Chapter 4

Visceral Leishmaniasis and Natural Infection Rates of *Leishmania* in *Lutzomyia longipalpis* in Latin America

Kárita Cláudia Freitas Lidani, Fabiana A. Andrade, Maria R.P.A. Tizzot, Magda C.V. Costa-Ribeiro, Marcia H. Beltrame and Iara J. Messias-Reason

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Abstract

Leishmaniasis, a neglected disease caused by protozoans of the *Leishmania* genus, is still present in 98 countries with about two million new cases yearly worldwide. It is transmitted by female phlebotomine sandflies and presents itself as cutaneous, mucocutaneous and visceral clinical forms, depending on the *Leishmania* species and the parasite-host relationship. Visceral leishmaniasis (VL) is caused by *Leishmania (Leishmania) infantum chagasi*, endemic in 12 countries of Latin America, with 90% of the cases reported in Brazil. VL is characterized by irregular bouts of fever, weight loss, hepatosplenomegaly and pancytopenia, being highly fatal with no treatment. The main strategy in limiting the expansion of VL, besides the treatment of human cases, is the control of the vector *Lutzomyia longipalpis* and its reservoirs. There are only few studies on the natural infection of *Leishmania* species, especially in relation to its endemic distribution. Epidemiological studies of leishmaniasis may indicate the infection rate of parasites in sandflies in order to assess the populations at risk and to direct public health control strategies. In this context, we aimed to review the main features of VL with regard the distribution of disease cases and natural infection rates of *Leishmania* in *Lu. longipalpis* in Latin America.

Keywords: visceral leishmaniasis, natural infection, *Lu. longipalpis*, phlebotomine, *Leishmania (Leishmania) infantum chagasi*

1. Introduction

Leishmaniasis is a protozoan disease caused by the *Leishmania* genus, transmitted by female phlebotomine sandflies. Foxes and didelphid marsupials are the main rural reservoirs, and

domestic dogs the principal reservoir in urban areas [1]. The introduction in urban settings is due to multiple conditions such as migrations, inadequate living conditions, high population density and environment changes [2].

The disease presents itself in different clinical forms including cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL), depending on the species of *Leishmania* and the parasite-host relationship. In Latin America, VL is caused by the protozoan *Leishmania (Leishmania) infantum chagasi* and is the most severe form, characterized by intermittent fever, weight loss, hepatosplenomegaly and pancytopenia [3].

Since VL is no longer being characterized as a rural disease (1980s), [3] the main strategy to limit the expansion of the disease, besides the treatment of human cases, is the control of the vector *Lutzomyia longipalpis* and the parasite's reservoirs. In addition, molecular epidemiological studies of natural infection with species of *Leishmania*, especially in relation to its endemic distribution, may indicate the infection rate of parasites in sandflies in order to assess the populations at risk and to direct public health control strategies. In this context, we aimed in this chapter to review the main features of VL with regard the distribution of disease cases and natural infection rates of *Leishmania* in phlebotomine females in Latin America.

2. Leishmaniasis

Leishmaniasis is one of the most neglected diseases, present in at least 88 countries across the tropical and subtropical regions of Africa, Asia, Mediterranean, South Europe as well as South and Central Americas, with a global distribution of about two million new cases yearly worldwide [4]. The disease poses a great impact in public health contributing to 3.3 million disability adjusted life years [5]. It is a parasitic disease caused by the biphasic protozoan of the family Trypanosomatidae, order Kinetoplastida and genus *Leishmania*, which includes 35 species, being at least 13 of them considered human pathogens [6] (**Figure 1**).

