zoonotic diseases

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Brief Report

Canine Leishmaniasis in Southern Brazil: Diagnosis and Clinical Features in Domestic Dogs

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Simple Summary: Leishmaniasis is a human and animal disease caused by the protozoan parasites *Leishmania* spp. Visceral leishmaniasis is a very serious form of this disease and occurs only through infection by the species *Leishmania infantum*. Furthermore, it is a zoonotic disease transmitted by vectors, with a complex transmission cycle involving numerous reservoirs of domestic and wild animals, such as dogs, cats, and foxes. *L. infantum* detection in animals is necessary to prevent outbreaks of canine and human visceral leishmaniasis (CanL/HVL). Increasing knowledge of diagnostic techniques and the main clinical manifestations of CanL is also essential to diagnose and treat dogs with this disease.

Abstract: *Leishmania infantum* is a hemopathogen of importance for the health of domestic dogs (*Canis lupus familiaris*), causing canine leishmaniasis (CanL), and it is also the etiological agent of human visceral leishmaniasis (HVL). This parasite was not reported in southern Brazil until the early 2000s, but CanL and HVL were increasingly reported in the last 15 years, mainly in cities bordering Argentina. The present study aimed to detect *L. infantum* in domestic dogs and to determine the main clinical manifestations in infected animals from Uruguaiana, a city with a high incidence of CanL. Fifty-one dogs suspected of having CanL in the urban perimeter of the city were clinically examined by veterinarians and investigated for the occurrence of *L. infantum* with two immunoassays (rapid chromatography test and ELISA) and real-time PCR (polymerase chain reaction). Clinical signs were compared in positive and negative *L. infantum* animals. A total of 31 dogs (60.8%) were infected with *L. infantum*. The main clinical manifestations associated with CanL dogs were onychogryphosis and peeling ($p < 0.05$). *L. infantum* was frequently detected in urban dogs from Uruguaiana, highlighting the concerning situation regarding health in this city. The occurrence of some clinical signs (onychogryphosis/peeling) could help to detect CanL more frequently in the canine population.

Keywords: *Leishmania infantum*; dogs; parasites

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1. Introduction

Hemopathogens are relatively frequent in dogs (*Canis lupus familiaris*), causing different infectious diseases with variable clinical signs and outcomes. Many hemopathogens are protozoa transmitted by vectors (fleas, ticks, and insects). Among them, *Leishmania*

infantum (Kinetoplastida: Trypanosomatidae) is an important hemopathogenic protozoan that causes canine leishmaniasis (CanL). Infected dogs are reservoirs of *L. infantum* that can be transmitted to humans and cause visceral leishmaniasis (HVL, human visceral leishmaniasis) [1,2].

The transmission of this parasite occurs through the sandfly *Lutzomyia longipalpis*, which is endemic in central and northern parts of Brazil, while it occurs sporadically/focally in the south [3]. Until the early 2000s, few reports of CanL and HVL in southern Brazil were considered imported cases due to the absence of the leishmaniasis vector *Lu. Longipalpis*. The first CanL autochthonous case was reported in São Borja, a city bordering the province of Corrientes, Argentina, in 2008 [4]. Other studies detected CanL in geographical regions of Argentina bordering Brazil as well as in cities from the Brazilian southernmost states (Rio Grande do Sul and Santa Catarina) a few years later [5–8]. The recent widespread dispersion of *Lu. longipalpis* in south Brazil was attributed to its great capacity to adapt to different ecological niches, highlighting urban environments [3]. More recent studies are also raising the hypothesis that other insects could be vectoring *L. infantum* in Brazil [9,10].

As a result of the higher density of dogs and humans, the zoonotic role of dogs with CanL is far more concerning in urban areas. Amastigotes from this protozoon present in the skin of infected dogs can be transmitted first to sandflies vectors and then to humans [11,12]. In many Brazilian cities, CanL outbreaks have been reported to precede the onset of HVL cases [13,14].

