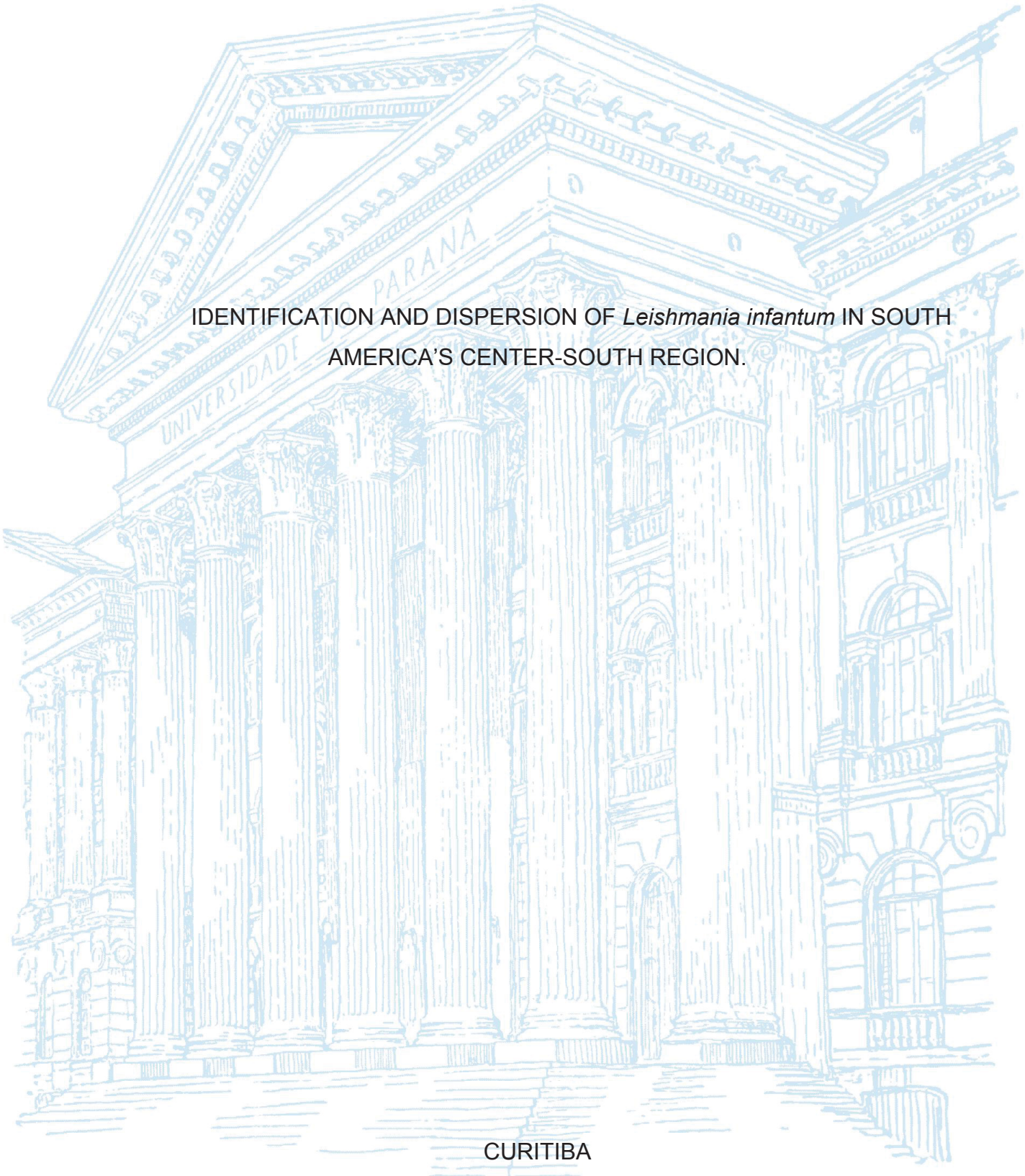
UNIVERSIDADE FEDERAL DO PARANÁ

ALINE KUHN SBRUZZI PASQUALI



IDENTIFICATION AND DISPERSION OF *Leishmania infantum* IN SOUTH AMERICA'S CENTER-SOUTH REGION.

CURITIBA

2018

ALINE KUHN SBRUZZI PASQUALI

IDENTIFICATION AND DISPERSION OF *Leishmania infantum* IN SOUTH AMERICA'S CENTER-SOUTH REGION.

Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnologia.

Orientadora: Profa. Dra. Vanete Thomaz Soccol

Coorientador: Prof. Dr. Rafael Antunes Baggio

CURITIBA

2018

Catalogação na Fonte: Sistema de Bibliotecas, UFPR
Biblioteca de Ciência e Tecnologia

P284i

Pasquali, Aline Kuhn Sbruzzi
Identification and dispersion of Leishmania infantum in South America's Center-South region / Aline Kuhn Sbruzzi Pasquali. – Curitiba, 2018.
115 p. : il. color.

Tese - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação Engenharia de Bioprocessos e Biotecnologia, 2018.

Orientador: Vanete Thomaz Soccol – Coorientador: Rafael Antunes Baggio.
Bibliografia: p. 89-115.

1. Leishmania. 2. Leishmaniose visceral. 3. Microssatélites (Genética). I. Universidade Federal do Paraná. II. Soccol, Vanete Thomaz. III. Baggio, Rafael Antunes. IV. Título.

CDD: 660.6

Bibliotecário: Elias Barbosa da Silva CRB-9/1894

MINISTÉRIO DA EDUCAÇÃO
SETOR TECNOLOGIA
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ALINE KUHN SBRUZZI PASQUALI** intitulada: **Identification and dispersion of Leishmania infantum in South America's Center-South region**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 29 de Março de 2018.

VANETE THOMAZ SOCCOL
Presidente da Banca Examinadora

JOÃO CARLOS MINOZZO
Avaliador Interno

ANDRÉ LUIZ GONCALVES
Avaliador Externo

WALTER ANTONIO PEREIRA BOEGER
Avaliador Externo

RAFAEL ANTUNES BAGGIO
Avaliador Externo

GILBERTO VINICIUS DE MELO PEREIRA
Avaliador Externo

Dedico essa tese aos meus pais Armelindo e Marlise, irmão Everson e esposo Darlan.

AGRADECIMENTOS

Agradecimentos à Deus, familiares e amigos.

À Deus, por guiar-me durante todo esse longo período de estudos e viagens para realização desse sonho.

Aos meus pais, Armelindo e Marlise Sbruzzi, por ensinarem a amar e respeitar o próximo, por estimularem a lutar pelo sonho de concluir o doutorado e estarem sempre de braços abertos para me receber com carinho e amor.

Ao meu irmão Everson Sbruzzi, pela parceira e amizade que somente um irmão pode fornecer.

Ao meu esposo Darlan Pasquali, por estar ao meu lado durante essa longa caminhada, por não desistir de nós mesmo quando a distância nos separava. Por confiar em nós e nunca impedir os meus sonhos.

Aos meus sogros João e Cleci Pasquali, meus cunhados Daiana e Albari Marques, Cristiane e Fabiano Panisson e Gabriela Naibo e aos meus sobrinhos Sofia e Julia Panisson e Kauan Marques, por me alegrarem, nos poucos momentos de lazer.

Às minhas amigas de longa data, Michelli Dacheri, Iana Arcari e Kellyn Marafon, por estarem ao meu lado em todas essas conquistas, juntas mostramos que amizade de infância é algo puro e verdadeiro.

À minha prima Angélica Pasquali e toda sua família, pelo amor e carinho recebido.

Às amigas que a Medicina Veterinária me presenteou, Alais Dall Agnol, Fernanda Ferreira e Eloiza Caldart, pelo companheirismo e amizade. Por nos permitirmos uma amizade em que evitávamos qualquer assunto de origem profissional.

À senhora Neli Mateus e sua filha Zizelane pelo acolhimento durante os três anos e meio de morada em Curitiba, fazendo com que eu sempre sentisse estar em família.

Muito obrigada, vocês foram essenciais para a finalização dessa etapa em minha vida!!!

Agradecimentos aos educadores e às Instituições de Ensino

À Profa. Dra. Vanete Thomaz Soccol, pela orientação durante o doutorado, por ensinar a importância de ética e profissionalismo no dia a dia.

Aos professores do Programa de Pós-Graduação em Engenharia e Bioprocessos da Universidade Federal do Paraná, pelo conhecimento e ensinamento repassado.

Ao Prof. Dr. João Carlos Minozzo, pelos ensinamentos repassados e manutenção dos coelhos para obtenção de sangue para o isolamento de *Leishmania*.

À secretária do Programa de Pós-Graduação em Engenharia e Bioprocessos da Universidade Federal do Paraná, Marta Szadkoski por estar sempre disposta a ajudar os alunos.

Aos colegas de mestrado e doutorado do Programa de Pós-Graduação em Engenharia e Bioprocessos da Universidade Federal do Paraná, em especial as colegas que se tornaram grandes amigas, Deborah Guedes, Ligia Barizon, Liliane Zoz e Bruna Coelho, pessoas que estarão para sempre em meu coração.

Ao Prof. Dr. Walter Boeger por fornecer as instalações do laboratório de Ecologia Molecular para realização das análises moleculares.

Ao pós-doutorando Rafael Baggio por todo ensinamento e ajuda no decorrer dos artigos.

À pós-doutoranda Luciana Patella pelo auxílio na edição de sequências.

À Secretaria de Saúde do Estado do Paraná, 9º Regional de Saúde e Centro de Controle de Zoonoses pelo apoio durante as coletas de amostras em campo.

Às médicas veterinárias Luciana Chyio e Eliane Pozzolo, pela coleta de amostras biológicas de cães usadas nesta pesquisa.

Às instituições de apoio à pesquisa: Capes, CNPq, Fundação Araucária e IDRC, pelo auxílio financeiro para realização das pesquisas.

O período de maior ganho em conhecimento e experiência é o período mais difícil da vida de alguém. (DALAI LAMA)

RESUMO

Leishmaniose é uma zoonose causada por *Leishmania* Ross, 1903 protozoário que é transmitido aos hospedeiros vertebrados (mamíferos) por vetores (flebotomíneo). São doenças negligenciadas de grande importância em saúde pública e com ampla distribuição mundial. O presente trabalho teve por objetivo identificar a(s) espécie(s) de *Leishmania* responsável pela doença em cães, na região Extremo Oeste do estado do Paraná usando como ferramenta o marcador espaçador interno transcrito (ITS) e avaliar a dispersão de *Leishmania (Leishmania) infantum* na região Centro Sul da América do Sul com o uso de marcadores microssatélites. Após o isolamento do prasito em cães, estes foram analisados por Reação em Cadeia de Polimerase (PCR) usando como marcador o ITS. Todas as amostras de *Leishmania* foram identificadas como *L. (L.) infantum.* Para testar o poder de discriminação do marcador ITS, um total de 486 sequências de 13 espécies de *Leishmania* presentes no Novo Mundo, mais *Leishmania donovani* e *L. (L.) infantum* foram obtidas do Genbank, somadas aos nossos isolados de campo foram analisadas usando o método de agrupamento Neighbor-Joining. Nossos resultados sustentam que o marcador ITS foi capaz de discriminar dois grupos. O primeiro grupo inclui os complexos *L. donovani* e *L. mexicana* do subgênero *Leishmania* e no segundo grupo inclui todas as espécies do subgênero *Viannia* que se mostrou menos efetivo na separação das atuais espécies descritas no Novo Mundo. Os isolados do parasito obtidos na região oeste do Paraná agruparam-se no complexo *L. donovani*, entre *L. donovani* e *L. (L.) infantum*. Numa segunda etapa foram usados marcadores microssatélites que mostraram que na região oeste do Paraná circulam duas populações de *L. (L.) infantum*. A comparação com isolados provenientes de outros estados do Brasil e do Paraguai foi possível separar três populações de *L. (L.) infantum*. Os dados levantados na literatura associado aos dados obtidos neste estudo permitiram verificar que *L. (L.) infantum* teve quatro movimentos de expansão na região Centro Sul da América do Sul. O primeiro movimento foi graças à migração populacional humana do Nordeste para a região Sudeste. O segundo deu-se com a construção do gasoduto Bolívia-Brasil passando por três estados da região Centro-Oeste do Brasil. O terceiro movimento de expansão foi a entrada de LV na tríplice fronteira via Argentina↔Brasil↔Paraguai. E,

enfim, foi possível constatar a entrada de uma “nova” população na região oeste de Santa Catarina e central do Paraná.

Palavras-chave: 1. Dispersão 2. ITS 3. *Leishmania (Leishmania) infantum* 4. Leishmaniose visceral 5. Microssatélites

ABSTRACT

Leishmaniasis is a zoonosis caused by *Leishmania* Ross, 1903 protozoan which is transmitted to vertebrate hosts (mammals) by vectors (sandfly). They are neglected diseases, of great importance in public health and with wide distribution worldwide. The objective of the present work was to identify the *Leishmania* species responsible for the disease in dogs in the extreme west region of the state of Paraná using the transcribed internal spacer marker (ITS) as a tool and to evaluate the dispersion of *Leishmania (Leishmania) infantum* South America's Center-South region with the use of microsatellite markers. After isolation of the prasite in dogs, these were analyzed by Polymerase Chain Reaction (PCR) using the ITS marker. All *Leishmania* samples were identified as *L. (L.) infantum*. To test the discriminating power of the ITS marker, a total of 486 sequences from 13 *Leishmania* species present in the New World plus *Leishmania donovani* and *L. (L.) infantum* were obtained from Genbank, plus our field isolates were analyzed using the Neighbor-Joining grouping method. Our results support that the ITS marker was able to discriminate two groups. The first group includes the *L. donovani* and *L. mexicana* complexes of the subgenus *Leishmania* and in the second group includes all species of the subgenus *Viannia* that proved less effective in separating the present species described in the New World. The isolates of the parasite obtained in the western region of Paraná were grouped in the *L. donovani* complex, between *L. donovani* and *L. (L.) infantum*. In a second step microsatellite marker were used that showed that in the western region of Paraná two populations of *L. (L.) infantum* circulate. Comparison with isolates from other states of Brazil and Paraguay allowed to separate three populations of *L. (L.) infantum*. The data collected in the literature associated with the data obtained in this study allowed us to verify that *L. (L.) infantum* had four expansion movements in the South America's Center-South region. The first movement was due to the human population migration from the Northeast to the Southeast region. The second occurred with the construction of the Bolivia-Brazil gas pipeline passing through three states of the Center-West region of Brazil. The third movement of expansion was the entrance of LV in the triple border via Argentina↔Brazil↔Paraguai. And, finally, it was possible to verify the entry of a "new" population in the western region of Santa Catarina and central Paraná.

Keywords: 1. Dispersion 2. ITS 3. *Leishmania (Leishmania) infantum* 4. Microsatellites 5. Visceral Leishmaniasis

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS OU SIGLAS

DNA	- Ácido desoxirribonucleico
pb	- pares de base
fg	- fentograma

LISTA DE SÍMBOLOS

© - copyright

µL - microlitros

® - marca registrada

µm - micrometros

% - porcentagem

SUMÁRIO

1 INTRODUCTION

Leishmaniases are anthropozoonoses caused by the protozoan *Leishmania* Ross, 1903, transmitted to vertebrate hosts (mammals) by vectors (phlebotomine). There are two clinical forms of the disease: visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) (Moreno; Alvar; 2002; Who, 2016a).

CL is a worldwide distributed disease and has been drawing attention from the world public health authorities, due to the increasing number of cases since 2005. In the year of 2015 were reported 214,096 cases (Who, 2017). The main species of New World *Leishmania* are: *Leishmania* (*Viannia*) *braziliensis, L.* (*V*) *peruviana, L.* (*V*) *panamensis, L.* (*V*) *guyanensis, L.* (*V*) *naiffii, L.* (*V*) *lainsoni, L.* (*V*) *shawi, L. (Leishmania) amazonensis and L. (L) mexicana* which causes CL and *L. (L) infantum* (sinonímia *L. chagasi)* causing VL (Lainson, 1983; Thomaz-Soccol *et al.,* 1993, 2009; Who, 2017). In Brazil, the parasite transmission occurs in several municipalities in all states. Since 1985, it has been increased CL cases in the country and, in the last two decades, the transmission peaks have been observed approximately every five years. The number of CL cases in humans raised from 3,000 in 1980 to 37,710 in 2005. From 1985 to 2010, it was registered an annual average of 35,000 autochthonous cases (Brasil, 2010).

VL is a neglected disease present in Asia, Africa, Europe and Americas with a large geographic distribution and an increasing number of cases (OPAS, 2018). Depending on the geographic regions, VL can be caused by the species *L.* (*L.*) *donovani* (in Asia and Africa); *L.* (*L.*) *infantum* (in Asia, Europe, Africa and Americas) (Brasil, 2006). Ninety percent of cases of VL occur in Bangladesh, Brazil, Ethiopia, India and Sudan (Who, 2013; Brasil, 2014a). It is estimated that 1.69 billion people are living in VL transmission areas worldwide (Pigott et al., 2014). In 2015, a worldwide incidence of 2.27 cases per 100,000 habitants was stipulated by the World Health Organization (WHO), of which 95.1% were reported in Brazil (Who, 2017).

VL has undergone a change in the transmission scenario, both in the Old and New World (Maia-Elkhoury et al., 2008; Ready, 2010; Gradoni, 2013). In the Old World countries, where VL was not previously reported, epidemics occurred and, in those where the disease was already endemic, there was an expansion in geographical areas. An example of this is the epidemiological surveillance data published in the Eurosurveillance journal of five endemic countries in southern Europe (Bulgaria, Greece, Croatia, Italy and France), which recorded an increase in the number of cases. In Spain there was outbreak of VL in Madrid (Gradoni, 2013; Lachaud et al., 2013).

In the Americas, the countries are classified according to the epidemiological scenario for VL (Who, 2016b), which are: sporadic transmission (Costa Rica, Guatemala, Honduras, Nicaragua, Bolivia, Guyana and Mexico), and controlled disease (Colombia and Venezuela) (Argentina, Brazil and Paraguay). The expansion of VL observed in the New World occurred in countries where autochthony of the parasitic cycle wasn't previously verified, such as United States and Uruguay (Cousiño, 2006; Duprey et al., 2006; Salomon et al., 2008; 2009; 2011), and internally there was a disease expansion to areas previously indene.

In Brazil, until 1980, VL was more common in wild and rural environments where the population had a low level of education (Gama et al., 1998; Gontijo, Melo, 2004, Brazil, 2010), being endemic especially in the North East region. Since 1980s, the disease dispersal has changed this paradigm with the registration of VL cases in periphery aereas of large cities and urban centers, and becoming a disease found in urban and peri-urban areas (Figueredo et al., 2010). In the 1990s, several epidemic outbreaks were reported especially in the southeastern and midwestern regions of the country. High rates of VL cases were registered in dogs (urban parasite reservoirs) followed by clinical cases in humans in the municipalities of Belo Horizonte (Minas Gerais state), Campo Grande (Mato Grosso do Sul state) and Araçatuba (São Paulo state), confirming the urbanization of the disease in Brazil (Maia-Elkhoury et al., 2008; Savani et al., 2011; Paulan et al., 2012; Cunha et al., 2014; Oliveira et al., 2016; Sevá et al., 2017). In the state of São Paulo, VL showed an increase of cases number in both dogs and humans in new geographical areas, such as Ilha Solteira, Campinas, Araçatuba, Bauru and other cities (Savani et al., 2011; Paulan et al. al., 2012, Oliveira et al., 2016). At the state of Mato Grosso do Sul, environmental changes such as the construction of BR 262 and the Bolivia-Brazil pipeline, parallel to this highway, contributed to the rapid expansion of VL in the state (Antonialli et al., 2007). In the southern region of the country VL, which until then was considered imported, now has records of autochthonous cases. First, it was observed in Rio Grande do Sul in 2008 followed by Santa Catarina, which recorded the first case of VL canine in 2010. And finally, the state of Paraná that recorded the first autochthonous case in dogs in 2012 (Souza; Santos; Andrade Filho, 2009; Steindel et al., 2013; Dias et al., 2013).

Exogenous factors have helped for this dispersal and change of the VL scenario, being fundamental for the occurrence and dispersion of leishmaniases in both sides of the World. Important exogenous factors are: migration of hosts, changes in socioeconomic patterns, introduction of infected hosts in the presence of sandflies, deforestation and changes in the ecosystem, whether or not caused by man (Evans et al., 1992; Alvar et al., 1996; 1997; Antonialli et al., 2007; Kuhls et al., 2011).

The main challenges to control these endemics or epidemics can be the adaptation of the vectors to the conditions existing in the cities and consequent urbanization of parasites transmission; changes in the clinical-epidemiological profile of the disease; operational difficulties such as early diagnosis; reduction of infected reservoirs; control of the vector population; degree of effectiveness of the measures employed; and high financial cost required for control (Brasil, 2006). Studies on the dispersion of *L.* (*L.*) *infantum* and of which parasite populations are involved are necessary to establish prevention and control measures, especially in South America's Center-South region, which is recent. Some points in relation to this expansion need to be considered and understood, such as:

1. would the construction of Bolivia-Brazil and Bolivia-Argentina gas pipeline in the Central-South / Southeast region of the continent have helped the dispersion of VL to the Central South region with reflections in southern Brazil and adjacent countries?

2. would the population flow on the triple border of Brazil, Argentina and Paraguay at Foz do Iguaçu city have aided the entry of *L.* (*L.*) *infantum* in Brazil or vice versa?

3. the migration of Italian and German settlers from the southern region of Brazil to the Southeast and Central West regions would have been relevant for the parasite dispersion, through dogs, to areas where there were no *L. (L.) infantum*?

Thus, the present work proposes, firstly, the identification of *Leishmania* species that cause canine VL in dogs from the extreme west of Paraná. And, subsequently test a panel of 14 microsatellites in a local population compared to populations of different regions. In this way, we try to understand the entrance and dispersion of *L.* (*L.*) *infantum* and consequently the disease in South America's Center-South region.

1.1 OBJECTIVE

1.1.1 General objetive

To identify *Leishmania* species causing canine LV in the Far West of Paraná and to evaluate the dispersion of *L.* (*L.*) *infantum* in the South America's Center-South region, using microsatellite markers.

1.1.2 Specific objetive

- Identify *Leishmania* species in the extreme west region of Paraná state;
- Check how the ITS marker separates *Leishmania* populations;

• Study the dispersion of the parasite in South America's Center-South region by genotyping *L.* (*L.*) *infantum* isolates in dogs, humans and sandflies from several regions of Brazil and Paraguay;

• Study the dispersion of the parasite in South America's Center-South region by means of a bibliographical survey of the first reports of canine and human VL;

• Test hypotheses for the entry of the VL causal agent in the extreme west region of Parana state and its dispersion to other municipalities by phylogenetic analyzes.

2 LITERATURE REVIEW

2.1 ETIOLOGICAL AGENT

Leishmania is a protozoan that requires vertebrate and invertebrate hosts to complete the biological cycle. This parasite has two forms of development: amastigote present in vertebrate hosts (mammals) and promastigote in invertebrate hosts (sand flies) (Peters; Killick-Kendrick, 1987).

The amastigote form has an oval or round body with a nucleus, kinetoplast and axoneme, measuring 2 to 5 μm in diameter and parasitizing the cells of the monocytic phagocytic system (MPS) of mammals. The promastigote form has an elongated body, an axoneme that extends beyond the border of the cell and transforms it into a flagellum, measure 14 to 20 μm, and is present in the digestive tract of sand flies (Pessôa; Martins, 1982, Peters; Killick-Kendrick, 1987). *Leishmania* has kinetoplast, a DNA rich organelle (kDNA) structured in the form of thousands of intertwined circles, called maxicircles and minicircles (Lukes et al., 2002). The genus *Leishmania* has clonal reproduction and are diploid organisms (Tibayrenc, Kyllberg; Ayla, 1990; Birky, 1996; Normark, 1996; Tibayrenc; Ayla, 2002; Balloux; Lehmann; de Meeus, 2003).

2.2 SYSTEMATIC

One of the most accepted classifications for the genus *Leishmania* is proposed by the International Society of Protistologists (Levine et al., 1980):

Domain: Eucaryota
Kingdom: Protista
Sub-kingdom: Protozoa
Phylum: Sarcomastigophora
Class: Zoomastigophorea
Order: Kinetoplastida
Family: Trypanosomatidae
Genus: *Leishmania* Ross, 1903

At the infrageneric level the parasite is subdivided into two subgenera: *Leishmania* and *Viannia* (Lainson; Shaw, 1987). The classification based on molecular characters of the genus *Leishmania* comprises two groups: *EuLeishmania* (including the subgenus

Leishmania and *Viannia*) and *ParaLeishmania* (including species found only in wild animals) (Akhoundi et al., 2016).

The earliest references to leishmaniasis in the New World date back to the time of the Incas and later to descriptions made by the Spanish in the sixteenth century (Thomaz-Soccol, 1993). Already in the Old World, in 1756 Russell and Hassel Quist summarily describe the disease. In 1833 Guilhou, in his thesis, describes the disease with more details, showing that the "Button of Aleppo" is quite dispersed. In 1903, Ross proposed the binomial *Leishmania donovani* to the parasite responsible for kala-azar in India. In the same year the causal agent of the eastern button was discovered by Wright, 1903, being called *Leishmania tropica* Luhe, 1906. In 1905 the first case of kala-azar in the Mediterranean region was reported (Sergent et al., 1905). Gaspar Vianna in 1906, while investigating amastigote forms in cutaneous lesions of a patient in Minas Gerais, concluded that these were a different species of *L. tropica*, denominating *Leishmania brasilienses* (Vianna, 1911). In 1908, Nicolle isolated the parasite from a child with visceral lesion and named the parasite *Leishmania infantum*, differentiating visceral leishmaniasis in the Mediterranean caused by *L. (L.) infantum* of Indian kala-azar caused by *L. donovani*. Wenyon (1911) described *Phlebotomus* as the likely *Leishmania* vector in the Old World. In 1911, the name of the species "*L. brasilienses"* was changed to *L. braziliensis* (Matta, 1916). Velez in 1913, described that cutaneous and mucocutaneous leishmaniasis in Peru was caused by *L. peruviana*. The first report of American visceral leishmaniasis (AVL) in the Americas was made by Migone in 1913. In 1914, Yakimoff and Schokhor proposed *L. tropica minor* as the causative agent of CL for the dry form of the lesion and that occurred in the urban region and *L. tropica major* as the causative agent of CL for the wet ulcer form and that occurred in the rural region. Castelani and Chalmers (1919) named *L. donovani archibaldi* as the etiological agent of the lethal form of VL (Akhoundi et al., 2016).

Pena (1934) made the first description of VL in the Amazon region and Chagas (1936) described the first case of VL in a living patient in Brazil. Cunha and Chagas in 1937 isolated the etiologic agent of VL in Brazil and named it *L. chagasi*. Medina (1946) found a parasite that caused skin lesions in India piglet (*Cavia porcellus*) and *Leishmania enriettii* Muniz & Medina, 1948. Convit and Lapenta (1946) observed a different clinical form of cutaneous leishmaniasis in Venezuelan patients and the authors have termed diffuse cutaneous leishmaniasis, caused by *Leishmania pifanoi* (Medina, 1959).

In 1954, Floch called *Leishmania guyanensis* the causative agent of leishmaniasis in French Guiana. In 1962, Garnham called *Leishmania mexicana* the causative agent of "chiclero ulcer" in Central America (Garnham, 1962). Lainson and Shaw (1972) observed biological aspects of *L. mexicana*, found in Central America, similar to those found in Brazil

as the etiological agent of *L. mexicana amazonensis*. Herrer in 1971 named *Leishmania hertigi* the parasite found in pork thorn in Panama. Lainson and Shaw (1972) called *L. panamensis* the causative agent of leishmaniasis in Panama.

After the identification of two species of parasites belonging to the genus *Leishmania* in 1903 by Ross, and several other species, the first attempts to group the parasites in a linear system appear. However, the classification underwent in modifications over the years depending on the characters used. In the first stage extrinsic characters were used, and from the 80s the intrinsic characters became used. A first classification proposal was made in the nineteenth century based on extrinsic characters such as clinical form, geographical distribution and epidemiological cycle (Thomaz-Soccol, 1993). The first to worry about the grouping of the genus was Matta (1916) who proposed a first classification with five species: *L. donovani, L. infantum, L. braziliensis, L. furunculosa, L. nilotica.* In 1937, Chagas et al proposed a new classification based on etiopathogenesis and biogeography designating as species: *L. braziliensis, L. chagasi, L. donovani, L. infanum and L. tropica*. In 1949, Kirk classified species of the genus *Leishmania* according to morphology, clinical aspects in humans, epidemiological aspects, promastigotes forms in culture, cross immunity, serological tests and xenodiagnosis. The author proposed a complete nomenclature of the genus *Leishmania* with eleven species described at that time (*L. tropica, L. donovani, L. braziliensis, L. (L.) infantum, L. peruviana, L. tropica minor, L. tropica major, L. archicaldi, L. enriettii, L. chagasi, L. pifanoi*).

In 1961, Pessôa reviewed the classification of the species from the genus *Leishmania* and used a binominal or trinominal nomenclature and named the VL-causing species only as *L. donovani*. For Old World cutaneous leishmaniasis, *L. tropica minor* (dry form) and *L. tropica major* (wet form) were included. For the species of the New World the author proposed five subspecies within the species *L. braziliensis: L. b. braziliensis; L. b. peruvian L. b. Mexican; L. b. guyanensis and L. b. piphalo.*

Nicolli in 1964, taking as reference the development of the parasite in the gut of the insect vector proposed the division of the *L. donovani* species into six subspecies (*L. d. donovani, L. d. infantum, L. d. chagasi, L. d. archibaldi, L. d. sinensis* and *L. d. myoxi*).

In 1971, Garnham suggested a classification based on the development of the parasite in the vector and in the vertebrate host. In this way, the determination of the interspecific phylogenetic bonds and classifying the VL agent into four species, three of which are present in the Old World and one in the New World (*L. chagasi*). Lainson and Shaw (1987) subdivided *Leishmania* into two subgenus *Leishmania* (Suprapiloria) and *Viannia* (Peripiloria) according to the developmental pattern of the parasite in the gut of sandflies.

In 1979, Lainson and Shaw reviewed the classification of Leishmania species present in the New World based on the development pattern of the parasite in the sandflie *Lutzomia longipalpis*. The authors proposed the subdivision into three groups: Hipopilaria for *L. agamae* and *L. ceramodactyli*, Peripilaria for *L. braziliensis* and Suprapilaria for *L. donovani*, *L. mexicana*, *L. hertigi* and *L. tropica* complex. In 1986, Le Blancq and Peters described a new taxonomic classification taking into account intrinsic characters of the parasite using isoenzymes as a system for differentiation of *Leishmania* species. Rioux and colleagues in 1990 proposed a new classification for *Leishmania* spp. based on intrinsic and extrinsic characters and numerical methods, grouping the Linneana and Adansonian classifications. The World Health Organization, in the same year, admitted the species of *Leishmania* in three subgenres: *Leishmania, SauroLeishmania* and *Viannia*. Thomaz Soccol et al. (1993) proposed the evolutionary characterization of New World *Leishmania* using as a tool the enzymatic identification integrating the subgenus *Viannia* and *Leishmania* with phylogenetic models. The authors proposed some species as synonymy (such as *L. (L.) infantum/L. chagasi*) and suggested that the genus *Leishmania* is of monophyletic origin. In the same year, Momen and collaborators proposed the use of synonymy for *L. chagasi* and *L. (L.) infantum*. Shaw in 1994, suggested that *Leishmania* encompass 30 species that infect mammals including the 21 species that infect humans. Cupolillo, Grimaldi and Momen (1994) confirmed the subgenus *Viannia* as monophyletic. In 1999, Dedet and colleagues, drawing on the evolutionary history of the Leishmania classifications, proposed the division of classifications into four periods: Linnaean, Adansonian, phenetic and phylogenetic.

At the beginning of the 21st century, proposals for new classifications for *Leishmania* composed of two groups: *EuLeishmania* (subgenus *Leishmania* and *Viannia*) and *ParaLeishmania* (for *L. hertigi, L. deanei, L. colombiensis, L. equatoriensis, L. herreri* and species of *Endotrypanum*) (Cupolillo et al., 2000; Schonian et al., 2000).

Currently there is concordance of phylogenetic classifications based on monophyletic concepts, parsimony and non-convergent characters. These concepts provided validation of the classification of these species according to extrinsic characteristics (geographical distribution, clinical signs and characteristics in the development of the sandfly gut) and intrinsic (biochemical, immunological and molecular markers) (Akhoundi et al., 2016).

2.3 BIOLOGICAL CYCLE OF *Leishmania*

Leishmania is a heteroxene parasite and requires two hosts to complete the biological cycle. Vertebrate hosts (mammals) can be classified as an accidental host or reservoir, while invertebrate hosts (hematophagous insects) are called vectors (Coura, 2005).

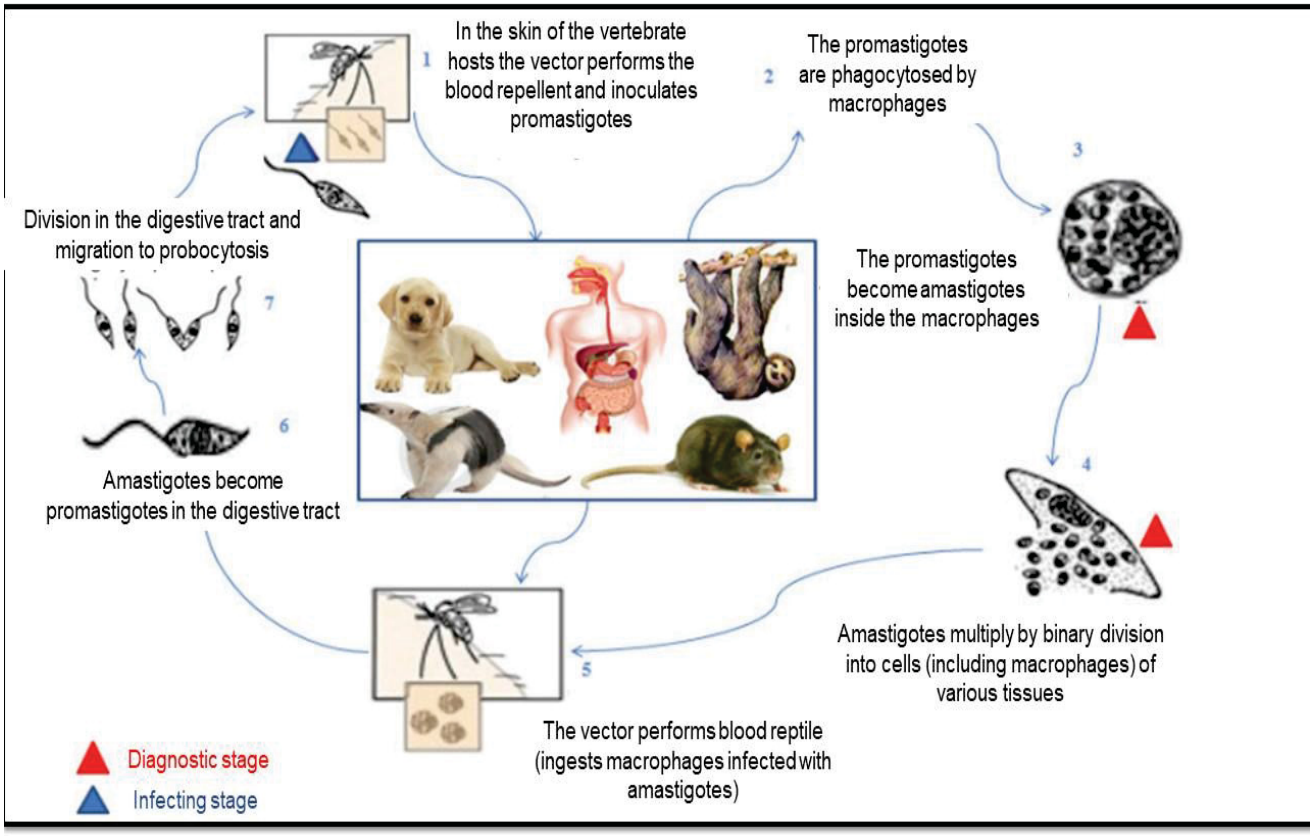
The reservoir of infectious agent is any mammal and arthropod where it lives and / or multiplies an infectious agent. In leishmaniasis, the main reservoir varies depending on the species of the parasite. Accidental hosts may be other mammals that help to maintain the pathogen (PAHO, 2010). For example, the reservoir of *L. (L.) infantum* of major relevance in Brazil is the domestic dog (*Canis familiaris*) and man is considered an accidental host (Coura, 2005). The dogs are primary reservoirs, but wild canids, marsupials and rodents have been described as possible secondary reservoirs of this parasite (Lainson, Rangel, 2005, Lainson, Shaw, 2005; Rotureau, 2006). High percentage of dogs are carriers of the parasite without clinical manifestation of the disease making it difficult to identify positive animals for *L. (L.) infantum* and corroborating with its role as reservoir (Coura, 2005).

