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Genetic and clinical characterization of canine leishmaniasis caused by *Leishmania (Leishmania) infantum* in northeastern Argentina

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ABSTRACT

Leishmaniases comprise zoonotic diseases caused by protozoan flagellates of the *Leishmania* genus. They are endemic to South America, and the visceral form has been recently reported in Argentina. Dogs can play different roles in the *Leishmania* transmission cycles, depending mainly on the species of parasite involved. Here we focused on the clinical characterization of canine leishmaniasis (CanL) in Northeast Argentina and on the molecular typing of its etiological agent. The nested polymerase chain reaction and sequence analysis of the *Leishmania* cytochrome *b (cyt* b) gene was performed on DNA templates purified from lymph nodes, bone marrow or spleen aspirates obtained from 48 dogs previously diagnosed by the observation of *Leishmania* amastigotes on smears from these aspirates. Their clinical and epidemiological data were also recorded. Systemic abnormalities were observed in 46 subjects (95.8%), most frequently lymphadenopathy, and emaciation (89.6 and 75%). Furthermore, 87% also presented tegumentary abnormalities, such as alopecia (54.2%) or secondary skin lesions (47.9%), among others. Twenty three dogs were positive for *cyt* b amplification. The sequence analysis showed the presence of two genotypes, LiA1 and LiA2, assigned to *Leishmania (Leishmania) infantum*, with 99.9 and 100% homology with the reference strain MHOM/TN/80/IPT1 respectively. LiA1 was identified in 18 cases (78.3%) and LiA2 in five (21.7%). Two *cyt* b variants of *L. (L.) infantum* were incriminated as the causative agents of CanL cases from three cities: Posadas, Garupá, and Ituzaingó. All three cities are located in the northeastern area of the country, where these parasites seem to be spreading in urban areas.

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1. Introduction

The leishmaniases are among the world's most neglected diseases. They are endemic in 98 countries, with 350 million people at risk and an estimated incidence of two million new cases per year. Mortality and morbidity from leishmaniases worldwide show a worryingly increasing trend (WHO, 2010). The diseases are caused by 20 species of protozoan flagellates of the genus *Leishmania* and comprise some clinical syndromes, including the tegumentary form and also visceral leishmaniasis (VL) which is lethal if untreated (WHO, 2004).

Members of family Canidae are also susceptible to infection by genus *Leishmania*. Thus, dogs have been found to be naturally infected with species such as *Leishmania* (*Viannia*) *peruviana, L. (Leishmania) major,* and *L. (L.) tropica* among others, in several countries (Dantas-Torres, 2007). In Argentina, *L. (V.) braziliensis* and *L. (L.) infantum* have been incriminated as the causal agents of canine leishmaniasis (CanL) in the cities of Orán and Posadas, northwest and northeast (NE) areas of the country, respectively (Cruz et al., 2010; Marco et al., 2005). The CanL clinically display a wide range of nonspecific and variable forms, affecting tissues and organs such as bone marrow, spleen, liver, lymph nodes, and the tegumentary system, where several types of alterations/lesions have been reported (Amusategui et al., 2003; Padilla et al., 2002).

In an epidemiological context, the role of the dog in the transmission cycles of *Leishmania* spp. is still in debate. It can vary from the primary reservoir as is the case of *L. (L.) infantum* in endemic areas of VL in Europe, to incidental host for *L. (V.) braziliensis* in several areas of tegumentary leishmaniasis of South America (Dantas-Torres, 2007, 2011; Marco et al., 2005).

The pleomorphic characteristics of the CanL indicate the importance of *Leishmania* genus typing in any endemic area, since the clinical presentation and the transmission cycles will mostly depend on the causal agent. Therefore, we have developed and established a DNA-based technique, the nested polymerase chain reaction (PCR) amplification and sequencing of cytochrome *b* (*cyt* b gene sequencing) aimed at the identification and typing of *Leishmania* spp. (Luyo-Acero et al., 2004). In addition, this technique has been validated against multilocus enzyme electrophoresis, the gold standard method, using *Leishmania* strains from several endemic areas of leishmaniasis around the world (Foulet et al., 2007; Marco et al., 2006a,b). This method has been successfully applied to DNA from clinical samples on Whatman FTA™ cards, suggesting that parasite isolation is not required, simplifying the sampling procedures. Moreover, the FTA technology allows easy collection, and storage the DNA from a variety of biological materials at room temperature, since it is fixed in a filter paper. It also facilitates the sample transportation while avoiding cross-contamination (Kato et al., 2010).