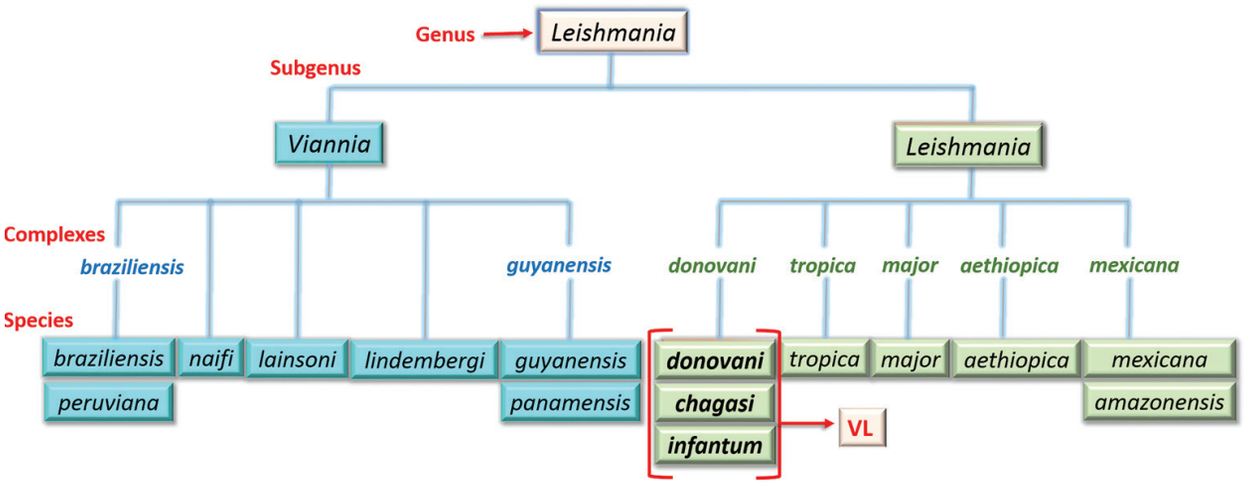

Figure 1. Main species of the *Leishmania* genus. Those causing visceral leishmaniasis (VL): *Leishmania (L.) donovani* in Asia, *Leishmania (L.) chagasi* in the Americas and *Leishmania (L.) infantum* in Asia, Europe and Africa. Note: *Leishmania (L.) infantum chagasi* nomenclature was proposed by Marcili et al. [7] using phylogenetic analysis of *Leishmania* species occurring in Latin America.

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Leishmania is transmitted by the bite of infected female sandflies of the Phlebotominea subfamily of the genus *Phebotomus* in the Old World and the genus *Lutzomyia* in the New World [8]. Sandflies become infected during blood meals by ingesting leishmanial amastigotes of infected cells. Amastigotes differentiated into dividing promastigotes (flagellate forms) to establish the parasite life cycle and multiplying in the gut of sandfly vector (in the hindgut and in the midgut for *Viannia* and *Leishmania* subgenus, respectively) [9, 10]. After digestion of the blood meal, successful infection in a sandfly vector results in the development of several promastigotes forms, named according to their morphology as procyclic, haptomonad, nectomonad, paramastigote and metacyclic forms. Only metacyclic forms transmitted through sandfly bites are able to begin an infection in vertebrate hosts, and thereby the transmission cycle completes [11, 12]. Thus, during blood meals, the vector injects the infective promastigotes in the host, which induce chemotaxis of neutrophils and macrophages. The parasites are then engulfed by macrophages and other types of mononuclear and polymorphonuclear phagocytic cells, becoming amastigotes, which is the tissue stage. Inside the cell, the amastigotes reproduce by binary fission, breakup the cell and are released to extracellular environment, being again engulfed by phagocytic cells and repeating the cycle [13–16] (**Figure 2**).

