RESEARCH ARTICLE

More than the eyes can see: The worrying scenario of canine leishmaniasis in the Brazilian side of the triple border

Vanete Thomaz Soccol1*, Aline Kuhn Sbruuzzi Pasquali1, Eliane Maria Pozzolo2, André de Souza Leandro3, Luciana Chiyo3, Rafael Antunes Baggio1, Mario Sergio Michaliszyn4, Carlos Silva5, Patrícia Hoerner Cubas3, Ricardo Peterlie6, Otacilio Lopes de Souza Paz7, Ivana Lucia Belmonte2, Alceu Bisetto-Junior2

1 UFPR—Graduate Program in Bioprocess Engineering and Biotechnology, Federal University of Paraná, Rua Francisco H dos Santos, Centro Politécnico, Curitiba, Paraná, Brazil, 2 SESA-Secretary of Health of the State of Paraná, Curitiba, Paraná, Brazil and Ninth Health Region, Foz do Iguaçu, Paraná, Brazil, 3 Zoonosis Control Center—CCZ, Foz do Iguaçu Paraná, Brazil, 4 Graduate Program in Environmental Engineering, Positive University, Curitiba, Paraná, Brazil, 5 Vigilância Sanitária, Santa Terezinha de Itaipu, Paraná, Brazil, 6 UFPR—Departamento de Saúde Comunitária, Curitiba, Paraná, Brazil, 7 Laboratório de Análise de Padrões Espaciais e Cartografia Temática (LAPE-CT), Laboratório Pedagógico de Geografia (LABOGE), Universidade Federal do Paraná, Curitiba, Paraná, Brazil

* vanetesoccol@gmail.com

Abstract

A cross-sectional epidemiological study in the extreme-west of the state of Paraná was carried out to access the prevalence, distribution, and risk variables of canine Visceral Leishmaniasis (cVL). This study was conducted in three areas, two cities of far west of Parana state: Foz do Iguaçu (FI) and Santa Terezinha de Itaipu (STI), and along two transects between these two municipalities. To sample the entire urban area, the cities (FI and STI) were divided into a grid of squares of 400 m² (patch). Among the 526 patches, 123 in FI, 40 in the transects and 33 in STI were selected according to the ‘worst scenario’ criterion. In the transect areas, in each 0.86 km five dogs from houses were surveyed to leishmaniasis. In each patch, blood of five dogs from houses (and from neighborhood when necessary) in the areas that seemed to be the most appropriate for the proliferation of vector were surveyed. The infection of the dogs by cVL were assessed using two serological tests were used (cELISA and TR-DPP®), and, for those seropositive for both methods, the PCR method were used. Moreover, dogs presenting clinical signs or cutaneous lesions were sampled to PCR. The identification of Leishmania species was confirmed using PCR-RFLP followed by DNA sequencing. Micro, meso and macro scale environmental variables were also surveyed and statistically analyzed. The prevalence rate Leishmania infantum was 23.8% in FI, 4.7% in STI and 9.1% in the transects areas. Among the extrinsic variables analysed, the number of vectors and the presence of infected dogs in neighbouring were positively correlated with the occurrence of infected dogs. Dog size was positively correlated with cVL infection, while the quality of the dog’s nutrition affected cVL negatively. As for cutaneous leishmaniasis (CL), the first registry of dogs infected with L. braziliensis in the region shows that there is potential for transmission in peri-urban areas, since environmental conditions allow the proliferation of vectors capable of transmitting this species of parasite. cVL is...
widely spread in FI, with high prevalence. This supports the hypothesis that the parasite has been present in the region for longer than previously believed, despite the fact that the presence of leishmaniasis in the region has only been recognized recently. It is important to control the population of dogs infected with *L. infantum* (parasite and non-antibodies) to prevent the spread of the disease to other dogs and also to people in the region.

**Introduction**

Visceral Leishmaniasis (VL) is a zoonotic disease caused by *Leishmania donovani* in the Old and by *L. infantum* in the Old and New World. Worldwide, domestic dogs are the primary reservoir of the disease, which is transmitted by the females of some species of sand flies (Phlebotominae) [1].

In the Brazil, from the 1950s to the 1980s, visceral leishmaniasis was only prevalent in disadvantaged rural areas of the Northeast. Since then, the disease has gradually expanded to peri-urban and urban areas, following the population migrations to these areas. From the 1990s, the disease has expanded fast to the Brazilian Southeast [2–7]. In the South region, LV is more recent. The first autochthonous cases of VL were reported in 2009 in São Borja, state of Rio Grande do Sul [8]. In the state of Santa Catarina, the first autochthonous cases of VL were reported in 2010, in the city of Florianópolis [9], and in 2013 in the western region (São Miguel do Oeste and Descanso) [10]. Concomitantly, VL has expanded its reach to neighboring countries (Argentina and Paraguay), where a gradual increase in human deaths due to it has been observed. Strong evidence supports the hypothesis that VL has expanded into urban areas in the south portion of South America [11–13].

The state of Paraná, in southern Brazil, was considered free of VL transmission until 2012, since neither cVL nor *Lu. longipalpis* had been reported there [14,15]. Vector of *Leishmania, Lu. longipalpis*, was sampled for the first time in Foz do Iguaçu in 2012, when the city was declared an area of risk to the disease. Later that year the first autochthonous cases of cVL were reported. Finally, in 2015, the first case of VL was documented, and *L. infantum* was confirmed as the aetiological agent [16–19].

The spatial distribution of a pathogenic agent at an area is important to monitor and to control the disease it causes. Since cVL in dogs usually precedes human cases, we used dogs as proxy to assess the spatial distribution and the risk factors for VL in the far west of the state of Paraná. To accomplish this, we conducted a cross-sectional epidemiological study in this area to determine the prevalence, distribution, and risk factors of cVL. To this end, three sampling sites were selected: Foz do Iguaçu (FI), a city adjacent to the triple frontier (Brazil, Argentina and Paraguay); Santa Terezinha do Itaipu (STI), to assess if the parasite has dispersed to nearby cities, and two transects (T1 and T2) between them, to assess if the disease is present or emergent, and how is the dissemination of cVL in this rural area.

**Materials and methods**

**Area studied**

The city of Foz do Iguaçu (FI, state of Paraná, southern of Brazil, 25°32′49″ S, 54°35′17″ W) is located in average 192 m above sea level [20]. For this study, the city of Foz do Iguaçu was divided into four areas (A—West; B—East; C—North; and D—South), consistent with the areas delimited by the Health Service. Area A has 64,864 habitants and 7,724 dogs, area B has 93,020 habitants and 16,146 dogs, area C has 123,128 habitants and 23,583 dogs, and area D has...
44,120 habitants and 7,530 dogs. The data from dogs was obtained at Zoonosis Control Center, CCZ of Foz do Iguacu, during the Rabies vaccination senses in August of 2014. The survey for cVL in FI was carried out from 06–22 October 2014.

The city of Santa Terezinha de Itaipu (STI, 25° 21' 44" S, 54° 29' 17" W) is located 218–312 m above sea level. It has an area of 248,133 Km$^2$, of which 96.0% are considered rural, 3.4% urban and 0.6% lake and forest. About 22,783 habitants live there, 90.38% of which live in urban areas [20]. The total dogs’ number was 5,696 (data obtained from Sanitaire Vigilance). For this survey dogs was sampled in November 2015.

Two Transects (T1 and T2) were drawn between Foz do Iguacu and Santa Terezinha de Itaipu, comprising only rural and forest areas. T1 ran from Foz do Iguacu to the natural reserve of Iguacu National Park, whereas T2 ran from this park to STI. In each 0.86 km five dogs from residences were surveyed from 03 to 21 of November 2014.