CanL can range from a total absence of symptoms to a severe clinical syndrome [15]. The most frequent clinical manifestations are cutaneous (alopecia, dermatitis, onychogryphosis) and may be seen along with other clinical symptoms or pathological abnormalities. CanL signs unrelated to cutaneous lesions include ocular alterations, epistaxis, lameness, and vascular and neurological disorders [16]. In the final stage of the disease, many organs are affected, most animals present cachexia, and death is the result of renal or hepatic failure [15].

L. infantum infection has been routinely diagnosed using serological tests recommended by the Brazilian Ministry of Health in the Visceral Leishmaniasis Control Program (PCLV, *Programa de Controle da Leishmaniose Visceral*) [17]. Currently, the complete diagnosis procedure includes a rapid chromatographic immunoassay (dual-path platform—DPP®) as a screening test, and the enzyme-linked immunosorbent assay (ELISA) is used as a confirmatory test. As serological tests have been questioned, especially regarding accuracy, PCR-based assays have also been included for a more comprehensive diagnosis [18].

Several CanL autochthonous cases have been detected in Uruguaiana, a city located on the border with Argentina in the southernmost Brazilian state (Rio Grande do Sul) in the last ten years. The aims of this study were to report the emergence of CanL in the urban area of the city, as well as to investigate the main clinical signs of this disease in infected dogs.

2. Materials and Methods

2.1. Study Area

The study was conducted in the urban perimeter of Uruguaiana (latitude −29.7495, longitude −57.0882, 29°44′58″ south, 57°5′18″ west), Rio Grande do Sul state, south Brazil. It is a city located in the province of Corrientes close to the Brazil/Argentina border (Figure 1). The municipality occupies an area of 5702.1 km^2 with a total population of 117,210 inhabitants (approximately 94% living in the urban area) according to the latest estimate by the Brazilian Institute of Geography and Statistic [19].

2.2. Animal Sampling

The population of this study was a convenience sample from a total of approximately 500 dogs (owned and free-ranged, with no record of previous anti-*Leishmania* immunization) living in the urban area of the city of Uruguaiana (Rio Grande do Sul, Brazil) and who were treated by veterinarians between August 2019 and February 2020. Demographic and

general information were also provided by the pet's tutors, answering an epidemiological survey form.