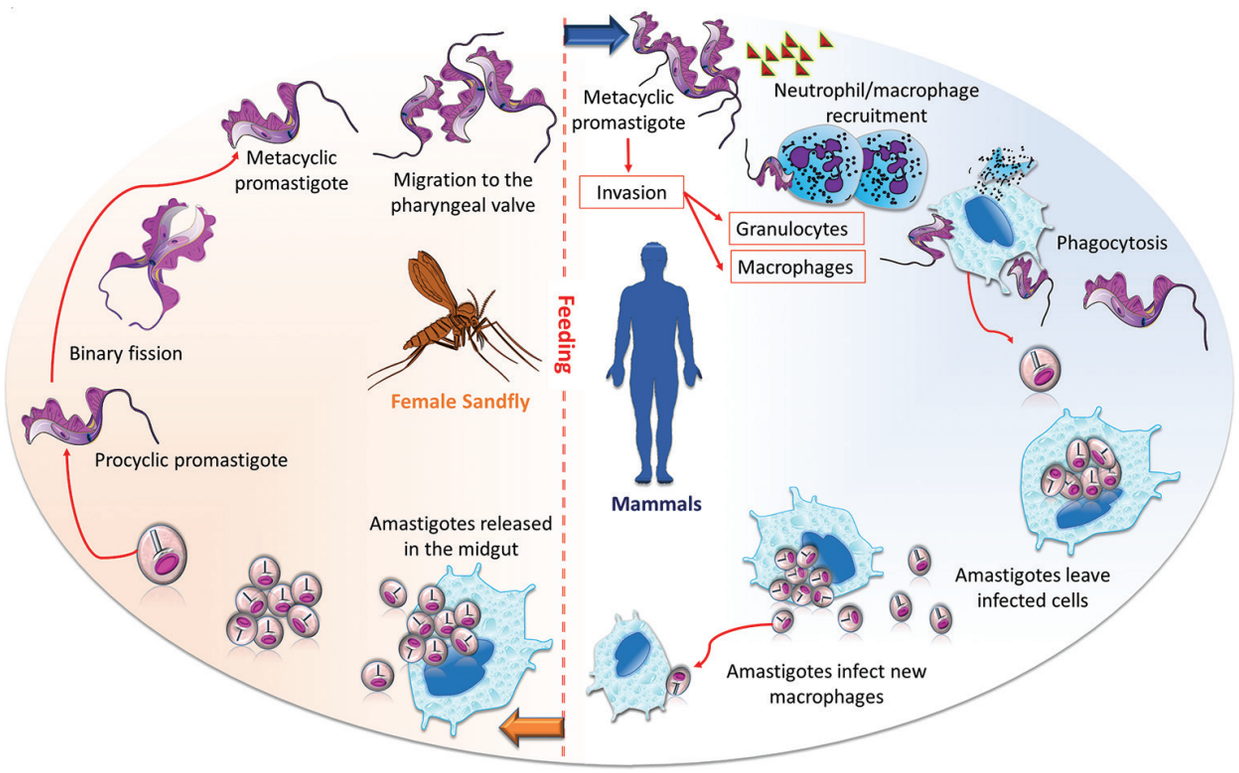

Figure 2. Life cycle of *Leishmania* spp. *Leishmania* parasites are transmitted by the bites of infected female sandflies during their blood meals. The vector injects the metacyclic promastigote forms, which are engulfed by phagocytic cells at the bite site. Inside the cells, promastigotes transform into amastigotes, the tissue stage of the parasite, which will then reproduce by binary fission and progress to infect other mononuclear phagocytic cells. Interactions between parasite, host and other factors will determine whether the infection progress to cutaneous or visceral leishmaniasis. Sandflies become infected by ingesting infected cells during blood meals. In the digestive tract of the vector, amastigotes differentiate into promastigotes and migrate to the proboscis, from where they are injected into the hosts during the bite.

Leishmaniasis in humans presents a wide diversity of clinical manifestations depending on the complex interactions between the parasite and the host immune responses, ranging from asymptomatic to severe and potentially lethal disease. The disease is classified into three main forms: cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL) [17].

CL is the most frequent clinical form, representing 75% of leishmaniasis total cases, and has an estimated yearly incidence of 0.7–1.2 million cases, being distributed in Afghanistan, Colombia, Brazil, Algeria, Peru, Costa Rica, Iran, Syria, Ethiopia and Sudan [4, 5]. CL is characterized by localized cutaneous nodules or lesions at the site of the sandfly bite (localized form). It has an incubation time of weeks to months, and initially has the appearance of an erythematous papule, which can evolve into a plaque or ulcer or can spontaneously heal in 2–10 months. These lesions are usually painless and without evident systemic symptoms or pruritus. Parasites can disseminate through the skin and form multiple non-ulcerative nodules (diffuse form), which is associated to an ineffective immune response, especially in patients infected with human immunodeficiency virus (HIV) [18, 19].

Moreover, *Leishmania* spp. can propagate through the lymphatic system, resulting in nasobronchial and oral mucosal tissue destruction (MCL form) [18, 19]. The MCL form affects both nasal and oral mucous membranes, leading to partial or total destruction. The VL is a systemic and chronic disease, and it is highly fatal if not treated [1].