Dogs sampling
The urban areas (FI and STI) were divided in 526 patches of 400 m$^2$ according to [21, 22]. Due to the limitation of number of CDC-LT traps, 123 patches from FI, 40 from the transects (in each 0.86 km) and 33 from STI were selected due to be the most appropriated for the proliferation of cVL according to the ‘worst scenario’ criterion [22, 23]. The patches (Fig 1) were geo-referenced using GPS equipment and the ArcGIS 10.1 software [24].

Five dogs from each patch (usually a house) have their blood sampled (irrespective of their clinical status). When five dogs were not available in this house, dogs from the neighbourhood were included, up to five. All sampled dogs were owned, and no stray dogs were collected. First, the dogs were examined for clinical signs of the disease and each of them was assigned an individual data file that included its identification, traits, behaviour, migration history and health-related issues. Each file included the following information: breed, gender, age, size, night resting place, and weather the dog was on a leash during the night or not, migration history, use and periodicity of repellent, clinical signs of leishmaniasis, and nutritional state. The presence of one or more of the following signs was taken as a clinical indication of cVL: lymphadenopathy, onychogryphosis, cutaneous lesions, weight loss, conjunctivitis and alopecia. In the second step, blood samples of the dogs were collected by venepuncture of the jugular or the cephalic using a disposable syringe and needle (25x7), transferred into 10 mL polypropylene tubes, and processed 3–4 h after collection. In the laboratory blood was centrifuged at 1000 x$g$ for 5 min and sera were separated and stored at $-20^\circ{\mathrm{C}}$ until analysed by two serological methods. Finally the extrinsic factors (environmental: vegetal cover, cement, bare soil cover + covered surface with unused materials, number of chickens, repellent, number of Lu. longipalpis and presence of infected dogs in the neighbourhood) (Table 1) were recorded.

All procedures involving dogs were conducted in strict accordance with the regulations outlined by the National Council for the Control of Animal Experimentation (CONCEA), and all efforts were made to minimise suffering.

The Animal Care and ethical Committee of the Federal University of Parana, under protocol no. 044/2014. The owners received the results of the examination, along with general prevention recommendations based on reducing effective vector-reservoir/vector-human contact.

Serological procedures and molecular Leishmania identification
Two serological tests were used in the serological survey: an immunoassay test (using crude antigen cELISA), as proposed by Mazieiro et al., 2014 [10], and the Dual Path Platform (DPP® cVL, Biomanguinhos).
Fig 1. In the 196 sites (pointed in the maps) from three areas of the extreme west of the Southern of Brasil (Foz do Iguacu, Santa Terezinha de Itaipu and transect between the two cities) dogs were sampled to determine the seroprevalence to leishmaniases. In each site blood of five (or more) dogs were sampled and examined serologically according to Brazil Health Ministry recommendation. The two test (DPP and Immunoassay) were realized simultaneously. We considered a positive animal when two serological exams were positives.

https://doi.org/10.1371/journal.pone.0189182.g001
As recommended by the Brazilian Ministry of Health, only dogs that tested seropositive in both tests were considered infected [25,26]. From these animals, popliteal lymph nodes were taken using a disposable syringe and needle (1.2 x 40 mm) and test for the presence of *Leishmania* parasites using Polymerase Chain Reaction (PCR). DNA extraction was performed using the Wizard Genomic DNA purification kit in accordance with the manufacturer’s recommendations. The DNA pellet was dissolved in 50 μL of Tris-EDTA buffer incubated in water bath at 65˚C for 30 min, and stored at −20˚C until analysis. PCR was performed with internal transcribed spacer (ITS) primers followed by RFLP as described by Schonian et al. 2003 [27] for *Leishmania* identification. The Cytochrome B1/B2 was used as an internal control to verify DNA amplification [28]. Positive PCR products (lymph nodes samples) with pattern of RFLP electrophoresis different from the reference *Leishmania* (i.e. the most common *Leishmania* species in the Brazil: *L. infantum*, *L. braziliensis*, *L. amazonensis*) were sequenced to confirm the identification. The sequencing was commercially performed by the Macrogen Inc. (Seoul, South Korea). The resulting sequences were deposited in the Genbank with the following accession numbers MF945579 to MF945584. The sequences were aligned using the MAFFT 7.0 [29] in the Guidance web server [30–32]. The final alignment was composed of 255 bp sequences, including the indel mutations. The identification was done through a Neighbor-Joining (NJ) tree constructed in MEGA 7.0 [33], using the substitution model (K80 – [34]) defined by the software jModeltest 2.1.10 [35], and the robustness of the NJ tree was assessed using 1,000 bootstrap replicates.

**Statistical analyses**

Each positive patch was recorded and transformed for input into the Geographic Information System (GIS) environment program. After the data were reclassified, they were converted to the raster format, to enable map algebra, highlighting the sites with the greatest number of

| Group | Variable Recorded | Explanation |
|-------|-------------------|-------------|
| Extrinsic | Site | Foz do Iguaçu, transects and Santa Terezinha de Itaipu |
| | Vegetal cover | Number of fruit trees of the household Percentage of vegetal cover of the patch |
| | Cement | Percentage of cement of the patch |
| | Bare soil cover + Covered surface with unused materials(intermedium) | Percentage of soil and surface unused of the patch |
| | Number of chicken | Number of chicken |
| | Repellent | Use of repellent |
| | Number of *Lu. longipalpis* | Number of *Lu. longipalpis* |
| | Infected neighbor dog | Presence of infected dogs in the neighborhood |
| Intrinsic | Age | Age of the dog |
| | Ambulate | Dog that moves from different places |
| | Autochthonous | Dog from the site |
| | Allocchthonous | Dogs from other places |
| | Sleep outside | Dogs that sleeps outside a house |
| | Size | Size of the dog |
| | Nutrition | Nutrition state of the dog |
| | Ticks | Presence of Ticks |
| | Fleas | Presence of Fleas |

https://doi.org/10.1371/journal.pone.0189182.t001
cases [24]. After the map algebra was completed, the data were converted to vector format using cross-tabulation.

To assess the clinical diagnostics of cLV, differences in clinical signals between seropositive and seronegative groups, recorded in the epidemiological questionnaire, were tested using the chi-square (for FI: without clinical signals, with clinical signals, state mind active, weight loss, adenomgalgy, alopecia, skin lesions, mucosae lesions, hiperkeratosis, muscular atrophy; for T1 + T2: without clinical signals, with clinical signals; all together: without clinical signals, with clinical signals, state mind active and apathic, weight loss, adenomgalgy, alopecia, skin lesions, mucosae lesions, hiperkeratosis, muscular atrophy) or Fisher’s exact test (F for FI: lethargic; for T1 + T2: eye injury; all sites: lethargic) in the R 3.3.3 [36]. The odds ratios (OR), with a confidence interval (CI) of 95%, was employed to measure the association between each clinical signal and the groups (seronegative and seropositive dogs).

The risk analysis started with pairwise correlations between infection and the 18 variables recorded: residence, % of trees, % of cement coverage, infected neighbor dogs, sleeps outside, presence of chickens, number of chickens, presence and abundance of Lutzomyia longipalpis, size of the animal, age, dog coming from the same neighborhood (autochthonous) or from other places (allochthonous), use of repellent, nutritional status, presence of ectoparasites. The variables with significant correlation were then tested in glm (generalized linear model) using a binary logistic regression. Odds Ratio (OR) and its 95% confidence Interval were calculated [37].