The present study has been focused on the characterization of CanL endemic in NE Argentina, particularly in the molecular typing of its causal agents, applied directly to biological samples from the subjects. The clinical and regional aspects of the disease are also discussed briefly.

2. Methods

2.1. Canine population and clinical examination

Forty-eight dogs with CanL and two healthy dogs were included in this descriptive study with prospective enrollment. They were recruited between 2008 and 2009, and diagnosed by veterinarians from the "Veterinaria del Oeste" clinic in the city of Posadas, Province of Misiones, NE Argentina. The subjects were examined while searching for clinical signs of CanL, such as systemic manifestations: apathy, weight loss, enlarged lymph nodes, hepatomegaly, splenomegaly, epistaxis, renal, neurological, digestive failures, and others. Tegumentary and ocular signs, such as alopecia, hypotrichosis, exfoliative dermatitis, cutaneous and mucocutaneous ulcers, crusted scars, and onychogryphosis were also recorded (Lima et al., 2014).

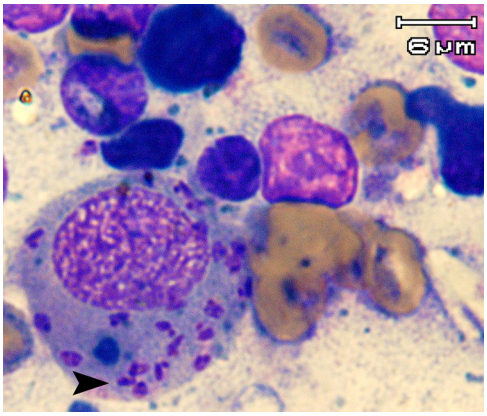

Fig. 1. Parasitological diagnosis of canine leishmaniasis. The arrow indicates intracellular amastigotes of *L. (L.) infantum* stained smears of an aspirate taken from an enlarged canine lymph node. Amplification: 1000X.

2.2. Sampling methodology and diagnosis of canine leishmaniasis

After the clinical examination, a puncture-aspiration was aseptically practiced on the dogs with 2.5 mL syringes and 21 G needles. In most of the cases the aspirates were taken from enlarged lymph nodes, specially the popliteal ones. When lymph nodes could not be found, the samples were taken from the bone marrow or the spleen. In dogs with tegumentary lesions, these were scraped with a sterile scalpel blade.

A fraction from each sample obtained by aspiration, or the scrapings were used for the parasitological diagnosis as described previously, with slight modifications (Marco et al., 2007). Briefly, a search for *Leishmania* amastigotes was done on smears of the materials, stained with Differential Quick Stain Kit N° 15 (Biopur Diagnostics, Rosario, Argentina), and examined under a light microscope at the maximum magnification, X 1000 (Fig. 1).

The remaining fraction of the aspirates was diluted with 0.3 mL of sterile saline solution and spotted onto FTA™ Classic Cards (Whatman International Ltd., Maidstone, Kent, UK) following the manufacturer's instructions. The cards were air dried and stored at room temperature until use (Kato et al., 2008).

2.3. PCR detection, direct sequencing, and analysis of the *Leishmania* cytochrome b gene

Disks of two-mm-diameter were punched out from the FTA cards, washed three times with FTA Purification Reagent (Whatman International Ltd.), and twice with TE^{-1} buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) in the PCR amplification tube. The disks were dried at 56 °C for 10 min, and subjected directly to PCR amplification using the TaKaRa Ex Taq DNA polymerase Hot Start Version (Takara-Bio, Shiga, Japan) and the primers L.cyt-AS (5-GCGGAGAGRARGAAAAGGC-3) and L.cyt-AR (5-CCACTCATAAATATACTATA-3) under the following conditions: initial denaturation at 95 °C for 10 min followed by 35 cycles of 95 °C, 50 °C and 72 °C for 1 min each, and a final extension of 7 min at 72 °C. One microliter of the PCR product was reamplified with L.cyt-S (5-GGTGTAGGTTTTAGTYTAGG-3) and L.cyt-R (5-CTACAATAAACAAATCATAATATRCAATT-3) primers in the same

conditions described above, except for the annealing temperature of 55 °C.