Vetor, according to the Pan American Health Organization (PAHO, 2010), is an insect that carries an infectious agent, from an individual or its excrement to the susceptible individual. The agent may or may not undergo transformation (mechanical vectors), multiply, or transform into the vector (biological vector). The vector for *Leishmania* spp. are females of hematophagous insects belonging to the Order Diptera, Suborder Nematocera, Family Psychodidae, Subfamily Phlebotominae. These insects develop in four evolutionary stages (egg, larva, pupa and adult) and only females are hematophagous (Coura, 2005). The egg phase to the pupa usually occurs in terrestrial breeders in the presence of organic matter and moisture (Aguiar and Medeiros, 2003). Phlebotomine females can be found in primary, secondary, peridomicile and domiciliary forest. In the last two habitats they feed mainly on the blood of domestic animals (Coura, 2005). The females of sandflies initiate the sanguine repast in the twilight and nocturne period, sheltering in humid and somber places. Humidity in the air and soil are important factors for reproduction and resting of sandflies (Ready, 2013).

The cycle begins with the parasite transmission at the moment of sandflies females carry out hematophagy, regurgitating the metacyclic promastigote forms in the dermis of the vertebrate host. These parasitic forms are phagocytosed by macrophages and transformed into amastigotes. Intracellularly the amastigote forms multiply by binary division, break the host cell and infect other macrophages. Phlebotomine performs hematophagy in this infected host (reservoir) and ingests the amastigote forms present in macrophages. The amastigote forms, in the vector, are taken to the midgut and become a

promastigote, continuing the cycle (Figure 1) (Sacks; Noben-Trauth, 2002; Coura, 2005; Montalvo et al., 2012).

Figure 1: – Evolutionary cycle of *Leishmania* spp.



Source: Adaptation of CDC - Bisetto Junior et al., 2015.

2.4 PATHOGENESIS AND CLINICAL MANIFESTATIONS

L. (L.) infantum is a parasite that mainly affects the skin and internal organs such as lymph nodes, spleens, liver and bone marrow (Solano-Gallego et al., 2011; Kaszak, Planellas, Dworecka-Kaszak, 2016).

In humans it is a disease that can present acutely evolving to chronic and systemic, being asymptomatic or symptomatic. When symptomatic, the patient presents weight loss, hepatosplenomegaly, anemia, long-term fever, among other clinical signs and can progress to death if not treated properly. The incubation period can be from ten days to two years. In endemic areas, the elderly, immunocompromised and children are at higher risk groups (Brazil, 2016). However, in recent cases, individuals of any age group are at risk of developing severe disease (Coura, 2005).

In dogs, the incubation period may vary from three months to several years (Brazil, 2016). The disease may present three clinical forms: 1. Asymptomatic (absence of clinical

signs suggestive of VL); 2. Oligosymptomatic (clinical signs such as weight loss, skin lesions, opaque hairs); 3. Symptomatic (clinical signs such as adenomegaly, onychogrifose, alopecia, hyperkeratosis, conjunctivitis, hepatomegaly, splenomegaly, convulsion, muscular atrophy, among other signs more common to the disease) (Solano-Gallego et al., 2011; Kaszak; Planellas; Dworecka- Kaszak, 2015).

2.5 EPIDEMIOLOGY

VL is a severe disease, in which 500,000 new cases are registered every year in the world and considered a neglected disease (Dedet et al., 2009; Who, 2013; 2017; PAHO, 2018). Ninety percent of the cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan (Who, 2016a). The incidence of the disease is estimated at 200 to 400 thousand cases/year with an average of 20 to 40 thousand deaths/year, leaving behind only by malaria the parasitic disease that causes more deaths (Who, 2013).

In the American continent, the disease and the parasite are recorded in both South and Central America (Lainson; Shaw, 1987, Baneth, 2006; Jeronimo; Souza; Pearson, 2007; Dedet et al., 2009; Who, 2017). The countries are classified according to epidemiological scenario: Costa Rica, Guatemala, Honduras, Nicaragua, Bolivia, Guyana and Mexico have sporadic transmission; Colombia and Venezuela are controlled; Argentina, Brazil and Paraguay the disease is expanding (Who, 2016b).

Factors such as climate and environmental changes, population rapid migration from rural to urban areas, rapidly expanding cities without infrastructure may be responsible for the urbanization of VL, since there was more interaction and mobilization of wild reservoirs and infected dogs to big centers (Lainson, 1989; Silva et al., 1997; Dias, Lorosa, Rebelo, 2003; Harhay et al., 2011).

It is known that 90% of the VL cases in the world occur in Brazil (Coura, 2005; Madeira et al., 2009), where the disease presents different geographic, climatic and social aspects, since it is present throughout the Brazilian territory. The first urban epidemic of VL occurred in Teresina, Piaui state in the 1980s. Since then there has been an expansion and urbanization of VL with human cases and positive dogs in large and medium-sized cities (Costa; Pereira, Araújo, 1990). São Luís (MA), Natal (RN), Aracaju (SE), Boa Vista (RR), Santarém (PA), Palmas (TO), Rio de Janeiro (RJ), Belo Horizonte Araçatuba (SP), Cuiabá (MT), Corumbá (MS), Três Lagoas (MS) and Campo Grande (MS) (Brazil, 2001; Brazil, 2006). For the last two decades the Northeast region present the highest VL prevalency in Brazil (Brasil, 2006).

In the southern region of the country, the first canine case of VL was recorded in 2008 and the first human case in 2009, both in São Borja, State of Rio Grande do Sul (Souza, Santos, Andrade Filho, 2009). In Uruguaiana (RS) the first canine case of VL was recorded in 2009 (Massia et al., 2015). In 2010, the first canine case of VL (Brazil, 2011a) and the first human case of VL in 2016 (CRMV, 2016) were registered in the city of Porto Alegre (RS). In the state of Santa Catarina, the first canine case of VL was recorded in 2010 in Florianópolis, (Steindel et al., 2013) and in 2017 the first case in humans (Steindel, 2017) in the same municipality. In the western region of Santa Catarina state, VL positive dogs were found by serology and polymerase chain reaction (PCR) in the cities of Descanso and São Miguel do Oeste in the year of 2014 (Mazieiro et al., 2014) and Erval Velho in 2016 (Pinto, 2017). In the state of Paraná, the first autochthonous case in dogs was described in Foz do Iguaçu in 2012 (Dias et al., 2013), followed by the first autochthonous case in humans in 2016 in the same municipality (Trench et al., 2016). However, allochthonous canine cases were already being reported between 2000 and 2009 together with an alert that in case of competent vectors entrance the parasite could be rapidly established and disseminated in the state of Paraná (Thomaz-Soccol et al., 2009).

2.6 PREVENTION AND CONTROL

For the prevention and control of VL in the human and canine population, actions directed to the man, vector, dogs and environment are necessary because it is a complex disease and because it involves several actors (Brasil, 2006).

In Brazil, the prevention forms against VL in humans proposed by the Ministry of Health (MS) are measures of individual protection and control in the residence such as: use of thin-bed nets, chemical repellents, screens on doors and windows and avoid access to areas of risk in crepuscular times. For dog population the VL control should be done by reducing the number of wandering dogs by means of castration and donation of these animals, use of collars impregnated with 4% deltrametrin. As for the vector control environmental management strategies is necessary for example , reduction of the accumulation of organic matter in the garden and vacant lots, and the use of insecticides in the environment should be avoided. However, this control is expensive and labor intensive. As for the individuals, it is necessary the diagnosis and early treatment of human cases, reduction of the population of sandflies, elimination of reservoirs and health education activities (Brasil, 2006).

One of the first steps to take control measures for VL is the classification of risk areas performed by competent bodies. In Brazil, teh Ministry Health proposes the following classifications: vulnerable areas, areas with first autochthonous VL in humans, areas of sporadic transmission, areas of moderate to intense transmission (Brasil, 2006).

Vulnerable areas are municipalities close to sites with cases of VL already confirmed in humans and canines, or the ones that are part of the same road flow and municipalities with intense migratory flow. In this area the entomological survey must be done, searching for the true vector (*Lu. Longipalpis*). If it is not found, the survey should be redone with an interval of no more than two years. If the vector is found the measures to be taken are: use of insecticides at sites with an initial radius of 500 m^2 in urban areas, and 1000 m^2 in rural areas (Brasil, 2007), canine population survey, search for stray dogs and environmental sanitation (Brasil, 2006). Insecticides are applied using local fumigation using residual-acting insecticides (Who, 2010).

In areas with a record of the first autochthonous case of human VL, the active search for suspected human cases, alert and applications of prevention and control measures to the human population and the entomological search of the vector should be made from the place of encounter of the human case. If the presence of the vector is verified, the chemical control with insecticides must be carried out at the transmission site and two new spray cycles should be programmed (the first cycle in the period of vector increase and the second three to four months after the first cycle) . If there is no vector, searches must be carried out every month until find it. Regarding the canine reservoir, the active search of dogs with clinical suspicion should be performed, an annual census survey at the transmission site and control of stray dogs performing serology of the same. In this case the seroreagent dogs must be euthanized and the negative ones monitored (Brasil, 2014a).

In areas with sporadic transmission of human VL, an entomological survey and verification of the presence of sand flies should be carried out, notification and investigation of human cases, follow-up of treatment and diagnosis of patients. For the dogs must be made the active search of animals with clinical suspicion, census survey performing serology, as previously mentioned, besides the control of the wandering population (Brasil, 2014a).

In areas with moderate to severe transmission of human VL, the diagnosis and treatment of infected humans should be investigated and accompanied by an entomological survey with indication of chemical control at the site of transmission. Regarding canine reservoirs, in areas with confirmed human cases, an annual census survey and serology of dogs should be carried out at the site of transmission. When in

non-human cases, the canine survey will be annual with serological tests, and when the prevalence of canine VL is greater than 2% positive animals should be euthanized and the negative ones monitored. When the prevalence is less than 2%, the annual census survey should be done with serological examination and euthanasia of the positive ones besides surveillance for new cases of canine VL (Brasil, 2014a).

In Brazil, the canine VL vaccine is available commercially, but it was not approved in the test phase III and its commercialization was suspended in 2014 (Brasil, 2014b). The test phase III for canine VL vaccine is based on the ability to stimulate immune responses in the dog, in estimating vaccine potency and carrier status; in testing doses, various schedules and different adjuvants; be safe for man against existing biological vectors; to allow the identification of immunological markers that allow the distinction between vaccinated and infected dogs with the monitoring of signs and clinical parameters vital to animal health, among other special features that may influence the vaccine response. At the end, the vaccine should have a minimum vaccination efficacy of 85%, being suitable for provisional licensing by the Ministry of Agriculture, Livestock and Supply (MAPA) (Brasil, 2005).

2.7 DIAGNOSTIC

The diagnosis of VL is based on clinical signs, parasitological, serological and molecular tests (Bañuls; Hide; Prugnolle, 2007).

The parasitological tests detect the parasite in direct examination (imprint), culture or inoculation in laboratory animals. In order to perform the parasitological examinations, materials such as leukocyte layer, biopsy or aspiration puncture of spleen, bone marrow, lymph node or liver (Peters, Killick-Kendrick, 1987) can be collected. Parasitological methods have high specificity (100%), but the sensitivity may vary according to the diagnostic method, material used, and trained professional (Sundar; Rai, 2002).

When visualization of the parasite is performed on a slide with the collected material (blood smear or biopsy imprint), it should be stained for visualization of the amastigote forms on a 100X objective lens. The dyes used are: Leishman, May-Grumald, Giemsa and Fast Pancho (Who, 2010). The direct smear is the simplest method, followed by printing on slides that can generate false negative results when the correct reading and processing of the material is not performed (Davies et al., 2000; Calvopina; Armijos; Hashiguchi 2004; Azevedo et al., 2011). This methodology is fast and inexpensive, but it has disadvantages such as being an invasive method to the patient because it requires a

biopsy to collect the material, the need og trained professionals and the impossibility to differentiate the species of *Leishmania* in the material (Akhoundi et al., 2017).

When the parasite isolation procedure is performed, the collected material is placed in culture medium for amastigote forms transformation into promastigotes. The culture media used may be monophasic or biphasic. The single-phase media available are: Schneider's, Grace's, Mitsuhashis-Manamosch's, LIBHIT-5, M199 and RPMI. The biphasic media are composed of agar, nutrient and rabbit blood, with Neal, Novy and Nicole (NNN) and Brain Heart Infusion (BHI) media being used more frequently, together adding a liquid medium that can be a physiological solution (NaCl 0.9%), BHI broth or Liver Infusion Triptose (LIT) (Rioux et al., 1986). The isolation of the parasite is a laborious method, which can take from 4 to 6 weeks to obtain the diagnosis, aseptic collection to reduce the contamination of the culture medium and equipment to perform the procedure (Calvopina; Armijos; Hashiguchi, 2004). The advantage of this method is that after isolation, identification and characterization of the parasite is possible using molecular techniques such as PCR, Multilocus Enzymatic (MLEE) or Multilocus microsatellite typing (MLMT) (Lanotte et al., 1981; Rioux et al. 1990; Bañuls, Hide, Prugnolle, 2007).

Serological tests evaluate the specific humoral response (Herwaldt, 1999). At the beginning of the infection, immunoglobulin M (IgM) should be investigated, while immunoglobulin G (IgG) should be investigated in the late phase or in cases of asymptomatic dogs. At the beginning of the infection, the production of IgG antibodies is low, tending to increase titration over time (Oliva et al., 2006). The most used tests are: indirect immunofluorescence reaction (IFAT), enzyme-linked immunosorbent assay (ELISA) and lateral flow tests (Gontijo; Melo, 2004; Grimaldi et al., 2012).

The IFAT is a sensitive test, however, it may present a cross-reaction with other trypanosomatids, reducing its specificity. This test requires a trained professional to interpret the result avoinding false-positive results. To perform this test it is used whole promastigote forms of the parasite (Peters; Killick-Kendrick, 1987).

The ELISA test has a high degree of sensitivity, mainly in asymptomatic hosts, and it is used for screening in seroepidemiological surveys at low cost and practicality (Who, 2003). This test is used primarily in population surveys and detects low antibody titers. The antigens are composed by promastigotes forms of *L. (L.) infantum*, which undergo rupture procedure, being called total antigens or crude antigens (Sundar; Rai, 2002; Mazieiro et al., 2014).

Side flow tests are indicated to be use in the field because of their practicality and ease of accomplishment. The main tests are based on the recombinant protein rk39 and rk26 (Gontijo, Melo, 2004, Brazil, 2006; Grimaldi et al., 2012). In Brazil, rk39 is the most

commonly used recombinant antigen, being specific for the *L. donovani* complex (Reed et al., 1990). In asymptomatic dogs of endemic area, these tests may present 96% sensitivity and 47% specificity. In the case of symptomatic dogs for VL the sensitivity is 98% (Grimaldi et al., 2012). In Brazil the TR DPP® test (Biomanguinhos) is available for dogs, in which peripheral blood can be used for analysis, with results in 15 minutes (Bio-Manguinhos, 2014).

According to the Ministry of Health (Brazil, 2014a), the diagnosis of VL in humans should be performed with the IT-LEISH® immunochromatographic rapid test.

For the diagnosis of canine VL, Joint Technical Note nº 01/2011-CGDT-CGLAB/DEVIT/SVS/MS was implemented, which provides for the application of the TR DPP® test (Biomanguinhos) in screening diagnosis, and ELISA cases of confirmatory diagnosis (Brasil, 2011b).

2.8 MOLECULAR TOOLS FOR DIAGNOSIS AND EPIDEMIOLOGICAL STUDY OF *LEISHMANIA (Leishmania) INFANTUM*

Leishmania species differentiation is considered a prerequisite for the correct diagnosis and treatment of the disease, as well as the implementation of control measures for VL (Akhoundi et al., 2017). PCR with DNA fragments evaluation has become an effective way for diagnosing VL from the 1990s (Smyth et al., 1992). With the beginning of the standardization of molecular techniques, it was used in the identification, classification and study of intra- or interspecific genetic variability of *Leishmania* complexes, resulting in an increase in the number of species described (Bañuls; Hide; Prugnolle, 2007).

According to the World Health Organization, accurate taxonomic knowledge is fundamental in practice when facts related to parasite and disease are characterized and classified (Who, 1984; Schonian et al., 2010).

Several DNA targets have been amplified for *Leishmania* such as: mini-circle of kDNA, rRNA, mini-exon and repeated genomic sequences (Arransay; Scoulica; Tselentis, 2000). Akhoundi and collaborators (2017) performed a survey showing that most of the studies used markers such as 18S, mini-exon and transcribed internal spaces (ITS); genes of proteins such as Hsp70, Hsp23, Hsp20, G6PDH and gp63 and kDNA with cytochrome B (cytB).

The 18S target is present in the small subunit (SSU) region of the rRNA and is a conserved region being a suitable marker for studies of reconstruction of phylogenetic relationships (Akhoundi et al., 2017).

The mini-exon gene is present in the order Kinetoplastidae, being composed of 39 highly conserved nucleotides, added to the 5'-end of nuclear mRNA (Marfut et al., 2003). This gene is able to differentiate *Leishmania* species from the New World, *Leishmania* and *L. chagasi* demotropic species with fragments sizes of 250, 350 and 400 bp respectively (Degrave et al., 1994; Fernandes et al., 1994). In a study evaluating phlebotomine infected with *L. (L.) infantum* the kDNA and mini-exon gene showed good markers, detecting 1 fg and 100 pg of DNA respectively. The mini-exon gene is useful in the rapid identification of *Leishmania*, since it requires a single primer to detect all species (Fernandes et al., 1994).

The ITS marker is between the 18S rRNA and 5.8S rRNA regions, which comprises a conserved region not decoded in DNA from 50 to 350 bp (Schonian et al., 2003). This marker has been used for diagnosis and identification of *Leishmania* species worldwide because of its high sensitivity and specificity (Schonian et al., 2003; 2011). According to Cupolillo et al. (1995) when using the ITS 1 and 2 markers, it is possible to differentiate the species from the subgenus *Viannia*, since these markers differ intra and interspecifically. However, Sampaio (2016) showed no intraspecific variability with the use of this marker and its use is not indicated for variability or phylogeography studies in the New World. The use of ITS as a marker followed by the restriction fragment length polymorphism (RFLP) technique has been widely used, since it could differentiate *Leishmania* species using the restriction enzyme HaeIII (Schannel et al., 2003; Rotureau et al., 2006).

The maxi-circle consists of DNA of Trypanosomatidae that codify mitochondrial protein genes and rRNA in conserved region and non-transcribed variable region (VR). The differences between the species of *Leishmania* are present in a large part of the VR, ranging from cytochrome oxidase I (highly conserved) to cytochrome oxidase III (less conserved) (Akhoundi et al., 2017). The cytochrome oxidase II gene (COII) has been used for *Leishmania* phylogeny studies, mainly of the *L. donovani* complex (Ibrahim; Barker, 2001; Cao et al., 2011).

The minicells that encode RNA are heterogeneous and are present in 95% of the kDNA (Cruz et al., 2005) being widely used as primers for PCR. The mini-circle of kDNA has a variable region that assists in the differentiation of *Leishmania* species (Lidiane, 2011).

The Hsp70 thermal shock protein gene has been used to discriminate New World *Leishmania* (Montalvo et al., 2012) by PCR followed by the use of RFLP (Garcia et al., 2004), as well as phylogenetic and taxonomic studies of the species (Akhoundi et al., 2017). Fraga et al. (2010) described a low differentiation of the *Leishmania* species of the

Viannia complex. The authors justify the low interspecific variability due to the occurrence of low ancestral gene flow in the geographic region studied.

The surface glycoprotein gp63 acts on the binding of *Leishmania* to the macrophage at the time of infection. In addition, *Leishmania* spp. is an important marker for the identification of *Leishmania* species, since it differs in terms of virulence factor among the species (Victoir et al., 1998; Mauricio et al., 1999; Guerbouj et al., 2001; 2001; 2007; Akhoundi et al., 2017).

The enzyme glucose-6-phosphate dehydrogenase (G6PD) is used for the identification of New and Old World *Leishmania* species by MLEE and Multilocus Sequence typing (MLST) techniques (Zemanova et al., 2007; Boite et al., 2012).

In addition to identifying the species, the study of genetic diversity has been extensively explored by MLEE and PCR techniques and their variants, among which are: RAPD, PCR-RFLP and MLMT (Ochsenreither et al., 2006; Silva et al., 2010). The MLST, MLMT and PCR-RFLP techniques are effective to examine intraspecific variation within the phylogenetic complexes of species (Akhoundi et al., 2016).

MLEE is the gold standard method, according to WHO, for identification and consequent classification of *Leishmania* isolates. After identification, the isoenzyme panel allows to group the isolates by zymodemas that have identical enzymatic patterns (Godfrey et al., 1976). This method is based on the mobility pattern of 15 isoenzymes (Rioux et al., 1990), in an electric field, thus verifying the difference in level of DNA sequences encoding the same (Hunter; Marketer, 1957; Murphy et al., 1990). When the band patterns of individuals differentiate, there may be a difference in the genetic basis (Murphy et al., 1990). However, the disadvantages of this method include the high cost and the fact that it is laborious (Rioux et al., 1990). The first study with isoenzymes for *L. (L.) infantum* was conducted in 1974 (Gardener et al., 1974). The use of isoenzymes in the *Leishmania* taxonomy allowed the discovery of different degrees of genetic diversity among the different species within the same complex (Kreutzer & Christensen, 1980; Grimaldi et al., 1991; Thomaz-Soccol et al., 1993; Tibayrenc et al., 1993).

The MLST method is based on the principles of MLEE. However, it is faster and easier to identify alleles at each locus of the DNA sequence (Enright; Spratt, 1999).

Molecular methods such as PCR-RFLP, RAPD and sequence-confirmed amplified region analysis (SCAR) are fast and simple methodologies widely used to verify the genetic diversity of *L. (L.) infatum* (Cuervo et al., 2004; Oliveira et al., 2004; Martin-Sanchez et al., 2004; de Castro et al., 2005; Bañuls, Hide, Prugnolle, 2007).

For the RAPD method prior knowledge of the DNA sequence is not required (Welsh; McClelland, 1990; Williams et al., 1990). This method identifies polymorphisms in

PCR amplified DNA fragments differentiating *Leishmania* isolates at the intra-species level (Hide; Bañuls; Tibayrenc, 2001; Zemanova et al., 2004). However, the electrophoresis bands are not homologous, and the technique is not reproducible (Bañuls; Hide; Prugnolle, 2007). In addition, the RAPD has markers that do not differentiate homozygous organisms from heterozygotes in a population study, since they are dominant markers (Wright, 1951; Weir; Cockerham, 1984; Meyer; Balloux, 2004; Bañuls; Hide; Prugnolle 2007; Tibayrenc, 2007).

Another accessible form of genotyping of *Leishmania* species is the use of single nucleotide polymorphisms (SNPs). These are genetic markers that can be used to evaluate the evolutionary history of populations (Brumfield et al., 2003), checking the rate of recombination, mapping and association of genomes (Bañuls; Hide; Prugnolle, 2007).

The RFLP method detects the variation between the patterns of DNA fragments produced by the digestion of DNA amplified with restriction enzymes. For the differentiation of *Leishmania* species this methodology is used with the ITS, Hsp70 and mini-exon markers (Minodier et al., 1997; Marfurt et al., 2003; Schonian et al., 2003; Montalvo et al., 2008; Akhoundi et al., 2013; Akhoundi et al., 2017).

Microsatellites are small (2 to 6 bp) sequences repeated in tandem and randomly distributed in the genome of eukaryotic cells. They are known as Short Tandem Repeats (STR) Variable Number of Tandem Repeats (VNTR) polymorphisms. Microsatellites are an important tool in genetic studies involving the differentiation of species from different pathogens (Tóth; Gaspari; Jurka, 2000). These genetic markers are used for genome mapping, genetic linkage analysis and population study (Tautz, 1989; Weber, 1990; Weissenback, 1993).

Microsatellite studies were implemented in the search for a marker able to show differences between populations of *Leishmania*. Microsatellite analysis allows differentiation of *L. (L.) infantum* isolates from different sites, such as region, country or continent, providing relevant information on the epidemiology and characterization of the agent in different geographic areas (Schwenkenbecher et al., 2006; Montoya et al., 2007; Al-Jawabreh et al., 2008; Alam et al., 2009; Oddone et al., 2009; Mahnaz et al., 2011; Downing et al., 2012; Gouzelou et al., 2012; Aluru et al., 2015).

The first microsatellite study to differentiate populations of *L. (L.) infantum* and *L. donovani* was performed by Rossi et al. (1994). From then on, several markers have been designed and evaluated. The first study with microsatellite designs based on the Leishmania DNA sequence was done by Jamjoom and collaborators in 2002. In the last two decades, several studies with markers were performed (Table 1) and the markers that obtained the best results and applicability were: LIST7031, LIST7039 (Jamjoom et al.,

2002), Lm2TG, TubCA, Lm4TA, Li41-56, Li46-67, Li22-35, Li23-41, Li45-24, Li71-33, Li71-5/2, Li71-7 (Ochsenreither et al., 2006) and CS20 (Kuhls et al., 2007).

Kuhls et al. (2007) identified six populations of *L. (L.) infantum* with a high degree of genetic isolation with the microsatellite panel. The genetic differentiation observed between populations of the Mediterranean and New World regions suggested the existence of two species (*L. donovani* and *L. infantum*) previously defined by other intrinsic characters (Rioux et al., 1990; Ashford, 2000; Jamjoom et al. 2002; Kuhls et al., 2007). In another study, Kulhs et al. (2008) analyzed isolates of zymodeme MON-1 with microsatellites, being able to identify important facts of the epidemiology of *L. (L.) infantum* whose isolates were grouped into a single zymodeme (MON-1).

Thus, the microsatellite markers are applicable to the phytogeography of *L. (L.) infantum*, since it comprises a tool with hypervariable, genetically neutral and co-dominant markers being ideal for fine-scale analysis of recent genetic alterations (Kuhls et al. al., 2007).

Studies with microsatellites in populations of *L. (L.) infantum* have been used also in the New World. Ferreira and collaborators (2012) analyzed isolates of *L. (L.) infantum* from Brazil with 14 markers and the results corroborate with the hypothesis described by Antonialli et al. (2007) that the recent VL epidemic observed in the states of Mato Grosso do Sul and São Paulo was disseminated due to the construction of the Bolivia-Brazil gas pipeline with the arrival of workers and their dogs from Bolivia to Mato Grosso do Sul and later São Paulo and Minas Gerais states (Motoie et al., 2013).

Microsatellites were also used in the differentiation of *L. (L.) chagasi* and *L. (L.) infantum* isolates from different regions of the world. The results obtained suggest that *L. (L.) chagasi* is a subpopulation of *L. (L.) infantum* imported from southern Europe and introduced into the Americas by dogs, probably in the 16th century (Kuhls et al., 2011; Leblois et al. Aluru et al., 2011).

Currently the migration of people or dogs with VL to endemic regions with the presence of sand flies is characterized as an important risk factor for the dispersal of *L. (L.) infantum* (Who, 2012). Marker studies that are capable of understanding the dispersion of the parasite are of supreme importance especially in areas of inter-country borders.

Table 1: List of works with microsatellite markers according to the analyzed material (isolated from *L. (L.) infantum* or strain bank) and parents of origin.

Year	Usats	Isolates or strain	Country	Reference
2002	LIST7021, LIST7022, LIST7023, LIST7024, LIST7025, LIST7026, LIST7027, LIST7028, LIST7029, LIST7030, LIST7031, LIST7032, LIST7033, LIST7034, LIST7035, LIST7036, LIST7037, LIST7038, LIST7039, LIST7040	Strain	Sudan, Spain. Brazil, Iran, Ethiopia, UK and Bangladesh	Jamjoom et al., 2002
2006	Li22-35, Li23-41, Li41-56, Li45-24, Li 46-67, Li71-5/2, Li71-7, Li71-33, Lm2TG, Lm4TA, TubCA, Li21-34, Li71-19, Li71-42, Li72-14, Li72-17/2, Li72-20	Strain	Tunisia. France, Spain, Portugal, Greece, China. Turkey, Israel, Panama, Brazil, Costa Rica, Italy, Malta, Sudan, India, Ethiopia	Ochsenreither et al., 2006
2007	Lm2TG, Li41-56, Li46-67, Li22-35, Li23-41, Li45-24, Li71-33, Li71-5/2, Li71-7, TubCA, Lm4TA, CS19, CS20, LIST7031, LIST7039	Strain	East Africa, India, Mediterranean Region	Kuhls et al., 2007
2008	Lm2TG, Li41-56, Li46-67, Li22-35, Li23-41, Li45-24, Li71-33, Li71-5/2, Li71-7, TubCA, Lm4TA, CS20, LIST7031, LIST7039	Strain	Spain, Portugal, France. Italy, Malta, Greece, Turkey, Israel, Tunisia	Kuhls et al., 2008
2008	Lm2TG, TubCA, Lm4TA, Li 41-56, Li 46-67, Li 22-35, Li 23-41, Li 45-24, Li 71-33, Li 71-5/2, Li 71-7, LIST7031	Strain	Tunisia, Algeria, France, Spain, Portugal	Seridi et al., 2008
2009	Lm2TG, Li41-56, Li46-67, Li22-35, Li23-41, Li45-24, Li71-33, Li71-5/2, Li71-7, TubCA, Lm4TA, CS20, LIST7031, LIST7039	Strain	Tunisia, France, Germany, Spain, Algeria, Turkey	Chargui et al., 2009
2011	Lm2TG, Li41-56, Li46-67, Li22-35, Li23-41, Li45-24, Li71-33, Li71-5/2, Li71-7, TubCA, Lm4TA, CS20, LIST7031, LIST7039	Strain	Costa Rica, Panama, Honduras, Venezuela, Colombia, Paraguay, Brazil. Spain, Portugal, France, Italy, Greece, Turkey, Malta, Tunisia, Algeria, Israel, Palestine, Uzbekistan, China, Sudan, Ethiopia, Kenya, India	Kuhls et al., 2011
2011	Lm2TG, Li41-56, Li46-67, Li22-35, Li23-41, Li45-24, Li71-33, Li71-5/2, Li71-7, TubCA, Lm4TA, CS20, LIST7031, LIST7039	Strain	Europe, Africa , Asia, Honduras, Panama, Costa Rica, Colombia, Venezuela, Paraguay and Brazil	Leblois et al., 2011
2012	Li22-35, Li23-41, Li45-24, Li71-33, Lm2TG, Lm4TA, TubCA.	Isolates	Brazil	Batista et al., 2012
2012	Lm2TG, Lm4TA, Li 41–56, Li 46–67, Li 22–35, Li 23–41, Li 45–24, Li 71–33, Li 71–5/2, Li 71–7, LIST7031, LIST7039, TubCA, CS20.	Strain	Brazil	Ferreira et al., 2012
2012	Li22-35, Li23-41, Li45-24, Li71-33, Lm2TG, Lm4TA and TubCA	Isolates	Brazil	Segato et al., 2012
2013	Li71-5/2, Li72-14, List7023, List7040, List7029, Li71-19, List7039, Li71-33, List7022, List7028, Li71-7, List7030, ISA136, Li45-24, ST436, Lm4TA	Isolates	Brazil	Motoie et al., 2013
2014	Lm2TG, Lm4TA, Li 41–56, Li 46–67, Li 22–35, Li 23–41, Li 45–24, Li 71–33, Li 71–5/2, Li 71–7, LIST7031, LIST7039, TubCA, CS20	Isolates	Portugal	Cortes et al., 2014
2014	Li22-35, Li41-56, Li46-67, Li71-7, Li71-33	Isolates	Ethiopia	Gelanew et al., 2014

2015	Li22-35, Li45-24, Li71-5/2, Li72-20, LiBTA, LiBTG, List7021, List7024, List7025, List7026, List7028, List7031, List7033, List7035, List7037, List7038, List7039, Rossi1, Rossi2, TubCA	Isolates	Spain	Tomás-Perez et al., 2015
2016	LiBTG, LiBTA, LIST7021, LIST7025, LIST7026, LIST7031, LIST7033, Li22-25, Li45-24, TubCA, Li71-5/2, Rossi2	Strain	France	Pomares et al., 2016

3 ARTICLE 1

THE ITS1 AS BARCODE IN LEISHMANIA SPECIES IDENTIFICATION IN A NEW FOCUS OF VISCERAL LEISHMANIASIS

Short Title: Identification of *Leishmania* in the triple frontier

Aline Kuhn Sbruzzi Pasquali[a,b], Rafael Antunes Baggio[a], Luciana Chiyo[c], Vanete Thomaz-Soccol[a,b] *

a. Laboratório de Biologia Molecular, Departamento de Engenharia de Bioprocessos e Biotecnologia, Universidade Federal do Paraná, Brazil.
b. Programa de Pós-Graduação em Bioprocessos e Biotecnologia, Universidade Federal do Paraná, Brazil.
c. Centro de Controle de Zoonoses, Prefeitura Municipal de Foz do Iguaçu, Paraná, Brazil

Manuscript formatted as the guidelines of PLos One journal

3.1 ABSTRACT

Leishmaniasis is a zoonosis caused by the protozoan parasite *Leishmania* Ross, 1903, of which there 22 species are present in the New World Region. There are several ways of identifying species of *Leishmania,* and in nowdays several molecular tools are used preferentially. Among thenhighlighting is give to the internal transcribed spacer 1 (ITS1). For this reason, we used this marker to identify *Leishmania* isolates obtained from dogs in the western region of Parana and compared the results with to isolates from Brazil, European and African countries. To complete the analysis the results of our isoltes were compared to sequences of *Leishmania* species deposited on GenBank for this marker. All isolates of the parasites obtained were grouped into the *L. donovani* complex. The use of the sequence of this marker didn´t allow the separation of the *Leishmania* phylogenetic complexs previously described. The genetic distances and Neighboor Joining analyses support that the ITS 1 is an effective marker to assing New World *Leishmania* parasites to species groups, but it´s of limited utility most species.

Key-words: Dogs, Foz do Iguaçu, ITS1, *Leishmania*, leishmaniasis, Neotropical region, rRNA.

3.2 INTRODUCTION

Leishmaniases are zoonosis caused by the protozoan parasite *Leishmania* Ross, 1903. The disease is present in 98 countries in the tropical, subtropical and Mediterranean regions, with 350 million people at risk and 12 million cases of infection [1,2]. There are two principal clinical manifestation forms: cutaneous (CL) and visceral leishmaniasis (VL) [1,3]. Both forms are present in 12 countries of the Americas, with 70% of CL cases and 96% of VL reported in Brazil [4].

Specifically, the extreme west of Paraná state, Brazil, was considered free of VL until 2012, when the first case of autochthonous canine VL was reported [5-10], followed by the first human VL case in 2015 [7-10]. The clinical cases were initially attributed to *Leishmania infantum*, but more recently another species *L. braziliensis* was recorded by Thomaz-Soccol *et al.* [11].

The parasite taxonomic caracterization is important to understand the clinical manifestations of the disease and to make a correct diagnosis and prognosis, aiding in the treatment and control of the disease [12]. According to Lainson and Shaw [13] and Lainson [14], there are about 22 species of *Leishmania* in the New World, distributed in two subgenera: *Leishmania* and *Viannia*. Among them, *Leishmania amazonensis, L.*

mexicana, L. braziliensis, L. peruviana, L. guyanensis, L. panamensis, L. lainsoni, L. naiffi, L. shawi, L. colombiensis and *L. lindenbergi* cause CL, while *L. (L.) infantum* cause VL.