Moreover, to assess the direct cross influence among all variables, including the infection, two Path Analyses was performed using the package ‘plspm’ 0.4.7 [38] in R 3.3.3. First, an analysis using only the environmental variables (see Table 1) was performed to test their effects on the proportion of infected dogs at each patch. This analysis assessed the factor that provided opportunities for a dog to become infected by the parasite. Second, a Path Analysis was carried out using only the intrinsic characteristics of the dogs (see Table 1) to test the risk factors that predisposed dogs to get infected with L. infantum. This analysis tested which factors affect the dogs’ likelihood of becoming infected by the parasite. In both analyses, the abundance data were logarithmized and the critical p values were corrected using the B-Y method [39].

Results

cVL seroprevalence in the extreme-west of Parana state

In the three areas a total of 196 patches were surveyed: 123 in FI, 33 in STI and 40 in the transects (T1 + T2). The number of patches with seropositive dogs were 67 (54.4%), 16 (48.5%) and 9 (27.3%), respectively. In the rural areas fewer dogs were positive for the infection. In fact, infection rates were always higher at urban sites (Table 2).

Table 2. Number of positive patches to cVL (before the bar), number of total patches (after the bar) and percentage of positive patches to cVL from three strata from Foz do Iguacu, Santa Terezinha de Itaipu and the transects between two cities. —No urban area; * small community.

| Area          | Foz do Iguacu | Transects | Santa Terezinha de Itaipu | TOTAL |
|---------------|---------------|-----------|---------------------------|-------|
| Urban         | 53/93         | -         | 08/29                     | 61/122|
|               | (56.9)        | -         | (27.6)                    | (50.0)|
| Peri-urban    | 13/27         | 06/40     | 1/4                       | 20/71 |
|               | (48.1)        | (15.0)*   | (25.0)                    | (28.2)|
| Rural         | 1/3           | 10/40     | -                         | 11/43 |
|               | (33.4)        | (25.0)    | -                         | (25.6)|
| TOTAL         | 67/123        | 16/40     | 09/33                     | 92/196|
|               | (54.4)        | (25.0)    | (27.3)                    | (47.0)|

https://doi.org/10.1371/journal.pone.0189182.t002
Among the 1,129 dogs sampled, 785 (69.5%) did not present clinical signals and 344 (30.5%) presented clinical signals. The percentage of seropositive dogs was 23.8% (185 of a total of 777 dogs) in FI, 9.1% (16 of a total of 176) in the transects, and 5.1% (9 of a total of 176) in STI (Table 3).

Clinical signs of the cVL

Of the 21 clinical signs surveyed that were compatible with visceral leishmaniasis, 11 were statistically significantly different between seropositive and seronegative groups: State mind (active, apatic and lethargic) weight loss, adenomegaly, alopecia, skin lesions, mucosae lesions, hyperkeratosis, onychogryphosis, muscular atrophy, eye injury, gastrointestinal disorder. The majority of dogs presented more than one clinical signal (Table 4).

Parasite identification

In Foz do Iguaçu, of the 124 dogs that tested positive in the two serological tests, the PCR of lymph node samples were also positive in 111 (89.8%). The PCR-RFLP test present bands consistent with the pattern produced by the *L. infantum* reference strain in 110 dogs, and the NJ tree supported their identification. One dog from Foz do Iguaçu presented RFLP pattern similar to *L. braziliensis*, and the ITS1 NJ tree supported its identification (Fig 2). Of the 37 seronegative dogs in both serological test and with clinical signs of cVL, 14 (37.8%) were positive to *L. infantum* by PCR-RFLP.

In the transect areas, 16 seropositive dogs were tested using PCR-RFLP and samples from six (37.5%) dogs yielded bands compatible with *Leishmania* spp. The PCR-RFLP showed that five of these dogs presented patterns similar to *L. infantum*, all of which were allochthonous (dogs coming from FI). In one dog autochthonous its PCR-RFLP pattern was consistent with *L. braziliensis* (the parasite of this dog was not sequenced).

In STI, of the nine dogs that were positive for both serological tests, seven yielded a PCR-RFLP pattern consistent with *L. infantum*, and its identification were corroborated by the NJ tree. However, among the seven dogs that tested positive by PCR-RFLP, five were from FI, and only two were authoctonous.

**The spatial distribution of the seropositive dogs**

In FI, the highest prevalence of cVL was in areas A and D (Table 3, Fig 3). In area B, seropositive dogs were present on both sides of BR-277 (largest Brazilian road in this region). In STI,

---

**Table 3.** Dogs sampled (N), seropositive dogs to cVL (N+) and its percentage in three sites in the extreme west of the Paraná state, Southern of Brazil: The Foz do Iguaçu (FI) city was devised in four areas (A, B, C and D), Santa Terezinha do Itaipu (STI) in two areas (A = north and B south), and two transects (T1 and T2) between the two cities (FI and STI). A total of 1129 dogs were sampled.

|            | **Foz do Iguaçu** | **Transects** | **Santa Terezinha de Itaipu** |
|------------|------------------|---------------|-------------------------------|
| **Area**   | **N**  | **N+** | **Area** | **N**  | **N+** | **Area** | **N**  | **N+** |
| A          | 170   | 48    |         |       |       |         |       |       |
|            | (28.2)|       | T1      | 90    | 11    | A        | 60    | 4     |
|            |       |       |         | (13.0)|       |         | (7.1) |       |
| B          | 157   | 44    |         |       |       | T2      | 86    | 5     |
|            | (28.0)|       |         |       |       |         | (5.9) | (3.7) |
| C          | 273   | 44    |         |       |       |         |       |       |
|            | (16.1)|       |         |       |       |         |       |       |
| D          | 177   | 49    |         |       |       |         |       |       |
|            | (27.6)|       |         |       |       |         |       |       |
| **Total**  | 777   | 185   |         |       |       | 176     | 16    | 9     |
|            | (23.8)|       |         |       |       |         | (9.1) | (4.7) |

https://doi.org/10.1371/journal.pone.0189182.t003
Table 4. Clinical classification of seropositive dogs in three regions in the extreme west of Parana state, Southern Brazil. OR: Odds Ratio.–Non observed date. * seropositive dogs in ELISA and DPP tests / total of dogs with clinical signals.

