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The Epidemiology and Ecology of Leishmaniasis

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Notice

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Meet the editor

David Claborn is an associate professor in the Master of Public Health Program at Missouri State University where he teaches graduate courses in international health and environmental health. He obtained his Doctor of Public Health (DrPH) from the Uniformed Services University of the Health Sciences in 2001, and his dissertation dealt with the reemergence of malaria in South Korea. Prior to his academic career, he was a military officer, retiring from the US Navy in 2008. In 1991, he was a medical entomologist for the First Marine Expeditionary Force during Desert Shield and Desert Storm. He lives in Springfield, Missouri, with his wife and daughter.

Contents

Preface

Leishmaniasis is a disease of great variability. It manifests in a variety of forms, from cutaneous to the deadly visceral forms. It is endemic to a variety of habitats, from deserts to jungles, though it is usually considered a disease of the developing world. It occurs in multiple locations throughout the world, from Latin America to North Africa to the Middle East and other areas. Although there are cases in which the disease is spread by unusual means such as blood transfusions or organ transplants, most transmission occurs through the bite of a phlebotomine sand fly. Like the disease, the fly occupies a variety of habitats, from desert rodent burrows to the canopy of tropical forests.

This variety in transmission and endemicity of the disease is reflected in the different epidemiologies of the disease throughout the world. The reservoir may be a sloth in South America or a dog in Southern Europe or a rodent in the Middle East. Multiple species of sand flies, with a variety of habitats, can serve as the disease vector for leishmaniasis.

Although leishmaniasis is indeed considered a neglected tropical disease primarily of the developing world, it does pose a threat outside those regions. For a variety of reasons, it is considered an emerging disease. The journal *Emerging Infectious Diseases* has published articles on human or canine cases of this disease in Germany, Spain, Italy, Israel, Canada, France, Japan, and the United States. Many of the cases were diagnosed in travelers, including military personnel or dogs who were deployed to endemic regions; however, autochthonous transmission was confirmed in several places outside the developing world. It is obvious that leishmaniasis is indeed an important infectious disease: emerging, neglected, and often deadly.

Any writing which attempts to describe the epidemiology of leishmaniasis must, therefore, acknowledge the fact that there are multiple epidemiologies of this complex disease resulting from the diversity of local cultures, the biology of the regional vectors and reservoirs, and the composition of the local habitats. The opening section of the book contains chapters on the application of the epidemiological method to the study of leishmaniasis. The second section consists of several chapters on the epidemiology of the disease in different sections of the world including North Africa, Central America, and South America. The chapters will demonstrate the truly complex epidemiology of the disease provided by the varying vectors, agents, and habitats involved in the transmission of leishmaniasis.

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Section 1

Basic Epidemiology

Chapter 1

Living on the Edge: Border Countries Should Have Strict Veterinary and Health Policy on Leishmaniasis

Tina Kotnik and Vladimir Ivović

Additional information is available at the end of the chapter

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Abstract

First human and canine cases as well as presence of competent Phlebotomine fly vectors are reported for the first time in Slovenia. Number of infected dogs in Slovenia has been increasing in the last few years. Having increased number of infected dogs and a presence of proven Phlebotomine fly vectors at the same time in a climatically suitable region may lead to endemic spread of the disease. And that is the kind of situation calling for governmental regulation. Basic preparedness and rapid response mechanisms should be in place. Leishmaniasis cases should be detected early and reaction should be quick. In epidemic-prone areas and before the anticipated outbreak season, the responsibilities of the outbreak task force members should be defined; the necessary needs for response, surveillance, and control should be assessed; the surveillance system should be reinforced; criteria for epidemic alert should be set up; and all health facilities should be provided with minimum stocks of basic diagnostic and treatment supplies. Successful preventive measures should include regular veterinary checks of all imported dogs and dogs traveling outside the country, vector control, use of effective repellents, sleeping indoors with nets on the windows, and antileishmanial vaccination of dogs.