3. Visceral leishmaniasis

3.1. Epidemiology

VL is recognized by the World Health Organization (WHO) as one of the most important zoonoses, due to its high incidence and mortality. Every year about 500,000 new cases of VL are reported, with 40,000–50,000 deaths worldwide [20]. The disease is endemic in 65 countries, including Bangladesh, India, Brazil, Nepal, Ethiopia and Sudan. In Latin America, VL is present in 12 countries and is caused by the protozoan *Leishmania (L.) infantum chagasi*, with 90% of the cases being reported in Brazil, especially in the Northeast and Southeast regions, representing a significant public health concern [3, 21]. In Brazil, the average number of cases of VL increased from 2866 in 1990–2000 to 3353 in 2001–2014 [22], with a fatality rate of about 7% in 2014 [23].

The disease has shown significant changes in the pattern of transmission, initially with a predominantly rural distribution, which fly has expanded to peri-urban and large urban areas [20, 24]. Although the main route of transmission is associated to hematophagous sandfly vectors, there are other routes which are important to be reported, including sexual, vertical and hematogenic [16].

Although the infection can affect people of all ages, in endemic areas, most reported cases are children below 10 years old. This is probably due to their immunological immaturity aggravated by malnutrition, which is common in these areas [3, 20]. Over 60% of the affected people are males [21, 25].

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3.2. Clinical features

VL is also known as kala-azar or "black fever/disease", which is a reference to the skin hyperpigmentation by melanocyte stimulation during infection. In addition, other terms are used to describe VL, such as Dumdum fever, Assam fever and infantile splenomegaly. It is the most severe leishmaniasis form and generally affects the spleen, liver, bone marrow or other lymphoid tissues. The syndrome is characterized by fever, weight loss, hepatosplenomegaly, pancytopenia and hypergammaglobulinemia. The fever can be continuous or remittent, and also characteristically described as periods with and without pyrexia, becoming intermittent at a later stage. Patients may also report night sudoresis, weakness, diarrhea, malaise and anorexia [26].

The onset of VL can be insidious or sudden, and the incubation period varies from 3 to 6 months, depending on the patient's age and immune status, as well as the species of *Leishmania*. If untreated, it is frequently fatal within 2 years. Death may be related to hemorrhage, severe anemia, immunosuppression and/or secondary infections. Interestingly, some successfully treated VL cases may develop maculopapular or nodular rashes, named post-kala-azar dermal leishmaniasis [17, 19] and classified into three types: depigmented macules, erythematous patches, and yellowish pink nodules [27]. Complications of VL include amyloidosis, glomerulonephritis [28] and cirrhosis [29]. In HIV patients coinfected with VL, atypical symptoms include gastrointestinal ulcerations, pleural effusion and odynophagia [30].

3.3. Diagnosis and treatment

The diagnosis of VL is still a challenge, especially in needy regions. Even though serological and molecular tests have improved the laboratory diagnosis of VL considerably, none of the available methods present 100% sensitivity and specificity [31]. The gold standard diagnosis method is still the identification of the parasite, with visualization of amastigotes from bone marrow or visceral aspirates, which holds 100% specificity. However, the sensitivity of the parasitological test varies depending on the sample, and aspirations are invasive and can cause life-threatening hemorrhages. Serological methods, on the other hand, are highly sensitive but with varying specificity [32], showing cross-reactivity with trypanosomiasis, malaria, tuberculosis, brucellosis and typhoid fever [31]. In addition, antileishmanial antibodies can be found in asymptomatic individuals and are still present after treatment and recovery, making the evaluation of therapeutic response difficult [33, 34]. Molecular techniques are remarkably sensitive and specific and can differentiate asymptomatic from clinically active infection even in HIV coinfected patients, but are costly [35, 36].

The first choice of treatment for VL is the antimonial N-methyl glucamine followed by amphotericin B (AmpB) and derivatives [37] (**Table 1**). The AmpB isolated in 1955 as a natural antibiotic was first reported as having antileishmanial activity in the early 1960s. Currently, its liposomal formulation is used to treat VL with a 95% cure rate for a single-course therapy [38, 39]. Although there are no absolute contraindications against the use of AmpB, nephrotoxicity [40] and hematotoxicity [41] should be considered [42].