| Clinical classification | Foz do Iguacu | Transects | Santa Terezinha de Itaipu | Total |
|------------------------|--------------|----------|--------------------------|------|
|                        | % positive /total * | OR  | p value | % positive /total * | OR  | p value | % positive /total * | OR  | p value | % positive /total * | OR  | p value |
| Without signals        |              |       |         |              |       |         |              |       |         |              |       |         |
| positive               | 11.7%        | 0.31  | 0.000   | 0.9%         | 0.09 | 0.020   | 4.9%         | 1.71  | 1.000   | 8.9%         | 0.32 | 0.000  |
| (62/528)               |              | (0.21–0.45) |       | (1/115)      | (0.01–0.86) |       | (7/142)     | (0.20–14.93) |       | (70/785) | (0.22–0.45) |       |         |
| With signals           | 29.7%        | 3.17  | 0.000   | 8.2%         | 10.17| 0.020   | 2.9%         | 0.58  | 1.000   | 23.7%        | 3.09 | 0.000  |
| (74/249)               |              | (2.17–4.64) |       | (5/61)       | (1.16–89.21) |       | (1/34)      | (0.06–4.91) |       | (80/344) | (2.18–4.39) |       |         |
| State mind             |              |       |         |              |       |         |              |       |         |              |       |         |
| Active                 | 16.6%        | 9.79  | 0.003   | 3.0%         | 0.24 | 0.273   | 4.0%         | 0.08  | 1.000   | 12.5%        | 0.30 | 0.000  |
| (123/742)              |              | (0.16–0.68) |       | (5/167)      | (0.02–2.36) |       | (7/173)     | (0.00–1.04) |       | (135/1082) | (0.16–0.57) |       |         |
| Apathetic              | 18.2%        | 1.18  | 0.766   | 11.1%        | 4.05 | 0.273   | 33.3%        | 11.85%| 1.000   | 26.3%        | 2.42 | 0.030  |
| (4/22)                 |              | (0.39–3.56) |       | (1/9)        | (0.42–38.86) |       | (1/3)       | (0.95–146.89) |       | (10/38)  | (1.15–5.10) |       |         |
| Lethargic              | 55.6%        | 6.07  | 0.010   | -            | -    | -       | -            | -    | -       | 55.6%        | 8.40 | 0.003  |
| (5/9)                  |              | (1.61–22.94) |       |              |       |         |              |       |         | (5/9)        | (2.23–31.66) |       |         |
| Weight loss            | 36.7%        | 3.00  | 0.000   | 0            | 0    | 1.000   | 0            | 0    | 1.000   | 28.6%        | 2.83 | 0.000  |
| (18/49)                |              | (1.62–5.54) |       |              |       |         |              |       |         | (18/63)      | (1.56–5.03) |       |         |
| Adenomegaly            | 45.2%        | 5.44  | 0.000   | 4.6%         | 1.41 | 0.556   | 0            | 0    | 1.000   | 37.0%        | 5.12 | 0.000  |
| (46/101)               |              | (3.47–8.54) |       | (1/22)       | (0.15–12.74) |       | (1/3)       | (0.95–146.89) |       | (47/127) | (3.38–7.75) |       |         |
| Alopecia               | 33.7%        | 2.83  | 0.001   | 10.00%       | 4.22 | 0.139   | 0            | 0    | 1.000   | 25.7%        | 2.64 | 0.000  |
| (33/98)                |              | (1.77–4.53) |       | (2/20)       | (0.72–24.69) |       | (1/3)       | (0.95–146.89) |       | (35/136) | (1.71–4.07) |       |         |
| Skin lesions           | 36.2%        | 2.90  | 0.001   | 3.5%         | 1.01 | 1.000   | 0            | 0    | 1.000   | 23.1%        | 2.08 | 0.014  |
| (17/47)                |              | (1.55–5.44) |       | (1/29)       | (0.11–9.01) |       | (1/3)       | (0.95–146.89) |       | (18/78)  | (1.19–3.64) |       |         |
| Mucosa lesions         | 36.8%        | 2.95  | 0.003   | 3.4%         | 1.01 | 1.000   | 0            | 0    | 1.000   | 35.0%        | 3.77 | 0.000  |
| (14/38)                |              | (1.48–5.86) |       | (1/29)       | (0.11–9.01) |       | (1/3)       | (0.95–146.89) |       | (14/40)  | (1.92–7.40) |       |         |
| Hyperkeratosis         | 35.7%        | 2.74  | 0.019   | 0            | 0    | 1.000   | 0            | 0    | 1.000   | 32.3%        | 3.25 | 0.005  |
| (10/28)                |              | (1.23–6.09) |       |              |       |         |              |       |         | (10/31)      | (1.50–7.06) |       |         |
| Onychogryphosis        | 23.3%        | 1.65  | 0.302   | 0            | 0    | 1.000   | 0            | 0    | 1.000   | 14.6%        | 1.28 | 0.496  |
| (7/30)                 |              | (0.69–3.95) |       |              |       |         |              |       |         | (7/48)       | (0.56–2.92) |       |         |
| Muscular atrophy       | 60.0%        | 7.35  | 0.003   | 0            | 0    | 1.000   | -            | -    | -       | 50.0%        | 6.75 | 0.002  |
| (6/10)                 |              | (2.04–26.41) |       |              |       |         |              |       |         | (6/12)       | (2.15–21.23) |       |         |
| Eye injury             | 12.9%        | 0.77  | 0.804   | 20.0%        | 10.1 | 0.039   | 6.2%         | 1.45  | 0.541   | (7/57)       | 1.04 | 0.834  |
| (4/31)                 |              | (0.26–2.26) |       | (2/10)       | (1.60–63.74) |       | (1/16)      | (0.16–12.65) |       | (7/57)  | (0.46–2.35) |       |         |
| Gastrointestinal disorder | 50.0%       | 5.33  | 0.292   | 0            | 0    | 1.000   | 0            | 0    | 1.000   | 20.0%        | 1.86 | 0.468  |
| (1/2)                  |              | (0.33–86.01) |       |              |       |         |              |       |         | (1/5)        | (0.20–16.81) |       |         |

https://doi.org/10.1371/journal.pone.0189182.t004
Seropositive dogs were found in the two studied areas (right and left side of BR-277 road). In the transects, the dogs that tested seropositive for \textit{L. infantum} originated from FI. The dogs that tested seropositive for \textit{L. braziliensis} were observed near the Iguacu National Park.

Risk analyses

The glm analysis supported that percentage of trees, percentage of cement, infected neighbour, animal size, nutritional status, outside sleeping, presence of ectoparasites and abundance of \textit{Lu. longipalpis} significantly affected dogs’s infection rates by cVL in FI. In STI, the presence of infected dogs in the neighbourhood influenced the rate of cVL infection the most. In the transects, the behaviour (living on the street) and the presence of infected dogs in the neighbourhood influenced dogs’ infection rates the most. When all animals at all sites were evaluated, eight variables were significant (Table 5).

The Path Analysis of extrinsic (environmental) factors supported that the presence of infected dogs in the neighborhood (path coefficient of 0.75, \( p < 0.01 \)) and the abundance of \textit{Lu. longipalpis} (path coefficient of 0.16, \( p < 0.01 \)) had a positive influence on the proportion of infected dogs at each patch (\( R^2 \) of the model = 60\%) (Fig 4). The Path Analysis of intrinsic dog characteristics supported that dog size was positively correlated with the probability of a dog becoming infected with \textit{Leishmania} spp. (path coefficient of 0.09, \( p < 0.01 \)) and nutrition was negatively correlated with it (better nutrition less risk and vice versa) (path coefficient of -0.11, \( p < 0.01 \). (\( R^2 \) of the model = 4\%) (Fig 5).

Discussion

In the three far-west sites surveyed in the state of Paraná, 210/1,129 (18.6\%), dogs were seropositive for \textit{Leishmania} infection. The highest prevalence was observed in FI (23.8\%),...
especially in areas A, D and B (with approximately 28% of the dogs infected). In area C, the prevalence rate was lower (16.13%). But, seropositive dogs were still present in the patches sampled (26.2% of the patches), indicating that the introduction of cVL in this area is more recent. Several authors have suggested that, in the new and old worlds, the prevalence of cVL varies according to the geographical region, parasite pressure and diagnostic method. For example, in the old world the rates of infection ranged from 5% to 80% in Italy, Spain, Portugal and France, averaging 23.3%. However, there are sites in the first three countries where there is a prevalence of 80%, while the greatest total prevalence rate of 43% was found in France [40–42].