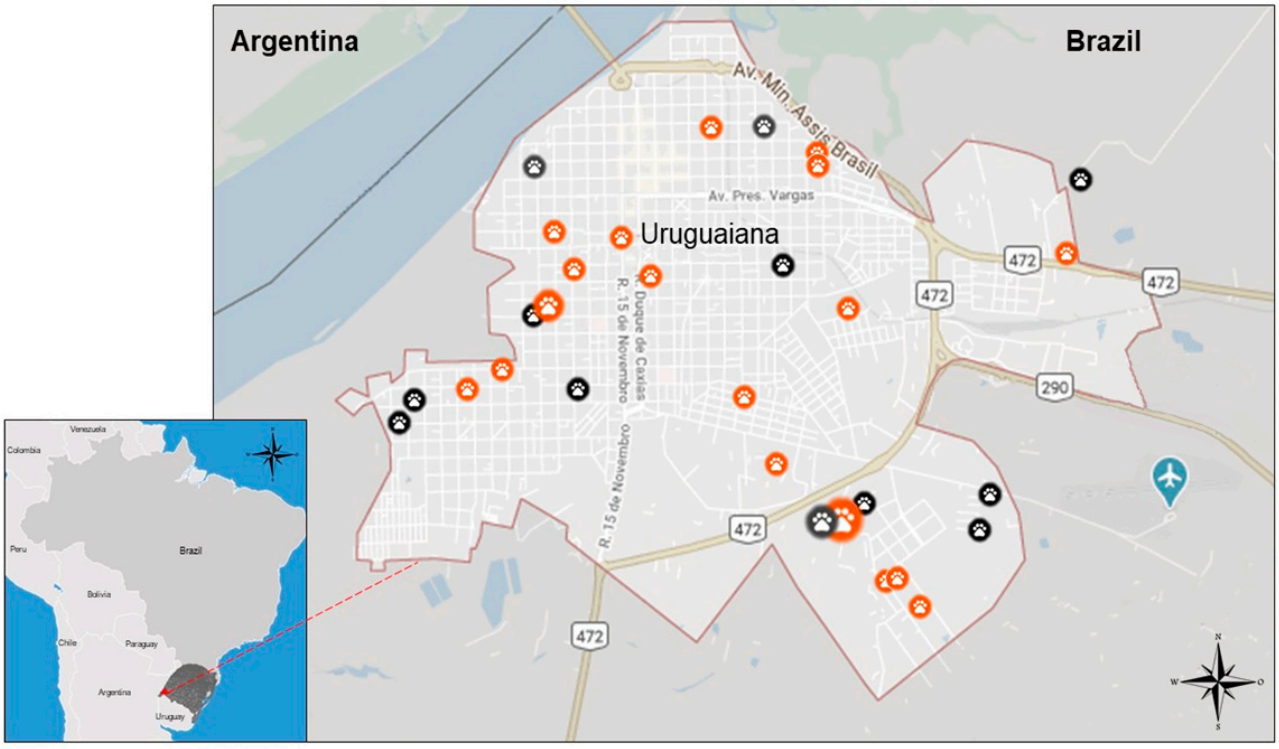

Figure 1. Map of the sample collection site in the city of Uruguaiana (orange dots: positive dogs; black dots: negative dogs).

The whole study was approved by the Ethics Committee on the Use of Animals (CEUA) of the Lutheran University of Brazil (CEUA-ULBRA nº 2017/355).

2.3. Laboratory Analysis

Blood samples from the 51 selected dogs were collected by venipuncture and stored in two aliquots of around 1 mL, one in a tube without anticoagulant (serum) for the immunological tests and another in a sterile tube containing EDTA (whole blood) for real-time PCR (qPCR). The tubes for the immunological tests were kept refrigerated until processing, and the others (for DNA analyses) were stored at −20 °C until the molecular detection procedure.

The rapid screening test Immunochromatographic TR DPP® (DPP) was performed according to the manufacturer's instructions (Biomanguinhos-FIOCRUZ, Rio de Janeiro, Brazil). The DPP test consists of a device impregnated with recombinant antigen rK28 (a chimaera combining antigens K9, K26, and K39) to detect the presence of IgG antibodies recognizing these specific *L. infantum* antigens. Positive samples in the DPP test were sent to a confirmatory ELISA assay (Biomanguinhos-FIOCRUZ, Rio de Janeiro, Brazil) performed at a reference laboratory (Laboratorio Central do Rio Grande do Sul/LACEN-RS, Porto Alegre, RS, Brazil).

The qPCR detection of *L. infantum* was carried out with commercial reagents. DNA was extracted by silica adsorption methodology using the NewGene® Prep and PreAmp commercial reagents according to the manufacturer's instructions (Simbios Biotecnologia, Cachoeirinha, RS, Brazil). *L. infantum* DNA was specifically detected by qPCR with LV-CAmp NewGene® reagents (Simbios Biotecnologia, Cachoeirinha, RS, Brazil). Control samples were used in all PCR assays, including water and DNAs extracted from healthy dogs (negative controls), as well as synthetic DNA fragments with oligonucleotide sequences hybridizing to the LVCAmp-specific target gene (gBlock, IDT, Coralville, IA, USA)

and DNAs extracted from dogs with CanL (positive controls). All reactions were performed on the StepOnePlus™ real-time PCR system (Applied Biosystems, Palo Alto, CA, USA) with the following amplification conditions: a cycle of 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Negative and positive DNA *L. infantum* controls were used in all runs. A standard curve with 10-fold dilutions of the DNA-positive control was carried out in some analyses to estimate the parasitemia. The positive result values were expressed in cycle thresholds (Cts) and parasites/mL.