The PCR products were visualized in 2% agarose gels and then purified by using Sephadex G-50 spin columns (Amersham Biosciences, NJ). The purified DNA was examined by direct sequencing with an Applied Biosystems-HITACHI 3130 Genetic Analyzer using the Big Dye Terminator v1.1 Cycle Sequencing kit (Perkin Elmer, Wellesley, MA). The sequencing primers used were L.cyt-S, L.cyt-R, LCYTB F4L (5′-TGTTATTGAATATGAGGTAGTG-3′) and LCYT B R4 (5′-GAACTCATAAAATAATGTAAACAAAA-3′). The obtained sequences of 817 bp, assembled and edited by Genetyx Mac 11.0.0 (Software Development Co. Ltd., Japan) were compared with the Argentinean and WHO reference strains of *Leishmania* reported previously (Foulet et al., 2007; Marco et al., 2006b). The MLSTest 1.0 software was used for building a dendrogram from the consensus sequences using the neighbor joining method with 1000 bootstrap repetitions (Tomasini et al., 2013).

2.4. Ethics statement

The owners of the dogs voluntarily requested the medical attention of their animals. Under clinical suspicion of the disease, they gave their informed consent to include the case in this study. The dogs infected with *Leishmania* spp. were treated or euthanized following local committee recommendations (Echenique, 2010). The procedures were approved by the Bioethics Committee of the Faculty of Agricultural and Veterinary Sciences, the Catholic University of Salta (April 23th, 2014), Argentina.

2.5. Statistical analysis

The clinical and demographic data were tabulated and analyzed independently with Office Excel®, 2007 software, Microsoft Corporation, CA. The association between sex, age, or breeds and the frequency of clinical manifestations or genotypes; and between the disease signs and symptoms and genotypes was statistically analyzed by Fisher exact test in GraphPad prism5 (GraphPad Software Inc., CA).

3. Results

Out of 50 dogs included in this study, 48 were diagnosed with CanL by the identification of *Leishmania* spp. amastigotes on smears of 36 lymph nodes, 11 bone marrows, and one spleen aspirates (Fig. 1). The remaining two dogs were healthy subjects included as controls, from which aspirates were taken from their bone marrows.

Of the 48 dogs with CanL, 26 were males (54, 2%), and distributed by age range as follows: 14 (29.2%) were less than two years old, 17 (35.4%) between two and eight, and the other 17 were over eight years old. Dogs belonged to 18 breeds, where Boxer (18.8%), Mongrel (16.7%), and Rottweiler (10.4%) were the most frequent ones. Of the dogs, the 89.6% resided in urban area, with 38 (79.1%) in Posadas (27°22′00″S; 55°53′49″W), one in Oberá (27°29′00″S; 55°08′00″W), Province of Misiones; one in Ituzaingó (27°36′00″S; 56°40′00″W), Province of Corrientes; one in Reconquista (29°14′00″S; 59°56′00″O), Province of Santa Fe, Argentina; and two in Encarnación (27°20′00″S; 55°52′00″W), the neighboring Paraguayan city to Posadas. The remaining 10.4% of patients resided in suburban areas of Posadas, Santo Tomé and Garupá (27°29′00″S; 55°50′00″W), Misiones.

3.1. Clinical manifestations

Forty-six out of 48 patients of the set (95.8%) presented clinical manifestations associated with CanL. They were generally classified as systemic and tegumentary, and their specific frequencies are shown in Tables 1 and 2and in Fig. 2. At least one of the systemic manifestations was present in 46 CanL cases, while the tegumentary signs were observed in 43 (89.6%). The presence of tegumentary lesions and no systemic manifestations of the disease or signs of recent infection (such as primary skin lesions or epistaxis) were not observed by us in any CanL case. These observations are in agreement with results reported by Lima et al. (2014).