The regional prevalence rates of cVL in Brazil also vary in the local and regional scales: from 6.7 to 29.3% in the Northeast; from 47.8 to 59.3% in the North; from 10 to 45.2% in the...
Table 5. Intrinsic and extrinsic variables that showed significance to canine visceral leishmaniasis. NS: non significant values.

| Variables       | Foz do Iguacu |           | Transects |           | Santa Terezinha de Itaipu |           | Total |           |
|-----------------|---------------|-----------|-----------|-----------|---------------------------|-----------|--------|-----------|
|                 | OR (95% CI)   | p-value   | OR (95% CI) | p-value | OR (95% CI) | p-value | OR (95% CI) | p-value |
| Trees           | 0.99 (0.98–0.99) | 0.041     | NS        |           | NS                        |           | 0.99 (0.98–0.99) | 0.033   |
| Infected neighbours |               |           |           |          |                           |           |        |           |
| no              | Reference      |           | Reference |           | Reference                 |           | Reference |           |
| yes             | 3.15 (2.09–4.82) | 0.000     | 3.36 (1.33–8.78) | 0.011 | 7.36 (2.88–22.75) | 0.000 | 3.46 (2.45–4.94) | 0.000   |
| Lu. longipalpis |               |           |           |          |                           |           |        |           |
| no              | Reference      |           | Reference |           | Reference                 |           | Reference |           |
| yes             | 1.86 (1.25–2.75) | 0.001     | NS        |           | NS                        |           | 1.79 (1.25–2.55) | 0.001   |
| Size            |               |           |           |          |                           |           |        |           |
| small           | Reference      |           | Reference |           | Reference                 |           | Reference |           |
| medium          | 1.72 (1.11–2.65) | 0.014     | NS        |           | NS                        |           | 1.63 (1.13–2.34) | 0.008   |
| big             | 2.67 (1.58–4.47) | 0.000     | NS        |           | NS                        |           | 1.97 (1.26–3.04) | 0.002   |
| Nutrition       |               |           |           |          |                           |           |        |           |
| good            | Reference      |           | Reference |           | Reference                 |           | Reference |           |
| regular         | NS            |           | NS        |           | NS                        |           | 1.96 (1.15–3.28) | 0.011   |
| bad             | 4.20 (1.40–12.18) | 0.008    | NS        |           | NS                        |           | 2.88 (1.01–7.67) | 0.037   |
| Fleas           |               |           |           |          |                           |           |        |           |
| no              | Reference      |           | Reference |           | Reference                 |           | Reference |           |
| yes             | 1.78 (1.19–2.66) | 0.004     | NS        |           | NS                        |           | 1.51 (1.07–2.11) | 0.017   |

Fig 4. Path analysis with extrinsic (environmental) characteristics that affect the infection rate in dogs from western region of the Paraná State, Brazil. Blue arrows represent positive effect, and red arrows represent negative effects.

https://doi.org/10.1371/journal.pone.0189182.t005

https://doi.org/10.1371/journal.pone.0189182.g004
Southeast; and from 9.3 to 65% in the Center-West. In the South (where LV is more recent) a survey in Rio Grande do Sul (2009 to 2010), conducted in 34 municipalities and using 5,430 dogs, found a prevalence rate of 20.8% [4, 7, 43–48]. In the neighboring countries around our study area, Argentina and Paraguay, cVL has a prevalence of 7.2 to 16.8 and 3.1 to 11.8% respectively. However, in those countries, only the rk39 test (lateral-flow assay) was used. The sensitivity and specificity of this test is not consistent with the recommendations of Brazil’s Health Ministry [21, 22, 49]. According Quinnell et al., [50], the sensitivity and specificity of the rk39 varied across studies, but their combined sensitivity to detect the clinical disease was 86.7% and to detect infection was 59.3%. Grimaldi et al., [51] showed that the sensitivity of the TRDPP, one of the methods used in our study, was 98% (59/60 samples) for the detection of dogs with with disease, but lower (47%) in identifying parasite-positive dogs without signs of cVL. In the extreme west of the state of Paraná, among the 1,129 dogs sampled, 28.1% presented clinical signs compatible with cVL, and 50.8% of them were seropositive. On the other hand, even dogs without clinical signals were positive for serological tests (8.1%). Several studies have shown that the prevalence of clinical signs is between 3 to 10% [4,52–55]. However, we found an alarmingly greater prevalence (about 30%). There are other diseases with clinical signs like cVL, including ehrlichiosis, dermatophytosis and canine scabies. For this reason, fast and better diagnoses are essential to understand the epidemiology, to control VL, and to confirm all seropositive cases of cVL. In addition, to determine the magnitude of the infection, we employed a PCR-RFLP approach to complement the diagnoses.

In general, molecular techniques are efficient to detect infections by *L. infantum*, even in the early stages, while serological techniques are more efficient at the advanced stages, when IgG production is established and can be more easily detected. In a longitudinal study, Quinnell et al. [56] reported a 98% sensitivity of the PCR in parasite-positive samples in the initial stage, but this decreased to 68% in the chronic phase. In our study, using lymph nodes samples the PCR technique supported that 37.8% of the dogs with clinical signals, but seronegative, were infected by *L. infantum*. It supports the necessity of working with different techniques to complete the diagnosis and reduce the infected number of animals. The RFLPs technique with ITS marker, showed more bands that suggested in the original article [27] in some isolates, and they were sequenced, confirming *L. infantum* as principal specie (Fig 2). Alternatively, *L. braziliensis* parasites were found in an autochthonous dog in the transect areas and other in

---

**Fig 5.** Path analysis with intrinsic characteristics of the dogs that affect their probability of infection in the western region of the Paraná State, Brazil. Blue arrows represent positive effect, and red arrows represent negative effects.

https://doi.org/10.1371/journal.pone.0189182.g005
Foz do Iguaçu. This is the first record of *L. braziliensis* in dogs from this region. This supports that both *L. infantum* and *L. braziliensis* are sympatrically and perhaps syntopically distributed in this region, especially in peri-urban areas.

In the Brazilian side of the triple border, the widespread distribution of seropositive dogs, the abundance of the vector *Lu. longipalpis* and the high percentage of older dogs infected throughout FI suggest that the parasite cycle can be established in this region, and that cVL may be considered endemic to this area. Moreover, authochthonous cVL in STI cases suggests that Foz do Iguaçu is a gateway (BR-277) for cVL in the western portion of the state of Paraná. Furthermore, the presence of infected dogs in the transects, although originated allochthonous, supported that this road is a possible dispersion route for the disease between these cities. Besides this rural road in the transects, the highway BR-277, connects both cities and runs to the city of Paranaguá, in the extreme east of the state of Paraná, is likely an important route of dispersion of infected dogs, and consequently the parasite and the disease, to other regions of the state.

The geo-referencing data of seropositive dogs showed that the sectors with high rates of canine positivity and the highest canine infection rates were associated with forest fragments and streams (see Fig 3). As part of the urbanisation of Foz do Iguaçu, Atlantic Forest vegetation was preserved in fragments in the urban area, especially in areas A and D which, in turn, support the phytophysionomic characteristics of semideciduous forests. Similarly, from the 1980s, with the development of the city that emerged in conjunction with the construction of the Itaipu hydroelectric power plant, and now, with the Municipal Plan for Urban Forestation (in its final steps), there has been a tendency to combine vegetated areas with buildings and urban structures. Thus, area C is currently booming with the construction of residential condominiums. Moreover, more recently an avenue linking areas A and B was built, passing through a forest reserve. The relationship between forest and urban occupation is a historical feature and was socially constructed by the population of Foz do Iguaçu. In particular, the maintenance of green areas in the urban portion is a capital of the city. The green areas can maintain the cycle of *Lu. longipalpis* and make it difficult to control cVL.