Initially, these parasites were identified using the symptoms and the geographic area of the transmition. However, symptoms can vary among individuals and can be confused with symptoms caused by other etiological agents. Furthermore, the geographic area cannot be used reliably for species identification, since more than one species may occur at an area, and *Leishmania* parasites are easily transported, even transcontinentally, by their reservoirs and vectors. The morphological identification of *Leishmania* species is problematic since species are externally similar [15]. To aid in species identifications, genetic tools have been developed. In the beginning of 90s Thomaz-Soccol et al. [16] using such as Multi-Locus Enzyme Electrophoresis (MLEE) protocol propose a new taxonomy to *Leishmania* species, confirming the two subgenera and separing in phylogenetic complexes. Even though it is considered a reliable identification tool, this technic is slow, expensive and laborious [17,18,12]. More recently, other methods based on Polymerase Chain Reaction (PCR) and its variants, as such PCR-RAPD, PCR-RFLP, PCR followed by sequencing and microsatellites have been proposed [19-21]. Specifically, the rRNA has been described as the Trypanosomatidae barcode [22], and the internal transcribed spacer 1 (ITS1) region is one of the most used fragments to identify species of *Leishmania* [23-29]. However, to our knowledge, there has been no comprehensive study assessing the genetic differentiation among *Leishmania* species using large data sets from different geographic areas, to test the effectiveness of the ITS1 for species identifications. The use of these molecular tools is important to understand the CL and VL epidemiological scenario, because it permits discriminate the entry of different species and verify their sympatric distribution and disease propagation through vectors and reservoirs.

This paper aimed to isolate and identify *Leishmania* species that have caused canine leishmaniasis in the extreme west of the Paraná State (Brazil). For this, we sampled dogs with leishmaniasis from the extreme west of Paraná state (Brazil), to identify which *Leishmania* species are present in this region. The discriminatory power of the ITS marker was then evaluated through sequences deposited in the GenBank.

3.3 MATERIAL AND METHODS

3.3.1 *Leishmania* specimens isolation and identification

The project was submitted and approved by the Ethics Committee of the Federal University of Parana (protocol no. 044/2014).

To prospect the species of *Leishmania*, dogs with clinical signs of leishmaniasis (onychogrifose, lymphadenomegaly, weight loss) from Foz do Iguaçu and Santa Terezinha de Itaipu were sampled between 2013 and 2016. Bone marrow, lymph nodes aspirates and leukocyte layer were collected from positive dogs in serological tests. To isolate the parasite the material was inoculated in Neal, Novy and Nicole (NNN) culture medium with saline solution 0.9% for one week at 24ºC. After isolation, the parasites are cultured in Brain Heart Infusion (BHI) with saline solution 0.9% at 24ºC.

Additionally, *L. (L.) infantum* coming from the collection of the Molecular Biology Laboratory, Department of Engineering of Bioprocesses and Biotechnology, Federal University of Parana, previously identified by RAPD-PCR and K26F/R primers [30], and isolates from the European and African continent gently donated by the researcher Jean Pierre Dedet in the Centre de Ressources Biologiques des *Leishmania* (CRB du CHU de Montpellier, France) were included in the analyses.

The promastigote forms were cultivated in BHI with saline solution 0.9% at 24ºC until reach 10^7 parasites/ mL [30]. The parasites were harvested by centrifugation and washed first with saline solution 0.9%, followed by a washed with 0.3% saline solution and finally with 0.9%. In each step the parasites were recovered by centrifugation at 5,000*g* for 10 min at 4º.C. DNA extraction was performed by phenol/chloroform/isoamyl alcohol method [31-33].

3.3.2 Genetic Analyses

The 350 pb sequence of the ITS1 fragment were amplified using the primers LITSR and L5.8S [15]. The 24 µL PCR amplifications contained 1.4 mM $MgCl_2$, 1 µg BSA, 0.2 mM dNTP, 0.1 pmol each primer, 0.9 UI of *Taq* polymerase (Invitrogen®, USA) and 10 ng DNA. The PCR conditions started with initial denaturation at 94ºC for 4 min, followed by 40 cycles with a denaturation step at 94ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 1 min, and a final extension cycle at 72ºC for 10 min. The cycle procedure was performed in a thermocycler Biocycler, MJ96 (Biosystems®). DNA of *L. infantum* reference strain was used as positive control, and water as the negative control in each reaction. The products of the PCR amplification were subjected to electrophoresis in 1.5% agarose gel to confirm the amplification and visualized after ethidium bromide and staining visualized under UV light. The positive amplifications were purified through precipitation with ammonia acetate and absolute ethanol [33]. The PCR purified product was amplified with marked dNTP and precipitate with ammonium acetate and ethanol PA. The sequencing was performed in 3.500 XL Genetic Analyzer (Applied Biosystems).

All DNA samples sequenced were aligned using MAFFT 7.0 [34] in the Guidance web server and Geneious 4.0.4 software [35-37]. The final alignment was composed of 320 bp sequences, including the indel mutations.

A tree was constructed using all the GenBank sequences in MEGA 7.0 [38], using the Neighbor-Joining (NJ) algorithm. The NJ algorithm has been the DNA Barcode identification method of choice, since it is able to discriminate among large numbers of specimens more quickly than other algorithms [39]. The substitution model (TrN – [40], with gamma rate variation) was defined by the software jModeltest 2.1.10 [41], and the robustness of the NJ tree was assessed using 1,000 bootstrap replicates.

3.4 RESULTS AND DISCUSSION

3.4.1 *Leishmania* specimens isolation and identification

A total of 98 *Leishmania* isolates were obtained from lymph nodes, bone marrow and leukocyte layer puncture. From these isolates 96 dog's samples were from Foz do Iguaçu and two samples from Santa Terezinha do Itaipu.

All isolates were positives for *Leishmania* in PCR using ITS (Fig. 2). After sequencing, the fragments showed 100% of similarity with *L. infantum* sequences deposited in GenBank (accession numbers MF945579 to MF945584).

a)

b)

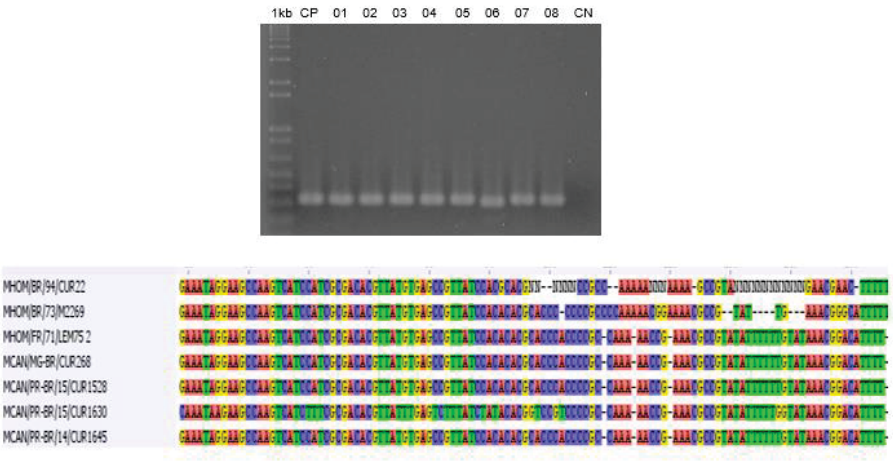


Figure 2: a) Example of amplified PCR product using ITS1. b) Example of alignment performed using Genious basic 4.0.4 software.

In the second step we compared the 98 strains with others isolates coming from other stats of Brazil. In the total 117 strains was identified (Table 2).

Table 2: Number of strains isolates in infected dogs with *Leishmania* and strains coming from another region

City/State/Country	N
Foz do Iguaçu/Paraná/Brazil	96
Santa Terezinha de Itaipu/Paraná/Brazil	2
Maringá/Paraná/Brazil	1
São Paulo/Brazil	3
Sergipe/Brazil	1
Minas Gerais/Brazil	4
Mato Grosso/Brazil	1
Ceará/Brazil	1
Mato Grosso do Sul/Brazil	1
France	2
Argelia	1
Spain	2
Portugal	1
Egypt	1
Total	**117**

In addition, after amplification by PCR and sequencing, our isolates were compared with sequences deposed on GenBank. A total of 486 ITS1 sequences were obtained (Table 3). ITS1 sequences of *L. aristidesi*, *L. enriettii*, *L. forattinii*, *L. hertigi*, *L. deanei*, *L. colombiensis*, *L. garnhami*, *L. pifanoi*, *L. equatorensis* are not present in that database. These are the Neotropical species of *Leishmania* according to Lainson [14].

Moreover, considering the similarity between *L. (L.) infantum chagasi*, *L. (L.) infantum* and *L. donovani* in the *L. donovani* complex [18,14], 188 sequences of *L. (L.) infantum* and 133 of *L. donovani* were also used.

Table 3: Principal species and number of sequences from GenBank.

Species	Number of sequences
L. amazonensis	25
L. braziliensis	38
L. guyanensis	20
L. (L.) infantum chagasi	29
L. lainsoni	4
L. Lindenberg	1
L. Mexicana	26
L. naiffi	4
L. panamensis	10
L. peruviana	5
L. shawi	1
L. utingensis	1
L. venezuelensis	1
L. (L.) infantum	188
L. donovani	133

The average genetic distance within American *Leishmania* species (only species with more than 10 sequences in the Genbank) ranged between 0.000 ± 0.000 (*L. infantum chagasi*) and 0.010 ± 0.003 (*L. amazonensis*). The average genetic distance between species ranged between 0.001 ± 0.001 (between *L. guyanensis* and *L. panamensis*) and 0.233 ± 0.039 (between *L. guyanensis* and *L. amazonensis*). The genetic distances separated species into three groups, with low genetic distance between the species within these groups (lower than 0.020). But, large genetic differentiation between species from different groups (values between 0.107 ± 0.022 – *L. mexicana* x *L. donovani*, and 0.233 ± 0.039 - *L. guyanensis* and *L. amazonensis*). The three groups were here named as follows: *L. donovani* cluster (*L. donovani, L. (L.) infantum* and *L. (L.) infantum chagasi*), *L. mexicana* cluster (*L. mexicana* and *L. amazonensis*), and *Viannia* cluster (*L. braziliensis*, *L. panamensis* and *L. guyanensis*).

In NJ tree were included the 98 sequences isolated from *L. (L.) infantum* of Foz do Iguaçu (96) and Santa Terezinha do Itaipu (02), Paraná. All sequences were grouped in the *L. donovani* cluster among *L. (L.) infantum, L. infanum chagasi* and *L. donovani* (Fig. 3). The identification of our isolates supported that *L. (L.) infantum* is the principal species of *Leishmania* circulating in western region of Paraná. Our work corroborates the results of Thomaz-Soccol et al. [11] that supported the cVL prevalance in Foz do Iguaçu and its

dispersion to the neighbor county Santa Terezinha de Itaipu. These results indicate that this region is endemic for VL.

The NJ tree and the genetic distances between the American species of *Leishmania* using ITS1 sequences from Genbank supported three groups of species. The first one, the *L. mexicana* cluster, is composed of *L. mexicana* and *L. amazonensis*, with each of the two species on a unique and exclusive branch, and high bootstrap support (higher than 0.8). The second group includes the species of the subgenus *Viannia: L. braziliensis*, *L. guyanensis*, *L. lainsoni*, *L. lindenberg*, *L. naiffi*, *L. panamensis*, *L. peruviana*, *L. shawi* and *L. utingensis*. The third one, the *L. donovani* cluster, includes *L. (L.) infantum*, *L. (L.) infantum chagasi* and *L. donovani*.

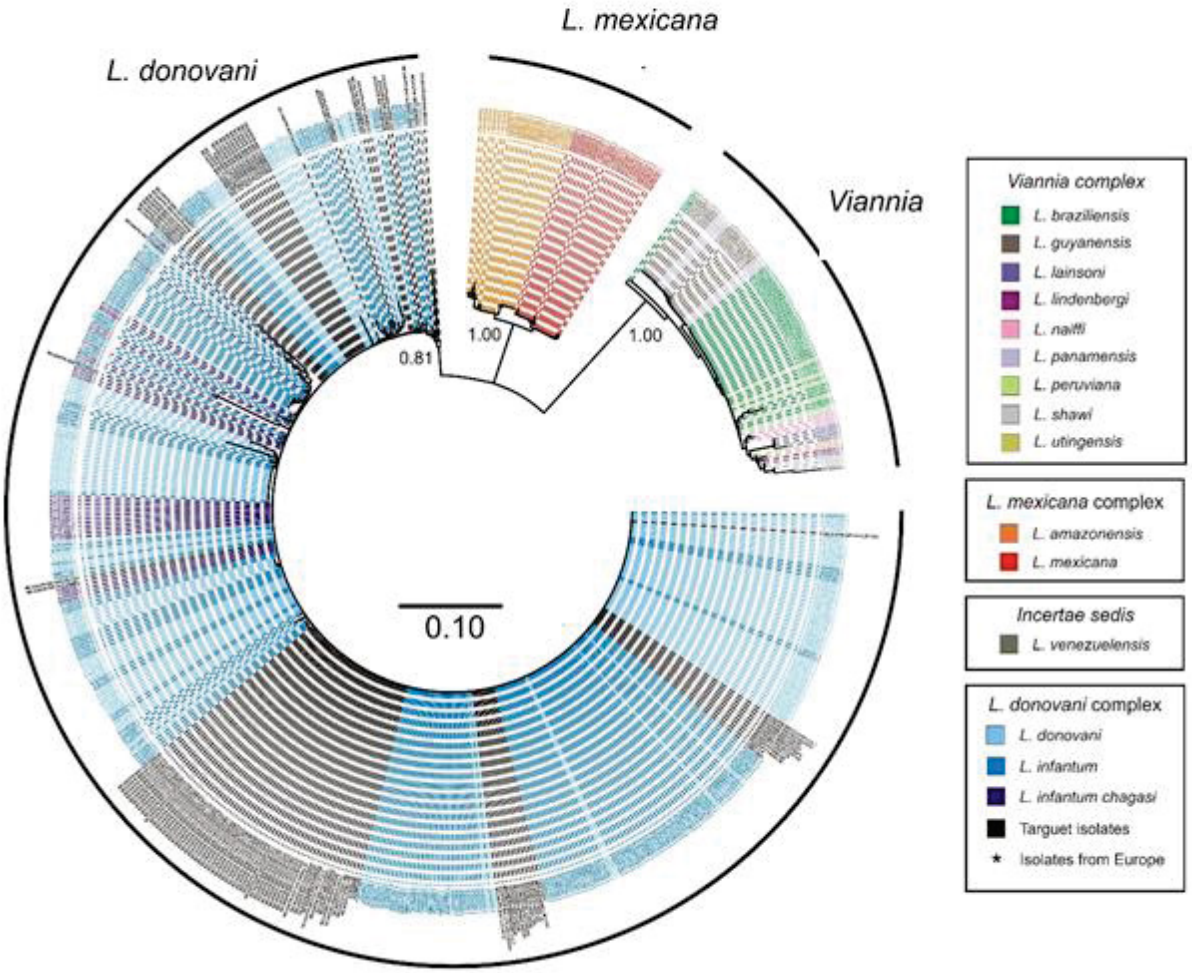


Figure 3: Synthetized Neighbor-joining tree with *Leishmania* target isolates. Only a subset of the individuals of each species and the target parasites from dogs and Phlebotominae are shown.

The rRNA has been described as the Trypanosomatidae barcode, our results support that ITS1 sequences are only effective to assign *Leishmania* parasites to species groups, being less suitable for species identifications. This may be due to the fact that

ITS1 is conserved at the level of species with low mutation rates, and/or that species definitions/limitations need to be reviewed. These facts represent a great problem in the definition of species in parasites with clonal reproduction. Schonian et al. [15], developed a method to identify *Leishmania* species using PCR-RFLP on ITS1 fragments in two steps. The authors found a genetic differentiation between *Viannia* subgenus (i.e. *L. braziliensis*, *L. guyanensis* and *L. panamensis*). However, in this study we evaluated ITS1 PCR fragments, the results showed that these species separation is not supported by the sequencing approach. Our results are supported by the work Marcili et al. [22], using rRNA and gGAPDH gene, and Harkins et al. [42], using phylogenomics, founded a similar topology that comprises these three groups of New World *Leishmania*.

According to our results, the identification of species in the *L. donovani* cluster using ITS1 sequences is unreliable. The sequences of *L. (L.) infantum*, *L. (L.) infantum chagasi* and *L. donovani* clustered in interleaved branches with low support. The taxonomic validity of these species has been debated [14,15,18,43-45]. According to the phylogenetic concept of species (see [46] for details), *L. (L.) infantum* and *L. (L.) infantum chagas*i are not distinct from *L. donovani* and, therefore, they cannot be considered three different species. It is very likely that *L. donovani*, *L. (L.) infantum* and *L. (L.) infantum chagasi* are one species, provisionally referred to as the *L. donovani* complex, with regional genetic differentiation, as proposed by Lukes et al. [18].

Differently from the other groups, the distinction between the branches of *L. mexicana* and *L. amazonensis* in the *L. mexicana* complex were well supported. This suggests that the ITS1 sequences are efficient for the identification of these two species. The validity of these species, however, when tested with other markers, has been questioned. For example, Fotouhi-Ardakani et al. [46] found two non-monophyletic groups of *L. mexicana* using nuclear and mitochondrial genes. Fraga et al. [45] did not find exclusive branches for each of these species using the conserved hsp70. Alternatively, they were distinguished in the results of Berzunza-Cruz et al. [47], using ITS rDNA sequences. The distributions of these species are discontinuous: *L. mexicana* is present in Central America, Mexico and southern USA, while *L. amazonensis* is present in northern South America, Brazil and Paraguay [14]. Although most of these studies, including our, have found that the branches that cluster these species are short, they are strongly supported, suggesting that they may represent different lineages.

In our reconstruction, a large number of New World *Leishmania* species clustered in more than one unique and exclusive branch (i.e. *L. (L.) infantum*, *L. (L.) infantum chagasi*, *L. donovani* and *L. braziliensis*, *L. guyanensis*, *L. panamensis*). Since GenBank staff members do not confirm the identification of the specimens from which sequences are

submitted, it is possible that the some of the sequences deposited there and used in this study came from incorrectly identified specimens. This could potentially introduce error in the analysis, for instance sequences from one species clustering in different branches. We do not believe that this has been a problem in our study, since most of the GenBank sequences we used had been obtained from isolates of reference *Leishmania* species. Moreover, the branches in the *L. donovani* complex and in the *Viannia* group were weakly supported, and this result would persist even if the sources of some of these sequences were incorrectly identified. Furthermore, fixing occasional mistakes would not increase support for the branches found in our study. In conclusion, possible errors in the identification of specimens and sequence attribution on GenBank does not affect our conclusion that ITS1 sequences are of limited use in the identification of most New World *Leishmania* species.

Concluding, the genetic distances and NJ analyses support that the ITS1 is an effective marker to assign Neotropical *Leishmania* parasites to species groups, but it is of limited utility to identify most species. The delimitation and identification of *Leishmania* species is very important in the development of strategies to prevent the spread of Leishmaniasis.

3.5 ACKNOWLEDGEMENTS

We thank the International Development Research Centre (IDRC-Canada grant number 107577-002); the National Council for Scientific and Technological Development (CNPq grant number Grant No. 307387/2011-9 and 480292/2012-4); the Paraná Araucaria Foundation for Scientific and Technological Development for the financial support (Grant No. 122/2010 -protocol 17401); the Secretariat of Health of the State of Paraná. RAB are fellows of CNPq, CAP and AKSP are fellows of Capes.

3.6 AUTHOR CONTRIBUTIONS

Project administration: Vanete Thomaz Soccol.
Conceived and designed the experiments: Vanete Thomaz-Soccol, Rafael Antunes Baggio.
Performed the experiments: Aline Kuhn Sbruzzi Pasquali, Rafael Antunes Baggio, Luciana Chiyo, Vanete Thomaz-Soccol.

Analyzed the data: Aline Kuhn Sbruzzi Pasquali, Rafael Antunes Baggio, Vanete Thomaz Soccol.

Writing – original draft: Aline Kuhn Sbruzzi Pasquali, Rafael Antunes Baggio.

Writing – review and editing: Aline Kuhn Sbruzzi Pasquali, Rafael Antunes Baggio, Vanete Thomaz-Soccol.

3.7 REFERENCES

1 Moreno J, Alvar J. Canine leishmaniasis: epidemiological risk and the experimental model. Trends Parasitol. 2002;18: 399–405. http://dx.doi.org/ 10.1016/S1471-4922(02)02347-4 PMID: 12377257

2 Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis Worldwide and global estimates of its incidence. PLoSOne. 2012; 7: e35671.https://doi.org/10.1371/journal.pone.0035671 PMID: 22693548

3 World Health Organization. Leishmaniasis: Informe Epidemiológico de las Américas. Informe Leishmaniasis. 2016; 4. Avaliable from: http://www.paho.org/hq/index.php?option=com_topics&view=readall&cid=6722&Itemid=40754&lang=es

4 WHO, World Health Organization. Leishmaniases: Epidemiological report of the Americas. Leishmaniases report. 2017; 5. Avaliable from: http://www.paho.org/hq/index.php?option=com_topics&view=readall&cid=6722&Itemid=40754&lang=es

5 Silva EA, Andreotti R, Dias ES, Barros JC, Brazuna JCM. Detection of *Leishmania* DNA in phlebotomines captured in Campo Grande, Mato Grosso do Sul, Brazil. Exp Parasitol. 2008;119(3): 343–348. https://doi.org/10.1016/j.exppara.2008.03.011

6 Thomaz-Soccol V, Castro EA, Navarro IT, Farias R, Souza LM, Carvalho Y, et al. Casos alóctones de leishmaniose visceral canina no Paraná, Brasil: implicações epidemiológicas. Rev Bras Parasitol Vet. 2009;18(3): 46–51. http://doi.editoracubo.com.br/10.4322/rbpv.01803008

7 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. http://dx.doi.org/10.1590/S0037-86822012000500019

8 Dias RCF, Thomaz-Soccol V, Bisetto Jr A, Pozzolo EM, Chiyo L, Freire RL, et al. Occurrence of anti-*Leishmania* spp. antibodies in domiciled dogs from the city of Foz do Iguaçú, state of Paraná, Brazil. In: WORLD CONGRESS ON 5, Porto de Galinhas. Abstract. Porto Galinhas: Soc Brasil Med Trop. 2013: 875–876.

9 Thomaz-Soccol V, Luz E, Bisetto-Jr A, Castro EA, Ferreira-Costa ES, Navarro IT. Visceral and Cutaneous in the Paraná State, Southern of Brazil border with Argentina and Paraguay. In: WORLD CONGRESS ON 5, Porto de Galinhas. Abstract. Porto Galinhas: Soc Brasil Med Trop. 2013: 76–77.

10 Trench FJP, Ritt AG, Gewehr TA, de Souza Leandro A, Chiyo L, Gewehr MR, et al. First Report of Autochthonous Visceral Leishmaniosis in Humans in Foz do Iguaçu, Paraná State, Southern Brazil. Ann Clin Cytol Pathol. 2013;2(6): 1041. https://www.jscimedcentral.com/ClinicalCytology/ clinicalcytology-2-1041.pdf

11 Thomaz-Soccol V, Pasquali AKS, Pozzolo EM, André Souza Leandro AS, Chiyo L. et al. More than the eyes can see: the worrying scenario of canine leshmaniasis in the Brazilian side of the triple border. PloSOne. 2017;12(12) https://doi.org/10.1371/journal.pone.0189182

12 Schonian G, Mauricio I, Cupolillo E. Is it time to revise the nomenclature of *Leishmania*?. Trend Parasitol. 2010; 26: 466–469. http://dx.doi.org/10.1016/ j.pt.2010.06.013 PMID: 20609626

13 Lainson R. Shaw JJ. New World Leishmaniasis. In: Cox FEG. Wakelin D. Gillespie SH. Despommier DD. Topley & Wilson's Microbiology and Microbial Infections. London: Wiley & Blackwell. 2005: 313–349. DOI: 10.1002/9780470688618.taw0182

14 Lainson R. The Neotropical *Leishmania* species: a brief historical review of their discovery, ecology and taxonomy. Rev Pan-Amer Saude. 2010; 1: 13–32. https://doi.org/10.5123/S2176-62232010000200002

15 Schonian G, Naserddin A, Dinse N, Scheweynoch C, Schallig HDFH, Presber W, et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis. 2003;47(1): 349–358. https://www.ncbi.nlm.nih.gov/pubmed/12967749 PMID: 12967749

16 Thomaz-Soccol V, Lanotte G, Rioux JA, Pratlong F, Martini-Dumas A, Serres E. Phylogenetic taxonomy of new world *Leishmania*. Ann Parasitol Hum Comp. 1993;68(2): 104–106. https://www.ncbi.nlm.nih.gov/pubmed/ 7692803 PMID: 7692803

17 Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. Ann Parasitol Hum Comp 1990;65(3): 111–125. https://doi.org/10.1051/ parasite/1990653111 PMID: 2080829

18 Lukes J, Mauricio IL, Schonian M, Dujardin JC, Soterladou K, Dedet JP, et al. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. Proc Natl Acad Sci USA. 2007;104(22): 9375–9380. http://www.pnas.org/content/104/22/9375 PMID: 17517634

19 Ochsenreither S, Kuhls K, Schaar M, Presber W, Schonian G. Multilocus microsatellite typing as a new tool for discrimination of *Leishmania infantum* MON-1 strains. J Clin Microbiol. 2006;44(2): 495–503. http://jcm.asm.org/ content/44/2/495 PMID: 16455904

20 Silva SDEO, Wu AA, Evans DA, Vieira LQ, Melo MN. *Leishmania* sp. isolated from human cases of cutaneous leishmaniasis in Brazil characterized as *Leishmania* major-like. Acta Trop. 2009;112(3): 239–248. https://doi.org/10.1016/j.actatropica.2009.07.026 PMID: 19660430

21 da Silva SM, Rabelo PFB, Gontijo NF, Ribeiro RR, Melo MN, Ribeiro VM, et al. First report of infection of *Lutzomyia longipalpis* by *Leishmania* (*Leishmania*) *infantum* from a naturally infected cat of Brazil. Vet Parasitol. 2010;174(1-2): 150–154. https://doi.org/10.1016/j.vetpar.2010.08.005 PMID: 20832944

22 Marcili A, Sperança MA, Da Costa AP, Madeira M de F, Soares HS, Sanches CdeOCC, et al. Phylogenetic relationships of *Leishmania* species based on trypanosomatid barcode (SSU rDNA) and gGAPDH genes: Taxonomic revision of *Leishmania (L.) infantum chagasi* in South America. Infec Genet Evol. 2014;25: 44–51. https://doi.org/10.1016/j.meegid.2014. 04.001 PMID: 24747606

23 el Tai NO, Osman OF, el Fari M, Presber W, Schoenian G. Genetic heterogeneity of ribosomal internal transcribed spacer (ITS) in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphism (SSCP) and sequencing. Trans R Soc Trop Med Hyg. 2000; 94: 575–579. https://www.ncbi.nlm.nih.gov/ pubmed/11132393 PMID: 11132393

24 Schonian G, Akuffo H, Lwein S, Maasho K, Nylen S, Pratlong F, et al. Genetic variability within the species *Leishmania aethiopica* does not correlate with clinical variations of cutaneous leishmaniasis. Mol Biochem Parasitol. 2000; 106(2): 239–248. https://www.ncbi.nlm.nih.gov/ pubmed/10699253 PMID: 10699253

25 Schonian G, Schnur L, Fari EM, Oskam L, Kolesnikoy AA, Sokolowska-Kohler W, et al. Genetic heterogeneity in the species *Leishmania tropica* revealed by different PCR-based methods. Trans R Soc Trop Med Hyg. 2001; 95(2): 217–224. https://www.ncbi.nlm.nih.gov/pubmed/11355565 PMID: 11355565

26 Cupolillo E, Brahim LR, Toaldo CB, Oliveira-Neto MP, Brito MEF, Falqueto A; et al. Genetic polymorphism and molecular epidemiology of *Leishmania (Viannia) braziliensis* from different hosts and geographic areas in Brazil. J Clin Microbiol. 2003; 41(7): 3126–3132. http://jcm.asm.org/content/ 41/7/3126.full

27 Kuhls K, Mauricio IL, Pratlong F, Presber W, Schonian G. Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex. Microbes Infect. 2005; 7(11-12): 1224–1234. https://doi.org/10.1016/j.micinf.2005.04.009 PMID: 16002315

28 Almeida ABF, Sousa VR, Boa Sorte EC, Figueiredo FB, Paula DAJ, Pimentel MF, et al. Use of parasitological culture to detect *Leishmania* (*Leishmania*) *chagasi* in naturally infected dogs. Vector Borne Zoonotic Dis. 2011; 11(12): 1555–1560. https://doi.org/10.1089/vbz.2011.0723 PMID: 21919725

29 Ghatee MA, Sharifi I, Kuhls K, Kanannejad Z, Harandi MF, de Almeida ME, et al. Heterogeneity of the internal transcribed spacer region in *Leishmania tropica* isolates from southern Iran. Exp Parasitol. 2014; 144: 44–51. https://doi.org/10.1016/j.exppara.2014.06.003 PMID: 24932536

30 Saloe SB. Variabilidade genética de isolados de *Leishmania infantum* x *L. chagasi* procedentes de várias regiões do Brasil. M.Sc. Thesis, The Federal University of Parana. 2010. Avaliable from: http://acervodigital.ufpr.br/ bitstream/handle/1884/24977/variabilidade%20genetica%20de%20isolados%20de%20L.infantum%20L.chagasi.pdf?sequence=1

31 Sambrook J, Fritch EF, Maniatis T. Molecular cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1989.

32 Bañuls AL. Apport a la genetique evolutive a l'epidemiologie et a la toxonomie du gene *Leishmania*. Montpellier, França. Docthor thesis Montpellier University. 1998; 196f.

33 Sambrook J, Russell RW. Molecular cloning: A laboratory manual, 3th ed. Cold Spring Harbor Laboratory Press; 2001.

34 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/mst010 PMID: 2332 9690

35 Landan G, Graur D. Local reliability measures from sets of co-optimal multiple sequence alignments. Pacific Symposium on Biocomputing 2008;13(15): 15–24. https://www.ncbi.nlm.nih.gov/pubmed/18229673 PMID: 18229673

36 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. https://doi.org/10.1093/nar/gkq443 PMID: 20497997

37 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. Https://doi.org/ 10.1093/nar/gkv318

38 Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evolut. 2016;33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

39 Hebert PDN, Cywinska A, Ball SL, de Waard JR. Biological identifications through DNA barcodes. Proc R Soc Lond B Biol Sci. 2003;270(1512): 313–321. http://rspb.royalsocietypublishing.org/content/270/1512/313.e-letters PMID: 12614582

40 Tamura K, Nei, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 1993;10(3): 512–526 https://www.ncbi.nlm.nih.gov/pubmed/8336541 PMID: 8336541

41 Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012;9(8): 772. http://www.nature.com/nmeth/journal/v9/n8/full/nmeth.2109.html?foxtrotcallback=true PMID: 22847109

42 Harkins KM, Schwartz RS, Cartwright RA, Stone AC. Phylogenomic reconstruction supports supercontinent origins for *Leishmania*. Infect, Genet Evolut. 2016;38: 101–109. https://doi.org/10.1016/j.meegid.2015.11.030 PMID: 26708057

43 Kuhls K, Keilonat L, Ochsenreither S, Schaar M, Schweynoch C, Presber W, et al. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. Microbes Infect. 2007;9(3): 334–343. https://doi.org/ 10.1016/j.micinf.2006.12.009 PMID: 17307010

44 Kuhls K, Alam MZ, Cupolillo E, Ferreira GEM, Mauricio IL, Oddone R, et al. Comparative microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. Plos Negl Trop Dis. 2011;5(6): 1–12. https://doi.org/10.1371/journal. pntd.0001155 PMID: 21666787

45 Fraga J, Montalvo AM, de Doncker S, Dujardin JC, Van der Auwera G. Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. Infect Genet Evolut. 2010;10(2): 238–245. https://doi.org/10.1016/j.meegid.2009.11.007 PMID: 19913110

46 Wiley EO, Liebermann B. Phylogenetics: Theory and Practice of Phylogenetic Systematics. 2 Ed. John Wiley & Sons, Hoboken, NJ, USA. 2011; 432 p.

47 Berzunza-Cruz M, Cabrera N, Crippa-Rossi M, Cabrera TS, Pérez-Montfort R, Becker I. Polymorphism analysis of the internal transcribed spacer and small subunit of ribosomal RNA genes of *Leishmania mexicana*. Parasitol Res. 2002;88(10): 918–925. https://link.springer.com/article/ 10.1007%2Fs00436-002-0672-x PMID: 12209333

Table 4: GenBank accession number of the ITS1 sequences of New World *Leishmania species u*sed in this study.