Table 1
Frequency of systemic abnormalities observed in Argentinean canine leishmaniasis cases.

Abnormality	Cases	(%)
Generalized lymphadenopathy	43	89,6
Emaciation	36	75,0
Mild	6	12,5
Moderate	19	39,6
Advanced	11	22,9
Sadness	17	35,4
Splenomegaly	12	25,0
Anemia	3	6,3
Hepatomegaly	1	2,1
Severe Locomotor difficulties	1	2,1
Polyarthritis	1	2,1
Total	46	95,8

Table 2
Frequency of tegumentary abnormalities observed in Argentinean canine leishmaniasis cases.

Sign	Cases	(%)
Alopecia	26	60,5
Generalized	11	22,9
Localized	4	8,3
Hypotrichosis	11	22,9
Secondary lesions	23	53,5
Crusted	16	37,2
Ulcerative	5	11,6
Crusted/ulcerative	1	2,3
Papular	1	2,3
Desquamation	17	35,4
Onychogryphosis	17	35,4
Seborrhea	14	32,6
Bilateral conjunctivitis	9	20,9
Bilateral blepharitis	5	11,6
Folliculitis	1	2,3
Hyperkeratosis	1	2,3
Total	43	89,6

No statistical associations were found among sex, age, or breeds with the frequency of clinical manifestations ($p > 0.05$).

3.2. Cytocrhome b variants of Leishmania (Leishmania) infantum incriminated as etiological agents of Argentinean canine leishmaniasis

The nested PCR was carried out directly on the FTA cards containing material of the aspirates mentioned above. Amplicons for gene sequencing were obtained from 23 out of 48 CanL cases, and in any of the parasitologically negative aspirates of the two healthy local dogs.

The *cyt* b sequence analysis resulted in two different variants, differing by a unique single nucleotide polymorphism (SNP). The cyt *b* genotypes defined by these sequences were termed LiA2 and LiA1, and assigned to *L. (L.) infantum,* since they showed a 99.9% and 100% homology with MHOM/TN/80/IPT1, the WHO reference strain for this species (Foulet et al., 2007; Luyo-Acero et al., 2004). The prevalent genotype was found in 18 out of 23 CanL cases (LiA1, 78.3%), and the other in the remaining five cases (LiA2, 21.7%). Fig. 3 shows a dendrogram built with several cyt *b* sequences of Argentinean and WHO references strains used in the analysis. No