Medication	Molecular formula	Presentation	Dose/route administration
Antimonial N-methylglucamine	$C_7H_{20}NO_9Sb$	Pentavalent antimony (Sb+5) Ampoules 5 mL (300 mg/mL)	20 mg/Sb+5/kg/day, once daily, endovenosa or intramuscular for 30 days. Max dose of 3 ampoules per day.
Amphotericin B	$C_{47}H_{73}NO_{17}$	Amphotericin B deoxycholate Bottle with 50 mg (lyophilized)	1 mg/kg/day by infusion for 14–20 days*.
Liposomal amphotericin B	$C_{47}H_{73}NO_{17}$	Bottle/ampoule with 50 mg (lyophilized)	3 mg/kg/day by infusion for 7 days or 4 mg/kg/day for 5 days single dose.

* The duration of treatment should be based on clinical outcome, considering the speed of response and the presence of comorbidities.
Source: Ministério da Saúde [46].

Table 1. Medvications for treatment of VL according to molecular formula, presentation, dose and route of administration recommended in Brazil.

The liposomal form of AmpB is ideal in the treatment of leishmaniasis, since enables the drug to concentrate specifically at the site of infection, reducing the concentration in others organs [43, 44]. More recently, other drugs such as miltefosine, paromomycin and pentamidine have been used in the treatment of VL in some countries of Africa and Asia, but the efficacy and required dosage of several of these medicines have not been demonstrated in all endemic areas and may differ between these areas [20].

Some criteria need to be observed for the choice of treatment, such as assessment and stabilization of clinical conditions and comorbidities present at the diagnosis of VL and electrocardiogram. The use of methylglucamine antimoniate has been especially critical in cases where resistance against pentavalent antimonials is widely spread [45].

Unfortunately, the majority of the population affected by VL is of low income, having no access to diagnosis and treatment options, thereby increasing the mortality rate due to the infection. In endemic areas, VL diagnosis is in most cases based only on clinical characteristics and epidemiologic aspects. Despite the urgent needs, research and development on leishmaniasis have been regrettably neglected.

4. Natural infection of phebotomine with Leishmania

4.1. Vectors of *L. (Leishmania) infantum chagasi*

According to Killick-Kendrick [47], four criteria must be fulfilled before incriminating a given specie as a vector for a zoonotic disease: feeding on humans and in the animal reservoir, supporting the parasites after ingestion, displaying indistinguishable parasites from those isolated from patients and transmitting the parasite by biting.

Lutzomyia (Lutzomyia) longipalpis is the most competent vector for *L. (L.) infantum chagasi* in VL Latin American foci; however, other sandflies species may be acting in the cycle of VL, mainly in areas where *Lu. longipalpis* is absent [48, 49]. In fact, *Pintomyia (Pifanomyia) evansi* has been related to VL transmission in Colombia [50–53] and Venezuela [54, 55].

Other reports from Argentina and Brazil associated the presence of *Migonemyia migonei* with autochthonous cases of VL [49, 56, 57]. Recent studies using quantitative polymerase chain reaction (qPCR) [58] and experimental infection [59] confirmed *Mg. migonei* as a potential vector of VL in Latin America. In addition, *Nyssomyia antunesi* [60] and *Lu.* (*Lutzomyia*) *cruzi* [61] were found naturally infected with *L. chagasi* in Brazil. Montoya-Lerma et al. [62] observed an association between *Pi. evansi* and *L. (L.) infantum chagasi* infection, and indicated that *Pi. evansi* represents a potential vector for VL in Colombia and Venezuela.

Evidence of transmission of VL by *Lu. cruzi* in the area of Jaciara, State of Mato Grosso in Brazil was confirmed by Missawa et al. [63]. *Lu. cruzi* and *Lu. (Lutzomyia) forattinii* are potential VL vectors in the area of Corumbá (Brazil), where notifications of the disease in humans and dogs have increased over the last two decades [64].

CL vectors such as *Nyssomyia neivai* were found infected with *L. (L.) infantum chagasi* in the city of Florianópolis (in the South region of Brazil) [65] and in an urban area of Minas Gerais state (in the Central region of Brazil), with no records of human VL and no data available for canine VL [66]. Similarly, as observed in Brazil, natural infection of *Mg. migonei* and *Nyssomyia whitmani* were found in Argentina [67].

Note: The classification and abbreviation of sandflies were used here according to Galati [68] and Marcondes [69], respectively.