Species	GenBank Accession Number
L. amazonensis	AJ000314.1, AJ000315.1, AJ000316.1, DQ182536.1, DQ300179.1, DQ300180.1, DQ300181.1, DQ300182.1, DQ300183.1, DQ300184.1, DQ300185.1, DQ300186.1, DQ300187.1, DQ300188.1, DQ300189.1, DQ300190.1, DQ300191.1, DQ300192.1, DQ300193.1, DQ300194.1, FJ753371.1, FJ753372.1, FJ753373.1, KF985162.2, KP274862.1
L. braziliensis	AJ300483.1, AJ300484.1, DQ182537.1, FJ753374.1, FJ753375.1, FJ753376.1, FJ753377.1, FJ753378.1, FJ753379.1, FJ753380.1, FJ753381.1, FJ753382.1, FJ753383.1, FJ753384.1, FJ753385.1, FN398333.1, FN398334.1, FN398335.1, FN398336.1, FN398337.1, FN398338.1, JN936955.1, JQ061322.1, JQ397604.1, JX448547.1, JX448548.1, JX448549.1, KF985166.2, KP274863.1, KU550586.1, KU550587.1, KU550588.1, KU550589.1, KU550590.1, KU550591.1, KU550592.1, KU550593.1, KU550594.1
L. donovani	AB725909.1, AJ000290.1, AJ000291.1, AJ000292.1, AJ000293.1, AJ000294.1, AJ000296.1, AJ000297.1, AJ249612.1, AJ249613.1, AJ249614.1, AJ249615.1, AJ249616.1, AJ249617.1, AJ249618.1, AJ249619.1, AJ249620.1, AJ249621.1, AJ249622.1, AJ276258.1, AJ276259.1, AJ276260.1, AJ634356.1, AJ634357.1, AJ634358.1, AJ634359.1, AJ634360.1, AJ634365.1, AJ634366.1, AJ634367.1, AJ634368.1, AJ634372.1, AJ634373.1, AJ634374.1, AJ634375.1, AJ634376.1, AJ634377.1, AJ634378.1, AM901447.1, AM901448.1, AM901449.1, AM901450.1, AM901451.1, AM901452.1, AM901453.1, EU326228.1, EU753225.1, EU753226.1, EU753227.1, EU753228.1, EU753229.1, EU753230.1, EU753231.1, EU753232.1, FJ753386.1, FN182206.1, FN182207.1, FN182208.1, FN182209.1, FN182210.1, FN398344.2, FN677363.1, FN677364.1, GQ367489.1, GU045589.1, GU045590.1, HM130608.1, HQ830354.1, HQ830358.1, JQ730001.1, JQ730002.1, KF500031.1, KF525783.3, KF543268.2, KF543269.3, KF543270.3, KF673344.1, KF673345.1, KF815213.1, KF815214.1, KF815215.1, KF815216.1, KJ002560.1, KJ018017.1, KJ465104.1, KJ465105.1, KJ465106.1, KJ465107.1, KJ465108.1, KM982538.1, KM982539.1, KP246847.1, KP780081.1, KP780082.1, KP780083.1, KP780084.1, KP780085.1, KP780086.1, KP780087.1, KP780088.1, KP780089.1, KR858307.1, KT152805.1, KT152806.1, KT152807.1, KT152808.1, KT152809.1, KT175573.1, KT175574.1, KT175575.1, KT273402.1, KT273403.1, KT273404.1, KT273405.1, KT273406.1, KT273407.1, KT273408.1, KT921417.1, KU975140.1, KU975141.1, KU975142.1, KU975143.1, KU975144.1, KU975145.1, KU975146.1, KU975147.1, KU975148.1, KU975149.1, KU975150.1, KU975151.1, KU975152.1, KU975153.1, LC086292.1
L. guyanensis	AJ000300.1, DQ182538.1, DQ182539.1, DQ182540.1, DQ182541.1, FJ753387.1, FJ753388.1, FJ753389.1, FJ753390.1, FN398329.1, FN398330.1, FN398331.1, FN398332.1, HF968630.1, HG512905.1, HG512915.1, HG512935.1, HG512960.1, HG512961.1, JN671917.1
L. (L.) infantum	AJ000288.1, AJ000289.1, AJ000295.1, AJ000303.1, AJ634339.1, AJ634340.1, AJ634341.1, AJ634342.1, AJ634343.1, AJ634344.1, AJ634345.1, AJ634346.1, AJ634347.1, AJ634348.1, AJ634349.1, AJ634350.1, AJ634351.1, AJ634352.1, AJ634353.1, AJ634354.1, AJ634355.1, AJ634361.1, AJ634362.1, AJ634363.1, AJ634364.1, AJ634369.1, AJ634370.1, AJ634371.1, EU326227.1, EU604810.1, EU810776.1, EU810777.1, FJ497004.1, FJ555210.1, FJ940891.1, FJ940892.1, FJ940893.1, FM164416.1, FM164417.1, FM164418.1, FM164419.1, FM164420.1, FN398341.2, FN398342.1, FN398343.2, GQ367486.1, GQ367487.1, GQ367488.1, GQ444144.1, GU045592.1, GU591397.1, HQ535858.1, HQ830353.1, JQ362410.1, JX151015.1, JX289852.1, JX289853.1, JX289879.1, JX289880.1, JX448535.1, JX448536.1, JX448537.1, JX448538.1, JX448539.1, JX448540.1, JX448541.1, JX448542.1, JX448543.1, JX448544.1, JX448545.1, JX448546.1, JX945644.1, JX945645.1, KC347299.1, KC347300.1, KC347301.1, KC355188.1, KC477100.1, KC570454.1, KC686340.1, KC686341.1, KC998879.1, KF705513.1, KF705514.1, KF705515.1, KF985164.1, KF985169.2, KF985170.2, KF985171.2, KJ002555.1, KJ002556.1, KJ002557.1, KJ002558.1, KJ364133.1, KJ417496.1, KJ567480.1, KJ567481.1, KJ567482.1, KJ573795.1, KM408430.1, KM677128.1, KM677129.1, KM677130.1, KM677131.1, KM677132.1, KM677133.1, KM677134.1, KM677135.1, KM677136.1, KM677137.1, KM677138.1, KM677139.1, KM677140.1, KM677141.1, KM677142.1, KM677143.1, KM677144.1, KM677145.1, KM677146.1, KM925005.1, KM925006.1, KM925007.1, KP274860.1, KP274861.1, KP738165.1, KP738166.1, KR081260.1, KR081261.1, KR081262.1, KR081263.1, KR081264.1, KR081265.1, KT003211.1, KT026221.1, KT153644.1, KT153645.1, KT153646.1, KT153647.1, KT153648.1, KT153649.1, KT966381.1, KT966382.1, KU550595.1, KU550596.1, KU550597.1, KU550598.1, KU550599.1, KU550600.1, KU550601.1, KU555887.1, KU680856.1, KU975154.1, KU975155.1, KU975156.1, KU975157.1, KU975158.1, KU975159.1, KX492916.1, KX492917.1, KX492918.1, KX492919.1, KX492920.1, KX580706.1, KX664449.1, KX664450.1, KX664451.1, KX664452.1, KX664453.1, KX664454.1, KX712139.1, KX808124.1, KY046309.1, KY658228.1, KY658229.1, KY658230.1, KY658231.1, KY658232.1, KY658233.1, KY658234.1, KY658235.1, KY973655.1, KY973656.1, KY973657.1, KY973658.1, KY973659.1, KY973661.1, KY973662.1, LC028234.1
L. (L.) infantum chagasi	AJ000304.1, AJ000305.1, AJ000306.1, GQ332357.1, GU045591.1, KT751245.1, KT751246.1, KT751247.1, KT751248.1, KT751249.1, KT751250.1, KT751251.1, KT751252.1, KT751253.1, KT751254.1, KT751255.1, KT751256.1, KT751257.1, KT751258.1, KT751259.1, KT751260.1, KT751261.1, KT751262.1, KT751263.1, KT751264.1, KT751265.1, KT751266.1, KT751267.1, KT751268.1, KT751269.1,

L. lainsoni	FN398154.1, HG512895.1, HG512899.1, HG512904.1
L. lindenbergi	FN398151.1
L. mexicana	AB558238.1, AB558239.1, AB558240.1, AB558241.1, AB558242.1, AB558243.1, AB558244.1, AB558245.1, AB558246.1, AB558247.1, AB558248.1, AB558249.1, AB558250.1, AB558251.1, AF466380.1, AF466381.1, AF466382.1, AF466383.1, AJ000312.1, AJ000313.1, FJ948432.1, FJ948433.1, FJ948434.1, FJ948435.1, FJ948436.1, FJ948437.1
L. naiffi	FN398152.1, HG512903.1, HG512939.1, HG512950.1
L. panamensis	AJ000298.1, FJ948438.1, FJ948439.1, FJ948440.1, FJ948441.1, FJ948442.1, FJ948443.1, FJ948444.1, FJ948445.1, FJ948446.1
L. peruviana	FN398339.1, FN398340.1, HG512896.1, HG512900.1, HG512902.1
L. shawi	FN398328.1
L. utingensis	FN398153.1
L. venezuelensis	AF339752.1

4 ARTICLE 2

DISPERSION OF LEISHMANIA INFANTUM ON SOUTH AMERICA' SOUTH-CENTRAL: EVIDENCE FROM AN INTEGRATIVE APPROACH

Short Title: *Leishmania infantum* dispersion

Aline Kuhn Sbruzzi Pasquali[a,b], Rafael Antunes Baggio[a,c], Walter Antonio Boeger[c], Deborah Carbonera Guedes[a,b], Nilsa González-Britez[d], Vanete Thomaz-Soccol[a,b] *

a. Laboratório de Biologia Molecular, Departamento de Engenharia de Bioprocessos e Biotecnologia, Universidade Federal do Paraná, Brazil.
b. Programa de Pós-Graduação em Bioprocessos e Biotecnologia, Universidade Federal do Paraná, Brazil.
c. Laboratório de Ecologia Molecular, Departamento de Zoologia, Universidade Federal do Paraná, Brazil
d. [3]Departamento de Medicina Tropical. Instituto de Investigaciones en Ciencias de la Salud, Universidad Nacional de Asunción (IICS-UNA). Paraguay

*vanetethomaz@gmail.com

Manuscript formatted as the guidelines of PLos One journal

4.1 ABSTRACT

Leishmania infantum is the parasite responsible for visceral leishmaniasis (VL), a worldwide distribution zoonosis. VL has been undergoing changes in the transmission scenario in the Old and New World. Previously VL was characterized as a disease restricted to rural areas, but nowadays it's urbanized. The objective of this study is understanding the dispersion of *L. (L.) infantum* in South America's center, using microsatellite markers of individuals dogs', humans' and sandflies' isolates from Brazil and Paraguay associated with historical data. The results of microsatellites in South America's central-south region and historical data (Colombia, Paraguay, Argentina, Chile, Uruguay and Brazil) supported four *L. (L.) infantum* dispersion events of in this region: 1. Colonization of Northweast (Rio Grande do Norte, Piaui, Ceará, Sergipe, Maranhão, Pernambuco, Paraiba, Alagoas and Bahia) to southeast states from Brazil (Rio de Janeiro, Espírito Santo, São Paulo and Minas Gerais) and center west (Mato Grosso, Mato Grosso do Sul and Goias); 2. Construction of Bolivia-Brazil pipeline, starting in Bolivia and expanding through the states of Mato Grosso do Sul, São Paulo, Rio de Janeiro and Minas Gerais; 3. VL dispersion from Paraguay to Brazil via triple border (Brazil, Argentina and Paraguay) in Foz do Iguaçu city, Parana state; 4. Dispersion of VL in Santa Catarina state and to Pato Branco (Paraná). The knowledge of these *L. (L.) infantum* dispersion routes is necessary to implement effective measures of VL prevention and control. The use of molecular tools, such as microsatellites, can aid in these approaches of searching *L. (L.) infantum* dispersion routes on South- America's central-south.

Key-words: Dogs, Humans, *Leishmania infantum,* Microsatellites, Visceral Leishmaniasis.

4.2 INTRODUCTION

Leishmania infantum is the protozoan that causes visceral leishmaniasis (VL) in humans and canines. The domestic dogs are reservoirs and present greater relevance in VL epidemiology [1]. The presence of the vector *Lutzomia longipalpis* is also necessary for *L. (L.) infantum* transmission [1], however Thomaz-Soccol et al., showed that the Stockholm Paradigm can be applied specially on the border focus and their importance in the elaboration of public health policies in international border areas [2]. On average, 90% of the VL cases in the world concentrated in Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan [3]. In Brazil, although known since 1920s, *L. (L.) infantum* possibly

arrived firstly in northeastern region, with dogs and rodents that came from Portugal and Spain with colonizers [4-7].

Since the description of visceral disease, in 1903, the epidemiological profile has been changing. Until 80s, the disease was present mainly in rural areas. After this period, the disease has adapted in peri urban regions and, nowadays, it is signaled in urban area in both old and new world [8-12].

Between 1920 and 1980, VL was a disease restricted to rural areas and endemic in Northeast Brazil [13-15]. In the years of 1981 and 1982 there was an epidemic of VL in Teresina city, Piaui state, and São Luis do Maranhão city, Maranhão state, characterizing the agent's progress to urban areas [8]. In the 1990s several epidemic outbreaks were reported especially in the southeastern and mid-western regions of the country, with highly rates of canine VL cases followed by clinical cases in humans in Belo Horizonte (Minas Gerais state), Campo Grande (Mato Grosso do Sul state) and Araçatuba (São Paulo state), demonstrating the urbanization of the disease in Brazil [16-21].

In the south-central of Brazil, *L. (L.) infantum* is spread along Minas Gerais, São Paulo, Mato Grosso, Mato Grosso do Sul, Rio de Janeiro and Espirito Santo, besides in the bordering countries as Paraguay (eastern region), Argentina (northern), Uruguay (northern) [22]. In this region, epidemics began in the 90s with the construction of the east/west route of the Bolivia-Brazil gas pipeline [23,24]. Its building allowed the dispersion of *L. (L.) infantum* on center-south region of Brazil, through the migration of workers and infected dogs and deforestation [24-27,22]. This scenario was worsted by the migration of humans and dogs by highways and railroads. Deforestation and climate or environmental changes may have assisted the expansion of VL in different parts of Brazil [28,24].

In southern region the entry of VL is more recent. The first registry of dog and human VL cases happened in Rio Grande do Sul in 2006 and 2008 respectively; in Santa Catarina in 2011 and Paraná in 2012, when dogs were firstly diagnosed, and the vector described. In 2016, human cases of VL were diagnosed in Paraná [29-32].

The recent urban dissemination of VL in medium and large cities, and the expansion to other Brazilian regions show the worst scenarios in the future. The movement of people and their infected dogs [33], climate change and the lack of joint policies in countries bordering Brazil (approximately 8 thousand km) are risks for VL dispersion to south region of Brazil. For instance, Foz do Iguaçu located in the triple border (Brazil-Argentina-Paraguay), is one of the main touristic cities in Brazil and presents an increase in the number of VL cases [34,32,1].

The use of microsatellites assists in *L. (L.) infantum* isolates differentiation and its region, country or continent; as well as provides information about epidemiology and agent

characterization in different geographic areas [35-43]. The use of microsatellites in studies addressing the dispersion of *L. (L.) infantum* helps to explain their distribution and potential future. These tools can be used to assess the hypothesis of *L. (L.) infantum* dispersion is associated to the construction of pipelines and highways; migration of people and their dogs, presence of nature reserves in urban centers with vector maintenance, or deforestation and natural disasters. Therefore, it is necessary to understand the parasite and vectors migration to assure control and vigilance measures.

The first objective of this study was to evaluate the dispersion the *L. (L.) infantum* in Foz do Iguaçu and Santa Terezinha do Itaipu, Paraná state. The second objective is to understand the dispersion of *L. (L.) infantum* in central South America. The knowledge of *L. (L.) infantum* dispersion in South America's center we used a multiapproach as molecular identification (using microsatellite markers of *Leishmania* isolates in dogs, humans, and sand flies from Brazil and Paraguay) associated with historical from South America's center.

4.3 MATERIAL AND METHODS

4.3.1 Sampling, parasite culture and DNA extraction

To test the dispersion of *L. (L.) infantum* in South America's Center-South using microsatellite markers, 132 isolates from dogs, humans and sandflies were evaluated. Among them, 70 samples were collected in Foz do Iguaçu in 4 areas (A: 3 isolates; B: 16; C: 21; D: 30) (Fig. 4 - see Thomaz Soccol *et al.* [1] for details), and 4 samples from dogs were collected in Santa Terezinha de Itaipu, in extreme western Paraná State, between 2013 and 2016 (see molecular identification in the Cap. 1).

The other isolates (41 samples origin of Curitiba, Maringa and Pato Branco, Paraná state; São Miguel do Oeste and Descanso, Santa Catarina state; Andradina and Bauru, São Paulo state; Tres Lagoas and Campo Grande Mato Grosso do Sul state; Mato Grosso state; Belo Horizonte, Minas Gerais state; Tocantins state; Aracaju, Sergipe state; Fortaleza, Ceara state) belong to Molecular Biology Laboratory of Graduate Program in Biprocess Engineering and Biotechnology (Federal University of Parana- UFPR). Additionally, 10 samples were from Paraguay, which were kindly given for Nilsa Gonzalez Britez, Parasitologia y Entomologia Medica, *Instituto de Investigaciones en Ciencias de la Salud*, *Universidad Nacional de Asuncion*. Seven samples are from Europe (France, Spain

and Portugal), Africa (Argelia and Egito) kindly given from Molecular Ecologie Laboratory of Medicine Faculty of University of Montpelier, France.

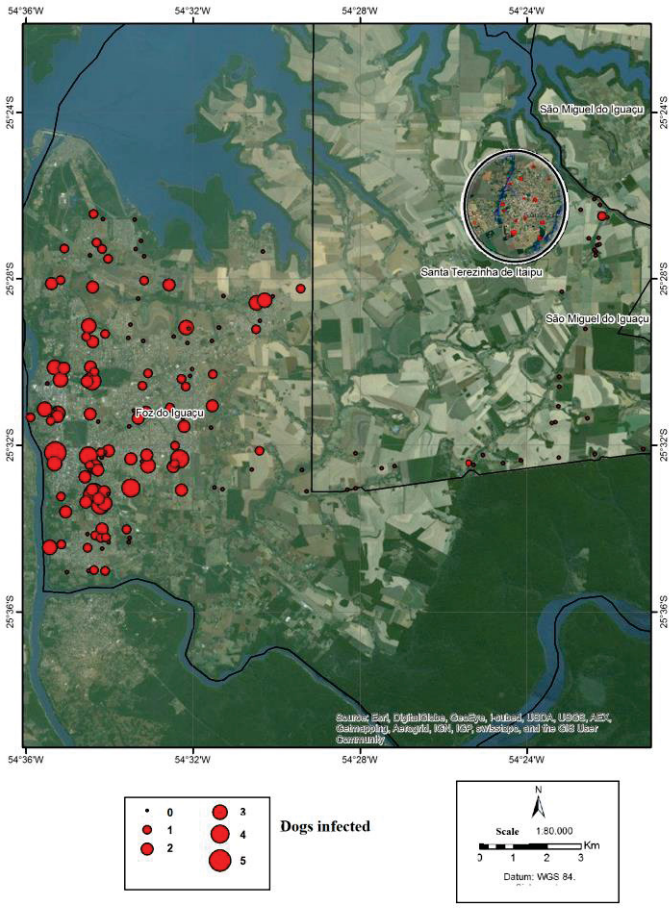


Figure 4: Geographical localizations where the dogs were sampled in Foz do Iguaçu and in Santa Terezinha de Itaipu (see Thomaz-Soccol et al., [1] from more details)

Samples were collected from bone marrow, aspiration of lymph nodes and leukocyte layer of dogs domiciled in Foz do Iguaçu and Santa Terezinha de Itaipu (Paraná state) for isolation of *L. (L.) infantum*. The material was inoculated in Neal, Novy and Nicole (NNN) culture medium with saline solution 0.9% for four weeks at 24ºC. The promastigote cultures from the other states were cultivated in BHI (Brain Heart Infusion) with saline solution 0.9% at 24ºC [44]. After culture the parasites were harvested by centrifugation at 5,000*g* at 4°.C, and washed three times: first with saline solution 0.9%, second with saline solution 0.3% and third with 0.9%.

The DNA of cultured promastigotes and biological *Leishmania* samples was extracted by phenol/chloroform/isoamyl alcohol method [45-47]. The DNA was dosed at Nanodrop® and standardized in the 10 ng/μL concentration.

4.3.2 Genotyping

Fourteen microsatellite markers (Li46-67, Li41-56, Li71-7, Li71-33, Li23-41, Li22-35, Lm2TG, Lm4TA, Li45-24, CS20, Li71-5/2, TubCA, List7031, List7039, (see Table 1) described by Jamjoom *et al.* [48], Ochsenreither *et al.* [49], Kuhls *et al.* [50] were selected to assess the genetic profile of the population from South America's center-south. PCR reaction was performed using 10 µL of a mix composed by 0.3 pmol of forward primer (loci Li46-67, Li41-56, Li 71-7, Li71-33, Lm2TG, CS20, Li71-5/2, TubCA and List7031) and 0.5 pmol of reverse primer (Li23-41, Li22-35, Lm4TA, Li45-24 and List7039), 10 ng of DNA template (5 ng for the loci Li71-7 and Lm2TG), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.3 units of Taq polymerase platinum (Invitrogen®) and ultrapure water to complete the reaction final volume. The PCR cycles were set to run for 3 min at 95 ºC; 35 cycles of 30 s at 95º C, 60 s at 50 ºC (Li46-67, Li41-56, Li71-7 and Li71-33), 52 ºC (Li23-41 and Li22-35), 54 ºC (Lm4TA and Li45-24), 55º C (Lm2TG), 56 ºC (CS20, Li71-5/2 and List7039) e 58 ºC (TubCA and List7031); 60 s at 72 ºC; and a final extension at 72 ºC for 60 min. PCR was performed with fluorescence conjugated forward primers and the amplified products were analyzed in an automated capillary sequencer ABi 3130 (Applied Biosystems®).

4.3.3 Data analyses

To evaluate *L. (L.) infantum* dispersion in Foz do Iguaçu and Santa Terezinha do Itaipu, parasites populations from these regions were analyzed by Pairwise Fst method in Arlequin 3.5. software and were assignment analyzed in Structure 2.3.3 software.

The amplification of the 14 microsatellite markers and their fragment size were assessed using the Gene Marker V2.4.2 (SoftGenetics). The presence of null alleles, allele dropout and scoring errors was analyzed with Micro-Checker 2.2.3 [51]. The Hardy-Weinberg disequilibrium, linkage disequilibrium, diversity (gene diversity, Ho and He) and genetic differentiation (Fst and AMOVA were performed only for population with more than 5 individuals, Fis) analyses were performed using the software Arlequin 3.5 [52]. Allelic richness was calculated in the Fstat 2.9.3.2 [53]. The critical p value was corrected using the B-Y method [54] in analyses with multiple comparisons.

The number of genetic populations was assessed using the assign method implemented in the STRUCTURE 2.3.3 [55] in eight runs for each *K* (K between 1 and 8), composed by a burn-in period of 500,000 itineration's and 5,000,000 Markov Chain Monte Carlo (MCMC) iterations. The ad hoc method of Evanno *et al.,* [56], implemented on the online tool Structure Harvester [57], was used to assess the most likely value of K.

Samples with genetic profile assigned in more than 75% of a cluster were considered pure individuals. Isolates assigned in 25 to 75% of their genome for more than one cluster were considered hybrids. The dendrogram was built with software Populations 1.2.32 for populations analyses.

4.3.4 First records cases of VL in South America's South-Central

The first records of VL cases in dogs and humans in each city of the South America´s South Central (Bolivia, Argentina, Paraguay, Uruguay and Brazilian states: Mato Grosso, Mato Grosso do Sul, São Paulo, Goias, Minas Gerais, Rio de Janeiro, Espirito Santo, Paraná, Santa Catarina and Rio Grande do Sul) were assessed using Google Scholar, Scielo, Scopus and PubMed data base from 1913 to 2017. The following key-words were used: "first case visceral leishmaniasis", "visceral leishmaniasis in dogs", "visceral leishmaniasis in human" or "*Leishmania infantum*". Moreover, human records of VL between 2001 and 2015 available in SINAN (Sistema de Informação de Agravos de Notificação) database, were also considered. Data from 2001 to 2015 were used, since it is only these periods with records filed in the SINAN platform.

After a bibliographical survey, tables with geographical coordinates, of the municipalities with VL record, were plotted on the map with routes of pipelines described by Abegas [58] and EPE [59] in the program ArcGis 10.2.2.

4.4 RESULTS

4.4.1 Microsatellite markers

All isolates were analysed for the panel of 14 microsatellites, observing the presence of polymorphic loci (Fig 05). The mean heterozygosis observed (Ho) was 0.125 and heterozygosis expected (He) 0.497. The most diverse markers (Table 05) were Li 23-41 (Ho: 0.133 He: 0.125), List 7039 (Ho: 0.370 He: 0.470) and Li 71-33 (Ho: 0.405 He: 0.533). Among the 14 microsatellite markers, the loci List 7031, Li 41-56, Li 45-24 and TubCA presented recurrent evidences of null alleles for some populations. Then, they were removed of the genetic structure and assignment analyses.

Table 5: Microsatellite markers, primer sequences, fragment size, heterozygosity observed (Ho) and heterozygosity expected (He), used to assess the dispersion of *Leishmania infantum* in the South America's center-south.

Marker	Forward	Reverse	Fragment (bp)	Ho	He
Li46-67	TCTTCTTTCGTTAGCTGAGTGC	CTGTATCACCCATGAGGGGC	76	0.000	0.511
Li41-56	TTGCTTCATGATAACAACTTGG	CCTGTTGGTGTGAGTTCGTG	86	0.000	0.555
Li71-7	GCTGCAGCAGATGAGAAGG	GTGAGAAGGCAGGGATTCAA	98	0.047	0.491
Li71-33	CTCCTTTCACACCGCCTCT	GAGAGAAGACGAGCCGAAGT	106	0.405	0.533
Li23-41	GATCGGAGGTGACAGCGT	CCTTTAACTGCCAGTGCG	88	0.133	0.129
Li22-35	CTTGATGTTCGGGTTAGCAAGT	ATGCACACCAAAAATCATGTG	96	0.036	0.462
Lm2TG	AAAAAGCGAGGAATGAAAGAA	TCCCTCCCCTCTACAACCTT	144	0.147	0.450
Lm4TA	TTTGCCACACACATACACTTAG	GTAGACGACATCGCGAGCAC	78	0.260	0.528
Li45-24	GCGCCTACAGGCATAAAGGA	CTGGCGCATCAACGGTGT	98	0.000	0.371
CS20	CGTTGGCTGTTGATT GTGTA	GCGTGGCAATCTCT CATT	84	0.142	0.802
Li71-5/2	GCACGGTCGGCATTTGTA	GATAAACGAGATGGCCGC	108	0.142	0.472
TubCA	GGCGTGGTTGCTAAACTGAT	GCCTGCGCACACAGAGAC	74	0.028	0.656
LIST7031	CCACTGGTGGAAATAGAAAGACT	GGAGAACTAAAACGAGCAGCA	170	0.035	0.524
LIST7039	CTCGCACTCTTTCGCTCTTT	GAGACGAGAGGAACGGAAAA	204	0.370	0.470

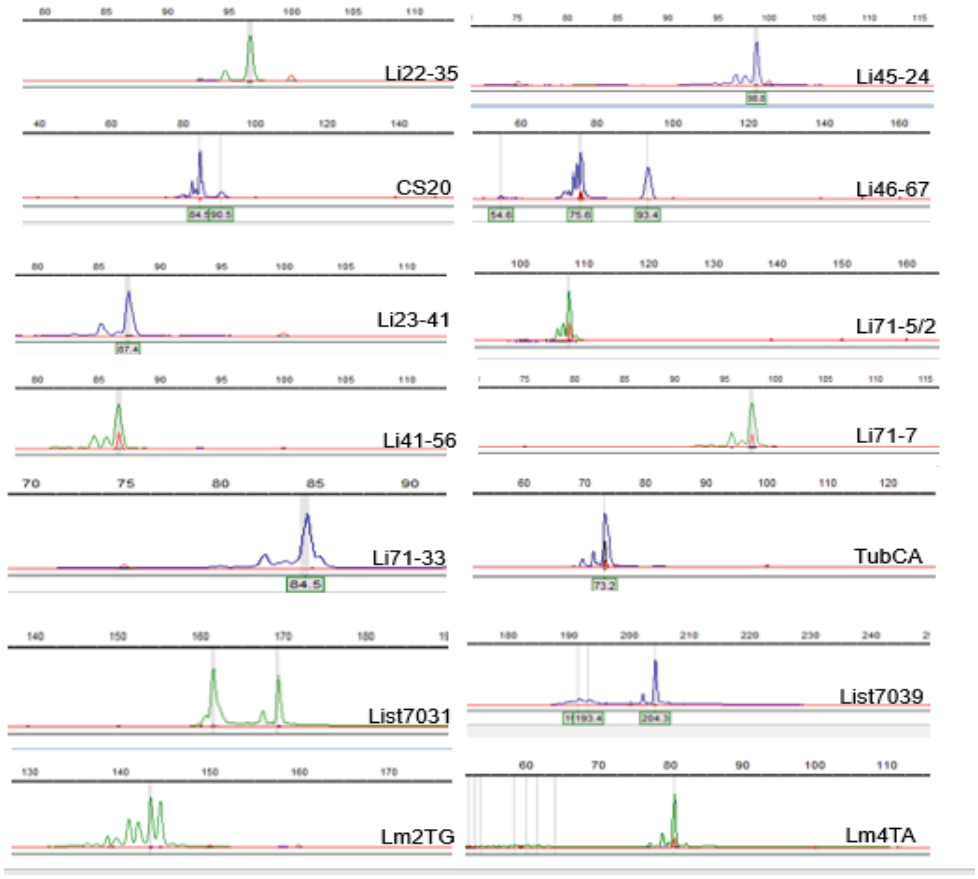


Figure 5: Peaks of the alleles for the 14 microsatellite markers used to study *Leishmania infantum* genetic variability.

The genetic differentiation among the parasites from areas of Foz do Iguaçu supported that the populations from different area are not genetically different ($p < 0.014$ after B-Y correction), but they are genetically different from those of Santa Terezinha de Itaipu – PR (Table 06 and Fig. 06).

Greater allelic diversity was observed in Brazilian populations from Campo Grande (MS) and Foz do Iguaçu (PR) (Table 07). The Lm2TG, Lm4TA and List7039 markers presented greater intra-population allelic diversity. Populations from Foz do Iguaçu - PR and Paraguay presented greater intra-population allelic diversity.

The genetic structure analysis supported 67.43% of the genetic variation of populations is from inter-population level (Fst = 0.674, p value = 0.000). The pairwise genetic differentiation presented significant genetic differentiation ($p < 0.017$ after B-Y correction) in every comparison to populations with more than five individuals genotyped (i.e. Assumpcion - Py, Belo Horizonte - MG, Foz do Iguaçu - PR, São Miguel do Oeste and Descanso - SC), except between Belo Horizonte and Assumpcion, Belo Horizonte and Foz do Iguaçu, and São Miguel do Oeste and Descanso (Table 08).

Table 6: FST and p value (in parenthesis) between Leishmania infantum populations sampled in four areas from Foz do Iguaçu and Santa Terezinha de Itaipu.

	Area A	Area B	Area C	Area D
Area B	-0.065 (0.990)			
Area C	-0.082 (0.990)	-0.001 (0.729)		
Area D	-0.071 (0.909)	-0.015 (0.774)	0.002 (0.540)	
Santa Terezinha do Itaipu	**0.376 (0.018)**	**0.662 (0.000)**	**0.674 (0.000)**	**0.756 (0.000)**

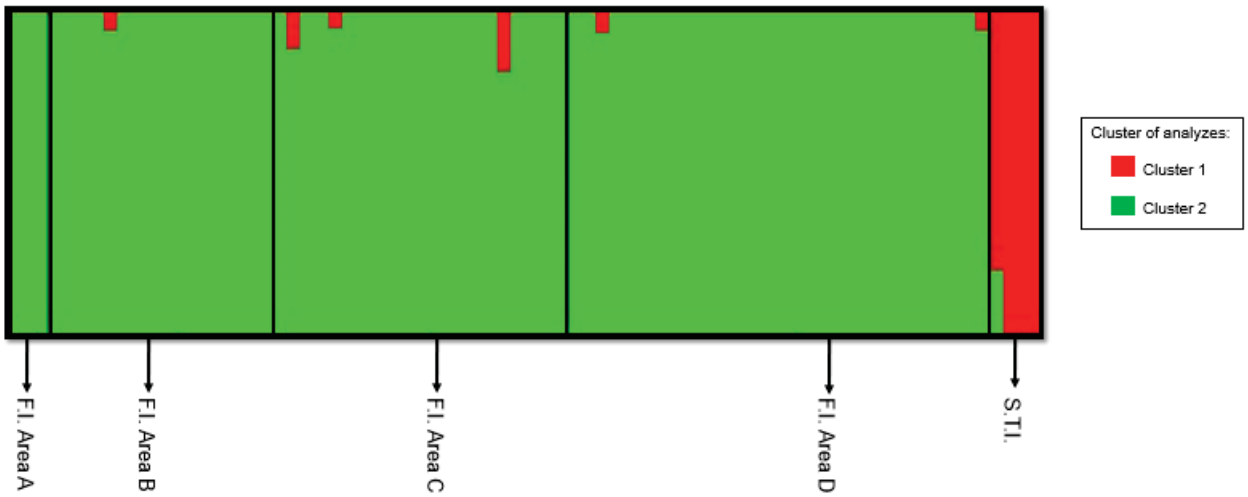


Figure 6: Genetic assignment of five *Leishmania infantum* populations of four areas (A, B, C and D) from Foz do Iguaçu (F.I.) and Santa Terezinha de Itaipu (S.T.I.).

Table 7: Genetic diversity (H), inbreeding coefficient (Fis) and allelic richness of 10 microsatellite markers for all individuals (N) of 18 *L. (L.) infantum* populations from South America's center-south.

				Allelic rich										
Population	N	H	Fis	Li 46-67	Li 71-7	Li 71-33	Li 23-41	Li 22-35	Lm2TG	Lm4TA	CS20	Li 71-5/2	List 7039	Average population
Europe*	7	0.58	0.58	2.00	2.43	3.36	2.43	5.30	3.97	5.29	4.58	2.70	3.80	3.59
Paraguay	10	0.08	0.00	1.00	1.00	1.00	2.00	1.00	2.00	3.00	1.00	1.00	3.00	1.60
Aracaju/Sergipe	1	0.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Fortaleza/Ceará	1	0.20	0.00	1.00	1.00	2.00	1.00	1.00	1.00	1.00	1.00	1.00	2.00	1.20
Tocantins	2	0.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mato Grosso	1	0.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Três Corações/ Mato Grosso do Sul	2	0.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Campo Grande/Mato Grosso do Sul	3	0.15	0.00	1.00	1.00	2.00	1.00	1.00	3.00	2.00	1.00	1.00	1.00	1.40
Belo Horizonte/Minas Gerais	8	0.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Bauru/São Paulo	3	0.07		1.00	1.00	1.00	1.00	1.00	2.00	1.93	1.00	1.00	1.00	1.19
Andradina/São Paulo	1	0.11		1.00	1.00	1.00	1.00	1.00	1.00	2.00	1.00	1.00	1.00	1.10
Curitiba/Paraná	1	0.00			1.00	1.00	1.00	1.00		1.00				1.00
Maringá/Paraná	1	0.00			1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Santa Terezinha do Itaipu/Paraná	4	0.05		1.00	1.00	1.00	1.00	1.00	1.79	1.00	1.00	1.00	1.00	1.08
Foz do Iguaçu/Paraná	70	0.02	0.67	1.00	1.00	1.00	1.70	1.91	2.80	2.70	1.00	1.00	1.91	1.60
São Miguel do Oeste/Santa Catarina	7	0.00	1.00	1.67	1.67	1.30	1.00	1.55	1.00	1.00	1.00	1.00	1.00	1.22
Descanso/Santa Catarina	9	0.00	1.00	1.00	1.53	1.00	1.00	1.43		1.00		1.00	1.00	1.12
Pato Branco/Paraná	1	0.00			1.00	1.00	1.00	1.00		1.00				1.00
Average locis		0.07	0,46	1.11	1.15	1.26	1.17	1.34	1.64	1.61	1.24	1.11	1.42	1.28

*Populations with only homozygote individuals; **France, Spain, Portugal, Egypt and Argelia

Table 8: Parwise genetic differation (Fst) and their significance (between parenthesis) of *Leishmania infantum* populations with more than 5 individuals genotyped for 10 microsatellites.

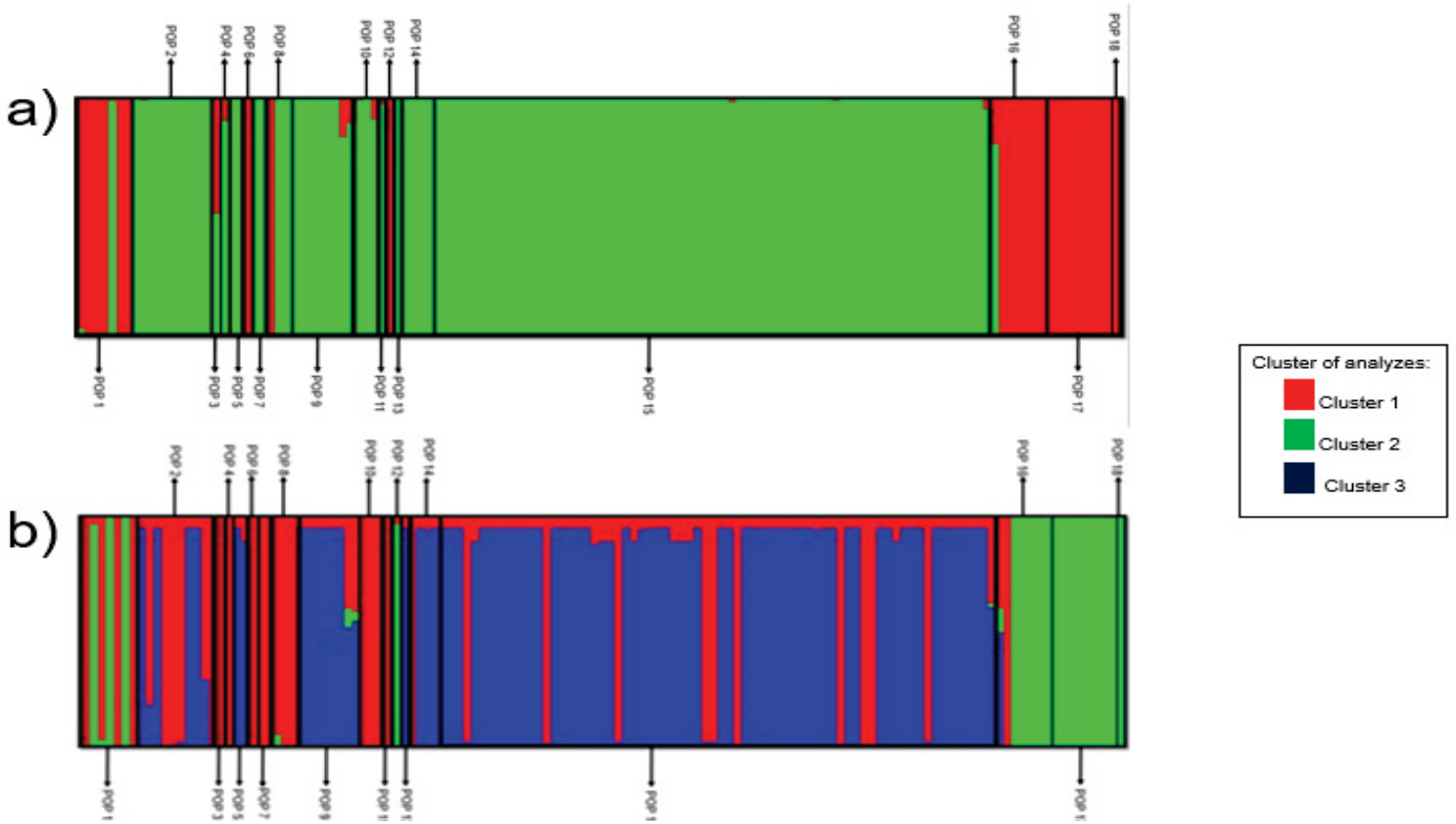
	Fst/pvalue pair by pair analyzes			
Population	Assumpcion/ Paraguay	Belo Horizonte/ MG	Foz do Iguaçu/ PR	São Miguel do Oeste/ SC
Belo Horizonte/MG	0.464 (0.00)			
Foz do Iguaçu/PR	**0.695 (0.00)**	-0.021 (0.018)		
São Miguel do Oeste/SC	**0.522 (0.00)**	**0.597 (0.00)**	**0.841 (0.00)**	
Descanso/SC	**0.682 (0.00)**	**0.919 (0.00)**	**0.940 (0.00)**	0.251 (0.117)

The genetic assignment analysis method of Evanno *et al.,* [56] supported that K=2 (ΔK: 52.99) is the most probable number of clusters (Fig 7). This analysis supported that *L. (L.) infantum* populations from Paraguay, Belo Horizonte (MG), Bauru (SP), Andradina (SP), Fortaleza (CE), Aracaju (SE), Tocantins, Campo Grande (MS), Três Lagoas (MS), Santa Terezinha do Itaipu and Foz do Iguaçu (PR) presented similar genetic profile. The populations of São Miguel do Oeste, Descanso (SC) and Pato Branco (PR) present similarity to each other and difference toother populations. The results of K=3 analysis were also informative (ΔK = 1.02, Fig 7), and showed that the populations are grouped as follow:

1. Foz do Iguaçu and Santa Terezinha do Itaipu (PR), Belo Horizonte (MG), Tocantins and Paraguay were similar to isolates from France, Portugal and Spain characterized as MON-1 by MLEE identification;

2. Três Lagoas and Campo Grande (MS), Mato Grosso (MT), Andradina and Bauru (SP), Fortaleza (CE) and Aracaju (SE) were similar to isolates from France, Portugal and Spain characterized as MON-1 by MLEE identification;

3. São Miguel do Oeste and Descanso (SC), and Pato Branco (PR) were similar to isolates from France, Spain and Argelia characterized as MON-108, MON-198 and MON-24 respectively. Both zymodemes characterized by MLEE.