The search for risk variables for the occurrence of cVL has shown that both intrinsic and extrinsic variables are important in the maintenance of the endemism of this disease. Our Path Analysis of the extrinsic variables supported that the abundance of *Lu. longipalpis* and the presence of infected dogs in the neighbourhood are the variables that affect the presence of infected dogs. Infected reservoirs are a source of parasites, and while *Lu. longipalpis* facilitates the transmission of the parasite between dogs. Vertical transmission of the disease is also possible, e.g. during pregnancy [57]. In this way, the proximity of infected dogs and the abundance of *Lu. longipalpis* provide the opportunity for *L. infantum* to parasitize a non-infected dog.

On the other hand, a factor that affects the capacity of dogs to avoid the infection is their nutritional status and size. Large dogs have larger areas to attract the vector and to be bitten by the infected Phlebotominae. Malnourished dogs have weaker immune response, which is further compromised by the cVL infection. Moreover, other factors, especially dogs’ capacity to migrate, have a greater importance on the dispersion of VL to different regions. Similarly, the presence of fleas increases the infection rate (1.51 in all regions and 1.78 in dogs from FI). Fleas are known to transmit diseases between individuals see Mencke 2013 [58]. Specifically, in recent years, Dantas-Torres (2011 [59]) and Paz (2013 [60]) postulated that fleas and ticks may act as *Leishmania* vectors. In this way, the influence of the presence of fleas on the infection of cVL may be resulted from direct transmission of *Leishmania* by the insect, or resulted from the decrease in the health of the dog by the bite of fleas or transmission of other diseases. Thus, once sand flies have been registered in the three areas surveyed, fleas could amplify the prevalence of the cVL.
Alternatively, our results did not support that chickens affect the probability of infection of dogs by *Leishmania*. Some studies supported that the blood of chickens are most preferred food supply by *Lu. longipalpis*, followed by blood of dogs [61,62]. Furthermore, chickens may be important to the maintenance of sand fly populations, and amplify the leishmaniasis prevalence [63]. However, although about 21% of the patches in FI presented chickens, 62% in the transects and 46% in STI, our results does not supported this hypothesis. Thus, the dogs, as a food supply, may be enough to maintain the cycles of the vector and the parasite.

In endemic areas, cVL represents only a small part of the problem [64], because the disease is complex, with several variables involved as such environmental, socio-cultural and geopolitics. VL is a zoonosis, and the domestic dog participates in the biological and epidemiological cycles. In a social survey conducted in the city of Foz do Iguacú, epidemiological records confirm that the average number of dogs per household was 1.35, while the number of people per household was 4.0. In developing countries, companion animals currently play a central role in families living in large cities, and this tendency has been observed in Brazil. With the decreasing number of individuals per household, dogs and cats have become more important to help people with mental disorders, and other disabilities [65]. Thus, more reliable diagnostic approaches and better drugs for treatment are essential to identify seropositive dogs and dogs that are parasitized, since isolating them, or treating them as soon as possible, would reduce parasite pressure, reducing the risk for the human population and sparing the rest of the canine population. In the example studied here, controlling the disease in the infected dogs would spare the other, healthy dogs (75% of the population). However, if the disease is not controlled, the continuous infection of vectors and hosts will allow for the propagation of LV. Therefore, it is urgent that researchers in areas that are considered endemic for visceral leishmaniasis discuss this issue in international forums specific to *Leishmania* and leishmaniasis and seek a global consensus to avoid the unnecessary suffering of people and animals.

The increased prevalence of cVL poses a public health problem that needs to be dealt with. This includes the decision to treat or not to treat infected animals. If treatment is the choice of action, who will pay for it in developing countries? Which organizations will monitor the treatments, and how to organize a public veterinary service for the care of dogs that belong to low-income people? All those issues need to be discussed. For over 10,000 years the dog has been considered man’s best friend. Therefore, it would seem inconceivable for society to kill its best friend. Avoiding the unnecessary death of animals is one of the most popular goals of today’s affluent Brazilian society.

**Conclusions**

Our results support that cVL is endemic in the extreme west of the state of Paraná, in areas bordering Argentina and Paraguay. For this reason, border surveillance systems are the key to avoiding this silent disease. The high prevalence of cVL in dogs (23.8%) and the widespread dispersion in FI and the next city (STI) indicates that cVL is endemic to the west area. It is also possible to estimate that in FI approximately 13,085 dogs may be infected with *L. infantum* out of a total of 54,983. This suggests a worrying scenario to the establishment of human visceral leishmaniasis and the spread of the disease by dogs to other areas of this region.

**Acknowledgments**

The authors would like to thank the Secretariats of Health of the state of Paraná; municipality of Foz do Iguacú and Santa Terezinha de Itaipu for the infra-structure provided. The authors are grateful to the Ministry of Health for providing the TRDPP® test. The authors would also
like to thank the staff of the Zoonosis control centre of Foz do Iguaçu for their cooperation, logistical support, and special dedication to this work.

**Author Contributions**

**Conceptualization:** Vanete Thomaz Soccol, Eliane Maria Pozzolo, Ivana Lucia Belmonte, Alceu Bisetto-Junior.

**Formal analysis:** Rafael Antunes Baggio, Ricardo Peterle, Otacilio Lopes de Souza Paz.

**Funding acquisition:** Vanete Thomaz Soccol.

**Investigation:** Vanete Thomaz Soccol, Aline Kuhn Sbruzzi Pasquali, Eliane Maria Pozzolo, André de Souza Leandro, Luciana Chiyo, Rafael Antunes Baggio, Mario Sergio Michaliszyn, Carlos Silva, Patrícia Hoerner Cubas.

**Project administration:** Vanete Thomaz Soccol.

**Resources:** André de Souza Leandro.

**Supervision:** Vanete Thomaz Soccol, Mario Sergio Michaliszyn.

**Validation:** Aline Kuhn Sbruzzi Pasquali.

**Writing – original draft:** Vanete Thomaz Soccol.

**Writing – review & editing:** Vanete Thomaz Soccol, Rafael Antunes Baggio.

**References**

1. World Health Organization. Leishmaniasis in high-burden countries: an epidemiological update based on data reported in 2014. No. 22, 2016; 91: 285–296. Available from: http://www.who.int/neglected_diseases/resources/who_ward22/en/

2. Pan American Health Organization: Epidemiological Report of the Americas. Leishmaniasis: Washington; 2017. Available from: http://www.paho.org/hq/index.php?option=com_topics&view=article&id=29&Itemid=40754.

3. Bevilacqua PD, Paixão HH, Modena CM, Castro MCPS. Urbanization of visceral leishmaniose in Belo Horizonte, Brazil. Arq Bras Med Vet Zootec. 2001; 53: 1–8 (in Portuguese).

4. Maia-Elkhoury ANS, Alves WA, Sousa-Gomes ML, Sena JM, Luna EA. Visceral leishmaniasis in Brazil: trends and challenges. Cad Saúde Pública. 2008; 24: 2941–2947. PMID: 19082286

5. Cerbino-Neto J, Wernerck GL, Costa CHN. Factors associated with the incidence of urban visceral: an ecological study in Teresina, Piauí State, Brazil. Cad Saúde Pública. 2009; 25: 1543–1551. PMID: 19578575

6. Brazil. Guide to Health Surveillance. 2016. Available from: http://portal.saude.gov.br/portal/arquivos/pdf/no10_n02_sit_epidemiol_zoonoses_br.pdf (in Portuguese).

7. Silva DA, Madeira MF, Figueiredo FB. Geographical expansion of canine visceral in Rio de Janeiro State, Brazil. Rev Inst Med Trop. 2015; 57(5): 435–438.

8. Teixeira MC, Freitas TD, Bisol J, Rocha AC, Tartarotti AL, Ramos R, et al. Occurrence of seropositivity for Leishmania infantum chagasi in dogs from built-up areas of the city of Porto Alegre, Rio Grande do Sul–Brazil. Fifth World Congress on Leishmaniosia, 2013; Porto de Galinhas. 2013. p. 854.