4.2. Methods for detecting naturally infected vectors

The report of natural infection by *L. (L.) i. chagasi* in female phlebotomine sandflies is an important tool for epidemiological investigation, being indispensable for appropriate VL control strategy. Distinct techniques have been applied to identify parasitic infection in the insect, including classical and molecular methods.

The classical method to detect natural infection is based on the direct observation of parasites under microscopy, after sandfly gut dissection. However, this *in loco* identification is laborious, time consuming and requires experience. Another limiting factor is the difficulty in processing the large number of samples required in epidemiological studies [70, 71]. In addition, since other flagellated parasites can be found in the digestive tract of the insects, infection needs to be confirmed by in vitro culture of *Leishmania* or by inoculation into laboratory animals [72, 73]. Furthermore, low parasitemia may underestimate the rates of natural sandfly infection, which are usually about 0.2% using the classical approach, often contrasting with the high frequency of VL in endemic areas [64, 74–76]. However, the dissection method has the advantage of allowing to determine the course and location of infection by *Leishmania* in the sandfly digestive tract [77].

Alternatively, molecular approaches represent a more specific and sensitive technique, allowing the DNA detection of a single *Leishmania* parasite, regardless of its stage and localization in the insect gut [78, 79]. Indeed, PCR-based technique was eight times more efficient in detecting trypanosomatids than the dissection method and two times more efficient in identifying natural infection by *Leishmania* [80]. However, molecular methods have the dis-

advantage of not being able to distinguish between viable and dead parasites [81]. To access the genetic material of the parasite, DNA/RNA is extracted generally using a pool of about 10 female phlebotomine sandflies [82, 83].

Multiple molecular markers from nuclear and kinetoplast *Leishmania* DNA have been used to detect naturally infected phlebotomines, including the miniexon-derived RNA gene, rRNA gene, repeated genomic sequences and the kinetoplast minicircle DNA (kDNA), which is present at thousands of copies per cell [84–87]. These molecular markers are assessed by PCR methods using specific primers to amplify conserved regions, with kDNA amplification having greater reliability as a marker for the parasite when compared to miniexon and 18S rRNA [88]. Currently, PCR assays are able to detect and identify the parasite (*L. (L.) i. chagasi*) and vector (*Lu. longipalpis*) responsible for VL [82, 89–91]. Besides that, qPCR combines the identification of genetic material with the quantification of parasites present in the phlebotomine, which is important for VL transmission and the establishment of infection [83].

4.3. Disease cases and natural infection rates in Latin America

The magnitude of VL in Latin America is not completely known, mainly because most countries do not have effective surveillance systems [92–94]. VL was reported in at least 12 countries in Latin America, with Brazil having the highest number of cases, followed by Paraguay, Argentina and Colombia [21, 25] (**Figure 3**).

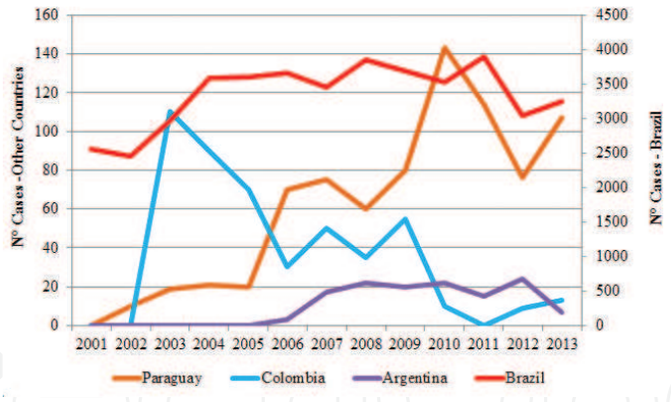

Figure 3. Visceral leishmaniasis cases in four Latin American countries: Brazil, Paraguay, Colombia and Argentina (2001–2013). Source: PAHO/WHO [21, 25].

The Brazilian Ministry of Health declared a total of 78,444 VL cases in 25 years of notification (1990–2014), with approximately 67% of them in the Northeast region. In this period, the annual mean in the country was 3137 cases and the incidence was two cases/100,000 inhabitants [22]. In addition, an increase of 3.2–6.6% in mortality rate caused by leishmaniasis was reported in Brazil from 2000 to 2014 [23].