POP 1 (France, Spain, Portugal, Egypt and Argelia); POP 2 (Assumpcion, Paraguay); POP 3 (Aracaju, Sergipe); POP 4 (Fortaleza, Ceará); POP 5 (Tocantins); POP 6 (Mato Grosso); POP 7 (Três Lagoas, Mato Grosso do Sul); POP 8 (Campo Grande, Mato Grosso do Sul); POP 9 (Belo Horizonte, Minas Gerais); POP 10 (Bauru, São Paulo); POP 11 (Andradina, São Paulo); POP 12 (allochthonous case with notification in Curitiba, Paraná); POP 13 (allochthonous case with notification in Maringá, Paraná); POP 14 (Santa Terezinha do Itaipu, Paraná);

Figure 7: Genetic assignment of 18 *Leishmania infantum* populations genotyped with 10 microsatellites. (a) considering k=2. (b) considering k=3

The contribution of individuals of each cluster (see material and methods for details) was plotting in the map (Fig 08) to better visualize the isolates distribution. Similarity, it can be observed in the populations:

1. Aracaju (SE), Fortaleza (CE), Mato Grosso, Campo Grande and Três Lagoas (MS), Andradina and Bauru (SP);

2. Tocantins, Belo Horizonte (MG), Paraguay, Foz do Iguaçu, Santa Terezinha do Itaipu (PR) and Belo Horizonte (MG);

3. São Miguel do Oeste and Descanso (SC) and Pato Branco (PR).

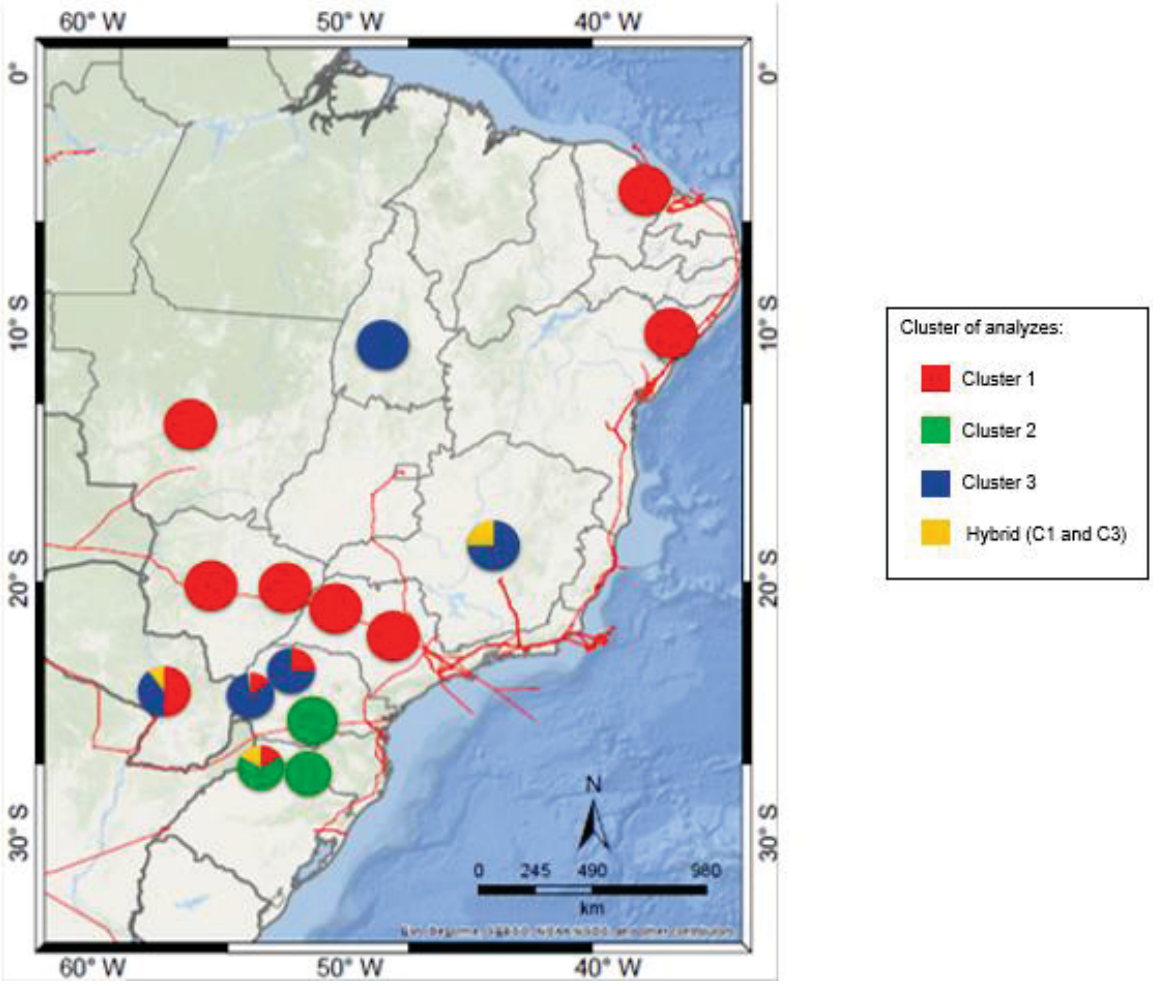


Figure 8: Populations distribution maps and pipeline construction expansion or operation according to percentage of genetic profile of each population. Samples with genetic profile assigned of more than 75% in a cluster were considered pure individuals.

After genetic profile test, the dendogram was analyzed, where the population groupings can be observed (Fig. 9): 1. Isolates of Bauru, São Paulo state (POP 10)

and Campo Grande, Mato Grosso do Sul state (POP 8); 2. Andradina, São Paulo state (POP 11) and Três Lagoas, Mato Grosso do Sul state (POP 7); 3. Mato Grosso state (POP 6), São Miguel do Oeste and Descanso, Santa Catarina state (POP 16) and Aracaju, Sergipe state (POP 3); 4. Europe and African (POP 1); 5. Maringa, Parana state (POP 13), Tocantins state (POP 5) and Belo Horizonte, Minas Gerais state (POP 9); 6. Foz do Iguaçu and Santa Terezinha de Itaipu, Parana state (POP 14 and 15) and Paraguay (POP 2); 7. Fortaleza, Ceara state (POP 4) and Curitiba, Paraná state (POP 12).

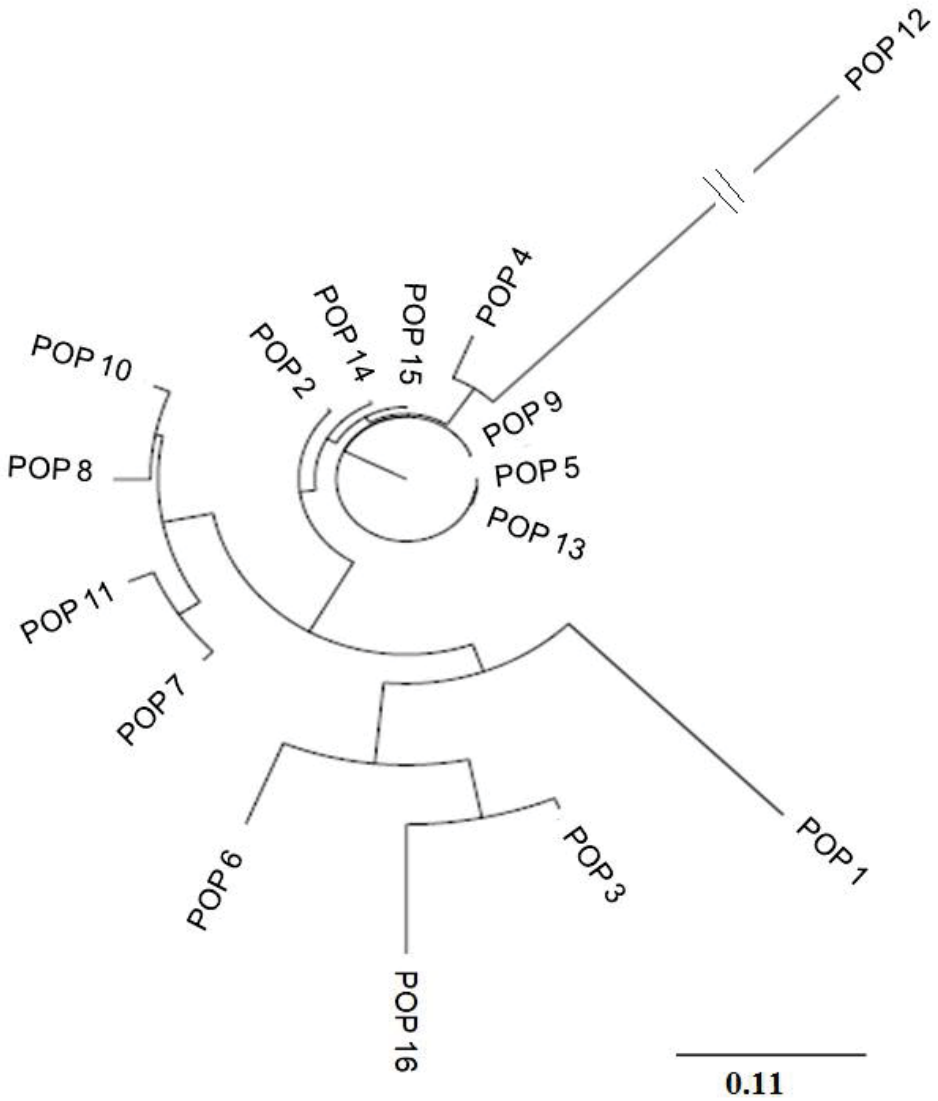


Figure 9: Dendogram building with software, and NJ for populations genetic grouping analysis. (POP 1: Europe and African; POP 2: Paraguay; POP 3: Aracaju, Sergipe state; POP 4: Fortaleza, Ceara state; POP 5: Tocantins state; POP 6: Mato Grosso state; POP 7; Três Lagoas, Mato Grosso Sul state; POP 8: Campo Grande, Mato Grosso do Sul state; POP 9: Belo Horozinte, Minas Gerais state; POP 10: allocthones case of Curitiba, Paraná state; POP 11: allocthones case of Maringa, Parana state; POP 14: Foz do Iguaçu, Parana state; POP 15: Santa Terezinha de Itaipu, Parana state; POP 16: São Miguel do Oeste and Descanso, Santa Catarina state.

4.4.2 First records of VL in South America's center-south: literature recovered date

The search of first records of VL cases in dogs and humans resulted in 52,029 articles, in which 350 were preselected because had epidemiology information or reported first cases of VL South America's center-south. Among these articles 55 were selected due to report human or canine VL cases in South America's center-south region (Colombia, Paraguay, Argentina, Chile, Uruguay and Brazil) between 1913 and 2017.

A total of 656 sites were described with human and canine VL cases in, of which 483 were human records between 2001 at 2015 available in the SINAN and 173 records in literature consultation.

These records were plotted on map separated by historical periods of VL, as follows:

1- 1913 to 1980: populational migration from northwest to southeast (16 counties recording VL case – Fig 10A);

2- 1981 to 1997: beginning of rural exodus, with migration of people and their animals from rural to urban areas (11 counties case records Fig 10B);

3- 1998 to 2005: construction of Bolivia-Brazil pipeline and migration of employees and their pets to Mato Grosso do Sul and São Paulo state, migration for epidemics of VL to big centers of São Paulo, Minas Gerais and Mato Grosso do Sul states (283 counties with case records - Fig 10C);

4- 2006 to 2010: VL cases registered in Paraguay, Argentina and in the border of Rio Grande do Sul state with Argentina (175 counties Fig 10D);

5- 2011 to 2017: Expansion in south Brazil (VL cases in Santa Catarina and Paraná states) and VL cases on border cities between Uruguay and Brazil (171 counties) (Fig. 10E)

All information about the cases recovered are presented in Table 9 and Fig 10.

Table 9: Number of sites per country and Brazilian state with cases of human and canine visceral leishmaniasis in the South America's Center south region.

	1913-1980	1981-1997	1998-2005	2006-2010	2011-2017
Chile	0	0	1	0	0
Bolivia	0	1	2	0	0
Argentina	2	0	1	11	1
Paraguay	0	0	1	0	3
Uruguay	0	0	0	1	1
Brazil	**14**	**10**	**279**	**163**	**166**
Mato Grosso	4	4	17	10	13
Mato Grosso do Sul	2	0	26	8	7
Goias	0	0	19	23	22
São Paulo	5	4	101	52	63
Minas Gerais	2	2	102	59	41
Espirito Santo	0	0	8	3	3
Rio de Janeiro	1	0	6	5	7
Parana	0	0	0	0	4
Santa Catarina	0	0	0	0	4
Rio Grande do Sul	0	0	0	3	2
Total	16	11	284	175	171

In map (Fig. 10F) is possible to observe several dispersions of *L. (L.) infantum* in South America's center south region, being:

1) the initial dispersion from northeast region to Minas Gerais state,

2) dispersion along construction Bolivia-Brazil pipeline,

3) dispersion from Paraguay to Brazil by triple frontier in Foz do Iguaçu city,

4) the entrance of new population by Santa Catarina state (or maybe coming from Argentina).

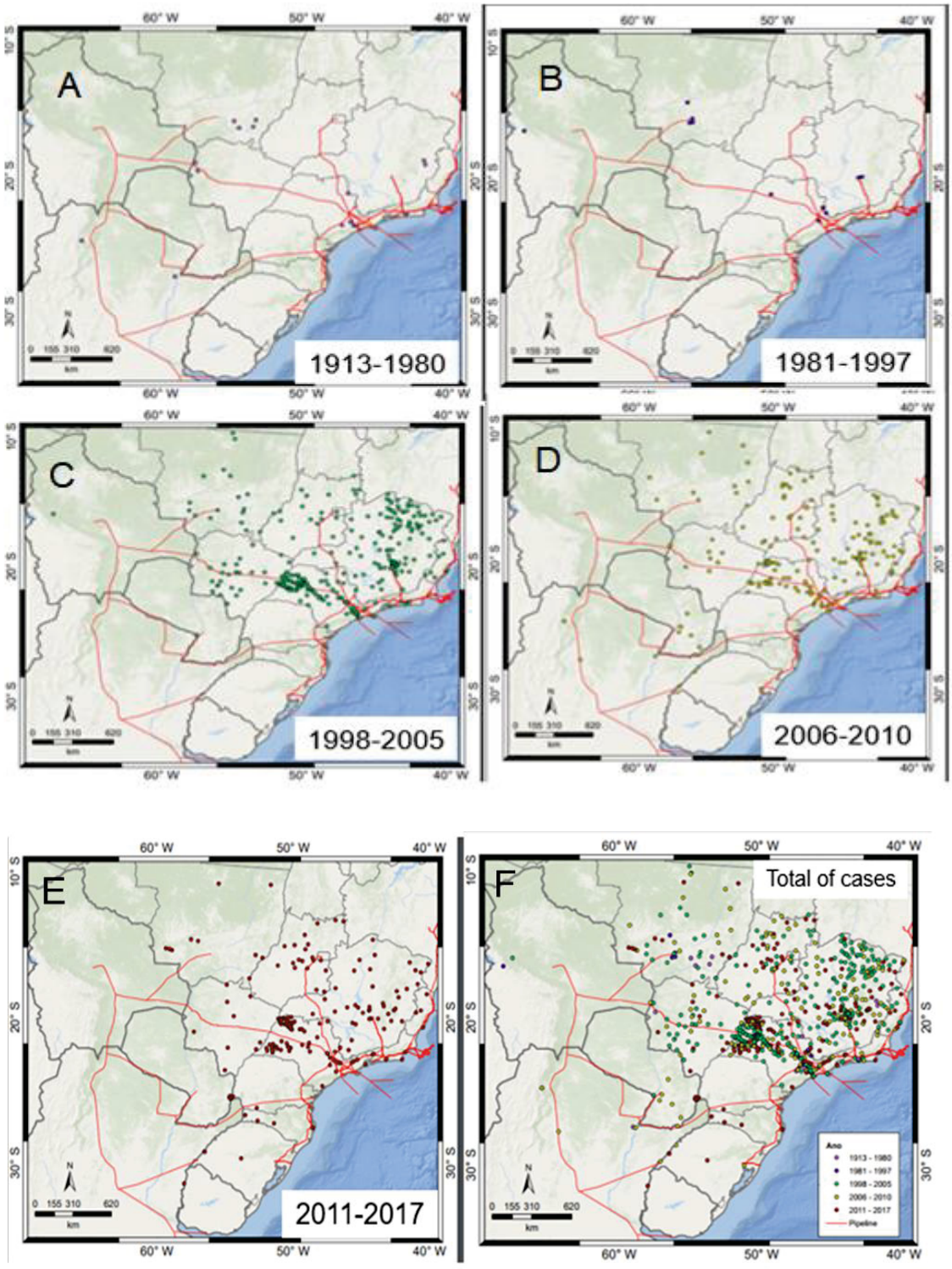


Figure 10: Period of the first VL cases and cVL cases in South America's center-south region (bibliographic survey) and pipeline construction expansion or operation. (A) Registered cases from 1913 to 1980; (B) Registered cases from 1981 to 1997; (C) Registered casesfrom 1998 to 2005; (D) Registered cases from 2006 to 2010; (E) Registered cases from 2011 to 2017 and (F) all dates together.

4.5 DISCUSSION

The municipality of Foz do Iguaçu is located on west of Paraná state (Brazil), bordering two other countries (Argentina and Paraguay), considered a potential place for VL dispersion because has an intensive people flow, environment with abundant vegetation, intense humidity and temperature media above 21.6ºC throughout the year. In addition, the presence of *Lu. longipalpis* was described in Foz do Iguaçu characterizing the first vector appearance in that region. This fact also corroborates to identify the region as a potential VL dispersion focus [60]. Actually, the vector is dispersed on all sites in Foz do Iguaçu and Santa Terezinha de Itaipu [61]. In the same way, VL autochthonous cases in dogs were signaled in 2013 and actually is distributed on all regions of Foz do Iguaçu and Santa Terezinha de Itaipu [29,1]. Besides that, in 2016 the first human autochthonous case was registered [32]. Foz do Iguaçu is now an endemic county for *L. (L.) infantum* due to the generalized distribution of seropositive dogs, abundance of *Lu. longipalpis* and human cases in all areas of the city [1]. However, the question is from where the key actors come to complete the parasite cycle?

Based on the results of our work, which used microsatellites approach to identify VL parasites dispersion, and on recorded data from literature it can be proposed four events that contributed to *L. (L.) infantum* dispersion in South America's center-south region: 1. Population migration and rural exodus; 2. Pipeline construction; 3. Brazilian-Argentine currency in Santa Catarina; 4. Triple border. The microsatellites are an interesting tool to study the population diversity and the dispersion of the disease. Several studies of *L. (L.) infantum* populations were developed in Brazil using microsatellite as tools [64-66,26] with the purpose of verifying the dispersion of the disease. And the literature dates are currently used to niche study, giving consistency to our study.

The first event, population migration and rural exodus started in the colonization event of Brazilian states in northeast region, going to north, southeast and center west was supported by the presence of the same *L. (L.) infantum* group between Aracaju (SE), Fortaleza (CE), Mato Grosso, Campo Grande and Três Lagoas (MS), Andradina and Bauru (SP). The beginning of the rural exodus occurred during this period of 60s, having dispersed the population to the center-west and southeast region, looking for new opportunities and thus disseminating diseases in

new places [61]. From 1981 to 1997, people and their animal's migration from rural to urban areas led to the dispersal of VL cases in peripheries of large cities. The first major urban epidemic of VL occurred in Teresina (Piaui), resulting with more than 1000 human cases reported between 1981 and 1986 [8]. In the following years VL cases began to be registered in large and medium cities, including São Luís (MA), Natal (RN), Aracaju (SE), Boa Vista (RR), Santarém (PA), Palmas (TO), Rio de Janeiro (RJ), Belo Horizonte (MG), Montes Claros (MG), Araçatuba (SP), Cuiabá (MT), Corumbá (MS), Três Lagoas (MS), Campo Grande (MS) [62,63].

The second event, construction of the Bolivia-Brazil gas pipeline, starting in Bolivia and expanding to Mato Grosso do Sul, São Paulo, Rio de Janeiro and Minas Gerais may have assisted in the dispersal of VL. The review of cases and the isolates of *L. (L.) infantum* demonstrated genetic similarity of population from Mato Grosso do Sul and São Paulo states. Probably the displacement of employees and their pets by the course of gas pipeline construction initiated in 1998, as well as deforestation and ecological imbalance resulted in VL dispersion in these states [24,65,26,27]. The deforestation of green areas for gas pipeline construction causes ecological imbalance resulting in the dispersion of *Lu. longipalpis* vector for urban areas [67,68]. Another ecological relevant factor associated to *L. (L.) infantum* dispersion is the possible parasite capacity of changing to other vectors [2,69-80].

The third event, Brazilian-Argentine currency in Santa Catarina indicates that other dispersion of *L. (L.) infantum* occurred in Brazil through the Brazilian-Argentine currency in Santa Catarina. More parasite isolation is necessary to confirm this hypothesis.

The fourth event, triple border the isolates from Paraguay, Foz do Iguaçu and Santa Terezinha de Itaipu (PR) and Belo Horizonte (MG) indicate the possible entry of *L. (L.) infantum* through the triple border Brazil, Argentina and Paraguay more specifically by Foz do Iguaçu (PR). In this region there is an intense flow of people and animals, and an outbreak of VL was observed in Paraguay, Colombia, Argentina and Paraguay (four periods 2006-2010), where VL cases have been reported in Argentina, Paraguay, and Uruguay [80-84,34] and in Rio Grande do Sul, Brazil [29]. In 2011 VL cases were reported in Santa Catarina, and in 2012 in Paraná characterizing the fifth period (2011-2017) [30-32,84,1]. Other possible VL entry in Foz do Iguaçu was by the migration of military and their pets residing in Minas Gerais to work in Foz do Iguaçu, since the city is bordered by countries and requires greater

military protection. The first case of human VL in Minas Gerais state was diagnostic in 1992 possible because of military migrations [9].

In the analyses of historical cases, most cases of VL in humans are not reported at the place of occurrence, thus limiting the search for information and may increase the prevalence in large centers due to omission of information. In other cases, the patient ends up dying without confirmation of the diagnosis, since VL is often not the main clinical suspicion evaluated.

In resume, microsatellite results support four dispersion events of *L. infantum* in South America's Center South region: 1. Migratory flow and rural exodus; 2. Pipeline construction; 3. New parasite population group in Santa Catarina state and 4. Triple border – populations from Paraguay, MG and/or MS.

4.6 ACKNOWLEDGEMENTS

We thank to International Development Research Centre (IDRC-Canada grant number 107577-002); the National Council for Scientific and Technological Development (CNPq grant number Grant No. 307387/2011-9 and 480292/2012-4); the Paraná Araucaria Foundation for Scientific and Technological Development for the financial support (Grant No. 122/2010 -protocol 17401); the Secretariat of Health of the State of Paraná. RAB are fellows of CNPq, AKSP are fellows of Capes.

4.7 AUTHOR CONTRIBUTIONS

Project administration: Vanete Thomaz Soccol.
Conceived and designed the experiments: Vanete Thomaz-Soccol, Rafael Antunes Baggio.
Performed the experiments: Aline Kuhn Sbruzzi Pasquali, Rafael Antunes Baggio, Deborah Carbonera Guedes, Nilce Gonsalez, Vanete Thomaz-Soccol.
Analyzed the data: Aline Kuhn Sbruzzi Pasquali, Rafael Antunes Baggio, Walter Antonio Boeger, Vanete Thomaz Soccol.
Writing – original draft: Aline Kuhn Sbruzzi Pasquali, Rafael Antunes Baggio.
Writing – review and editing: Aline Kuhn Sbruzzi Pasquali, Rafael Antunes Baggio, Vanete Thomaz-Soccol.

4.8 REFERENCES

1. Thomaz-Soccol V, Pasquali AKS, Pozzolo EM, André Souza Leandro AS, Chiyo L. Baggio R, Michaliszyn MS, Silva C, Cubas PH, Peterlle R, Paz OLS, Belmonte IL, Bisetto Jr A. More than the eyes can see: the worrying scenario of canine leshmaniasis in the Brazilian side of the triple border. Plos One. 2017;12(12) https://doi.org/10.1371/journal.pone.01891822.
2. Thomaz-Soccol V, Gonçalves AL, Piecknick CA, Antunes RA, Boeger WA, Buchman TL, Michaliszyn M, et al. Hidden danger: unexpected scenario in the vector-parasite dynamics of leishmanioses in the Brazil side of triple border (Argentina, Brazil and Paraguay). Plos Neglected Tropical Disease. 2018;
3. WHO, World Health Organization. Leishmaniasis Burden and distribution. 2013. Avaliable from: http://www.who.int/leishmaniasis/burden/en/
4. Kilick-Kendrick R, Molyneux DH, Rioux JA, Lanotte G; Leaney AJ. Possible origins of *Leishmania chagasi*. Ann Trop Med Parasitol. 1980;74(5):563-565. https://www.ncbi.nlm.nih.gov/pubmed/7469570 PMID: 7469570
5. Moreno G, Rioux JA, Lanotte G, Pratlong F, Serres E. Le complexe *Leishmania donovani* s.l. Analyse enzymatique et traitment numérique. Individualisation du complexe *Leishmania infantum.* Corollaires biogéographiques et phylétiques. A propos de 146 souches originaires de l'Ancien et du Nouveau Monde. In: Colloque International CNRS/INSERM, 1984. *Leishmania*. Taxonomie et Phylogenése. Applications éco-épidémiologiques. Montpellier: IMEEE, 1986:105-117.
6. Thomaz-Soccol V, Lanotte G, Rioux JA, Pratlong F, Martini Dumas A, Serres E. Phylogenetic taxonomy of New World *Leishmania*. Annales de Parasitologie Humaine et Comparée. 1993;68(1):104-106. Avaliable from:https://www.researchgate.net/profile/Vanete_ThomazSoccol/publication/15505968_Phylogenetic_taxonomy_of_New_Word_Leishmania/links/0a85e531a2685c4e71000000.pdf
7. Kuhls K, Alam MZ, Cupolillo E, Ferreira GEM, Mauricio IL, Oddone R, Feliciangeli MD, Wirth T, Miles MA, Schönian G. Comparative microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. PLoS Negl Trop Dis. 2011;5(6). 10.1371/journal.pntd.0001155 PMID: 21666787

8. Costa CHN, Pereira HF, Araújo MV. Epidemia de leishmaniose visceral no estado do Piauí, Brasil., 1980- 1986. Rev Saúde Públic. 1990;24(5):361-372. http://dx.doi.org/10.1590/S0034-89101990000500003
9. Bevilacqua PD, Paixão HH, Modena CM, Castro MCPS. Urbanização de leishmaniose visceral em Belo Horizonte. Arq Bras Med Vet Zootec. 2001; 53(1). http://dx.doi.org/10.1590/S0102-09352001000100001
10. Costa CHN. Characterization and speculations on the urbanization of visceral leishmaniasis in Brazil. Cad Saúde Pública. 2008; 24(12)-2959-2963. http://dx.doi.org/10.1590/S0102-311X2008001200027
11. Souza VAFde, Cortez LRPdeB, Dias RA, Amaku M, Neto JSF, Kuroda RBdosS, Ferreira F. Space time cluster analysis of American visceral leishmaniasis in Bauru, São Paulo state, Brazil. Cad Saúde Pública. 2012; 28(10):1949-1964. http://dx.doi.org/10.1590/S0102-311X2012001000013
12. Brazil RP. The dispersion of *Lutzomia longipalpis* in urban areas. Rev Soc Bras Med Trop. 2013; 46(3). http://dx.doi.org/10.1590/0037-8682-0101-2013
13. Gama MEA, Barbosa JS, Pires B, Cunha AKB, Fretias AR, Ribeiro IR, Costa JML. Avaliação do nível de conhecimento que populações residentes em áreas endêmicas têm sobre leishmaniose visceral, Estado do Maranhão, Brasil. Cad Saúde Pública. 1998;14(2):381-390. http://dx.doi.org/10.1590/S0102-311X1998000200014
14. Gontijo CMF, Melo MN. Leishmaniose visceral no Brasil: quadro atual, desafios e perspectivas. Rev Bras Epidemiol. 2004; 7(3). http://dx.doi.org/10.1590/S1415-790X2004000300011
15. Brasil, Ministério da Saúde – Secretaria de Vigilância em Saúde. Manual de vigilância da Leishmaniose tegumentar americana: Série A, normas e manuais. Brasília – DF, 2010;2. Avaliable from: http://bvsms.saude.gov.br/bvs/publicacoes/manual_vigilancia_leishmaniose_tegumentar_americana.pdf
16. Maia Elkhoury ANS, Alves WA, Sousa Gomes ML, Sena JM, Luna EA. Visceral leishmaniasis in Brazil: trends and challenges. Cad Saúde Pública. 2008; 24(12): 2941–2947. http://dx.doi.org/10.1590/S0102-311X2008001200024
17. Savani ESM, Presotto D, Roberto T, Camargo MCGO, D´auria N, Sacramento DV. First ocurrence of na autochthonous canine case os *Leishmania (Leishmania) infantum chagasi* in the minucipality of Campinas, state of São paulo, Brazil. Rev Inst

Med Trop São Paulo. 2011;54(4):227-229. https://www.ncbi.nlm.nih.gov/pubmed/21915468 PMID: 21915468

18. Paulan SC, Silva HR, Lima EACF, Flores EF, Tachibana VM, Kanda CZ, Noronha Junior ACF, Dobre PR. Spatial distribuition of canine visceral Leishmaniasis in Ilha Solteira, São Paulo, Brazil. Eng Agríc. 2012;32(4). http://dx.doi.org/10.1590/S0100-69162012000400016
19. Cunha RC, Andreotti R, Cominetti MC, Silva EA. Detection of *Leishmania infantum* in *Lutzomyia longipalpis* captured in Campo Grande, MS. Rev Bras Parasitol Vet. 2014;23(2):269-273. Avaliable from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S198429612014000200269 PMID: 25054512
20. Oliveira AM, Vieira CP, Dibo MR, Guirado MM, Rodas LAC, Chiaravalloti-Neto F Dispersal of *Lutzomyia longipalpis* and expansion of canine and human visceral leishmaniasis in São Paulo state, Brazil. Acta Trop. 2016;164:233-242. 101610.1016/j.actatropica.2016.09.014 PMID: 2764032
21. Sevá ADP, Mao L, Galvis-Ovallos F, Tucker Lima JM, Valle D. Risk analysis and prediction of visceral leishmaniasis dispersion in São Paulo State, Brazil. PLoS Neg Trop Dis. 2017; 11(2). https://doi.org/10.1371/ journal.pntd.0005353
22. Who, World Health Organization. *Leishmania*ses. Epidemiological Report of the Americas. 2017; 5. Avaliable from: http://iris.paho.org/ xmlui/handle/123456789/34111
23. Oliveira AG, Galati EAB, Oliveira O, Oliveira GR, Espindola IAC, Dorval MEC, Brazil RP. Abundance of *Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae) and urban transmission of visceral leishmaniasis in Campo Grande, state of Mato Grosso do Sul, Brazil. Memórias do Instituto Oswaldo Cruz. 2006;101:869–874. http://dx.doi.org/10.1590/S0074-02762006000800008
24. Antonialli SAC, Torres TG, Paranhos Filho AC, Tolezano JE. Spatial analysis of American visceral leishmaniasis in Mato Grosso do Sul State, central Brazil. J Infect. 2007; 54(5): 509–514. 10.1016/j.jinf.2006.08.004 PMID: 1697924
25. Werneck GL. Forum: geographic spread and urbanization of visceral leishmaniasis in Brazil. Introduction. Cad Saude Publica. 2008;24:2937–2940. http://dx.doi.org/10.1590/S0102-311X2008001200023
26. Motoie G, Ferreira GE, Cupolillo E, Canavez F, Pereira-Chioccola VL. Spatial distribution and population genetics of *Leishmania infantum* genotypes in São Paulo State, Brazil, employing multilocus microsatellite typing directly in dog infected

tissues. Infect Genet Evol. 2013;18:48-59. 10.1016/j.meegid.2013.04.031 PMID: 23665466

27. Cardim MF, Guirado MM, Dibo MR, Chiaravalloti FN. visceral leishmaniasis in the state of Sao Paulo, Brazil: spatial and space-time analysis. Rev Saúde Pública. 2016; 50. http://dx.doi.org/10.1590/S1518-8787.2016050005965
28. Evans TG, Teixeira MJ, Mcauliffe IT, Vasconcelos I, Vasconcelos AW, Couza AA, Lima JW, Pearson RD. Epidemiology of visceral Leishmaniasis in Northeast Brazilian. J Infect Dis. 1992;166(5):1124-1132. https://www.ncbi.nlm.nih.gov/pubmed/1402024 PMID: 1402024
29. Souza GD, Santos E, Andrade Filho JD. The first report of the main vector of visceral *Leishmania*sis in America, *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae: Phlebotominae), in the state of Rio Grande do Sul, Brazil. Mem Inst Oswaldo Cruz. 2009;104(8):1181-1182. http://dx.doi.org/10.1590/S0074-02762009000800017
30. Dias RCF, Thomaz-Soccol V, Bisetto Júnior A, Pozzolo EM, Chiyo L, Freire RL, Breganó RM, Pasquali AKS, Alban S, Fendrich RC, Caldart ET, Navarro IT. Occurrence of anti-*Leishmania* spp. antibodies in domiciled dogs from the city of Foz do Iguaçu, State of Paraná, Brazil. In: World Congress On Leishmaniasis, 5., 2013, Porto de Galinhas. Abstract. Porto Galinhas: Soc Brasil Med Trop. 2013:875-876
31. Steindel M, Menin A, Evangelista T, Stoco PH, Marlow MA, Fleith RC, Pilati C, Grisard EC. Outbreak of autochthonous canine visceral leishmaniasis in Santa Catarina, Brazil. Pesq Vet Bras. 2013;33(4):493-496. http://dx.doi.org/10.1590/S0100-736X2013000400013
32. Trench FJP, Ritt AG, Gewehr TA, de Souza Leandro A, Chiyo L, Gewehr MR, Ripoli M, Bisetto A, Pozzolo EM, Thomaz Soccol V. First Report of Autochthonous Visceral Leishmaniosis in Humans in Foz do Iguaçu, Paraná State, Southern Brazil. Ann Clin Cytol Pathol. 2013;2(6): 1041. https://www.jscimedcentral.com/ClinicalCytology/clinicalcytology-2-1041.pdf
33. Furlan MBG. Epidemia de leishmaniose visceral no Município de Campo Grande-MS, 2002 a 2006. Epidemiol. Serv. Saúde. 2010;19(1):15-24. http://dx.doi.org/10.5123/S1679-49742010000100003
34. Salomón OD, Basmajdian Y, Fernández MS, Santini MS. *Lutzomyia longipalpis* in Uruguay: the first report and the potential of visceral leishmaniasis

transmission. Mem Inst Oswaldo Cruz. 2011;106(3):381-382. http://dx.doi.org/10.1590/S0074-02762011000300023

