be localized to specific areas as they were shared among 2 subjects each, and present exclusively
188 in municipalities of the B area. Likewise, pattern 16 was found only in 2 dogs in 2 municipalities
189 of area D at a distance of 4.8 km. The other 10 patterns were shared among two individuals (dogs
190 and/or humans) thus resident in different study areas. Among the analyzed patterns, only 4 were
191 shared among individuals resident in the same municipality (patterns 6,11,13 and 14). A perfect
192 agreement (k=1) between RFLP and in-silico RFLP was recorded, as the same pattern resulted for
193 all tested samples with both techniques.

194 **3. Discussion**

195 The epidemiology of Leishmaniasis has been changing rapidly over the past decades. In Europe
196 and in the Mediterranean basin, *L. infantum* has spread to higher latitudes and elevations (Ballart
197 et al.,2012; Guernaoui et al., 2006) and has become endemic in areas with continental climate with
198 active foci registered both in humans and dogs (Biglino et al., 2009; Ferroglia et al., 2005). The
199 entomological survey confirmed the local presence of competent phlebotomine sandflies vectors
200 in all 5 study areas, with trapping density values within the range of those obtained in the same
201 area in 2000-2001 (Ferroglia et al., 2005) while capture frequency of *P. perniciosus* increased

202 from 21.8% in 2000-2001 (Ferroglia et al., 2005) to 42.86% of positive trapping stations. The
203 survey was conceived as a single sampling during seasonal abundance peak to maximize capture
204 sensitivity. No inference can be made on eventual differences in seasonal dynamics nor abundance
205 among the study areas. When the first stable foci of infection were identified in the study area
206 (Northwestern Italy) in the years 2000-2001, seroprevalence by indirect fluorescent antibody test
207 (IFAT) in dogs ranged from 3.9% to 5.8% (Ferroglia et al., 2005) and reached 7.41% by WB in
208 asymptomatic healthy humans (Biglino et al., 2009). Data collected within this study, refer to the
209 period 2007-2009 and recorded a marked increase in prevalence in both canine and human
210 population. The overall prevalence of *L. infantum* by PCR reached 40% in dogs and exceeded 48%
211 in autochthonous dogs, with animals living in areas B and D being significantly more infected by
212 *L. infantum*. Even the difference is not statistically significant, sandfly capture frequency in areas
213 B and D was higher than in the other study sites. We hypothesized that a more frequent presence
214 of competent vectors within the study area, increased the risk of host-vector contact and thus the
215 risk of *L. infantum* infection (Ferroglia et al., 2005; Ferroglia et al., 2006). The same risk factors
216 did not apply in regards of human infection. Antibodies against *L. infantum* were detected by WB
217 in more than 16% of the tested individuals, and the DNA of the parasite was detected in 73% of
218 the seropositive subjects. The only significant association with infection was found, as for dogs,
219 with the area of origin of the tested subjects. Humans from the two agricultural areas considered
220 (B and C), were significantly less infected than those from the other study sites. No direct relation
221 was indeed found between prevalence in dogs and humans from the same areas, and further
222 research is needed to assess the reasons behind the contemporary high prevalence recorded in dogs
223 and the low infection rate recorded in humans from the same area B. Notably, also owning a dog
224 positive to *L. infantum* was not a risk factor for human infection as shown by the subset of dogs

225 and human owners tested in parallel. This might suggest that at least in the first years after the
226 disease becoming endemic, the infection status of dogs in the immediate surroundings is not a
227 critical point for human infection. This hypothesis is also corroborated by RFLP pattern analysis.
228 The high number of circulating strains confirms the multiple-introduction origin of *L. infantum* in
229 the study area since as previously demonstrated, in classically endemic areas, the same high
230 variability of RFLP patterns was recorded (Millan et al., 2011)Especially those RFLP pattern
231 strains localized to limited areas like patterns 2,8,16 and 17 are suggestive of patterns that have
232 been somehow recently introduced into the territory or have been weakly spread among a small
233 number of subjects. Patterns that are found in a higher number of subjects (i.e. pattern 7, 11 and
234 14) are strains that have been circulating in the Piedmont territory for a longer time; Thanks to the
235 frequent travels and journeys to which the population is subject and to an expanding presence of
236 the vectors in terms of capture frequency, these are now circulating in much of the Piedmont
237 territory, probably in a stable manner considering the size of their presence area. The complete
238 agreement between RFLP and in-silico RFLP confirms the possibility of using either technique
239 with the same degree of accuracy in pattern determination.

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

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247 study as well as all the people that volunteered to participate as anonymous blood donors.

248 **Conflict of Interest**

249 None

250 **References**

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347 **Caption to figures**

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

350

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