2.4. Statistical Analysis

All data analysis was conducted with IBM SPSS (version 23.0) and RStudio (version 4.2.3 version). The demographic data and clinical signs were represented by their frequencies (absolute and relative). For continuous data, normality was assessed using the Anderson–Darling test. Bivariate analyzes were performed to evaluate the association between categorical variables and the outcome. Absolute and relative frequencies were estimated for categorical data using Pearson's chi-square test or Fisher's exact test. The pre-established significance level for the 5% alpha error, bilateral, and $p < 0.05$ were considered significant.

3. Results

3.1. Overall CanL Prevalence and Epidemiological Data

A total of 51 dogs, 10.2% of the approximately 500 dogs attended by veterinarians, were considered suspect to have CanL in the clinical exam in the period of the study. These suspect CanL-positive dogs were obtained in several locations in the city (Figure 1). Laboratorial tests demonstrated that 31 out of the 51 dogs were positive for *L. infantum* in at least one laboratorial detection method. Among them, a total of 22 (43.1%) dogs were positive in both rapid DPP and qPCR methods, though 6 (11.8%) were positive only in the DPP test and 3 (5.9%) were only positive in the qPCR test (Table 1). All six positive samples in the DPP test were also positive in the confirmatory ELISA. *L. infantum* parasitemia was evaluated in the 25 (49.0%) qPCR-positive samples, ranging from 3 to 52,700 parasites/mL of blood.

Table 1. Comparative analysis of IC rapid test and qPCR.

	DPP+	**DPP−**
qPCR+	22 (43.1%)	3 (5.9%)
qPCR−	6 (11.8%)	20 (39.2%)

The general epidemiological data demonstrated the occurrence of males (n = 29; 56.9%) and females (n = 22; 43.1%), pure (n = 9; 17.6%) and mixed (n = 41; 80.4%) breeds, and young (n = 23; 45.1%), adult (n = 15; 29.4%), and elderly (n = 11; 21.6%) dogs in all the examined animals. Among the 31 animals positive for CanL, 18 (58.1%) were males and 13 (41.9%) were females. In addition, 12 (38.7%) were puppies (0–2 years old), 11 (35.5%) were adults (3–5 years old), and 6 (19.4%) were elderly dogs (>6 years old). Further, animals with short fur and that live with other dogs represented 90.3% (28) of those positive for CanL (Table 2).

3.2. Clinical Signs

Different clinical signs were observed in the dogs, including lymphadenopathy (n = 26), dull hair coat (n = 26), alopecia (n = 22), desquamation (n = 20), eye lesion (n = 20), loss (n = 17), onychogryphosis (n = 16), muscle atrophy (n = 10), hyperkeratosis (n = 10), skin ulcer (n = 10), apathy (n = 6), hepatomegaly (n = 5), and diarrhea (n = 2). Among the clinical signs commonly observed in dogs with visceral leishmaniasis, desquamation and onychogryphosis were frequently observed (in 64.5% and 51.6% of positive animals, respectively), with a p value < 0.05 (Table 3).

Table 2. General demographic characteristics of the animals according to whether they were positive or negative for *L. infantum*.

Variables	Positive (n = 31)		Negative (n = 20)		OR (95% Cl)	*p*-Value
	n	%	n	%		
Age						
0–2 years	12	38.7	11	55.0	0.91 (0.22–3.84)	0.897 [a]
3–5 years	11	35.5	4	20.0	2.30 (0.44–11.91)	0.324 [a]
6 or more years	6	19.4	5	25.0	1.Ref	
Missing	2	6.4	0	0		
Sex						
Males	18	58.1	11	55.0	1.13 (0.36–3.52)	0.082
Females	13	41.9	9	45.0	1.Ref	
Breed						
Mixed bred	27	87.1	14	70.0	2.41 (0.55–10.42)	0.231 [a]
Purebred	4	12.9	5	25.0	1.Ref	
Missing	0	0	1	5.0		
Living with more dogs						
Yes	28	90.3	17	85.0	1.65 (0.30–9.10)	0.565 [a]
No	3	9.7	3	15.0	1.Ref	
Hair						
Short	28	90.3	17	85.0	1.65 (0.30–9.10)	0.565 [a]
Long	3	9.7	3	15	1.Ref	

[a] Fisher's exact test; Ref. Reference category; OR. Odds ratio.