35. Schwenkenbecher JM, Wirth T, Schnur LF, Jaffe CL, Schallig H, Al-Jawabreh A, Hamarsheh O, Azmi K, Pratlong F, Schönian G. Microsatellite analysis reveals genetic structure of *Leishmania tropica*. Int J Parasitol. 2006;36(2):237–246. 10.1016/j.ijpara.2005.09.10 PMID: 16307745
36. Montoya L, Gallego M, Gavignet B, Piarroux R, Rioux JA, Portus M, Fisa R. Application of microsatellite genotyping to the study of a restricted *Leishmania infantum* focus: different genotype compositions in isolates from dogs and sand flies. Am J Trop Med Hyg. 2007;76(5):888-895. Avaliable from; http://www.ajtmh.org/docserver/fulltext/14761645/76/5/0760888.pdf?expires=1518633186&id=id&accname=guest&checksum=F67641F863A0A9F335FF37C49F0D82F1 PMID: 17488911
37. Al-Jawabreh A, Diezmann S, Müller M, Wirth T, Schnur LF, Strelkova MV, Kovalenko DA, Razakov SA, Schwenkenbecher J, Kuhls K, Schönian G. Identification of geographically distributed sub-populations of *Leishmania* (*Leishmania*) major by microsatellite analysis. BMC Evol Biol. 2008:8(183). 2008. 10.1186/1471-2148-8-183
38. Alam MZ, Haralambous C, Kuhls K, Gouzelou E, Sgouras D, Soteriadou K, Schnur L, Pratlong F, Schönian G. The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. Microbes Infect. 2009;11(6-7):707–715. 10.1016/j.micinf.2009.04.009.
39. Oddone R, Schweynoch C, Schönian G, De Sousa CDos S, Cupolillo E, Espinosa D, Arevalo J, Noyes H, Mauricio I, Kuhls K. Development of a multilocus microsatellite typing approach for discriminating strains of *Leishmania* (*Viannia*) species. J Clin Microbiol. 2009;47(9):2818–2825., jul. 2009. 10.1128/JCM.00645-09
40. Mahnaz T, Al-Jawabreh A, Kuhls K, Schönian G. Multilocus microsatellite typing shows three different genetic clusters of *Leishmania major* in Iran. Microbes Infect. 2011;13(11):937–942. 10.1016/j.micinf.2011.05.005 PMID: 21664984
41. Downing T, Stark O, Vanaerschot M, Imamura H, Sanders M, Decuypere S, De Doncker S, Maes I, Rijal S, Sundar S, Dujardin JC, Berriman M, Schönian G. Genome-wide SNP and microsatellite variation illuminate population-level epidemiology in the *Leishmania donovani* species complex. Infect Genet Evol. 2012;12:149–159. 10.1016/j.meegid.2011.11.005 PMID: 22119748

42. Gouzelou E, Haralambous C, Amro A, Mentis A, Pratlong F, Dedet JP, Votypka J, Volf P, Toz SO, Kuhls K, Schönian G, Soteriadou K. Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic *L. donovani* sensu lato group. PLoS Negl Trop Dis. 2012;6(2). 10.1371/journal.pntd.0001507 PMCID: PMC3279343
43. Aluru A, Hide M, Michel G, Banuls AL, Marty P, Pomares C. Multilocus microsatellite typing of *Leishmania* and clinical applications: a review. Parasite. 2015;22,(16). 10.1051/parasite/2015016 PMID: 25950900
44. Rioux JA. Recommendations. In: *Leishmania*. Taxonomy and phylogeny. Applications to ecology and epidemiology. RIOUX, J.A. (Ed.). 1986:513-517.
45. Sambrook J, Fritch EF, Maniatis T. Molecular cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press. 1989.
46. Bañuls AL. Apport a la genetique evolutive a l'epidemiologie et a la toxonomie du gene *Leishmania*. Montpellier, França. Docthor thesis Montpellier University. 1998; 196f.
47. Sambrook J, Russell RW. Molecular cloning: A laboratory manual, 3th ed. Cold Spring Harbor Laboratory Press. 2001.
48. Jamjoom MB, Ashford RW, Bates P.A, Kemp SJ, Noyes HA. Towards a standard battery of microsatellite markers for the analysis of the *Leishmania donovani* complex. Ann Trop Med Parasitol. 2002;96(3):265-270. 10.1179/000349802125000790 PMID: 12061973
49. Ochsenreither S, Kuhls K, Schaar M, Presber W, Schonian G. Multilocus microsatellite typing as a new tool for discrimination of *Leishmania infantum* MON-1 strains. J Clin Microbiol. 2006; 44(2):495-503. 10.1128/JCM.44.2.495-503.2006 PMID: 16455904
50. Kuhls K, Keilonat L, Ochsenreither S, Schaar M, Schweynoch C, Presber W, Schonian G. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. Microbes Infect. 2007;9(3):334-343. 10.1016/j.micinf.2006.12.009 PMID: 17307010
51. Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. Program Note Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Molec Ecol Not. 2004;4:535–538. 10.1111/j.1471-8286.2004.00684.x

52. Excoffier l, Lischer HE. Arlequin Suite ver 3.5: a new series of programs to perform population genetics analyses under linux and windows. Mol ecol resour. 2010;10(3):564-7. 10.1111/j.1755-0998.2010.02847.x.
53. Goudet J. FSTAT Software, v. 2.9.3.2. 2002. Avaliable from: http://www2.unil.ch/popgen/softwares/fstat.htm
54. Narum SR. Beyond Bonferroni: Less conservative analyses for conservation genetics. Conserv Genet. 2006; 155(2):783-787. Avaliable from: https://link.springer.com/article/10.1007/s10592-005-9056-y
55. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155(2):945-959. Avaliable from: http://www.genetics.org/content/155/2/945.long PMID: 10835412
56. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol. 2005;14(8):611-20. 10.1111/j.1365-294X.2005.02553.x PMID: 15969739
57. Earl DA, Vonholdt, BM. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. 2012; 4(2):359-361. Available from: http://taylor0.biology.ucla.edu/structureHarvester/.
58. Abegas, Associação Brasileira das Empresas Distribuidoras de Gás Canalizado. Gasodutos. 2012. Avaliable from: http://www.abegas.org.br/Site/?page_id=842
59. Epe, Empresa de Pesquisa Energética. Zoneamento. 2016. Avaliable from:http://antigo.epe.gov.br/Petroleo/Documents/Zoneamento/ZNMT2013_15_Publ_10.1.mpk
60. 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. http://dx.doi.org/10.1590/S0037-86822012000500019
61. Barreto MP. Movimentos migratórios e sua importância na epidemiologia de doenças parasitárias no Brasil. Rev Soc Bras Med Trop. 1967;1(3):91-102. http://dx.doi.org/10.1590/S0037-86821967000300003
62. Brasil, Ministério da Saúde - Centro Nacional de Epidemiologia. Leishmaniose Visceral no Brasil: situação atual, principais aspectos epidemiológicos, clínicos e medidas de controle. Boletim Epidemiológico. 2001;6(13):1-11.

63. Brasil, Ministério da Saúde - Secretaria de Vigilância em Saúde. Manual de vigilância e controle da leishmaniose visceral. Brasília – DF, 2006. Avaliable from: http://bvsms.saude.gov.br/bvs/publicacoes/manual_vigilancia_controle_leishmaniose_visceral.pdf
64. Batista LF, Segatto M, Guedes CE, Sousa RS, Rodrigues CA, Brazunza JC, Silva JS, Santos SO, Larageira D, Macedo AM, Scriefer A, Veras OS. An Assessment of the Genetic Diversity of *Leishmania infantum* Isolates from Infected Dogs in Brazil. Am J Trop Med Hyg. 2012;86(5):799-806. 10.4269/ajtmh.2012.11-0300 PMID: 22556077
65. Ferreira GE, dos Santos BN, Dorval ME, Ramos TP, Porrozzi R, Peixoto AA, Cupolillo E. The genetic structure of *Leishmania infantum* populations in Brazil and its possible association with the transmission cycle of visceral leishmaniasis. PLoS One. 2012;7(5). 10.1371/journal.pone.0036242 PMID: 22606248
66. Segatto M, Ribeiro LS, Costa DL, Costa CH, Oliveira MR, Carvalho SF, Macedo AM, Valadares HMS, Dietze R, Brito CFA; Lemos EM. Genetic diversity of *Leishmania infantum* field populations from Brazil. Mem Instit Oswaldo Cruz. 2012;107(1):39-47. 10.1590/S0074-02762012000100006 PMID: 22310534
67. Zell R. Global climate change and the emergence/re-emergence of infectious diseases. Int J Med Microbiol. 2004;293(37):16–26. https://doi.org/10.1016/S1433-1128(04)80005-6
68. Harrus S, Baneth G. Drivers for the emergence and reemergence of vector-borne protozoal and bacterial diseases. Int J Parasitol 2005; 35:1309–1318. 10.1016/j.ijpara.2005.06.005 PMID: 16126213
69. Travi BL, Velez ID, Brutus L, Segura I, Jaramillo C, Montoya J. *Lutzomyia evansi*, an alternate vector of *Leishmania chagasi* in a Colombian focus of visceral leishmaniasis. Trans R SocTrop Med Hyg. 1990;84(5):676–677. Avaliable from: https://academic.oup.com/trstmh/article-abstract/84/5/676/1914099?redirectedFrom=fulltext PMID: 2278068
70. Santos SO, Arias J, Ribeiro A, Hoffmann MP, Freitas RA, Malacco MAF. Incrimination of *Lutzomyia cruzi* as a vector of American Visceral *Leishmania*sis. Medic Vet Entomol. 1998;12(3):315-317. 10.1046/j.1365-2915.1998.00104.x
71. Feliciangeli MD, Rodriguez N, De Guglielmo Z, Rodriguez A. The reemergence of American visceral leishmaniasis in an old focus in Venezuela. Parasite. 1999; 6(2):113–120. 10.1051/parasite/1999062113 PMID: 1041618

72. Lainson R, Rangel EF. *Lutzomyia longipalpis* and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil: a review. Mem Inst Oswaldo Cruz. 2005;100(8):811–827. 10.1590/S0074-02762005000800001 PMID: 16444411

73. Lainson R, Shaw JJ. New World Leishmaniasis. In: Cox FEG, Wakelin D, Gillespie SH, Despommier DD. Topley & Wilson's Microbiology and Microbial Infections. London: Wiley & Blackwell. 2005; 10:313–349.

74. Carvalho GML, Andrade Filho JD, Falcão AL, Lima ACVMR, Gontijo CMF. Naturally infected *Lutzomyia,* sandflies in a *Leishmania*-endemic area of Brazil. Vector-Borne Zoonotic Dis. 2008;8(3):407-414. 10.1089/vbz.2007.0180 PMID: 18429695

75. Pita-Pereira D, Cardoso Ma, Alves Cr, Brazil Rp, Britto C. Detection of natural infection in *Lutzomyia cruzi* and *Lutzomyia forattinii* (Diptera: Psychodidae: Phlebotominae) by *Leishmania infantum chagasi* in an endemic area of visceral Leishmaniasis in Brazil using a PCR multiplex assay. Acta Trop. 2008;107(1):66-69. 10.1016/j.actatropica.2008.04.015 PMID: 18502392

76. Saraiva L, Carvalho GML, Sanguinette CC, Carvalho DAA, Falcão AL, Andrade Filho JD. Sandflies (Diptera: Pychodidae: Phlebotominae) collected on the banks of the Velhas River in the state of Minas Gerais, Brazil. Mem Inst Oswaldo Cruz. 2008;103:843-846. http://dx.doi.org/10.1590/S0074-02762008000800018

77. Savani ES, Nunes VL, Galati EA, Castilho TM, Zampieri RA, Floeter-Winter LM. The finding of *Lutzomyia almerioi* and *Lutzomyia longipalpis* naturally infected by *Leishmania* spp in a cutaneous and canine visceral *Leishmania*ses focus in Serra da Bodoquena, Brazil. Vet Parasitol. 2009;160(1-9):18-24. 10.1016/j.vetpar.2008.10.090 PMID: 19062193

78. Rêgo FD, Shimabukuro PHF, Quaresma PF, Coelho IR, Tonelli GB, Silva KMS, Barata RA, Dias ES, Gontijo CMF. Ecological aspects of the Phlebotominae fauna (Diptera: Psychodidae) in the Xakriabá Indigenous Reserve, Brazil. Parasit Vectors. 2014;7(220). 10.1186/1756-3305-7-220 PMID: 24886717

79. Moya SL, Giuliani MG, Manteca Acosta M, Salomon OD, Liotta DJ. First description of *Migonemyia migonei* (Franca) and *Nyssomyia whitmani* (Antunes & Coutinho) (Psychodidae: Phlebotominae) natural infected by *Leishmania infantum* in Argentina. Acta Trop. 2015;152:181-184. 10.1016/j.actatropica.2015.09.015 PMID: 26409011

80. Duprey ZH, Steurer FJ, Rooney JA, Kirchhoff LV, Jackson JE, Rowton ED, Schantz, PM. Canine Visceral Leishmaniasis, United States and Canada, 2000–2003. Emerg Infect Dis. 2006;12(3):440–446. 10.3201/eid1203.050811 PMID: 16704782
81. Cousiño B. Vigilancia y control de la leishmaniasis en ParaguayIn Panaftosa, Informe final de la reunión de expertos OPS/OMS sobre Leishmaniasis Visceral en las Américas, Panaftosa, Rio de Janeiro. 2006:34-36, 2006.
82. Salomón OD, Ramos LK, Quintana MG, Acardi SA, Santini MS, Schneider, A. Distribución de vectores de Leishmaniasis Visceral en la provincia de Corrientes, 2008. Medicina. 2009;69:625-630. Avaliable from: http://www.scielo.org.ar/scielo.php?pid=S0025-76802009000700006&script=sci_arttext&tlng=pt
83. Salomon OD, Sinagra A, Nevot MC, Barberian G, Paulin P, Estevez JO, Riarte A, Estevez J. First visceral leishmaniasis focus in Argentina. Mem Instit Oswaldo Cruz. 2008;103(1):109-111. http://dx.doi.org/10.1590/S0074-02762008000100018
84. Steindel M. SC tem o primeiro caso de leishmaniose visceral humanaRSC portal (comunicação on line), 17 ago 2017. Avaliable from: https://www.rscportal.com.br/artigo/sc-tem-primeiro-caso-de-leishmaniose-visceral-humana

5 FINAL CONSIDERATIONS

L. (L.) infantum was isolated and identified as a parasite present in dogs with suspected VL in Foz do Iguaçu and Santa Terezinha de Itaipu, Paraná, through the ITS marker. This marker allowed to identify the circulating species in a recent transmission focus. However, it was not possible to observe intra-species genetic variability, and was therefore not suitable for population analysis of *L. (L.) infantum*. The use of other molecular tools such as microsatellites, for population studies and dispersion of VL cases in South America's Center South region, became necessary. With the use of fourteen microsatellite markers, associated with the historical survey of VL cases, it was possible to show that four events contributed to the spread of the disease in the South America's Center South region. The events were: colonization and occupation of Brazilian states, construction of pipelines and pipelines, population migration and a new entry of VL in Pato Branco (different genotype). These factors raise the hypothesis that this population has entered the state of Santa Catarina. In order to answer this question, it is necessary to study a larger group of isolates of *L. (L.) infantum*, including parasite samples from Argentina and the state of Rio Grande do Sul (Brazil) to confirm the entry of the parasite in the state of Paraná and understand the new VL scenario in Santa Catarina. With the knowledge of *Leishmania* species and the disease dispersal routes in the South America's Center South region it may be possible to implement measures of prevention and control of VL. For example, with the results obtained in the present work it is possible to predict that in the State of Paraná there will be two dispersion routes for VL. The first will be BR 277 and the second through BRs 158 and 373. It is therefore necessary to organize the primary, secondary and tertiary health services of these regions in the face of disease dispersal and to alert physicians and veterinarians to differentiate VL from other diseases with similar symptoms or clinical signs. In dogs VL should be differentiated from diseases such as demodicosis, erlichiosis, babesiosis because they cause similar clinical signs. In humans, risk groups (immunocompromised, children, elderly) and suspected cases such as clinical signs of high fever and rapid weight loss, differing from diseases such as leukemia, typhoid fever, malaria, histoplasmosis, and others should be considered. With actions and surveillance measures, an effective way of prevention and control of VL can be established based on the results and events presented here.

6 REFERENCE

AGUIAR, G. M.; MEDEIROS W.M. **Distribuição regional e hábitats das espécies de flebotomíneos do Brasil**, p.207- 255. In RANGEL, E.F.; LAINSON, R. Flebotomíneos do Brasil, Rio de Janeiro, Editora FIOCRUZ, 368p, 2003.

AKHOUNDI, M.; HAJJARAN, H.; BAGHAEI, A.; MOHEBALI, M., Geographical distribution of *Leishmania* species of human cutaneous leishmaniasis in Fars province, southern Iran. **Iranian Journal of Parasitology**, v. 8, n. 1; p. 85-91, 2013. Avaliable from: <https://www.ncbi.nlm.nih.gov/pmc/articles/ PMC3655245/pdf/IJPA-8-085.pdf>. Acesso em 15 feb. 2018.

AKHOUNDI, M.; KUHLS, K.; CANNET, A.; VOTYPKA, J.; MARTY, P.; PASCAL, D.; SERENO, D. A historical overview of the Classification, Evolution and Dispersion of *Leishmania* Parasites and Sandflies. **PLOS Negliected Tropical Diseases**, v. 10, n. 3, mar. 2016. DOI. 10.1371/journal.pntd.0004349. Avaliable from: <http://journals.plos.org/ plosntds/article?id=10.1371/journal.pntd.0004349>. Access: 10 dec. 2016.

AKHOUNDI, M.; DOWNING, T.; VOTYPKA, J.; KUHLS, K.; LUKES, J.; CANNET, A.; RAVEL, C.; MARTY, P.; DELAUNAY, P.; KASBARI, M.; GRANOUILLAC, B.; GRADONI, L.; SERENO, D. *Leishmania* infections: Molecular targets and diagnosis. **Molecular Aspects of Medicine**, v. 57, p. 1-29, 2017. DOI: 10.1016/j.mam.2016.11.012. Avaliable from: <https://www.sciencedirect.com/science/article/pii/S0098299716300450?via%3Dihub>. Access: 14 feb. 2018.

AKOPYANTS, N. S.; KIMBLIN, N.; SECUNDINO, N.; PATRICK, R.; PETERS, N.; LAWYER, P.; DOBSON, D. E.; BEVERLEY, S. M.; SACKS, D. L. Demonstration of genetic exchange during cyclical development of *Leishmania* in the sand fly vector. **Science** New York, v. 324, n. 5924, p. 265-268, abr. 2010. DOI:10.1126/science.1169464. Avaliable from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2729066/>. Access: 20 feb. 2017.

ALAM, M. Z.; HARALAMBOUS, C.; KUHLS, K.; GOUZELOU, E.; SGOURAS, D.; SOTERIADOU, K.; SCHNUR, L.; PRATLONG, F.; SCHÖNIAN, G. The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. **Microbes and Infection**, v. 11, n. 6-7, p. 707–715, mai. Jun. 2009. doi: 10.1016/j.micinf.2009.04.009. Avaliable from: <https://www.sciencedirect.com/science/article/pii/S128645790900080X?via%3Dihub>. Access: 30 jan. 2017.

AL-JAWABREH, A.; DIEZMANN, S.; MÜLLER, M.; WIRTH, T.; SCHNUR, L. F.; STRELKOVA, M. V.; KOVALENKO, D. A.; RAZAKOV, S. A.; SCHWENKENBECHER, J.; KUHLS, K.; SCHÖNIAN, G. Identification of geographically distributed sub-populations of *Leishmania* (*Leishmania*) *major* by microsatellite analysis. **BMC Evolutionary Biology**, v. 8, n. 183, jun. 2008. doi: 10.1186/1471-2148-8-183. Avaliable from: <https://bmcevolbiol.biomed central.com/articles/10.1186/1471-2148-8-183>. Access: 10 dec. 2016.

ALURU, A.; HIDE, M.; MICHEL, G.; BANULS, A. L.; MARTY, P.; POMARES, C. Multilocus microsatellite typing of *Leishmania* and clinical applications: a review. **Parasite**, v. 22, n. 16, 2015. DOI: 10.1051/parasite/2015016. Avaliable from: <https://www.parasite-journal.org/articles/parasite/pdf/2015 /01/parasite140122.pdf>. Access: 8 jan. 2017.

ALVAR, J.; SOLAR, B. G.; PACHON, I.; CALBACHO, E.; RAMIREZ, M.; VALLÉS, R.; GUILLÉN, J. L.; CAÑAVATE, C.; AMELA, C. AIDS and *Leishmania infantum*: new approaches for a new epidemiological problem. **Clinics in Dermatology**, v. 14, n. 5; p. 541-546, sep. oct. 1996. DOI: 10.1016/0738-081X(96)00046-6. Avaliable from: <http://www.cidjournal. com/article/0738-081X(96)00046-6/pdf>. Access: 23 feb. 2018.

ALVAR, J.; CAÑAVATE, C.; GUTIÉRREZ-SOLAR, B.; JIMÉNEZ, M.; LAGUNA, F.; LÓPEZ-VÉLEZ, R.; MOLINA, R.; MORENO, J. *Leishmania* and human immunodeficiency virus coinfection: the first 10 years. **Clinical Microbiological Reviews**, v. 10, n. 2, p. 298-319, 1997. Avaliable from: < https://www.ncbi.nlm.nih.gov/pmc/articles/PMC172921/>. Access: 7 mai. 2016.

ANTONIALLI, S. A. C.; TORRES, T. G.; PARANHOS FILHO, A. C.; TOLEZANO, J. E. Spatial analysis of American visceral leishmaniasis in Mato Grosso do Sul State, central Brazil. **Journal of Infection**, v. 54, n. 5, p. 509–514, mai. 2007. DOI: 10.1016/j.jinf.2006.08.004. Avaliable from: < http://www.journalofinfection.com/article/S0163-4453(06)00257-X/fulltext>. Access: 15 dec. 2017.

ARRANSAY, A. M.; SCOULICA, E.; TSELENTIS, Y. Detection and identification of *Leishmania* DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. **Applied and environmental microbiology**, v. 66, n. 5, p. 1933-1938, mai. 2000. AVALIABLE FROM: <https://www.ncbi.nlm.nih.gov/pmc/articles/ pmc101436/>. ACCESS: 6 feb. 2018.

ASHFORD, R. W. The *Leishmania*sis as emerging and reemerging zoonoses. **International Journal Parasitology**, v. 30, p. 1269-1281, 2000. DOI: 10.1016/S0020-7519(00)00136-3. Avaliable from: https://www.researchgate. net/publication/12215407_The_Leishmaniases_as_emerging_and_reemerging_zoon oses. Access: 7 mai. 2016.

AZEVEDO, E. M. R.; DUARTE, S. C.; COSTA, H. X.; ALVES, C. E. F.; SILVEIRA NETO, O. J.; JAYME, V. S.; LINHARES, G. F. C. Estudo da leishmaniose visceral canina no município de Goiânia, Goiás, Brasil. **Revista de Patologia Tropical,** v. 40, n. 2, p. 159-168, abr. jun. 2011. DOI: 10.5216/rpt.v40i2.14941 Avaliable from: <https://www.revistas. ufg.br/iptsp/article/view/14941 >. Access: 28 jan. 2018.

BALLOUX, F.; LEHMANN, L.; DE MEEÛS, T. The population genetics of clonal and partially clonal diploids. **Genetics**, v. 164, n. 4, p. 1635-1644, aug. 2003. Avaliable from: < http://www.genetics.org/content/164/4/1635>. Access: 10 jan. 2018.

BANETH, G. **Canine Leishmaniasis.** In Greene CE, editor. Infectious diseases of the dog and cat. Ed. Saunders/Elsevier, e. 3, p. 696–698, 2006.

BAÑULS, A. L.; HIDE, M.; PRUGNOLLE, F. *Leishmania* and the *Leishmania*ses: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. **Advances in Parasitology**, v. 64, n. 1–199, 2007. DOI: 10.1016/S0065-308X(06)64001-3. Avaliable from: < https://www.sciencedirect.com/science/article/pii/S0065308X06640013?via%3Dihub> . Access: 10 dec. 2017.

BATISTA, L. F.; SEGATTO, M.; GUEDES, C. E.; SOUSA, R. S.; RODRIGUES, C. A.; BRAZUNZA, J. C.; SILVA, J. S.; SANTOS, S. O.; LARAGEIRA, D.; MACEDO, A. M.; SCRIEFER, A.; VERAS, P. S. An Assessment of the Genetic Diversity of *Leishmania infantum* Isolates from Infected Dogs in Brazil. **The American Journal of Tropical Medicine and Hygiene**, v. 86, n. 5, p. 799-806, mai. 2012. DOI: 10.4269/ajtmh.2012.11-0300. Disponivel em: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC333568 3/pdf/tropmed-86799.pdf >. Access: 15 jan. 2017.

BIO-MANGUINHOS, Instituto de Tecnologia de Imunobiológicos. **Teste DPP® Leishmaniose Canina.** 2014. Avaliable from: <https://www.bio.fiocruz.br/index.php/produtos/reativos/testesrapidos/dpprleishmaniose-canina>. Access: 19 feb. 2018.

BIRKY, C. W. J. Heterozygosity, heteromorphy and phylogenetic trees in asexual eukaryotes. **Genetics,** v. 144, n. 1, p. 427-437, sep. 1996. Avaliable from: < http://www.genetics.org/content/144/1/427.long >. Access: 30 jan. 2018.

BISETTO JUNIOR, A.; PASQUALI, A. K. S.; POZZOLO, E. M.; NAVARRO, I. T.; CHIYO, L.; BREGANO, R. M.; DIAS, R. C. F.; FRIEDRICH, R.; FREIRE, R. L.; THOMAZ SOCCOL, V. **Manual técnico de leishmanioses caninas: Leishmaniose Tegumentar Americana e Leishmaniose Visceral.** Conselho Regional de Medicina Veterinária e Zootecnica, Paraná, 2015. Avaliable from: < http://www.crmv-pr.org.br/?p=inicial/pagina_adicional&id=169>. Access: 20 dec. 2016.

BOITE, M.C.; MAURICIO, I. L.; MILES, M. A.; CUPOLILLO, E. New insights on taxonomy, phylogeny and population genetics of *Leishmania (Viannia*) parasites based on multilocus sequence analysis. **PLoS Neglected Tropical Diseases**, v. 6, n. 11, nov. 2012. DOI: 10.1371/journal.pntd.0001888. Avaliable from: <http://journals.plos.org/plosntds/article/file?id=10.1371/journal.pntd.0001888&type=printable>. Access: 14 jan. 2018.

BRASIL, Ministério da Saúde - Centro Nacional de Epidemiologia. **Leishmaniose Visceral no Brasil: situação atual, principais aspectos epidemiológicos, clínicos e medidas de controle**. Boletim Epidemiológico, n. 6, v. 13, p. 1-11, 2001.

BRASIL, Ministério da Pecuária e Abastecimento. **Portaria nº 176, de 03 de outubro de 2005**. Brasília – DF, 2005. Avaliable from: <http://www.cfmv.org.br/portal/legislacao/outras_normas/portaria_0176.htm>. Access: 24 feb. 2018.

BRASIL, Ministério da Saúde - Secretaria de Vigilância em Saúde. **Manual de vigilância e controle da leishmaniose visceral.** Brasília – DF, 2006. Avaliable

from: <http://bvsms.saude.gov.br/bvs/publicacoes/manual_vigilancia _controle_leishmaniose_visceral.pdf>. Access: 20 mar. 2016.

BRASIL, Ministério da Saúde – Secretaria de Vigilância em Saúde. **Manual de vigilância da Leishmaniose Tegumentar Americana: Série A, normas e manuais.** Brasília – DF, e. 2, 2007. Avaliable from: <http://bvsms.saude.gov.br/bvs/publicacoes/manual_vigilancia_leishmaniose_2ed.pdf>. Access: 24 feb. 2018.

BRASIL, Ministério da Saúde – Secretaria de Vigilância em Saúde. **Manual de vigilância da Leishmaniose tegumentar americana: Série A, normas e manuais.** Brasília – DF, e. 2, 2010. Avaliable from: < http://bvsms.saude.gov. br/bvs/publicacoes/manual_vigilancia_leishmaniose_tegumentar_americana.pdf>. Access: 20 mar. 2016.

BRASIL, Secretaria da Saúde/Rio Grande do Sul. **Boletim epidemiológico**. V. 13, n. 1, 2011a.

BRASIL, Ministério da Saúde. **Nota Técnica Conjunta nº 01/2011-CGDT-CGLAB/DEVIT/SVS/MS**. 2011b.

BRASIL, Ministério da Saúde – Secretaria da Vigilância em Saúde. **Manual de vigilância e controle da Leishmaniose Visceral**. Brasilia – DF, e. 1, 2014a. Avaliable from: <http://bvsms.saude.gov.br/bvs/publicacoes/manual_ vigilancia_controle_ leishmaniose_visceral_1edicao.pdf >. Access: 15 dec. 2016.

BRASIL, Ministério da Pecuária e Abastecimento. **Nota Técnica nº 038/2014/DFIP/SDA.** Brasília – DF, 2014b. Avaliable from: <http://www. agricultura.gov.br/assuntos/insumosagropecuarios/insumospecuarios/produtos-veterinarios/arquivos-comunicacoes-e-instrucoes-tecnicas/nota-tecnica-dfip-38-14-leishmune.pdf>. Access: 24 feb. 2018.

BRASIL, Ministério da Saúde. **Guia de vigilância em saúde**. Brasília – DF, e. 1, 2016. Avaliable from: <http://portalarquivos.saude.gov.br/images/pdf/2016 /novembro/18/Guia-LV-2016.pdf>. Access: 10 dec. 2017.

BRUMFIELD, R.; BEERLI, P.; NICKERSON, D.; EDWARDS, S. The utility of single nucleotide polymorphisms in inferences of population history**. Trends in Ecology and Evolution**, v. 18, n. 5, p. 249-256, mai. 2003. DOI: 10.1016/S0169-5347(03)00018-1. Disponivel em: <http://www.cell.com/trends/ ecology-evolution/abstract/S0169-5347(03)00018-1>. Access: 15 jan. 2018.

CALVOPINA, M.; ARMIJOS, R. X.; HASHIGUCHI, Y. Epidemiology of leishmaniasis in Ecuador: current status of knowledge - A review. **Memórias do Instituto Oswaldo Cruz**, v. 99, n. 7, p. 663-672, nov. 2004. DOI: 10.1590/S0074-02762004000700001. Avaliable from: <http://www.scielo.br/sci elo.php?script=sci_abstract&pid=S007402762004000700001&lng=pt&nrm=iso&tlng=pt>. Access: 15 jan. 2018.

CAO, D. P.; GUO, X. G.; CHEN, D. L.; CHEN, J. P. Species delimitation and phylogenetic relationships of Chinese *Leishmania* isolates reexamined using kinetoplast cytochrome oxidase II gene sequences. **Parasitology Research**, v. 109, n. 1, p. 163-173, jul. 2011. Avaliable from: <https://link.sprin ger.com/article/10.1007%2Fs00436-010-2239-6>. Access: 15 feb. 2018.

CHAGAS, E.; CUNHA, A. M. da; CASTRO, G. de O.; FERREIRA, L. C. Leishmaniose Visceral Americana (Nova entidade morbida do homem na America do Sul): relatorio dos trabalhos realisados pela commissão encaregada do estudo da Leishmaniose Visceral Americana em 1936. **Memórias do Instituto Oswaldo Cruz**, v. 32, n. 3, 1937. DOI: 10.1590/S0074-02761937000300001. Avaliable from: < http://www.scielo.br /scielo.php?script=sci_arttext&pid=S007402761937000300001>. Access: 10 oct. 2017.

CHARGUI, N.; AMRO, A.; HAOUAS, N.; SCHONIAN, G.; BABBA, H.; SCHIMIDT, S.; RAVEL. C.; LEFEBVRE, M.; MASTIEN, P.; CHAKER, E.; AOUN, K.; ZRIBI, M.; KUHLS, K. Population structure of Tunisian *Leishmania infantum* and evidence for the existence of hybrids and gene flow between genetically different populations. **International Journal for Parasitology**, v. 39, n. 7, p. 801-811, jun. 2009. DOI: 10.1016/j.ijpara.2008.11.016. Disponivel em: <https://www.sciencedirect.com/science/article/pii/S0020751909000046? via%3Dihub>. Access: 18 jan. 2017.

CONVIT, J.; LAPENTA, P. Sobre un caso de leishmaniose tegumentaria de forma diseminada. **Revista Policlin Caracas**, v. 18, p. 153-158, 1946.

CORTES, S.; MAURICIO, I. L.; KUHLS, K.; NUNES, M.; LOPES, C.; MARCOS, M.; CARDOSO, L.; SCHONIAN, G.; CAMPINO, L. Genetic diversity evaluation on Portuguese *Leishmania infantum* strains by multilocus microsatellite typing. **Infection, Genetic and Evolution**, v. 26, p. 20-31, aug. 2014. DOI: 10.1016/j.meegid.2014.04.023. Disponivel em: <https://www.sciencedirect.com/science/article/pii/S1567134814001580?via%3Dihub >. Access: 8 dec. 2017.

COSTA, C. H. N.; PEREIRA, H. F.; ARAÚJO, M. V. Epidemia de leishmaniose visceral no estado do Piauí, Brasil., 1980- 1986. **Revista Saúde Pública**, v. 24, n.5, p. 361-372, 1990. DOI: http://dx.doi.org/10.1590/S0034-89101990000500003. Avaliable from: http://www.scielo.br/scielo.php?script =sci_arttext&pid=S003489101990000500003. Access: 28 dec. 2017.

COURA, J. R. **Dinâmica das Doenças infecciosas e parasitárias**. Ed. Guanabara, v. I, 2005.

COUSIÑO, B. **Vigilancia y control de la leishmaniasis en Paraguay**. In Panaftosa, Informe final de la reunión de expertos OPS/OMS sobre Leishmaniasis Visceral en las Américas, Panaftosa, Rio de Janeiro, p. 34-36, 2006.

CRMV, Conselho Regional de Medicina Veterinária do Rio Grande do Sul. Confirmação de caso autóctone de leishmaniose visceral em um paciente humano em Porto Alegre. Nota técnica nº11/2016, oct. 2016.

CRUZ, A. K.; TOSI, L. R. Molecular biology. **Clinics in Dermatology**, v. 14, n.5, p. 533-540, 1996. DOI: 10.1016/0738-081X(96)00043-0. Avaliable from: < https://www.sciencedirect.com/science/article/pii/0738081X96000430?via%3Dihub>. Access: 8 dec. 2017.

CUERVO, P.; CUPOLILLO, E.; NEHME, N.; HERNANDEZ, V.; SARAVIA, N.; FERNANDES, O. *Leishmania* (*Viannia*): genetic analysis of cutaneous and mucosal strains isolated from the same patient. **Experimental Parasitology**, v. 108, n. 1-2, p. 59–66, sep. Out 2004. DOI: 10.1016/j.exppara.2004.07.005. Avaliable from: <https://www.ncbi.nlm. nih.gov/pubmed/15491550 >. Access: 24 jan. 2018.

CUNHA, A. M.; CHAGAS, E. Nova espécie de protozoário do gênero *Leishmania* patogênico para o homem. *Leishmania chagasi,* **Nota prévia Hospital**, v. 11, n. 2, p. 148-152, 1937.

CUNHA, R. C.; ANDREOTTI, R.; COMINETTI, M. C.; SILVA, E. A. Detection of *Leishmania infantum* in *Lutzomyia longipalpis* captured in Campo Grande, MS. **Revista Brasileira de Parasitologia Veterinária**, v. 23, n. 2, p. 269-273, jun. 2014. DOI: 10.1590/S1984-29612014049. Avaliable from: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1984-29612014000200269>. Access: 20 jan. 2018.

CUPOLILLO, E.; GRIMALDI, G. J.; MOMEN, H. A general classification of New World *Leishmania* using numerical zymotaxonomy. **The American Journal of Trpical Medicina and Hygiene**, v. 50, n. 3, p. 250-311, 1994. Avaliable from: <http://ajtmh.org/content/journals/10.4269/ajtmh.1994.50.296> Access: 20 nov. 2017.

CUPOLILLO, E.; GRIMALDI JÚNIOR, G.; MOMEN, H.; BEVERLEY, S. M. Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of *Leishmania*. **Molecular and Biochemical Parasitology,** v. 73. N. 1-3, p. 145-155, jul. 1995. DOI: 10.1016/0166-6851(95)00108-D. Disponivel em: <https://www.sciencedirect.com/science/ article/pii/016668519500108D?via%3Dihub>. Access: 15 feb. 2018.