9. Steindel M, Menin A, Evangelista T, Stoco PH, Marlow MA, Fleith RC, et al. Outbreak of autochthonous canine visceral in Santa Catarina, Brazil. Pesq Vet Bras. 2013; 33: 490–496.

10. Maziero N, Thomaz-Soccol V, Steindel M, Link JS, Rossini D, Alban SM, et al. Rural-urban focus of canine visceral leishmaniasis in the far western region of Santa Catarina State, Brazil. Vet Parasitol. 2014; 206: 92–95. https://doi.org/10.1016/j.vetpar.2014.06.005 PMID: 25023635

11. Salomon OD, Sinagra A, Nevot MC, Barberian G, Paulin P, Estevez JO, et al. First visceral leishmaniasis focus in Argentina. Mem Inst Oswaldo Cruz. 2008; 103: 109–111. PMID: 18368242

12. Salomon OD, Quintana MG, Bruno MR, Quiriconi RV, Cabral V. Visceral leishmaniasis in border areas: clustered distribution of phlebotomine sand flies in Corinoida, Argentina. Mem Inst Oswaldo Cruz. 2009; 104: 801–804. PMID: 19620846
13. Salomon OD, Basmajian Y, Fernandez MS, Santini MS. *Lutzomyia longipalpis* in Uruguay: the first report and the potential of visceral leishmaniasis transmission. Mem Inst Oswaldo Cruz. 2011; 106: 381–382. PMID: 21655832

14. Silva AM, Camargo NJ, Santos DR, Massafera R, Ferreira AC, Postai C, et al. Diversity, distribution and abundance of sand flies (Diptera: Psychodidae) in Paraná State, Southern Brazil. Neotrop Entomol. 2008; 37: 209–225. (in Portuguese). PMID: 18506303

15. Thomaz-Soccol V, Castro EA, Navarro IT, Farias MR, Souza LM, Carvalho Y, et al. Allochthonous cases of canine visceral leishmaniasis in Paraná, Brazil: epidemiological implication. Rev Bras Parasitol Vet. 2009; 18(3): 46–51 (in Portuguese). PMID: 19772775

16. Santos DR, Ferreira AC, Bisetto-Junior A. The first record of *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) in the State of Paraná, Brazil. Rev Soc Bras Med Trop. 2012; 45: 643–645. PMID: 23152351

17. Thomaz-Soccol V, Luz E, Bisetto-Junior A, Castro EA, Ferreira-Costa ES, Navarro I, et al. Visceral and cutaneous in the Paraná State, Southern of Brazil border with Argentina and Paraguay. Fifth World Congress on Leishmaniosis, 2013; Porto de Galinhas, Brazil. Porto Galinhas: Rev Soc Bras Med Trop; 2013. p. 76–77.

18. Dias RCF, Thomaz-Soccol V, Bisetto-Júnior A, Pozzolo EM, Chiyio L, Freire RL, et al. Occurrence of anti-Leishmania spp. antibodies in domiciled dogs from the city of Foz do Iguaçu, state of Paraná, Brazil. Fifth World Congress on Leishmaniosis, 2013; Porto de Galinhas, Brazil. Porto Galinhas: Rev Soc Brasil Med Trop; 2013. p. 875–876.

19. Trench FJP, Ritt AG, Gewehr TA, de Souza Leandro A, Chiyo L, Gewehr MR, et al. First report of autochthonous visceral leishmaniasis in humans in Foz do Iguaçu, Paraná State, Southern Brazil. Ann Clin Cytol Pathol 2016; 2(6): 1041.

20. Brazilian Institute of Geography and Statistics (IBGE). Estatísticas sobre Foz do Iguaçu. Available from: <http://www.cidades.ibge.gov.br/painel/painel.php?lang=&codmun=410830&search=parana%7Cfoz-do-iguacu%7Cinfograficos:-dados-gerais-domunicipio>: Accessed on 23/12/2015 (in Portuguese).

21. Santini MS, Utges ME, Berrozpe P, Acosta MM, Casas N, Heuer P, et al. *Lutzomyia longipalpis* presence and abundance distribution at different microspatial scales in an urban scenario. PLoS Negl Trop Dis. 2015; 9(8): A037, 16.

22. Cruz I, Acosta L, Gutiérrez MN, Nieto J, Cañavate C, Deschutter J, et al. Canine pilot survey in an emerging focus of visceral: Posadas (Misiones, Argentina). BMC Infect Dis. 2010; 10: 342. https://doi.org/10.1186/1471-2334-10-342 PMID: 21122107

23. Berrozpe P, Lamattina D, Santini MS, Araujo AV, Utgés ME, Salomón OD. Environmental suitability for *Lutzomyia longipalpis* in a subtropical city with a recently established visceral leishmaniasis transmission cycle, Argentina. Mem Inst Oswaldo Cruz. 2017; 112(10):674–680. https://doi.org/10.1590/0074-02760170056 PMID: 28953995

24. Hengl T. Finding the right pixel size. Comput Geosci. 2006; 32: 1283–1298.

25. Brazil—CGDT, CGLAB, DEVIT, SV. Technical Note joint N˚ 01/2011 “Enlightenment on replacement of the diagnostic protocol of canine visceral”. 2011.

26. Brazil—CGDT/DEVIT/SV/MS. Informative Note no. 29 de 2015. 2015.

27. Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schalling HD, Presber W, et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infec Dis. 2003; 47(1): 349–358.

28. Oshaghi MA, Chavshin AR, Vatandoost H, Yaaghoo bi F, Mohtarami F, Noorjah N. Effects of postingestion and physical conditions on PCR amplification of host blood meal DNA in mosquitoes. Exp Parasitol. 2006; 112: 232–236. https://doi.org/10.1016/j.exppara.2005.11.008 PMID: 16364301

29. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30(4): 772–780. https://doi.org/10.1093/molbev/ms310 PMID: 23296990

30. Landan G, Graur D. Local reliability measures from sets of co-optimal multiple sequence alignments. Pacific Symp Biocomp. 2008; 13(15): 15–24.

31. Penn O, Privman E, Ashkenazy H, Landan G, Graur D, Pupko T. Guidance: a web server for assessing alignment confidence scores. Nucleic Acids Res. 2010; 38: 23–28.

32. Sela I, Ashkenazy H, Katoh K, Pupko T. Guidance2: accurate detection of unreliable alignment regions accounting for the uncertainty of multiple parameters. Nucleic Acids Res. 2015; 1(43): 7–14.

33. Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016; 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054 PMID: 27004904
Recognizing cLV in state of Paraná, Brazil

34. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980; 16(2): 111–120. PMID: 7463489
35. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012; 9(8): 772.
36. Dean JA, Coulombier D, Grendel KA, Armer TG, Dean AG. Epi-info, Version 7.1.5.2 Atlanta: CDC. 1994. Available from: https://www.cdc.gov/epiinfo/pc.html
37. R Development Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from: https://cran.r-project.org/bin/windows/base/.
38. Sanchez G, Trinchera L, Russolligo G. Pspm: Tools for partial least squares path modeling (PLS-PM). R package version 0.4.7. Available from: https://CRAN.R-project.org/package=plspm. 2015
39. Benjamini Y, Yekutieli D. The control of false discovery rate under dependency. Ann Stat. 2001; 29: 1165–1188.
40. Franco AO, Davies CR, Mylne A, Dedet JP, Gallego M, Ballart C, et al. Predicting the distribution of canine leishmaniasis in western Europe based on environmental variables. Parasitol. 2011; 138: 1878–1891.
41. Martín-Sánchez J, Morales-Yuste M, Acedo-Sánchez C, Barón S, Díaz V, Morillas-Márquez F. Canine in Southeastern Spain. Emerg Infect Dis. 2009; 15(5): 795–798. https://doi.org/10.3201/eid1505.080968 PMID: 19402973
42. Gálvez R, Dascalzo MA, Guerrero I, Miró G, Molina R. Mapping the current distribution and predicted spread of the leishmaniasis sand fly vector in the Madrid region (Spain) based on environmental variables and expected climate change. Vector Borne Zoonotic Dis. 2011; 11(7): 795–806. https://doi.org/10.1089/vbz.2010.0109 PMID: 21417927
43. Brazil—Health Ministry. Manual of surveillance and control of visceral leishmaniasis. Brasília: 2006. Available from: http://bvms.saude.gov.br/bvs/publicacoes/manual_vigilancia_controle_leishmaniase_visceral_1edicao.pdf. (in Portuguese).
44. Cardim MFM, Rodas LAC, Dibo MR, Guirado MM, Oliveira AM, Chiaraavalloti-Neto F. Introduction and expansion of human American visceral leishmaniasis in the state of Sao Paulo, Brazil, 1999–2011. Rev Saúde Pública. 2013; 47: 691–700. https://doi.org/10.1590/S0034-8910.2013047004454 PMID: 24346660
45. Barata RA, Peixoto JC, Tanure A, Gomes ME, Apolinário EC, Bodovan E, et al. Epidemiology of visceral in a remerging focus of intense transmission in Minas Gerais State, Brazil. BioMed Res Int. 2013; ID405083.
46. Teixeira MC, Freitas TD, Bisol J, Rocha AC, Tartarotti AL. Occurrence of seropositivity for Leishmania infantum chagasi in dogs from built-up areas of the city of Porto Alegre, Rio Grande do Sul–Brazil. Fifth World Congress on Leishmaniasis, 2013; Porto de Galinhas, Brazil. Porto Galinhas: Rev Soc Brasil Med Trop. 2013. p.854.
47. Peixoto HM, Oliveira MRF, Romero GAS. Serological diagnosis of canine visceral in Brazil: systematic review and meta-analysis. Trop Med Internat Health. 2015; 20 (3): 334–352.
48. Felipe IM, Aquino DM, Kuppinger O, Santos MD, Rangel ME, Barbosa D, et al. Leishmania infection in humans, dogs and sandflies in a visceral endemic area in Maranhão, Brazil. Mem Inst Oswaldo Cruz. 2011; 106: 207–211. PMID: 21537682
49. Canese J. Major increase in cases of visceral leishmaniasis in humans in Paraguay. Pediatr (Asunción). 2010; 37: 167–168 (in Spanish).
50. Quinnell RJ, Carson C, Reithinger R, Garcez LM, Courtenay O. Evaluation of rK39 rapid diagnostic tests for canine visceral leishmaniasis: longitudinal study and meta-analysis. PLoS Negl Trop Dis. 2013; 7(1): e1992. https://doi.org/10.1371/journal.pntd.0001992 PMID: 23326615
51. Menke N. Future challenges for parasitology: vector control and ‘one health’ in Europe: the veterinary medicinal view on CVBDS such as tick borellosis, rickettiosis and canine leishmaniasis. Vet Parasitol. 2013; 195: 256–271. https://doi.org/10.1016/j.vetpar.2013.04.007 PMID: 23680539
52. Grimaldi G Jr, Teva A, Ferreira AL, dos Santos CB, Pinto IS, de-Azevedo CT, et al. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2012; 106: 54–59. https://doi.org/10.1016/j.trstmh.2011.10.001 PMID: 22137538
53. Guimarães VCFV, Puzinova K, Sadlova J, Volfova V, Myskova J, Brandão Filho SP, et al. Lutzomyia migonei is a permissive vector competent for Leishmania infantum. Parasit Vectors. 2016; 9(1): 159.
54. Solano-Gallego L, Morell P, Arboix M, Alberola J, Ferrer L. Prevalence of Leishmania infantum infection in dogs living in an area of canine endemicity using PCR on several tissues and serology. J Clin Microbiol. 2001; 39: 560–563. https://doi.org/10.1128/JCM.39.2.560-563.2001 PMID: 11158106
55. Alvar J, Cañavate C, Molina R, Moreno J, Nieto J. Canine leishmaniasis. Adv Parasitol. 2004; 57: 1–88. https://doi.org/10.1016/S0065-308X(04)57001-X PMID: 15504537

56. Pérez-Cutilias P, Goyena E, Chitimia L, De la Rúa P, Bernal LJ, Fisa R, et al. Spatial distribution of human asymptomatic Leishmania infantum infection in southeast Spain: a study of environmental, demographic and social risk factors. Acta Trop. 2015; 146: 127–134. https://doi.org/10.1016/j.actatropica.2015.03.017 PMID: 25800329

57. Quinnell RJ, Courtenay O, Davidson S, Garcez L, Lambson B, Ramos P, et al. Detection of Leishmania infantum by PCR, serology and cellular immune response in a cohort study of Brazilian dogs. Parasitol. 2001; 122: 253–261.

58. Boggiatto PM, Gibson-Corley KN, Metz K, Gallup JM, Hostetter JM, Mullin K, et al. Transplacental Transmission of Leishmania infantum as a means for continued disease incidence in North America. PLoS Negl Trop Dis. 2011; 5(4): e1019. https://doi.org/10.1371/journal.pntd.0001019 PMID: 21532741

59. Dantas-Torres F. Ticks as vectors of Leishmania parasites. Trends Parasitol. 2011; 27: 155–159. https://doi.org/10.1016/j.pt.2010.12.006 PMID: 21227752

60. Paz GF, Reis IA, Avelar DM, Ferreira ECM, Werneck GL. Ectoparasites and anti-Leishmania antibodies: association in an observational case-control study of dogs from a Brazilian endemic area. Prev Vet Med. 2013; 112: 156–159. https://doi.org/10.1016/j.prevetmed.2013.07.012 PMID: 23932895

61. Afonso MMS, Gomes AC, Meneses CRV, Rangel EF. Studies on the feeding habits of Lutzomyia (L.) longipalpis (Lutz & Neiva, 1912) (Diptera:Psychodidae: Phlebotominae) populations from endemic areas of American leishmaniasis in northeastern Brazil. J Trop Med. 2012; 858657.

62. Soares BR, Souza AP, Prates DB, de Oliveira CI, Barral-Netto M, Miranda JC, et al. Serocconversion of sentinel chickens as a biomarker for monitoring exposure to visceral leishmaniasis. Sci Rep. 2013; 3:2352. https://doi.org/10.1038/srep02352 PMID: 23912591

63. Casanova C, Andrighetti MT, Sampaio SM, Marcoris ML, Colla-Jacques FE, Prado AP. Larval breeding sites of Lutzomyia longipalpis (Diptera:Psychodidae) in visceral leishmaniasis endemic urban areas in southeastern Brazil. PLoS Negl Trop Dis. 2013; 7: e2443. https://doi.org/10.1371/journal.pntd.0002443 PMID: 24069494

64. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniasis–new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 2008; 24(7): 324–330. https://doi.org/10.1016/j.pt.2008.04.001 PMID: 18514028

65. Friedmann E, Son H. The human-companion animal bond: how humans benefit. Vet Clin North Am Small Anim Pract. 2009; 39(2): 293–326. https://doi.org/10.1016/j.cvsm.2008.10.015 PMID: 19185195