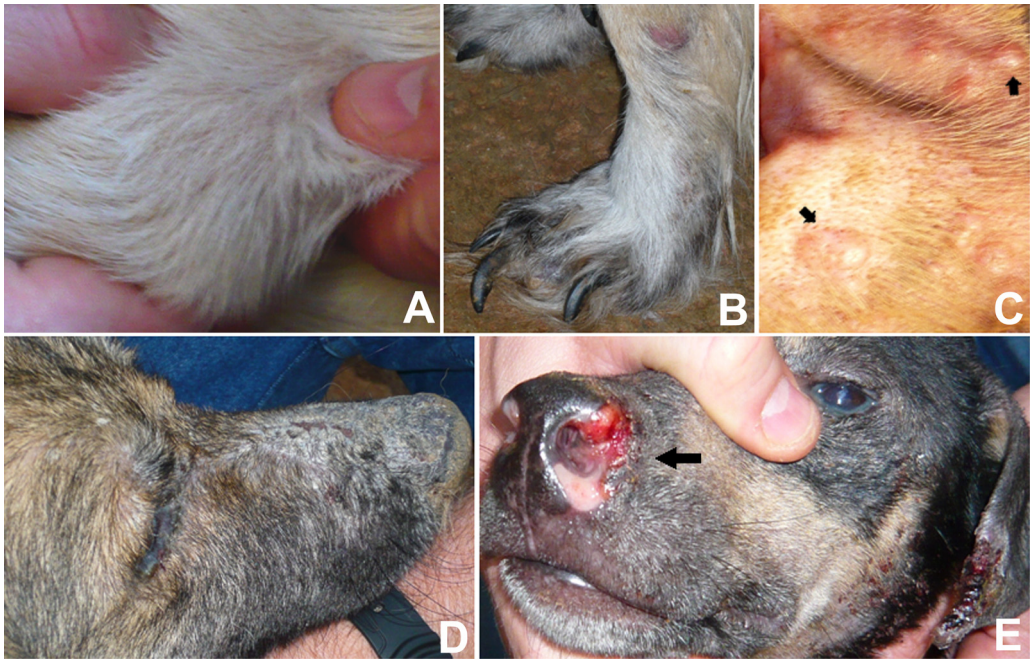

Fig. 2. Clinical manifestations of canine leishmaniasis caused by local genotypes of *L. (L.) infantum*. (A) lymphadenopathy, enlarged popliteal lymph node. (B) Onychogryphosis. (C) Secondary nodular lesions. (D) Seborrhea, desquamation, hypopigmentation and localized alopecia. (E) Mucocutaneous lesion (arrow), blepharitis, cutaneous hypopigmentation and secondary ulcerative cutaneous lesions.

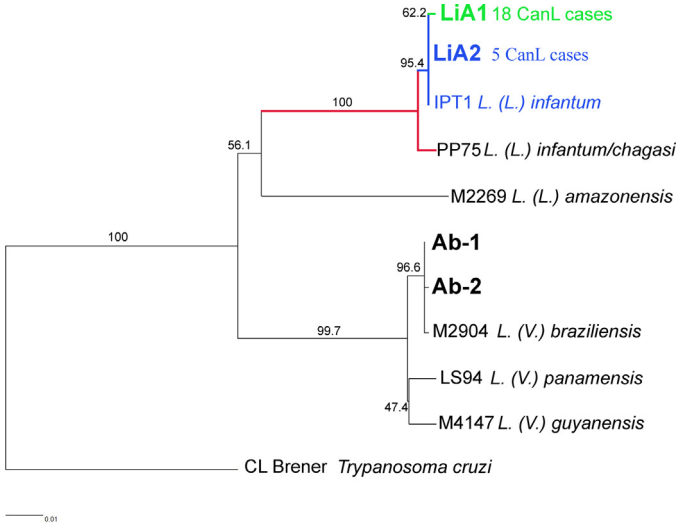

Fig. 3. Relationship among *Leishmania* cytochrome *b* gene sequences of Argentinean and WHO reference strains. The local genotypes LiA1 and LiA2 were assigned to *L. (L.) infantum*, while Ab-1 and Ab-2 to *L. (V.) braziliensis*. The WHO reference strains sequences included were MHOM/TN/80/IPT1 (*L. (L.) infantum*), LMHOM/BR/74/PP75 (*L. (L.) infantum* syn chagasi), MHOM/BR/73/M2269 (*L. (L.) amazonensis*), MHOM/BR/75/M2904 (*L. (V.) braziliensis*), MHOM/BR/75/M4147 (*L. (V.) guyanensis*), and MHOM/PA/71/LS94 (*L. (V.) panamensis*). The *Trypanosoma cruzi* strain sequence was considered as the outgroup. The dendrogram was built by the neighbor joining method with 1000 bootstrap repetitions using MLSTest 1.0 software.

association was found among the *L. (L.) infantum* variants with sex, age, breeds, or the frequency of clinical manifestations ($p > 0.05$).

4. Discussion

Leishmaniasis is classified by the WHO as a category I TDR, emerging and not controlled disease (WHO, 2004). In Argentina the leishmaniases are in the northern region, and seem to be spreading to the center of the country. Here we are reporting cases of CanL in several cities located at northeast of the country, with most of the cases originating in Posadas, and one in Reconquista, Santa Fe, the southernmost city of all the studies reported to date. Two cyt *b* genotypes, LiA1 and LiA2 of *L. (L.) infantum* are incriminated as the causal agents of CanL in the area. In particular, this species was found in autochthonous cases caused by LiA1 in Posadas and Garupá, of Misiones province, and by LiA2 in Posadas and Ituzaingó, in the province of Corrientes. This is the first report of autochthonous CanL cases due to *L. (L.) infantum* in the cities of Garupá and Ituzaingó. Moreover, at least one of them, LiA2, has also been associated with a human VL case in the country, suggesting a connection between these two hosts in an autochthonous transmission cycle (Barrio et al., 2012).