CUPOLILLO, E.; MEDINA-ACOSTA, E.; NOYES, H.; MOMEN, H.; GRIMALDI, G. J. A revised classification for *Leishmania* and *Endotrypanum*. **Parasitology Today**, v. 16, n. 4, p. 142-144, 2000. Avaliable from: <http://www.genomics. liv.ac.uk/tryps/papers/62CUPOLLIL.PDF>. Access: 1 dec. 2017.

DA SILVA, L. A.; DE SOUSA, C. dos S.; DA GRACA, G. C.; PORROZZI, R.; CUPOLILLO, E. Sequence analysis and PCR-RFLP profiling of the hsp70 gene as a valuable tool for identifying *Leishmania* species associated with human leishmaniasis in Brazil. **Infection, Genetics and Evolution**, v. 10, n. 1, p. 77-83, jan. 2010. DOI: 10.1016/j.meegid.2009.11.001. Avaliable from: <https://www.sciencedirect.com/science/article/pii/S1567134809002287>. Access: 8 feb. 2018.

DAVIES, C. R.; LLANOS-CUENTAS, E. A.; CAMPOS, P.; MONGE, J.; LEON, E.; CANALES, J. Spraying houses in the Peruvian Andes with lambda-cyhalothrin

protects residents against cutaneous leishmaniasis. **Facial Plastic Surgery Clinicis**, v. 94, n. 6, p. 631-636, nov. dec. 2000. DOI: 10.1016/S0035-9203(00)90214-1. Avaliable from: <http://www.facialplastic. theclinics.com/article/S0035-9203(00)902141/abstract>. Access: 22 jan. 2018.

DE CASTRO, E. A.; LUZ, E.; TELLES, F. Q.; PANDEY, A.; BISETO, A.; DINAISKI, M.; SBALQUEIRO, I.; THOMAZ-SOCCOL V. Eco-epidemiological survey of *Leishmania (Viannia) braziliensis* american cutaneous and mucocutaneous leishmaniasis in Ribeira Valley River, Parana State, Brazil. **Acta Tropica***,* v. 93, n. 2, p. 141-149, feb. 2005. DOI:10.1016/j.actatropica.2004.10.004. Disponivel em: < https://www.sciencedirect.com/ science/article/pii/S0001706X04002359 >. Access: 15 dec. 2017.

DEDET, J. P.; PRATLONG, F. LANOTE, G.; RAVEL, C. The parasite. **Clinics in Dermatology**, v. 17, n. 3, p. 261-268, mai. Jun. 1999. DOI: 10.1016/S0738-081X(99)00044-9. Avaliable from: <http://www.cidjournal.com/article/S0738-081X(99)00044-9/abstract>. Access: 20 nov. 2017.

DEDET, J. P.; DEREURE, J.; VANWAMBEKE, S. O.; MALÉ, P.; MARTINEZ, S.; PRATLONG, F.; BALARD, Y. The potential effects of global warming on changes in canine leishmaniasis in a focus outside the classical area of the disease in southern France. **Vector Borne Zoonotic Diseases**, v. 9, n. 6, p. 687-694, dec. 2009. DOI: 10.1089/vbz.2008.0126. Avaliable from: <http://online.liebertpub.com/doi/abs/10.1089/vbz.2008.0126>. Access: 10 dec. 2017.

DEGRAVE, W.; FERNANDES, O.; CAMPBELL, D.; BOZZA, M.; LOPES, U. Use of molecular probes and PCR for detection and typing of *Leishmania* - a mini-review. **Memorias do Instituto Oswaldo Cruz**, v. 89, n. 3, p. 463-469, jul. sep. 1994. DOI: 10.1590/S0074-02761994000300032. Avaliable from: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02761994000300032&lng=en&nrm=iso&tlng=en>. Access: 5 feb. 2018.

de MEEUS, T.; BALLOUX, F. Clonal reproduction and linkage disequilibrium in diploids: a simulation study. **Infections, genetics and evolution**, v. 4, n. 4, p. 345-351, jul. 2004. DOI: 10.1016/j.meegid.2004.05.002. Avaliable from: <https://www.sciencedirect.com/science/article/pii/S1567134804000528?via%3Dihub>. Access: 10 jan. 2018.

DIAS, F. O. P.; LOROSA, E. S.; REBELO, J. M. M. Fonte alimentar sangüínea e a peridomiciliação de *Lutzomyia longipalpis* (Lutz & Neiva, 1912) (Psychodidae, Phlebotominae). **Caderno Saúde Pública**, v. 19, n. 5, p. 1373-80, 2003. DOI: 10.1590/S0102-311X200300050001. Avaliable from: < http://www.scielo.br/scielo.php?pid=S0102-311X2003000500015&script=sci_abstract&tlng=pt>. Access: 20 nov. 2017.

DIAS, R. C. F.; THOMAZ-SOCCOL, V.; BISETTO JÚNIOR, A.; POZZOLO, E. M.; CHIYO, L.; FREIRE, R. L.; BREGANÓ, R. M.; PASQUALI, A. K. S.; ALBAN, S.; FENDRICH, R. C.; CALDART, E. T.; NAVARRO, I. T. Occurrence of anti-*Leishmania* spp. antibodies in domiciled dogs from the city of Foz do Iguaçu, State of Paraná, Brazil. In: WORLD CONGRESS ON LEISHMANIASIS, 5., 2013, Porto de Galinhas.

Abstract.Porto Galinhas: **Sociedade Brasileira de Medicina Tropical,** p. 875-876, mai. 2013.

DOWNING, T.; STARK, O.; VANAERSCHOT, M.; IMAMURA, H.; SANDERS, M.; DECUYPERE, S.; DE DONCKER, S.; MAES, I.; RIJAL, S.; SUNDAR, S.; DUJARDIN, J. C.; BERRIMAN, M.; SCHÖNIAN. G. Genome-wide SNP and microsatellite variation illuminate population-level epidemiology in the *Leishmania donovani* species complex. **Infection, Genetics and Evolution**, v. 12, p. 149–159, 2012. DOI: 10.1016/j.meegid.2011.11.005. Avaliable from: < https://dokupdf.com/queue/genome-wide-snp-and-microsatellite-variation-illuminate-population-level-epidemiology-in-the-leishmania-donovani-species-complex-_5a0258a0d64ab2b9bdb3a0a2_pdf?queue_id=-1>. Access: 15 dec. 2016.

DUPREY, Z. H.; STEURER, F. J.; ROONEY, J. A.; KIRCHHOFF, L. V.; JACKSON, J. E.; ROWTON, E. D.; SCHANTZ, P. M. Canine Visceral Leishmaniasis, United States and Canada, 2000–2003. **Emerging Infectious Diseases**, v. 12, n. 3, p. 440–446, mar. 2006. DOI: 10.3201/eid1203.050811. Avaliable from: < https://www.ncbi.nlm.nih.gov/pubmed/16704782>. Access: 20 feb. 2018.

ENRIGHT, M. C.; SPRATT, B. G. Multilocus sequence typing. **Trends in Microbiology**, v. 7, n. 12, p. 482-487, dec. 1999. DOI: 10.1016/S0966-842X(99)01609-1. Avaliable from: <http://www.cell.com/trends/ microbiology /fulltext/S0966-842X(99)016091?returnURL=http%3A%2F%2Flinkinghub. elsevier.com%2Fretrieve%2Fpii%2FS0966842X99016091%3Fshowall%3Dtrue>. Access: 8 jan. 2018.

EVANS, T.G.; TEIXEIRA, M. J.; MCAULIFFE, I. T.; VASCONCELOS, I.; VASCONCELOS, A. W.; COUZA, A. de A.; LIMA, J. W.; PEARSON, R. D. Epidemiology of visceral Leishmaniasis in Northeast Brazilian. **Journal Infection Diseases**, v. 166, n. 5, p. 1124-1132, 1992. Avaliable from: < https://www.ncbi.nlm.nih.gov/pubmed/1402024>. Access: 15 dec. 2017.

FELICIANGELI, M. D. Natural breeding places of phlebotomine sandflies. **Medical and Veterinary Entomology,** v. 18, n. 1, p. 71- 80, 2004. DOI: 10.1111/j.0269-283X.2004.0487.x. Avaliable from: <http://onlinelibrary. wiley.com/doi/10.1111/j.0269283X.2004.0487.x/abstract>. Access: 15 dec. 2017.

FERNANDES, O.; MURTHY, V. K.; KURATH, U.; DEGRAVE, W. M.; CAMPBELL, D. A. Mini-exon gene variation in human pathogenic *Leishmania* species. **Molecular Biochemical Parasitology**, v. 66, n. 2, p.261-271, aug. 1994. Avaliable from: <https://www.ncbi.nlm.nih.gov/pubmed/7808476>. Access: 18 dec. 2017.

FERREIRA, G. E.; DOS SANTOS, B. N.; DORVAL, M. E.; RAMOS, T. P.; PORROZZI, R.; PEIXOTO, A. A.; CUPOLILLO, E. The genetic structure of *Leishmania infantum* populations in Brazil and its possible association with the transmission cycle of visceral leishmaniasis. **PLoS One**, v. 7, n. 5, mai. 2012. DOI: 10.1371/journal.pone.0036242. Avaliable from: <http://journals.plos.org /plosone/article?id=10.1371/journal.pone.0036242>. Access: 20 dec. 2016.

FIGUEREDO, F. B.; BARBOSA FILHO, C. J. de L.; SCHUBACH, E. Y. P.; PEREIRA, S. A.; NASCIMENTO, L. D.; MADEIRA, M. de F. Relato de caso autóctone de leishmaniose visceral canina na zona sul do município do Rio de Janeiro. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 43, n. 1, p. 98-99, jan. feb. 2010. Avaliable from: < http://www.scielo.br/pdf/rsbmt/v43n1/a22v43n1.pdf>. Access: 5 jan. 2018.

FRAGA, J.; MONTALVO, A. M.; DE DONCKER, S.; DUJARDIN, J. C.; VAN DER AUWERA, G. Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. **Infection, Genetics and Evolution**, v. 10, n. 2, p. 238-245, mar. 2010. DOI: 10.1016/j.meegid.2009.11.007. Disponivel em: <https://www.sciencedirect.com/cience/article/pii/S1567134809002573?via%3Dihub> Access: 14 jan. 2018.

GAMA, M. E. A.; BARBOSA, J. S.; PIRES, B.; CUNHA, A. K. B.; FRETIAS, A. R.; RIBEIRO, I. R.; COSTA, J. M. L. Avaliação do nível de conhecimento que populações residentes em áreas endêmicas têm sobre leishmaniose visceral, Estado do Maranhão, Brasil. **Caderno de Saúde Pública**, v. 14, n. 2, p. 381-390, abr. jun, 1998. DOI: 10.1590/S0102-311X1998000200014. Avaliable from: <http://www.scielo.br/scielo.php?pid=S0102-311X1998000200014&script =sci_abstract&tlng=pt>. Access: 10 jan. 2018.

GARCIA, L.; KINDT, A.; BERMUDEZ, H.; LLANOS-CUENTAS, A.; DE DONCKER, S.; ARÉVALO J, TINTAYA, K. W. Q.; DUJARDDIN, J. C. Culture-independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes**. Journal of Clinical Microbiology**, c. 42, n. 5, p. 2294-2297, 2004. DOI: 2004;42(5):2294-7. DOI: 10.1128/JCM.42.5.2294-2297.2004. Avaliable from: <http://pubmed centralcanada.ca/pmcc/articles/PMC404633/pdf/1698-03.pdf>. Access: 10 jan. 2018.

GARDENER, P. J.; CHANCE, M. L.; PETERS, W. Biochemical taxonomy of *Leishmania*. II: Electrophoretic variation of malate dehydrogenase. **Annals of Tropical Medicine and Parasitology**, v. 68, n. 3, p. 317-325, 1974. Avaliable from: < https://www.cabdirect.org/cabdirect/abstract/19752903124>. Access: 20 nov. 2017.

GARNHAM, P. C. C. Cutaneous leishmaniasis in the New World, with special reference to *Leishmania mexicana*. **Science Report Institute Superior Sanitary**, v.2, p. 76-82, 1962.

GELANEW, T.; HAILU, A.; SCHONIAN, G.; LEWIS, M. D.; MILES, M. A.; YEO, M. Multilocus sequence and microsatellite identification of intra-specific hybrids and ancestor-like donors among natural ethiopian isolates of *Leishmania donovani*. **International Journal for Parasitology,** v. 44, n. 10, p. 751-757, sep. 2014. Doi: 10.1016/j.ijpara.2014.05.008. Avaliable from: <https://www.sciencedirect.com/science/article/pii/S0020751914001428?via%3Dihub >. ACCESS: 18 OCT. 2017.

GODFREY, D. G.; KILGOUR, V. Enzyme electrophoresis in characterizing the causative organism of Gambian trypanosomiasis. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 70, n. 3, p. 219-224, 1976. DOI:

10.1016/0035-9203(76)90043-2. Avaliable from: < https://www.sciencedirect.com/science/article/pii/ 0035920376900432>. Access: 22 nov. 2017.

GONTIJO, C. M. F.; MELO, M. N. Leishmaniose visceral no Brasil: quadro atual, desafios e perspectivas. **Revista Brasileira de Epidemiologia**, v. 7, n. 3, sep. 2004. DOI: 10.1590/S1415-790X2004000300011. Avaliable from: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1415790X2004000300011>. Access: 20 dec. 2017.

GOUZELOU, E.; HARALAMBOUS, C.; AMRO, A.; MENTIS, A.; PRATLONG, F.; DEDET, J. P.; VOTYPKA, J.; VOLF, P.; TOZ, S. O.; KUHLS, K.; SCHÖNIAN, G.; SOTERIADOU, K. Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic *L. donovani* sensu lato group**. PLoS Neglected Tropical Diseases**, v. 6, n. 2, feb. 2012. DOI: 10.1371/journal.pntd.0001507. Avaliable from: <https://www.ncbi. nlm.nih.gov/pmc/articles/PMC3279343/>. Access: 20 dec. 2016.

GRADONI, L. Epidemiological surveillance of leishmaniasis in the European Union: operational and research challenges. **Eurosurveeillance**, 2013. Avaliable from: <https://www.eurosurveillance.org/docserver/fulltext/euro surveillance/18/30/art20539en.pdf?expires=1520210598&id=id&accname=guest&checksum=4DEE15CA12 481AE3FE3A053D971284BB>. Access: 28 jan. 2018.

GRIMALDI, G.; MOMEN, H.; NAIFF, R. D.; MCMAHONPRAT, D.; BARRETT, T. V. Characterization and classification of leishmanial parasites from humans, wild mammals, and sand flies in the Amazon region of Brazil. **American Journal of Tropical Medicine and Hygiene**, v. 44, n. 6, p. 645–661, jun. 1991. DOI: 10.4269/ajtmh.1991.44.645. Avaliable from: < http://www.ajtmh.org/content/journals/ 10.4269/ajtmh.1991.44.645>. Access: 30 dec. 2017.

GRIMALDI JUNIOR, G.; TEVA, A.; FERREIRA, A. L.; SANTOS, C. B. dos; PINTO, I. de S.; AZEVEDO, C. T. de; FALQUETO, A. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. **Transactions of Royal Society of Tropical Medicine & Higiene**, v. 106, n. 1, p. 54-59, jan. 2012. DOI: 10.1016/j.trstmh.2011.10.001. Avaliable from: < https://academic.oup.com/trstmh/articleabstract/106/1/54/1885141?redirectedFrom=fulltext>. Access: 20 feb. 2018.

GUERBOUJ, S.; GUIZANI, I.; SPEYBROECK, N.; LE RAY, D.; DUJARDIN, J. C. Genomic polymorphism of *Leishmania infantum*: a relationship with clinical pleomorphism? **Infection, Genetics and Evolution**, v. 1, n. 1, p. 49–59, jul. 2001. DOI: 10.1016/S1567-1348(01)00008-9. Avaliable from: < https://www.sciencedirect.com/science/article/pii/S1567134801000089?via%3Dihub>. Acesso em 20 dec. 2017.

HAMAD, S. H.; KHALIL, E. A.; MUSA, A. M.; IBRAHIM, M. E.; YOUNIS, B. M.; ELFAKI, M. E.; EL-HASSAN, A. M. *Leishmania donovani*: genetic diversity of isolates from Sudan characterized by PCR-based RAPD**. Experimental Parasitology**, v.

125, n. 4, p. 389-393, aug. 2010. DOI: 10.1016/j.exppara.2010.03.008. Avaliable from: < https://www.sciencedirect.com/science/article/pii/S0014489410000937?via%3Dihub> . Access: 20 dec. 2017.

HARHAY, M. O.; OLLIARO, P. L.; COSTA, D. L.; COSTA, C. H. Urban parasitology: visceral leishmaniasis in Brazil. **Trends in Parasitology**, v. 27, n. 9, p. 403-409, sep. 2011. DOI: 10.1016/j.pt.2011.04.001. Avaliable from: < https://www.sciencedirect.com /science/article/pii/S147149221100064X>. Access: 8 jan. 2018.

HERRER, A. *Leishmania hertigi* sp.n., from the tropical porcupine, *Coendou rothschildi* Thomas. **Journal Parasitology**, v. 57, n. 3, p. 626-629, jun. 1971.

HERWALDT, M. D. B. L. Leishmaniasis. **The lancet,** v. 354, n. 9185, p. 1191-1199, oct. 1999. DOI: 10.1016/S0140-6736(98)10178-2. Avaliable from: < http://www.thelancet. com /journals/lancet/article/PIIS0140-6736(98)10178-2/fulltext >. Access: 10 dec. 2017.

HIDE, M.; BAÑULS, A. L.; TIBAYRENC, M. Genetic heterogeneity and phylogenetic status of *Leishmania* (*Leishmania*) *infantum* zymodeme MON-1: epidemiological implications. **Parasitology**, v. 123, n. 5, p. 425-432, nov. 2001. DOI:10.1017/S003118200100871X. Disponivel em: < https://www.cambridge.org/core/journals/parasitology/article/geneticheterogeneityn d-phylogenetic-status-of-leishmania-leishmania-infantum-zymodeme-mon-1epidemiologicalimplications/2D0A2CB8DA2638A29C1E922E7EA14786 >. Access: 7 dec. 2017.

HUNTER, R. L.; MARKERT, C. L. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. **Science New York**, v. 125, n. 3261, p. 1294-1295, jun. 1957. DOI: 10.1126/science.125.3261.1294-a. Avaliable from: <http://s cience.sciencemag.org/content/125/3261/1294.2.long >. Access: 20 jan. 2017.

IBRAHIM, M. E.; BARKER, D. C. The origin and evolution of the *Leishmania donovani* complex as inferred from a mitochondrial cytochrome oxidase II gene sequence. **Infection Genetics and Evolution**, v. 1, n. 1, p. 61-68, jul. 2001. DOI: 10.1016/S1567-1348(01)00009-0. Avaliable from: <https://www.sciencedirect.com/ science/article/pii/S1567134801000090 >. Access: 8 jan. 2018.

INDIANI DE OLIVEIRA, C.; TEIXEIRA, M. J.; TEIXEIRA, C. R.; RAMOS DE JESUS, J.; ROSATO, A. B.; DA SILVA, J. S.; BRODSKYN, C.; BARRAL-NETTO, M.; BARRAL, A. *Leishmania braziliensis* isolates differing at the genome level display distinctive features in BALB/c mice**. Microbes and Infection**, v. 6, n. 11, p. 977–984, sep. 2004. DOI: 10.1016/j.micinf.2004.05.009. Disponivel em: < https://www.sciencedirect.com/science/article/pii/S1286457904001856?via%3Dihub >. Access: 31 dec. 2017.

JAMJOOM, M. B.; ASHFORD, R. W.; BATES, P.A.; KEMP, S. J.; NOYES, H. A. Towards a standard battery of microsatellite markers for the analysis of the

Leishmania donovani complex. **Annals of Tropical Medicine & Parasitology**, v. 96, n. 3, p. 265-270, 2002. DOI: 10.1179/000349802125000790. Avaliable from: < https://pdfs.semanticscholar.org/8e4a/e4f430f565ac1a55ee66b117bafb32ae7bac.pdf >. Access: 20 nov. 2016.

JERONIMO, S. M. B.; SOUSA, A. de Q.; PEARSON, R. D. Leishmaniasis. In: Guerrant RL, Walker DH, Weller PF, editors. **Tropical infectious diseases**, ed. 2, p. 1095–1107, 2007.

KASZAK, I.; PLANELLAS, M.; DWORECKA-KASZAK, B. Canine leishmaniosis – an emerging disease. **Annals of Parasitology**, v. 61, n. 2, p. 69-76, 2015. Avaliable from: < https://www.ncbi.nlm.nih.gov/pubmed/263425 00>. Access: 10 feb. 2018.

KILLICK-KENDRICK, K. The biology and control of phlebotomine sand flies. **Clinics in Dermatology**, v. 17, n. 3, p. 279-289, mai. Jun. 1999. DOI: 10.1016/S0738-081X(99)00046-2. Avaliable from: < https://www.sciencedirect. com/science/article/pii/S0738081X99000462?via%3Dihub>. Access: 15 dec. 2017.

KIRK, R. The differentiation and nomenclature of Leishmania. **Parasitology**, v. 39, n. 3-4, p. 263-273, 1949. Avaliable from: <https://www.ncbi.nlm.nih. gov/pubmed/18112009>. Access: 5 jan. 2017.

KREUTZER, R. D.; CHRISTENSEN, H. A. Characterization of *Leishmania* spp. by isozyme electrophoresis. **The American journal of tropical medicine and hygiene**, v. 29, n. 2, p. 199-208, mar. 1980. DOI: 10.4269/ajtmh.1980.29.199. Avaliable from: < http://www.ajtmh.org/content/ journals/10.4269/ajtmh.1980.29.199>. Access: 20 jan. 2017.

KUHLS, K.; KEILONAT, L.; OCHSENREITHER, S.; SCHAAR, M.; SCHWEYNOCH, C.; PRESBER, W.; SCHONIAN, G. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. **Microbes and Infection**, v. 9, n. 3, p. 334-343, mar. 2007. DOI: 10.1016/j.micinf.2006.12.009. Avaliable from: < https://www.sciencedirect.com /science/article/pii/S1286457907000081 >. Access: 20 nov. 2016.

KUHLS, K.; CHICHARRO, C.; CAÑAVATE, C.; CORTES, S.; CAMPINO, L.; HARALAMBOUS, C.; SOTERIADOU, K.; PRATLONG, F.; DEDET, J. P.; MAURICIO, I.; MILES, M.; SCHAAR, M. OCHSENREITHER, S.; RADTKE, O. A.; SCHONIAN, G. Differentiation and gene flow among European populations of *Leishmania infantum* MON-1. **PLoS Neglected Tropical Diseases**, v. 2, n. 7, jul. 2008. DOI: 10.1371/journal.pntd.0000261. Avaliable from: < https://www.ncbi.nlm.nih.gov/ pmc/articles/PMC2438616/>. Access: 20 nov. 2016.

KUHLS, K.; ALAM, M. Z.; CUPOLILLO, E.; FERREIRA, G. E. M.; MAURICIO, I. L.; ODDONE, R.; FELICIANGELI, M. D.; WIRTH, T.; MILES, M. A.; SCHÖNIAN, G. Comparative microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. **PLoS Neglected Tropical Diseases**, v. 5, n. 6, jun. 2011. DOI: 10.1371/journal.pntd.0001155. Avaliable from: <http://journals.plos.org/ plosntds/article?id=10.1371/journal.pntd.0001155>. Access: 20 nov. 2016.

LACHAUD, L.; DEDET, J. P.; MARTY, P.; FARAUT, F.; BUFFET, P.; GANGNEUX, J. P.; RAVEL, C.; BASTIEN, P. The Working Group for the Notification of Human Leishmanioses in France. Surveillance of leishmaniases in France, 1999 to 2012. **Euro Surveillance**, v. 18, n. 29, 2013. Avaliable from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20534>. Access: 10 mar. 2018.

LAINSON, R. American Leishmaniasis: some observations their Ecology and Epidemiology. **Transactions of the Royal Society of Tropical Medicine & Hygiene**, v. 77, n. 5, p. 569-596, 1983. Avaliable from: <https://www.ncbi.nlm.nih.gov/pubmed/6197791>. Access: 28 feb. 2018.

LAINSON, R. **Demographic changes and their influence on the epidemiology of American leishmaniasis.** In: Service MW, editor. Demography and vector- borne diseases. Boca Raton: CRC Press; p. 85-106, 1989.

LAINSON, R.; RANGEL, E. F. *Lutzomyia longipalpis* and the eco-epidemiology of American visceral leishmaniasis, with particular reference to Brazil: a review. **Memórias do Instituto Oswaldo Cruz,** v. 100, n. 8, p. 811–827, dec. 2005. DOI: 10.1590/S0074-02762005000800001. Avaliable from: < http://www.scielo.br/scielo.php?script=sci_ arttext&pid=S0074-02762005000800001>. Access: 20 dec. 2017

LAINSON, R.; SHAW, J. J. Taxonomy of the New World *Leishmania* species. **Transactions of the Royal Society of Tropical Medicine & Hygiene**, v. 66, n. 6, p. 943-944, 1972.

LAINSON R.; SHAW J. J. **The role of animals in the epidemiology of South American Leishmaniasis**. In: Biology of the Kinetoplastida. London: Academic Press. v. 2, p. 1-116, 1979.

LAINSON, R.; SHAW, J. J**. Evolution, classification and geographical distribution**. In: PETERS, W.; KILLICK-KENDRICK, R. The leishmaniases in biology and medicine. London: Academic Press. pp 1–120, 1987.

LAINSON, R.; SHAW, J. J. **New World Leishmaniasis.** In: COX, F. E. G.; WAKELIN, D.; GILLESPIE, S. H.; DESPOMMIER, D. D. Topley & Wilson's Microbiology and Microbial Infections, 10 ed. London: Wiley & Blackwell. P. 313–349, 2005.

LANOTTE, G.; RIOUX, J. A.; MAAZOUN, R.; PASTEUR, N.; PRATLONG, F.; LEPART, J. The application of a numerical method to the taxonomy of the genus *Leishmania* Ross, 1903. The recognition of 146 original lines in the Old World. Use of allozymic characters. Epidemiological and phyletic significance. **Annales de Parasitologie Humaine et Comparée**, v. 56, p. 575-591, 1981.

LE BLANCQ, S. M.; PETERS, W. *Leishmania* in the Old World: 2. Heterogeneity among *L. tropica* zymodemes. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 80, n. 1, p. 113-119, 1986. DOI: 10.1016/0035-

9203(86)90208-7. Avaliable from: <https://www.
sciencedirect.com/science/article/pii/0035920386902087>. Access: 15 dec. 2017.

LEBLOIS, R.; KUHLS, K.; FRANÇOIS, O.; SCHÖNIAN, G.; WIRTH, T. Guns, germs and dogs: on the origin of *Leishmania chagasi*. **Infection, Genetics and Evolution**, v. 11, n. 5, p. 1091–1095, jul. 2011. DOI: 10.1016/j.meegid.2011.04.004. Avaliable from: <https://www.sciencedirect. com/science/article/pii/S1567 134811001109?via%3Dihub>. Access: 27 nov. 2017.

LEVINE, N. D.; CORLISS, J. O.; COX, F. E. G.; DEROUX, G.; GRAIN, J.; HONIGBERG, B. M.; LEEDALE, G. F.; LOEBLICH, A. R.; LOM, III. J.; LYNN, D.; MERINFLED, E. G.; PAGE, F. C.; POLJANSKY, G.; SPRAGUE, V.; VAVRA, J.; WALLACE, F. G. A Newly revised classification of the Protozoa. **Journal of Eucaryotic Microbiology**, v. 27, n. 1, feb. 1980. DOI: 10.1111/j.1550-7408.1980.tb04228.x. Avaliable from: <https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1550-7408.1980.tb04228.x>. Access: 20 abr. 2018.

LIDIANE, K. C. F. **Estudo molecular de cepas de *Leishmania (L.) infantum chagasi* isoladas de flebotomíneos *Lu. longipalpis* de área endêmica de leishmaniose visceral da Amazônia brasileira.** Dissertação (Mestrado em Medicina Interna) – Programa de Pós Graduação em Medicina Interna, Universidade Federal do Paraná, Curitina, Paraná, 2011. Avaliable from: < http://acervodigital.ufpr.br/handle/1884/32221?show=full>. Access: 10 feb. 2018.

LÜHE, M. **DIE im Blute schmarotzenden Protozoen und ihre nachsten Verwandren**. In: Mense C, Barth IA, editors. Hanbuch der Tropenkrankheiten, p. 203, 1906.

LUKES, J.; GUILBRIDE, D. L.; VOTÝPKA, J.; ZÍKOVA, A.; BENNE, R.; ENGLUND, P. T. Kinetoplast DNA network: evolution of an improbable structure. **Eukaryotic Cell**, v. 1, n. 4, p. 495-502, aug. 2002. DOI: 10.1128/EC.1.4.495-502.2002. Disponivel em: <http://ec.asm.org/content/1/4/495>. Access: 14 feb. 2018.

MADEIRA, M. F.; FIGUEIREDO, F. B.; PINTO, A. G. S.; NASCIMENTO, L. D.; FURTADO, M.; MOUTA-CONFORT, E.; PAULA, C. C. de; BOGIO, A.; GOMES, M. C. A.; BESSA, A. M. S.; PASSOS, S. R. L. Parasitological diagnosis of canine visceral leishmaniasis: Is intact skin a good target? **Research in Veterinary Science**, v. 87, n. 2, p. 260-262, oct. 2009. DOI: 10.1016/j.rvsc.2009.03.008. Avaliable from: < https://www.sciencedirect.com/science/article/pii/S003452880900054X?via%3Dihub >. Access: 28 dec. 2017.

MAHNAZ, T.; AL-JAWABREH, A.; KUHLS, K.; SCHÖNIAN, G. Multilocus microsatellite typing shows three different genetic clusters of *Leishmania major* in Iran. **Microbes and Infection**, v. 13, n. 11, p. 937–942, 2011. DOI: 10.1016/j.micinf.2011.05.005. Avaliable from: < https://www.sciencedirect .com/science/article/pii/S1286457911001250?via%3Dihub>. Access: 22 jan. 2017.

MAIA ELKHOURY, A. N. S.; ALVES, W. A.; SOUSA GOMES, M. L.; SENA, J. M.; LUNA, E. A. Visceral leishmaniasis in Brazil: trends and challenges. **Caderno de Saúde Pública**, v. 24, n. 12, p. 2941–2947, dec. 2008. DOI: 10.1590/S0102-311X2008001200024. Avaliable from: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0102-311X2008001200024>. Access: 20 feb. 2018.

MARFUT, J.; NASEREDDIN, A.; NEIDERWEISER, I.; JAFFE, C. L.; BECK, H. P.; FELGER, I. Identification and differentiation of *leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. **Journal of clinical microbiology**, v. 41, n. 7, p. 3147-3153, jul. 2003. Doi: 10.1128/jcm.41.7.3147-3153.2003. Avaliable from: < http://jcm.asm.org/content/41/7/3147.long>. Access: 5 feb. 2018.

MARTIN-SANCHEZ, J.; GRAMICCIA, M.; DI MUCCIO, T.; LUDOVISI, A.; MORILLAS-MARQUEZ, F. Isoenzymatic polymorphism of *Leishmania infantum* in southern Spain. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 98, n. 4, p. 228-232, abr. 2004. DOI: 10.1016/S0035-9203(03)00060-9. Avaliable from: < https://www.sciencedirect.com/science/article/pii /S0035920303000609>. Access: 15 dec. 2017.

MASSIA, L. I.; LAMADRIL, R. D. Q.; WELLICKS, J. R.; BITTENCOURT, D. G.; MARQUES, G. D.; CELIS, E. L. H.; PELLEGRINI, D. da C. P. Leishmaniose visceral canina em três bairros de Uruguaiana – RS. **Revista Visa em debate: sociedade, ciência e tecnologi**a, v. 4, n. 1, p. 113-119, jan. 2016.DOI: 10.3395/2317-269x.00679. Avaliable from: <https://visaemdebate.incqs.fiocruz.br/index.php/visaemdebate/article/download /.../298>. Access: 10 jan. 2018.

MATTA, A. A. Sur les leishmanioses tégumentaires. Classification générale des Leishmanioses. **Bull Soc Path Exot**., v.9, p. 494-503, 1916.

MAURICIO, I. L.; HOWARD, M. K.; STOTHARD, J. R.; MILES, M. A. Genomic diversity in the *Leishmania donovani* complex. **Parasitology**, v. 119, n. 3, p. 237-246, sep. 1999. Avaliable from: <https://www.cambri dge.org/core/journals/parasitology/article/genomic-diversity-intheleishmania-donovanicomplex/B56F440BB5AD8156CA01A6E4C70DFDA3>.Access: 15 feb. 2018.

MAURICIO, I. L.; GAUNT, M. W.; STOTHARD, J. R.; MILES, M. A. Genetic typing and phylogeny of the *Leishmania donovani* complex by restriction analysis of PCR amplified gp63 intergenic regions. **Parasitology**, v. 122, n. 4, p. 393-403, 2001. DOI: 10.1017/S0031182001007466. Avaliable from: < https://www.scopus.com/record/display.uri?eid=2-s2.00035072579&origin= inward&txGid=aee07e2554fb5d82 3661ec652c85c36e>. Access: 15 feb. 2018

MAURICIO, I. L.; GAUNT, M. W.; STOTHARD, J. R.; MILES, M. A. Glycoprotein 63 (gp63) genes show gene conversion and reveal the evolution of Old World Leishmania. **International Journal for Parasitology**, v. 37, n. 5, p. 565-576, abr. 2007. DOI: 10.1016/j.ijpara.2006.11.020. Avaliable from:

<https://www.sciencedirect.com/science/article/pii/S0020
751906004437?via%3Dihub>. Access: 15 feb. 2018.

MAZIERO, N.; THOMAZ-SOCCOL, V.; STEINDEL, M.; LINK, J. S.; ROSSINI, D.; ALBAN, S. M.; NASCIMENTO, A. J. Rural urban focus of canine visceral leishmaniosis in the far western region of Santa Catarina State, Brazil. **Veterinary Parasitology**, v. 205, n. 1-2, p. 92-95, sep. 2014. DOI: 10.1016/j.vetpar.2014.06.005. Avaliable from: < https://www.sciencedirect.com/science/article/pii/S03044017140 03422>. Access: 10 jan. 2017.

MAZZA, S. Leishmaniasis tegumentária y visceral. **Boletin Intituto Clínica Quirurgica**, v. 13, p. 208-216, 1926.

MEDINA, H. S. G. Estudos sôbre leishmaniose. I. Primeiros casos de leishmaniose espontânea observados em cobaios. **Brazilian Archives of Biology and Technology**, v. 1, p. 39-74, 1946.

MEDINA, R.; ROMERO, J. Estudio clínico y parasitológico de una nueva cepa de leishmania. **Archivos Venezolanos de Petologia Medica**, v. 3, p. 298-326, jul. 1959.

MIGONE, L. E. UN CASO DE KALA-ZAR A ASUNCIÓN (PARAGUAY). **Bulletin de la societe de pathologie exotique**, v. 6, p. 118-120, 1913.

MINODIER, P.; PIARROUX, R.; GAMBARELLI, F; JOBLET, C.; DUMON, H. Rapid identification of causative species in patients with old world leishmaniasis. **Journal of Clinical Microbiology**, v. 35, n. 10, p. 2551-2555, oct. 1997. Avaliable from: < https://www.scopus.com/record/ display.uri?eid=2s2.00030856031&origin=inward&txGid=c7d97d01f4a41bb96a70213 abe046085>. Access: 15 feb. 2018.

MOMEN, H.; PACHECO, R. S.; CUPOLILLO, E.; GRIMALDI JÚNIOR, G. Molecular evidence for the importation of Old World *Leishmania* into the Americas. **Biological Research**, v. 26, n. 1-2, p. 249-255, 1993. Avaliable from: < https://www.ncbi.nlm.nih.gov/pubmed/7545502>. Access: 20 dec. 2017.

MONTALVO, A. M.; MONZOTE, L.; FRAGA, J.; MONTANO, I.; MUSKUS, C.; MARÍN, M.; DE DONCKER, S.; VELEZ, I. D.; DUJARDIN, J. C. PCR-RFLP and RAPD for typing neotropical *Leishmania*. **Biomedica Revista Del Instituto Nascional de Salud**, v. 28, n. 4, p. 597-606, 2008. DOI: 10.7705/biomedica.v28i4.66. Avaliable from: <https://www.revistabio medica.org/index.php/biomedica/article/view/66>. Acesso em 14 feb. 2018.