Although resources have been invested in the VL control and establishment of protocols for specific treatment, important territorial expansion of VL in Latin America countries has been registered [21, 25, 95]. In Brazil, it was initially restricted to poor rural areas in the northeast

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of the country; however, since 1980s, the disease has gradually spread to major cities and peri-urban areas in North, Southeast, South and Midwest regions [3, 96], occurring in 23 of the 27 Brazilian states [97] (**Figure 4**).

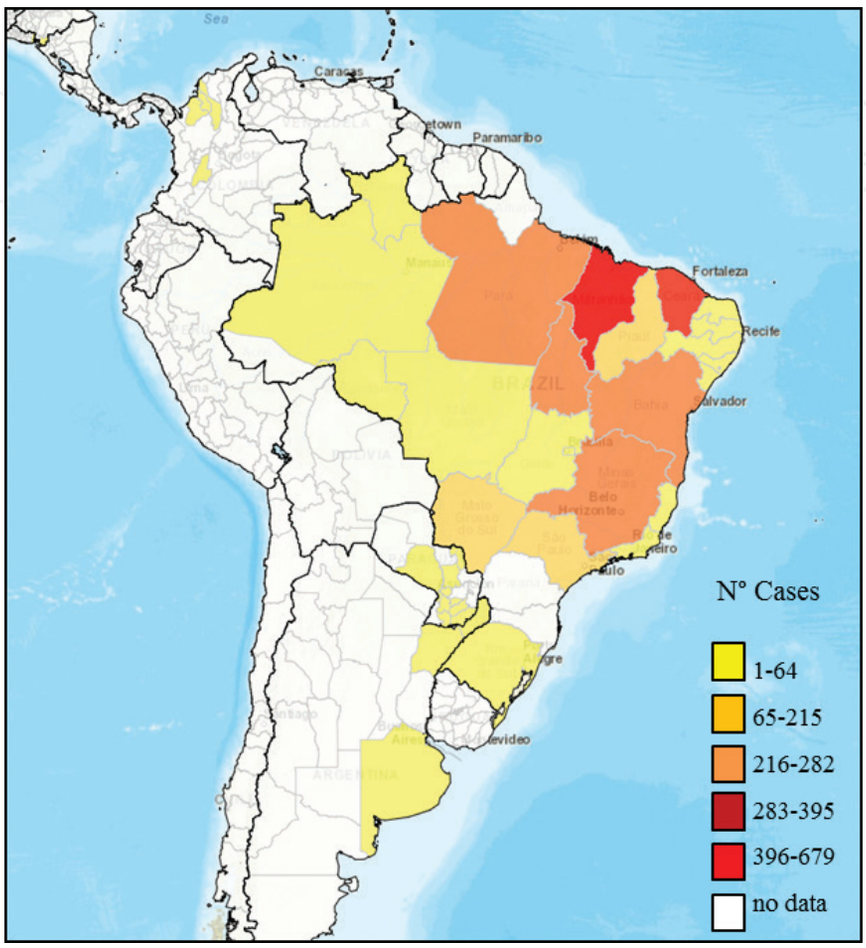

Figure 4. Distribution of visceral leishmaniasis cases in Latin America countries in 2013. Source: PAHO/WHO [97].

Current control strategies to limit the VL expansion are directed against the vector, using insecticides; the canine reservoir by serological screening, by euthanasia in seropositive dogs and by the use of vaccine in asymptomatic animals with negative serological results, in addition to the diagnosis and treatment of human cases. Unfortunately, the results of those interventions have been shown to be modest [3, 96]. Since VL epidemiological data are generally based only on the prevalence of human infection [98], surveillance strategies based on a better definition of transmission, risk areas and rates of naturally infected sandflies are necessary in order to provide better control of the disease.

Natural infection rates by *L. (L.) i. chagasi* in phlebotomine are still poorly investigated even in VL endemic areas (**Table 2**). Literature has shown that infection ratios are usually low, ranging around 1–3% in Latin America, often contrasting with the high incidence of the disease in these regions [74, 76, 99].