Table 3. Clinical variables according to cases and controls dogs, respectively.

Variables	Cases (n = 31)		Controls (n = 20)		OR (95% Cl)	*p*-Value
	n	%	n	%		
Apathy						
Yes	6	19.4	8	40	0.36 (0.10–1.27)	0.107
No	25	80.6	12	60	1.Ref	
Emaciation						
Yes	17	54.8	14	70	0.52 (0.16–1.70)	0.279
No	14	45.2	6	30	1.Ref	
Muscular Atrophy						
Yes	10	32.3	8	40	0.72 (0.22–2.30)	0.572
No	21	67.7	12	60	1.Ref	
Opaque hair						
Yes	26	83.9	14	70	0.23 (0.58–8.62)	0.245 [a]
No	5	16.1	6	30	1.Ref	
Alopecia						
Yes	22	71	10	50	2.44 (0.76–7.88)	0.131
No	9	29	10	50	1.Ref	
Peeling						
Yes	20	64.5	7	35	3.37 (1.04–10.95)	0.039
No	11	35.5	13	65	1.Ref	
Hyperkeratosis						
Yes	10	32.3	5	25	1.43 (0.40–5.04)	0.579 [a]
No	21	67.7	15	75	1.Ref	
Skin Ulcers						
Yes	10	32.3	10	50	0.48 (0.15–1.51)	0.205
No	21	67.7	10	50	1.Ref	
Ocular lesions						
Yes	20	64.5	12	60	1.21 (0.38–3.86)	0.745
No	11	35.5	8	40	1.Ref	
Onychogryphosis						
Yes	16	51.6	4	20	4.27 (1.16–15.69)	0.024 [a]
No	15	48.4	16	80	1.Ref	
Hepatosplenomegaly						
Yes	5	16.1	2	10	1.73 (0.30–9.92)	0.535 [a]
No	26	83.9	18	90	1.Ref	

Table 3. *Cont.*

Variables	Cases (n = 31)		Controls (n = 20)		OR (95% Cl)	*p*-Value
	n	%	n	%		
Lymphadenopathy						
Yes	26	83.9	17	85	0.92 (0.19–4.35)	0.914 [a]
No	5	16.1	3	15	1.Ref	
Epistaxis						
Yes	0	0	1	5	0.20 (0.1–5.32)	0.209 [a]
No	31	100	19	95	1.Ref	
Diarrhea						
Yes	2	6.5	2	10	0.62 (0.08–4.80)	0.645 [a]
No	29	93.5	18	90	1.Ref	
Limb paresis						
Yes	0	0	1	5	0.20 (0.01–5.32)	0.209 [a]
No	31	100	19	95	1.Ref	

[a] Fisher's exact test; Ref. Reference category; OR. Odds ratio.

4. Discussion

Visceral leishmaniasis is considered a neglected disease endemic in many Brazilian regions. This situation is still the subject of discussion in southern Brazil, as *L. infantum* has not yet been detected in several cities. In the urban perimeters of certain cities, dogs are the main reservoirs of the etiological protozoan *L. infantum*. Furthermore, these animals can present CanL and play an important role in the epidemiology of this zoonosis which is very concerning to health [13].