PCR amplification of the *cyt* b gene was not achieved in several of the parasitologically confirmed CanL cases included in the study. Although the sampling and PCR methodology could be improved, this fact may be related to the low parasitic burden observed in smears of aspirates of many CanL cases. This method reaches a 100% of positivity when it is applied on DNA templates form positive cultures of aspirates from different tissues o patients (Barroso et al., 2015; Locatelli et al., 2014). Nevertheless, *L. (L.) infantum* is the only species incriminated as the causal agent of CanL in this study.

The clinical patterns of the local CanL are basically defined by the systemic signs and symptoms, with lymphadenopathy present in most of the dogs. There were not cases with tegumentary signs and without systemic symptoms, in particular, the primary lesion typically presented as a circular ulcer with elevated active borders and a depressed center. All the lesions observed were chronic, most of them crusted and secondary to a visceral involvement (Fig. 2C and D). This indicates that the entrance door signs of the CanL due to *L. (L.) infantum* were spontaneously resolved by the host defense systems in most of the cases. On the other hand, the CanL cases from northwestern Argentina, caused by *L. (V.) braziliensis*, frequently presented primary lesion at the inoculation site of the parasites through the vector sand fly bite, without systemic manifestations (Marco et al., 2005; Marco et al., 2001; Padilla et al.,

2002). Therefore, primary ulcers of cutaneous form of the disease could be considered as unresolved entrance door signs, which may progress to more severe presentations such as the secondary muco-cutaneous leishmaniasis.

No statistical associations were found between the clinical forms of CanL and the different genotypes of *L. (L.) infantum* described in this work, in agreement with the study of Kuhls et al., who reported similar results after analyzing several isolates of the clade from different foci around the world by multilocus microsatellite typing (MLMT) (Kuhls et al., 2011). *Leishmania (L.) infantum* was recently and repeatedly imported from southwestern Europe by moving reservoirs with the ability to establish a transmission cycle in a new environment (Kuhls et al., 2011). This microsatellite analysis also showed that *L. (L.) infantum* isolates from American marsupials and foxes do not constitute a separate population from those infecting humans or dogs, indicating that its cycle not only has a domestic but also a wild component. Thus, during and after the colonization of America, *L. (L.) infantum* wild transmission cycles could have been established throughout the Argentinean forests, and maintained as zoonoses. An invasion of the cycles by an accidental host, for example in deforestation activities, could have triggered the occurrence of sporadic cases of human visceral leishmaniasis, such as happened in Salta province, in northwestern Argentina (Barrio et al., 2012). This parasite could also be transferred from its wild source to domestic animals, like the dogs, that in the appropriate circumstances, could change their status from accidental host to reservoirs, establishing an urban transmission cycle, as happened in Posadas city (Acardi et al., 2010). Both the urban and wild cycles could remain connected, hampering the control of human disease if only the domestic reservoirs are culled down. Thus, the incrimination of putative wild reservoirs such as wild canids, rodents, bats, or marsupials, is necessary and such potential reservoirs must be studied (Chaves et al., 2007; Dantas-Torres, 2007; Sobrino et al., 2008; Souza et al., 2014).

Finally, cyt b sequence analysis rendered higher genetic variability among local *L. (L.) infantum* population than metabolic genes providing similar insights to MLMT strategy (Marco et al., 2014; Kuhls et al., 2011; Souza et al., 2014).

In conclusion, cytochrome *b* showed to be a good molecular marker for future clinical and epidemiological analysis, since when applying the method in non-cultured samples, we found two genetic variants among the analyzed population, compared with a previous study that described a very homogenous population structure of this species in Brazil and Paraguay. These cyt *b* variants of *L. (L.) infantum* were incriminated as the causal agents of CanL in an area in which the disease seems to be geographically spreading or urbanizing. Thus, this species has been identified for the first time as the etiological agent of autochthonous cases in Garupá and Ituzaingó cities, both located south of Posadas where the main Argentinean focus of leishmaniasis caused by this species took place.

Conflict of interest

The authors have no conflicts of interest to declare.

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