Locality	Specimens (*N*)	Technique	Infection rates (%)	Period of collect	Reference
Brazil					
North region					
Pará, Barcarena	280	PCR	5.3–8.6	Nov 2003–Feb 2004	[88]
Pará, Barcarena	1451	Dissection	0.0	Oct–Dec 2007, Feb 2008 and Jan 2009	[100]
Northeast region					
Maranhão, São Luís Island	800*	PCR	0.25–1.25	Mar–Aug 2005	[101]
Maranhão, Raposa	448*	PCR	1.56	Aug 2006–Jul 2008	[98]
Piauí, Teresina	1832	Dissection	1.1	Feb 2004–Jan 2005	[102]
Ceará, Fortaleza	1220*	PCR	3.7	Feb 2009–Jan 2010	[83]
Midwest region					
Mato Grosso, Várzea Grande	420*	PCR	0.71	Jul 2004–Jun 2006	[99]
Mato Grosso do sul, Campo Grande	105*	PCR	1.9	Oct 2005–Sep 2006	[103]
Mato Grosso do Sul, Antônio João	81*	PCR	3.9	No data	[86]
	81	Dissection	1.24	No data	[86]
Southeast region					
Minas Gerais, Belo Horizonte	245*	PCR	19	Jul 2006–Jun 2007	[104]
	245	Dissection	1.22	Jul 2006–Jun 2007	[104]
Minas Gerais, Janaúba	1550*	PCR	3.9	Apr 2006–Mar 2007	[105]
Colombia					
Santander, Piedecuesta	1138*	PCR	1.93	May 1999–Sep 2000	[106]
El Callejon	681	Dissection	0.59	1986–1988	[107]
Venezuela					
Aragua, Guayabita	353	Dissection	0.28	Jan 1993–Jun 1994	[55]
Bolivia					
La Paz, Los Yungas	2578	Dissection	2.2–4.2	Oct–Nov 1982	[108]
Argentina					
Misiones, Posadas	211*	PCR	0.47	Jan–Feb 2009	[82]

Note: * *Lu. longipalpis* females evaluated in pools.

Table 2. Natural infection ratios by *Leishmania (L.) chagasi* in *Lu. longipalpis* females in Latin America.

According to Cimerman and Cimerman [109], transmission depends on the presence of high densities of *Lu. longipalpis*, as observed during outbreaks of the disease. Several factors may be associated with the difference between natural infection rates detected and VL human cases reported. However, it is possible that even low infection rates are sufficient to maintain

circulating infection, highlighting the importance of monitoring sandfly vectors in order to prevent the occurrence of VL, as well as for the definition of risk areas.

On the other hand, high rates of natural infection were observed by Freitas-Lidani et al. [88] and Saraiva et al. [104], with 8.6 and 19%, respectively, in Pará and Minas Gerais states (North and Southeast regions of Brazil). Both rates were determined using molecular approaches by individual vector analysis. The local incidences of VL for the same period were 281 (Pará state, Brazil) and 407 (Minas Gerais state, Brazil) cases [22], respectively. Although the assessment of individual vectors may be more laborious, the great advantage over pooled samples is the achievement of more informative rates of infected sandflies, especially in areas where new cases are beginning to emerge in dogs and humans.

5. Conclusion

The epidemiology of leishmaniasis is complex due to the diversity of protozoan, vector and reservoirs species, associated to a variety of clinical events. Early diagnosis and treatment of infected patients is crucial to direct public policies of VL control, especially because the disease has common clinical manifestations and geographic distributions with other infections such as Chagas disease, malaria, schistosomiasis, typhoid fever and tuberculosis. In this context, molecular approaches to determine rates of *Lu. longipalpis* naturally infected with *Leishmania* allows the estimation of the transmission risk for VL and vectorial capacity in areas where many species of phlebotomine sandflies coexist.

Author details

Kárita Cláudia Freitas Lidani[1]*, Fabiana A. Andrade[1], Maria R.P.A. Tizzot[1, 2], Magda C.V. Costa-Ribeiro[3], Marcia H. Beltrame[1, 4] and Iara J. Messias-Reason[1]

*Address all correspondence to: kari.lidani@gmail.com

1 Laboratory of Molecular Immunopathology, Federal University of Paraná, Curitiba, Paraná, Brazil

2 Health School, UniBrasil University, Curitiba, Paraná, Brazil

3 Laboratory of Molecular Parasitology, Federal University of Paraná, Curitiba, Paraná, Brazil

4 Department of Genetics, University of Pennsylvania, Philadelphia, USA

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