In the present study, 51 dogs were suspected of having CanL in a clinical exam by veterinarians in the period of seven months in Uruguaiana, a city bordering Argentina. The convenience sampling obtained here presented different genders, breeds, and ages of dogs, and it also included animals living in different neighborhoods in the urban perimeter of the city. A total of 31 (60.8%) of the 51 animals with clinical manifestations were confirmed to be positive for *L. infantum*. If we consider only these animals as positive, we would have a prevalence of approximately 6.2% (31/500) in the total domestic dogs population. But there are probably many other positive animals that did not show clinical signs; therefore, the prevalence of dogs with present or past infection with *L. infantum* must be even higher. In a previous survey including 965 domestic dogs from this city, 4.5% of the animal samples presented antibodies against *L. infantum* between 2009 and 2010 [20]. In view of this concerning epidemiological situation with an increasing number of dogs with CanL, other studies have detected more animal species with *L. infantum*, including wild and domestic mammals [21,22].

The present study also highlights the importance of using different *L. infantum* detection methods in diagnosing CanL in domestic dogs. Indirect immunological methods evaluate the occurrence of antibodies (mainly IgG) against this parasite in animal body fluids, while direct tests detect *L. infantum* nucleic acids (mainly DNA). The use of these different assays (immunological and molecular) is still not officially recommended, but this rule must be revised. A more accurate diagnosis could be reached by evaluating the antigenic response (detecting antibodies with rapid test and/or ELISA) and the presence of the parasite by PCR [17]. Noteworthy, the whole diagnostic performance is influenced by the time of infection, sample collection, vaccination status, immunosuppressing diseases, and other factors. Therefore, the use of two or more methods, including PCR, would be very welcome for a definitive diagnostic [18,23]. In addition, qPCR is a useful method to report the parasites load. This information has been proved to be necessary to monitor dogs with CanL, as well as to evaluate the response to medicaments [24–26].

Also, the clinical exam is very important to identify CanL in the dogs in an endemic region. The main clinical signs of the infected animals observed here were enlarged lymph nodes (82.4%) and dull coat (64.7%), but weight loss and skin diseases were also important clinical manifestations. Skin lesions were detected mainly in the ears (where there is less hair), being preferable for mosquitoes [15,16]. Skin peeling and onychogryphosis were, respectively, observed 3.4 and 4.3 times more often in dogs with leishmaniasis than in unin-

fected animals. Onychogryphosis is a clinical manifestation highly correlated with CanL, especially when associated with other cutaneous findings such as exfoliative dermatitis, as observed in this study. Although approximately half of the dogs with leishmaniasis presented onychogryphosis in this study, this clinical sign has already been described in up to 70% of dogs with this disease [27–31]. The complete pathogenesis is not completely known, but excessive nail growth has been associated with more severe parasitism [30,32,33]. The frequent observation of cutaneous findings (onychogriphosis and skin scaling) in this study highlights that the observation of these main clinical manifestations are strong indications of CanL in this endemic region. Veterinarians should pay attention to these specific clinical signs to diagnose new cases.

The results of the present study also reinforce the high prevalence of *L. infantum* in southern Brazil, on the border with Argentina. Previous diagnoses of CanL in south Brazil were preceded by cases of this same disease in nearby tropical regions of this neighboring country [34,35]. This international border region has been considered the entry point for *L. infantum* in southern Brazil, which is now spreading to other cities in the state of Rio Grande do Sul. HVL cases were already detected in the Rio Grande do Sul state in recent years [36]. Therefore, more effective prevention methods are necessary to control the dissemination of *L. infantum* to human and animals in all southernmost states from Brazil.

5. Conclusions

CanL was detected in 31 out of 51 (60.8%) domestic dogs, with the clinical signs being suggestive of this disease in the urban area of Uruguaiana city. These data demonstrate the high frequency of *L. infantum* in urban dogs from this city in recent years, reinforcing the introduction of this parasite in southern Brazil by the Argentinian border. It also highlights the importance of detecting dogs with CanL by clinical and laboratorial evaluation to prevent more widespread *L. infantum* dissemination in dogs, humans, and even other animals.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee on the Use of Animals (CEUA) of the Lutheran University of Brazil (CEUA-ULBRA nº 2017/355).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article; further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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