MONTALVO, A. M.; FRAGA, J.; MAES, I.; DUJARDIN, J. C.; AUWERA G. V. der Three new sensitive and specific heat-shock protein 70 PCRs for global *Leishmania* species identification. **European Journal Clinical Microbiology & Infection Diseases**, v. 31, n. 7, p. 1453-1461, jul. 2012. DOI: 10.1007/s10096-011-1463-z. Avaliable from: <https://link.springer.com/article/10.1007/s10096-011-1463-z>. Access: 10 feb. 2018.

MONTOYA, L.; GALLEGO, M.; GAVIGNET, B.; PIARROUX, R.; RIOUX, J. A.; PORTUS, M.; FISA, R. Application of microsatellite genotyping to the study of a restricted *Leishmania infantum* focus: different genotype compositions in isolates from dogs and sand flies. **The American Journal of Tropical Medicine and Hygiene**, v. 76, n. 5, p. 888-895, 2007. Avaliable from: <http://www.ajtmh.org/docserver/fulltext/14761645/76/5/0760888.pdf?expires=1518633186&id=id&accname=guest&checksum=F67641F863A0A9F335FF37C49F0D82F1>. Access: 26 dec. 2016.

MORENO, J.; ALVAR, J. Canine *Leishmania*sis: epidemiological risk and the experimental model. **Trends in Parasitology,** v. 18, n. 9, p. 399-405, sep. 2002. DOI: 10.1016/S1471-4922(02)02347-4. Avaliable from: <https://www.sciencedirect.com/science/article/pii/S1471492202023474>. Access: 15 nov. 2017.

MOTOIE, G.; FERREIRA, G.E.; CUPOLILLO, E.; CANAVEZ, F.; PEREIRA-CHIOCCOLA, V. L. Spatial distribution and population genetics of *Leishmania infantum* genotypes in São Paulo State, Brazil, employing multilocus microsatellite typing directly in dog infected tissues. Infection, **Genetics and Evolution**, v. 18, p. 48-59, mai. 2013. DOI: 10.1016/j.meegid.2013.04.031. Avaliable from: <https://www.sciencedirect. com/science/article/pii/S1567134813001809>. Access: 10 dec. 2017.

MURPHY, R. W.; SITES, J. W.; BUTH, D.G.; HAUFLER, C.H**. Proteins I: Isozyme electrophoresis**. In: HILLIS, D. M.; MORITZ, C. Molecular systematics. Sinauer Sunderland, 126, 1990.

NORMARK, B. B. Phylogeny and evolution of parthenogenetic weevils of the *Aramigus tessellatus* species complex (Coleoptera: Curculionidae: Naupactini): evidence from mitochondrial DNA sequences. **Evolution,** v. 50, n. 2, p. 734–745, abr. 1996. DOI: 10.1111/j.1558-5646.1996.tb03883.x. Avaliable from: < https://www.ncbi.nlm.nih.gov/pubmed/28568943 >. Access: 8 jan. 2018.

OCHSENREITHER, S., KUHLS, K., SCHAAR, M., PRESBER, W., SCHONIAN, G. Multilocus microsatellite typing as a new tool for discrimination of *Leishmania infantum* MON-1 strains. **Journal of Clinical Microbiology**, v. 44, n. 2, p. 495-503, feb. 2006. DOI: 10.1128/JCM.44.2.495-503.2006. Avaliable from: < http://jcm.asm.org/content/44/2/495.long>. Access: 15 dec. 2016.

ODDONE, R.; SCHWEYNOCH, C.; SCHÖNIAN, G.; DE SOUSA, C. Dos S.; CUPOLILLO, E.; ESPINOSA, D.; AREVALO, J.; NOYES, H.; MAURICIO, I.; KUHLS, K. Development of a multilocus microsatellite typing approach for discriminating strains of *Leishmania* (*Viannia*) species. **Journal of Clinical Microbiology**, v. 47, n. 9, p. 2818–2825, jul. 2009. DOI: 10.1128/JCM.00645-09. Avaliable from: <http://jcm.asm.org/content/ 47/9/2818.full>. Access: 15 dec. 2016.

OLIVA, G.; SCALONE, A.; FOGLIA MANZILLO, V.; GRAMICCIA, M.; PAGANO, A.; DI MUCCIO, T.; GRADONI, L. Incidence and time course of *Leishmania infantum* infections examined by parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons. **Journal of**

Clinical Microbiology, v. 44, n. 4, p. 1318-22, abr. 2006. DOI: 10.1128/JCM.44.4.1318-1322.2006. Avaliable from: <http://jcm.asm.org/content/44/4/1318.long>. Access: 15 feb. 2018.

OLIVEIRA, A. M.; VIEIRA, C. P.; DIBO, M. R.; GUIRADO, M. M.; RODAS, L. A. C.; CHIARAVALLOTI-NETO, F. Dispersal of *Lutzomyia longipalpis* and expansion of canine and human visceral leishmaniasis in São Paulo state, Brazil. **Acta Tropica**, v. 164, p. 233-242, dec. 2016. DOI: 10.1016/j.actatropica.2016.09.014. Avaliable from: <https://www.science direct.com/science/article/pii/S0001706X16301231?via%3 Dihub>. Access: 23 feb. 2018.

OPAS, Organização Pan-Americana da Saúde. **Módulo de princípios de epidemiologia e controle de enfermidades (MOPECE)**. V. 7, 2010. Avaliable from: < http://www.paho.org/bra/index.php?option=com_docman &view=download&category_slug=informacao-e-analise-saude096&alias =950-modulos-principios-epidemiologia-para-controle-enfermidadesmopece -modulo-2-0&Itemid=965>. Access: 1 jan. 2018.

OPAS, Organização Pan-Americana da Saúde. **OMS divulga lista de doenças e patógenos prioritários para pesquisa e desenvolvimento em 2018**. Feb. 2018. Disponivel em: < http://www.paho.org/bra/index .php?option=com_content&view=article&id=5595:oms-divulga-lista-de-doencas-e-patogenos-prioritarios-para-pesquisa-e-desenvolvimento-em-2018&Itemid=812>. Access: 15 feb. 2018.

PAULAN, S. de C.; SILVA, H. R.; LIMA, E. A. C. de F.; FLORES, E. F.; TACHIBANA, V. M.; KANDA, C. Z.; NORONHA JUNIOR, A. C. F. de; DOBRE, P. R. Spatial distribuition of canine visceral Leishmaniasis in Ilha Solteira, São Paulo, Brazil. **Engenharia Agrícola**, v. 32, n. 4, jul. aug. 2012. DOI: 0.1590/S0100-69162012000400016. Avaliable from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100691620120004000 16>. Access: 23 feb. 2018.

PENA, H. A. Leishmaniose visceral no Brasil. **Brazilian Medicine**, n. 48, p. 949-950, 1934.

PERFIL'EV, P. P. Phlebotomidae. Translation of Perfil'ev, 1966 Diptera: Family Phlebotomidae. **Fauna SSSR**. V. 93, p. 1-382, 1968.

PESSÔA, S. B.; MARTINS, A. V. **Parasitologia médica.** 11.ed. Rio de Janeiro: Guanabara Koogan, 872p., 1982.

PETERS, W.; KILLICK-KENDRICK, R. **The Leishmaniases in biology and medicine: clinical aspects and control**. V. 2, 1987.

PIGOTT, D. M.; BHATT, S.; GOLDING, N.; DUDA, K. A.; BATTLE, K. E.; BRADY, O. J.; MESSINA, J. P.; BALARD, Y.; BASTIEN, P.; PRATLONG, F.; BROWNSTEIN, J. S.; FRIEFELD, C. C.; MEKARU, S. R.; GETHING, P. W.; GEORGE, D. B.; MYERS, M. F.; REITHINGER, R.; HAY, S. I. Global distribution maps of the leishmaniases. **eLife**, v. 3, 2014. DOI: 10.7554/eLife.02851. Avaliable from:

<https://www.ncbi.nlm.nih.gov/ pmc/articles/PMC4103681/pdf/elife02851.pdf>. Access: 20 feb. 2018.

PINTO, A. de O. **Diagnóstico sorológico e molecular de leishmaniose visceral canina (LVC), em municípios do Vale do Rio do Peixe, Santa Catarina, Brasil**. 52f. Dissertação (Mestrado em Biociências e Saúde) – Programa de Pós-Graduação em Biociências e Saúde, Universidade do Oeste de Santa Catarina, Joaçaba, 2017. Avaliable from: < http://www.unoesc.edu.br/images/uploads/mestrado/DISSERTAÇÃO_ANDREA_DE_OLIVEIRA_PINTO_10._2017.pdf>. Access: 13 feb. 2018.

POMARES, C.; MARTY, P.; BANULS, A. L.; LEMICHEZ, E.; PRATLONG, F.; FAUCHER, B.; JEDDI, F.; MOORE, S.; MICHEL, G.; ALURU, S.; PIARROUX, R.; HIDE, M. Genetic Diversity and Population Structure of *Leishmania infantum* from Southeastern France: Evaluation Using Multi-Locus Microsatellite Typing. **Plos Neglected Tropical Diseases**, v. 10, n. 1, jan. 2016. DOI: 10.1371/journal.pntd.0004303. Disponivel em: <http://journals.plos.org/plosntds/article?id=10.1371/journal.pntd. 0004303>. Access: 10 oct. 2017.

READY, P. D. Leishmaniasis emergence in Europe. **Euro Surveillance**. V. 15, n. 10, 2010. Avaliable from: <http://www.eurosurveillance.org/ ViewArticle.aspx?Articleid=19505.PMid:20403308>. Access: 20 feb. 2018.

READY, P. D. Biology of phlebotomine sand flies as vectors of disease agents. **Annual Review of Entomology**, v. 58, p. 227–250, 2013. doi: 10.1146/annurev-ento-120811-153557. Avaliable from: < http://arjournals.annualreviews.org/doi/full/10.1146/annurev-ento-120811-153557?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3dpubmed>. Access: 10 feb. 2018.

REED, S. G.; SHREFFLER, W. G.; BURNS, J. M.; SCOTT, J. M.; ORGE, M. G.; GHALIB, H. W.; SIDDIG, M.; BADARO, R. An improved serodiagnostic procedure for visceral leishmaniasis. **The American Journal Tropical Medicine and Hygiene**, v. 43, n. 6, p. 632-639, dec. 1990. DOI: 10.4269/ajtmh.1990.43.632. Avaliable from: <http://www.ajtmh.org/content/journals/10.4269/ajtmh.1990.43.632>. Access: 20 dec. 2017.

RIOUX, J. A.; LANOTTE, G.; SERRES, E.; PRATLONG, F.; BASTIEN, P.; PERIERES, J. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. **Annales de Parasitologie Humaine et Comparée**, v. 65, n. 3, p. 111-125, 1986. DOI: 10.1051/parasite/1990653111. Avaliable from: < https://www.ncbi.nlm.nih.gov/pubmed/2080829 >. Access: 20 dec. 2017.

RODRIGUES, E. H. G. **Validação de abordagens moleculares para o diagnóstico da leishmaniose tegumentar americana em Pernambuco**. Dissertação (Mestrado em Saúde Pública) – Departamento de Saúde Coletiva, Fundação Oswaldo Cruz, Recife, 2000. Avaliable from: < https://www.arca.fiocruz.br/handle/icict/14715>. Access: 18 jan. 2018.

ROSSI, V.; WINCKER, P.; RAVEL, C.; BLAINEAU, C.; PAGÉS, M.; BASTIEN, P. Structural organisation of microsatellite families in the *Leishmania* genome and polymorphisms at two $(CA)_n$ loci. **Molecular and Biochemical Parasitology**, v. 65, n. 2, p. 271-282, jun. 1994. DOI: 10.1016/0166-6851(94)90078-7. Avaliable from: < https://www.science direct.com/science/article/pii/0166685194900787>. Access: 14 dec. 2016.

ROTUREAU, B. Ecology of the *Leishmania* species in the Guianan ecoregion complex. **The American Journal Tropical Medicine and Hygiene**, v. 74, n. 1, p. 81–96, 2006. Avaliable from: < http://www.ajtmh.org/docserver/fulltext/14761645/74/1/0740081.pdf?expires=1518478441&id=id&accname=guest&checksum=9F8D8883ED96321883BFF79BC459F066 >. Access: 20 dec. 2017.

ROTUREAU, B.; RAVEL, C.; COUPPIE, P.; PRATLONG, F.; NACHER, M.; DEDET, J. P.; CARME, B. Use of PCR-Restriction Fragment Length Polymorphism Analysis to Identify the Main New World *Leishmania* Species and Analyze Their Taxonomic Properties and Polymorphism by Application of the Assay to Clinical Samples. **Journal of Clinical Microbiology**, v. 44, n. 2, p. 459-467, feb. 2006. DOI: 10.1128/JCM.44.2.459-467.2006. Access: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1392689/>. Access: 8 feb. 2018.

ROUGERON, V.; DE MEEÛS, T.; HIDE, M.; WALECKX, E.; BERMUDEZ, H.; AREVALO, J.; LLANOS-CUENTAS, A.; DUJARDIN, J. C.; DE DONCKER, S.; LE RAY, D.; AYALA, F. J.; BAÑULS, A. L. Extreme inbreeding in *Leishmania braziliensis*. **Proceedings of the National Academy of Sciences of the United, States of America**, v. 106, n. 25, p. 10224-10229, jun. 2009. DOI: 10.1073/pnas.0904420106. Avaliable from: < http://www.pnas.org/content/106/25/10224.long>. Access: 28 dec. 2017.

SACKS, D.; NOBEM-TRAUTH, N. The immunology of susceptibility and resistance to *Leishmania major* in mice. **Nature reviews Immunology**, v. 2, p. 845-858, nov. 2002. DOI: 10.1038/nri933. Avaliable from: < https://www.nature.com/articles/nri933 >. Access: 5 jan. 2018.

SALOMON, O. D.; SINAGRA, A.; NEVOT, M. C.; BARBERIAN, G.; PAULIN, P.; ESTEVEZ, J. O.; RIARTE, A.; ESTEVEZ, J. First visceral leishmaniasis focus in Argentina. **Memórias do Instituto Oswaldo Cruz**, v. 103, n. 1, p. 109-111, feb. 2008. DOI: 10.1590/S0074-02762008000100018. Avaliable from: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762008000100018>. Access: 20 feb. 2018.

SALOMÓN, O. D.; RAMOS, L. K.; QUINTANA, M. G.; ACARDI, S. A.; SANTINI, M. S.; SCHNEIDER, A. Distribución de vectores de Leishmaniasis Visceral en la provincia de Corrientes, 2008. **Medicina**, v. 69, p. 625-630, 2009. Avaliable from: < http://www.scielo.org.ar/scielo.php?pid=S0025-76802009000700006&script=sci_arttext&tlng=pt>. Access: 10 feb. 2018.

SALOMÓN, O. D.; BASMAJDIAN, Y.; FERNÁNDEZ, M. S.; SANTINI, M. S. *Lutzomyia longipalpis* in Uruguay: the first report and the potential of visceral

leishmaniasis transmission. **Memórias do Instituto Oswaldo Cruz**, v. 106, n. 3, p. 381-382, mai. 2011. DOI: 10.1590/S0074-02762011000300023. Avaliable from: < http://www.scielo.br/scielo.php?script=sci_arttext& pid=S007402762011000300023>. Access: 20 feb. 2018.

SAMPAIO, B. M. **Variação intraespecífica e biogeografia de isolados brasileiros de *Leishmania infantum chagasi* baseado em genes nucleares e mitocondriais**. Dissertação (Mestrado em Epidemiologia Experimental Aplicada as Zoonoses) – Programa de Pós Graduação em Epidemiologia Experimental Aplicada as Zoonoses, Universidade de São Paulo, São Paulo – SP. 57f. 2016. Disponivel em: < http://www.teses.usp.br/teses/disponiveis/10/10134/tde-26092016-123454/pt-br.php>. Access: 8 jan. 2018.

SAVANI, E. S. M.; PRESOTTO, D.; ROBERTO, T.; CAMARGO, M. C. G. de O.; D´AURIA, N.; SACRAMENTO, D. V. First ocurrence of na autochthonous canine case os *Leishmania (Leishmania) infantum chagasi* in the minucipality of Campinas, state of São paulo, Brazil. **Revista do Instituto de Medicina Tropical de São Paulo**, v. 54, n. 4, p. 227-229, jul. aug. 2011. DOI: 10.1590/S0036-46652011000400010. Avaliable from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S003646652011000400010&lng=en&nrm=iso&tlng=en>. Acesso em 23 feb. 2018.

SCHONIAN, G.; AKUFFO, H.; LWEIN, S.; MAASHO, K.; NYLEN, S.; PRATLONG, F.; EISENBERGER, C. L.; SCHNUR, L. F.; PRESBER, W. Genetic variability within the species *Leishmania aethiopica* does not correlate with clinical variations of cutaneous leishmaniasis. **Molecular and Biochemical Parasitology**, v. 106, n. 2, p. 239-248, mar. 2000. DOI: 10.1016/S0166-6851(99)00216-9. Avaliable from: < https://www .sciencedirect.com/science/article/pii/S0166685199002169>. Access: 20 jan. 2018.

SCHONIAN, G.; NASEREDDIN, A.; DINSE, N.; SCHWEYNOCH, C.; SCHALLIG, H. D. F. H.; PRESBER, W.; JAFFE, C. L. PCR diagnosis and characterization of *leishmania* in local and imported clinical samples. **Diagnostic microbiology & infectious disease**, v. 47, n. 1, p. 349-358, sep. 2003. Avaliable from: < http://www.dmidjournal.com/article/s0732-8893(03)00093-2/fulltext>. Access: 8 jan. 2018.

SCHONIAN, G.; MAURICIO, I.; CUPOLILLO, E. Is it time to revise the nomenclature of *Leishmania*? **Trends in Parasitology**, v. 26, n. 10, p. 466-469, oct. 2010. DOI: 10.1016/j.pt.2010.06.013. Avaliable from: < http://www.cell.com/trends/parasitology/abstract/S1471-4922(10)00134-0>. Access: 30 nov. 2017.

SCHONIAN, G.; KUHLS, K.; MAURICIO, I. L. Molecular approaches for a better understanding of the epidemiology and population genetics of *Leishmania*. **Parasitology**, v. 138, n. 4, p. 405-425, abr. 2011. DOI: 10.1017/S0031182010001538. Avaliable from: <https://www.cambridge.org/core/journals/parasitology/article/molecular-approaches-for-a-better understanding-of-the-epidemiology-and-opulation-genetics-ofleishmania/A0AA0B017D4C7384118AE3394F8BCF9A>. Access: 15 feb. 2018.

SCHWENKENBECHER, J. M.; WIRTH, T.; SCHNUR, L. F.; JAFFE, C. L.; SCHALLIG, H.; AL-JAWABREH, A.; HAMARSHEH, O.; AZMI, K.; PRATLONG, F.; SCHÖNIAN, G. Microsatellite analysis reveals genetic structure of *Leishmania tropica*. **International Journal for Parasitology**, v. 36, n. 2, p. 237–246, feb. 2006. DOI: 10.1016/j.ijpara.2005.09.10. Avaliable from: <https://www.sciencedirect.com/science/article/pii/S0020751905003164?via%3Dihub>Access: 20 dec. 2017.

SEGATTO, M.; RIBEIRO, L. S.; COSTA, D. L.; COSTA, C. H.; OLIVEIRA, M. R.; CARVALHO, S. F.; MACEDO, A. M.; VALADARES, H. M. S.; DIETZE, R.; BRITO, C. F. A. de; LEMOS, E. M. Genetic diversity of *Leishmania infantum* field populations from Brazil. **Memorias do Instituto Oswaldo Cruz**, v. 107, n. 1, p. 39-47, feb. 2012. DOI: 10.1590/S0074-02762012000100006. Avaliable from: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074027620120001000006&lng=en&nrm=iso&tlng=en >. Access: 20 jan. 2017.

SEREDI, N.; AMRO, A.; KUHLS, K.; BELKAID, M.; ZIDANE, C.; AL-JAWABREH, A.; SCHONIAN. G. Genetic polymorphism of Algerian *Leishmania infantum* strainscrevealed by multilocus microsatellite analysis. **Microbes and Infection**, v. 10, n. 1-3, p. 1309-1315, oct. 2008. DOI: 10.1016/j.micinf.2008.07.031 Avaliable from: <https://www.sciencedirect.com/science/article/pii/S1286457908002256?via%3Dihub>. Access: 8 jan. 2018.

SEVÁ, A. D. P.; MAO, L.; GALVIS-OVALLOS, F.; TUCKER LIMA, J. M.; VALLE, D. Risk analysis and prediction of visceral leishmaniasis dispersion in São Paulo State, Brazil. **PLoS Neglected Tropical Disases**, v. 11, n. 2, feb. 2017. DOI: 10.1371/journal.pntd.0005353. Avaliable from: < http://journals.plos.org/plosntds/article/file?id=10.1371/journal.pntd.0005353&type=printable>. Access: 20 feb. 2018.

SILVA, A. R.; VIANA, G. M.; VARONIL, C.; PIRES, B.; NASCIMENTO, M. D.; COSTA, J. M. Leishmaniose visceral (calazar) na ilha de São Luís, Maranhão, Brasil: evolução e perspectivas. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 30, n. 5, p. 359-368, sep. oct. 1997. DOI: 10.1590/S0037-86821997000500002. Avaliable from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0037868219970005000002>. Access: 7 feb. 2018.

SILVA, S, de O.; WU, A. A.; EVANS, D. A.; VIEIRA, L. Q.; MELO, M. N. *Leishmania* sp. isolated from human cases of cutaneous *Leishmania*sis in Brazil characterized as *Leishmania major*-like. **Acta tropica** 112, 239-248, 2009. DOI: 10.1016/j.actatropica.2009.07.026. Avaliable from: < https://www.sciencedirect.com/science/article/pii/S0001706X0900206X?via%3Dihub >. Access: 2 nov. 2017.

SMYTH, A. J.; GHOSH, A.; HASSAN, M. Q.; BASU, D.; DE BRUIJN, M. H.; ADHYA, S.; MALLIK, K. K.; BARKER, D. C. Rapid and sensitive detection of *Leishmania* kinetoplast DNA from spleen and blood samples of kala-azar patients. **Parasitology**, v. 105, n. 2, p. 183-192, oct. 1992. DOI: 10.1017/S0031182000074096. Disponivel

em: <https://www.cambridge. org/core/journals/parasitology/article/rapid-andsensitive-detection-ofleishma nia-kinetoplast-dna-from-spleen-and-bloodsamples-ofkalaazarpatients /AC47C435593F876411AF9CD 043BEDC3D>. Access: 14 feb. 2018.

SOLANO-GALLEGO, L.; MIRO, G. M.; KOUTINAS, A.; CARDOSO, L.; PENNISI, M. G., FERRER, L.; BOURDEAU, P.; OLIVA, G.; BANETH, G. LeishVet guidelines for the practical management of canine leishmaniosis. **Parasites & Vectors**, v. 4, n. 86, mai. 2011. DOI: 10.1186/1756-3305-4-86. Avaliable from: <https://parasitesandvectors.biomedcentral.com/articles/ 10.1186/1756-3305-4-86>. Access: 10 dec. 2017.

SOUZA, G. D.; SANTOS, E. dos; ANDRADE FILHO, J. D. The first report of the main vector of visceral *Leishmania*sis in America, *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae: Phlebotominae), in the state of Rio Grande do Sul, Brazil. **Memória Instituto Oswaldo Cruz**, v. 104, n. 8, p. 1181-1182, dec. 2009. Disponivel em: <http://www.scielo.br/scielo. php?pid=S0074027620090008000017&script=sci _abstract>. Access: 5 dec. 2017.

STEINDEL, M.; MENIN, A.; EVANGELISTA, T.; STOCO, P. H.; MARLOW, M. A.; FLEITH, R. C.; PILATI, C.; GRISARD, E. C. Outbreak of autochthonous canine visceral leishmaniasis in Santa Catarina, Brazil. **Pesquisa Veterinária Brasileira**, v. 33, n. 4, p. 493-496, jan. abr. 2013. DOI: 10.1590/S0100-736X2013000400013. Avaliable from: < http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-736X2013000400013 >. Access: 10 jan. 2017.

STEINDEL, M. **SC tem o primeiro caso de leishmaniose visceral humana**. RSC portal (comunicação on line), 17 aug. 2017. Avaliable from: < https://www.rscportal.com.br/artigo/sc-tem-primeiro-caso-de-leishmaniose-visceral-humana>. Access: 13 feb. 2018.

STUART, K. D.; SCHNAUFER, A.; ERNST, N. L.; PANIGRAHI, A. K. Complex management: RNA editing in trypanosomes. **Trends in Biochemical Sciences**, v. 30, n. 2, p. 97-105, feb. 2005. DOI: 10.1016/j.tibs.2004.12.006. Avaliable from: < http://www.cell.com/trends /biochemical-sciences/fulltext/S09680004(04)00320-2>. Access: 14 feb. 2018.

SUNDAR, S.; RAI, M. Laboratory diagnosis of visceral Leishmaniasis. Clinical and Diagnostic Laboratory Immunology, v. 9, n. 5, p. 951-958, sep. 2002. DOI: 10.1128/CDLI.9.5.951-958.2002. Avaliable from: < https://www.ncbi.nlm.nih.gov/pmc/ articles/PMC120052/>. Access: 01 nov. 2017.

TAUTZ, D. Hypervariability of simple sequences as a general source for polymorphic dna markers. **Nucleic acids research**, v. 17, n. 16, p. 6463-6471, aug. 1989. Avaliable from: < https://www.ncbi.nlm.nih.gov/ pmc/articles/pmc318341/>. Access: 2 feb. 2018.

THOMAZ-SOCCOL, V. **Les *Leishmania* du Nouveau Monde. Analyse enzymatique. Démarche progressive phénétique cladistique**. **Rélations**

phylogénétiques avec les *Leishmania* de l'Ancien Monde. Doctoral Thesis, Médecine, Montpellier, 190 pp. 1993

THOMAZ-SOCCOL, V.; LANOTTE, G.; RIOUX, J. A.; PRATLONG, F.; MARTINI-DUMAS, A.; SERRES E. Monophyletic origin of the genus *Leishmania* Ross, 1903. **Annales de Parasitologie Humaine et Comparee**, v. 68, n. 2, p. 107-108, 1993. Avaliable from: < https://www.ncbi.nlm.nih.gov/pubmed/8215109>. Access: 5 oct. 2016.

THOMAZ-SOCCOL, V.; CASTRO, E. A.; NAVARRO, I. T.; FARIAS, R.; SOUZA, L. M.; CARVALHO, Y.; BISPO, S.; MEMBRIVE, N. A.; MINOZZO, J. C.; TRUPPEL, J.; BUENO, W.; LUZ, E. Casos alóctones de leishmaniose visceral canina no Paraná, Brasil: implicações epidemiológicas. **Revista Brasileira de Parasitologia Veterinária**, v. 18, n. 3, p. 46-51, jul. sep. 2009. DOI: 10.4322/rbpv.01803008. Avaliable from: < http://www.scielo.br/pdf/rbpv/v18n3/a08v18n3.pdf>. Access: 8 jan. 2017.

TIBAYRENC, M. Human genetic diversity and the epidemiology of parasitic and other transmissible diseases. **Advances in parasitology**, v. 64, p. 377-429, 2007. DOI: 10.1016/S0065-308X(06)64004-9 Avaliable from: <https://www.sciencedirect.com/science/article/pii/S0065308X06640049?via%3Dihub>. Access: 31 dec. 2017.

TIBAYRENC, M.; KJELLBERG, F.; AYALA, F.J. A clonal theory of parasitic protozoa: the population structures of *Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. **Proceedings of the National Academy of Sciences of the United States of America**, v. 87, n. 7, p. 2414-2418, abr. 1990. Avaliable from: < http://www.pnas.org/content/87/7/2414.long>. Access: 10 jan. 2018.

TIBAYRENC, M.; BEN ABDERRAZAK, S.; GUERRINI, F.; BANULS, A. *Leishmania* and the clonal theory of parasitic protozoa. **Archives de L'Institut Pasteur de Tunis**, v. 70, n. 3-4, p. 375-382, jul. oct. 1993. Avaliable from: < https://www.ncbi.nlm.nih.gov/pubmed/7802492>. Access: 15 dec. 2017.

TIBAYRENC, M.; AYALA, F. J. The clonal theory of parasitic protozoa: 12 years on. **Trends in Parasitology**, v. 18, n. 9, p. 405-410, sep. 2002. DOI: DOI: 10.1016/S1471-4922(02)02357-7. Avaliable from: < http://www.cell.com/trends/parasitology/fulltext/S14714922(02)023577?_returnURL=http%3A%2F%2Flinkinghub.elsevier.com%2Fretrieve%2Fpii%2FS1471492202023577%3Fshowall%3Dtrue>. Access: 10 jan. 2018.

TOMAS-PEREZ, M.; HIDE, M.; RIERA, C.; MOONTOYA, L.; BANULS, A. L.; RIBERA, E.; PORTUS, M.; FISA, R. Multilocus microsatellite typing of *Leishmania infantum* isolates in monitored *Leishmania*/HIV coinfected patients. **Parasites & vectors**, v. 8, n. 386, jul. 2015. DOI: 10.1186/s13071-015-0989-9. Disponivel em: <https://parasitesandvectors.biomedcentral. com/articles/10.1186/s13071-015-0989-9>. Access: 18 oct. 2017.

TOTH, G.; GASPARI, Z.; JURKA, J. Microsatellites in different eukaryotic genomes: survey and analysis. **Genome Research**, v. 10, n. 7, p. 967-981, jul. 2000. Avaliable from: <https://www.ncbi.nlm.nih.gov/pmc/ articles/PMC310925/>. Access: 5 nov. 2017.

TRENCH, F. J. P.; RITT, A. G.; GEWEHR, T. A.; SOUZA A. L. de; CHIYO, L.; GEWEHR, M. R.; RIPOLI, M.; BISETTO-JUNIOR, A.; POZZOLO, E. M.; THOMAZ-SOCCOL, V. First Report of Autochthonous Visceral Leishmaniosis in Humans in Foz do Iguaçu, Paraná State, Southern Brazil. **Annals of Clinical Cytology and Pathology**, v. 2, n. 6, p. 1041, oct. 2016. Avaliable from: <https://www.jscimedcentral.com/ClinicalCytology/ clinicalcytology-2-1041.pdf>. Access: 9 dec. 2017.

VELEZ, I. R. Uta et espundia. **Bulletin de La Societe de Pathologie Exotique.** V.6, p. 545, 1913.

VIANNA G. Sobre uma nova especie de *Leishmania* (Nota Preliminar). **Brazil Medico**, v. 25, p. 411, 1911.

VICTOIR, K.; BANULS, A. L.; AREVALO, J.; LLANOS-CUENTAS, A.; HAMERS, R.; NOEL, S.; DE DONCKER, S.; LE RAY, D.; TIBAYRENC, M.; DUJARDIN, J. C. The gp63 gene locus, a target for genetic characterization of *Leishmania* belonging to subgenus *Viannia.* **Parasitology**, v. 117, n. 1, p. 1-13, 1998. Disponivel em: < https://www.scopus.com/record/display. uri?eid=2-s2.014444272516&origin=inward&txGid=26141f80242106eadee9 20a16b08483d>. Access: 14 feb. 2018.

WEBER, J. L. Human DNA polymorphisms and methods analysis. **Current Opinion in Biotechnology**, v. 1, n. 2, p. 166-171, dec. 1990. DOI: 10.1016/0958-1669(90)90026-H. Disponivel em: < https://www.science direct.com/science/article/pii/095816699090026H?via%3Dihub>. Access: 5 feb. 2018.

WEIR, B. S.; COCKERHAM, C. C. Estimating F statistics for the analysis of population structure. **Evolutions**, v. 38, n. 6, p. 1358-1370, nov. 1984. Avaliable from: <https://www.jstor.org/stable/2408641?seq=1#page_ scan_tab_contents>. Access: 8 jan. 2018.

WEISSENBACH, J. A second generation linkage map of the human genome based on highly informative microsatellite loci. **Gene**, v. 135, n. 1-2, p. 275-278, dec. 1993. Doi: 10.1016/0378-1119(93)90077-g. Disponivel em: < https://www.sciencedirect.com/science/article/pii/037811199390077g>. Access: 25 jan. 2018.

WELSH, J.; MCCLELLAND, M. Fingerprinting genomes using PCR with arbitrary primers. **Nucleic Acids Research**, v. 18, n. 24, p. 7213-7218, dec. 1990. Avaliable from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC 332855/>. Access: 8 jan. 2018.

WHO, World Health Organization. **The Leishmaniasis: Report of a WHO expert committee.** Tech. Rep. Series WHO No. 701, World Health Organization, Geneva, 105-107, 1984.

WHO, World Health Organization. **TDR diseases/Diseases current portfolio**. 2003. Disponivel em: <http://www.who.int/tdr/diseases-topics/leishmaniasis/en/>. Access: 22 jan. 2018.

WHO – World Health Organization. **Control of the leishmaniasis. Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases,** Geneva, 22–26 March 2010. Avaliable from: <http://apps.who.int/iris/bitstream/10665/44412/1/WHO_TRS_949_eng.pdf>. Access: 12 dec. 2016.

WHO – World Health Organization. **The Post Kala-azar Dermal Leishmaniasis (PKDL) atlas: a manual for health workers**. 2012. Avaliable from: <http://apps.who.int/iris/bitstream/10665/101164/1/ 9789241504102_eng.pdf?ua=1>. Access: 8 dec. 2017.

WHO, World Health Organization. **Leishmaniasis Burden and distribution**. Feb. 2013. Avaliable from: http://www.who.int/leishmaniasis/ burden/en/ Access: 03 mai. 2015.

WHO, World Health Organization. **Leishmaniasis: Informe Epidemiológico de las Américas**. Informe Leishmaniasis, n. 4, jul. 2016a. Avaliable from: <http://iris.paho.org/xmlui/handle/123456789/34111>. Access: 15 dec. 2017.

WHO, World Health Organization. ***Leishmania*sis in high-burden countries: an epidemiological update based on data reported in 2014**. Weekly epidemiological record. N. 22, v. 91, p. 285-296, 2016b. Avaliable from: <http://www.who.int/wer/en/>. Access: 18 oct. 2017.

WHO, World Health Organization. ***Leishmania*ses. Epidemiological Report of the Americas.** N. 5, abr. 2017. Avaliable from: < http://iris.paho.org/xmlui/handle/123456789/34111 >. Access: 1 dec. 2017.

WILLIAMS, J. G. K.; KUBELIK, A. R.; LIVAK, K. J.; RAFALSKI, J.; TINGEY, S. V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. **Nucleic Acids Research**, v. 18, n. 22, p. 6531-6535, nov. 1990. Avaliable from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC33 2606/>. Access: 8 dec. 2017.

WRIGHT, J. H. Protozoa in a case of tropical ulcer ("Delhi sore"). **Journal Medicine Research**, v. 10, n. 3, p. 472-480, dec. 1903.

WRIGHT, S. The genetical structure of populations**. Annals of Eugenics**, v. 15, n. 4, p. 323-354, mar. 1951. Disponivel em: <https://www.ncbi.nlm.nih. gov/pubmed/ 24540312>. Access: 15 dec. 2017.

ZEMANOVA, E.; JIRKU, M.; MAURICIO, I. L.; MILES, M. A.; LUKES, J. Genetic polymorphism within the L*eishmania donovani* complex: correlation with geographic

origin. **The American journal of tropical medicine and hygiene**, v. 70, n. 6, p. 613-617, 2004. Disponivel em: < http://www.ajtmh.org/docserver/fulltext/14761645/70/6/0700613.pdf?expires=1518630833&id=id&accname=guest&checksum=CC73017669DF9EE0F622BEAE48E13F80 >. Access: 7 dec. 2017.

ZEMANOVA, E.; JIRKU, M.; MAURICIO, I. L.; HORAK, A.; MILES, M. A.; LUKES, J. The *Leishmania donovani* complex: genotypes of five metabolic enzymes (ICD, ME, MPI, G6PDH, and FH), new targets for multilocus sequence typing. **International Journal for Parasitology**, v. 37, n. 2, p. 149-160, feb. 2007. DOI: 10.1016/j.ijpara.2006.08.008. Avaliable from: <https://www.sciencedirect.com/science/article/pii/S0020751906002967?via%3Dihub >. Access: 15 feb. 2018.