Keywords: leishmaniasis, CanL, Phlebotomine, visceral leishmaniasis, *L. infantum*, *P. perniciosus*

1. Introduction

Leishmaniasis is a disease that affects humans, as well as wild and domestic animals. They are caused by parasites of the genus *Leishmania* (protozoa, trypanosomatidae) and are transmitted by Phlebotominae flies (Diptera, Psychodidae). Not all species of *Leishmania* parasites are of

medical importance; over 20 of them are considered pathogenic for humans and 10 have been isolated from dogs, including *L. infantum* (Syn. *L. chagasi*), *L. donovani*, *L. tropica*, *L. major*, *L. arabica*, *L. amazonensis*, *L. mexicana*, *L. braziliensis*, *L. peruviana*, and *L. colombiensis* [1]. *L. donovani* is the one that causes human visceral leishmaniasis (VL) with more than 90% of the cases occurring in India, Sudan, Bangladesh, and Brazil [2]. This species is involved in anthroponotic epidemiological cycle with anthropophilic vector and humans act as the reservoir of the disease. *L. tropica* causes anthropophilic cutaneous leishmaniasis (CL). Unique proven vector *Phlebotomus sergenti* is present mainly in urban areas, often at the periphery of old towns and cities, and in poor suburbs where low sanitary conditions facilitate breeding sites for this species. Zoophilic CL epidemics seem to be related to the fluctuations of the rodent populations and the accumulations of nonimmune people. This disease is found mainly in rural areas. The responsible parasite for zoonotic CL is *L. major* and the proven vector in the Mediterranean basin is *Phlebotomus papatasii* [3, 4]. *L. infantum* is involved in zoonotic epidemiological cycle with zoophilic vectors serving dogs as the reservoir of the disease [5] and is currently the predominant causative agent of VL in humans and dogs in the Mediterranean region. Majority of both suspected and proven vectors of this pathogen belongs to the *Larroussius* subgenus. With so many species of human-infective parasites, different reservoir and vector species in a wide range of topographically different foci, the ecology, and epidemiology of leishmaniases are without doubt the most diverse of all vector-borne diseases.

At the beginning of the twentieth century in Europe only sporadic human visceral leishmaniasis cases had been reported. The spread of the disease happened after 1975 and many of the European Union (EU) countries developed surveillance system around that time. The increased incidence of leishmaniasis in the Mediterranean region is due to several reasons including the influx of nonimmune population into the natural foci of transmission, changes in ecology of vectors and reservoir hosts, reduction in the use of residual insecticides for the control of mosquito populations, improvements in the diagnostic methods, and reporting of positive cases.

Human VL has long been considered a disease of young children but epidemiology of the disease after 1975 has changed with the increase of incidence in adults. This correlated to the emergence of HIV. However, in the last decade, numbers of VL infections in adults in many EU countries decreased. This can be attributed, among other measures, also to the use of a novel, highly active antiretroviral therapies (HAART) [6].

We are observing also changes of epidemiology of canine leishmaniasis (CanL). While foci of CanL including insecticide nontreated dogs of predisposed breeds traditionally were settled in the coastal districts, recent studies show that there are various risk factors for CanL, such as age 2 years or more, sleeping mostly outdoors, season of sampling (spring to autumn), and geographical origin [7, 8]. Today, leishmaniasis is endemic in all the countries of Southern Europe, with an incidence rate of 0.21 per 100,000 inhabitants and more than 750 autochthonous human cases reported each year [6, 9]. In the Mediterranean region, leishmaniasis is generally associated with *Leishmania infantum*, but other species autochthonous in Asia, the Middle East, and Africa, such as *L. donovani* and *L. tropica*, may colonize European Phlebotomine fly vectors as well.

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Slovenia is one of the smallest member countries of the European Union neighboring to Austria, Italy, and Croatia. Despite an area count of only 16,423 square kilometers, it has diverse landscape. The northern part of the country is composed of Alpine and the southern part is composed of Mediterranean landscape. Being the bridge between eastern and western part of the Mediterranean, this region hosts unknown *Leishmania* species and some of the most important Phlebotomine fly vectors.

From 2004 to 2011 a questionnaire-based multinational survey on canine leishmaniasis has been conducted in Europe. Slovenian veterinary clinics were among the 12,546 subjects that have been questioned. Reply rate of Slovenian veterinarians was satisfying (46.7%) and the survey had shown no endemic CanL case recognized in Slovenia up to that time [10, 11]. In spite of its vicinity to Italy and Croatia, both well-known endemic countries, all infected dogs were found to be imported to Slovenia or have previously traveled to one of the endemic regions. Autochthonous cases of both canine and human leishmaniasis in Slovenia were not reported until recently. There may be two main reasons: unrecognizing and underreporting of potential cases.

In January 2014, the first endemic CanL case was reported in Slovenia the same year when the first specimens of Phlebotomine flies were collected. During the survey in the Istrian peninsula, both Slovenian and Croatian side, five Phlebotomine fly species were identified, including *P. neglectus* and *P. perniciosus*, some of the most important vectors of *Leishmania* parasites [12]. Although only one CanL autochthonous case and one suspected autochthonous human case (data not published) have been reported [13], recent information indicates increase in the number of infected dogs settled in Slovenia.

Registration of CanL cases in certain region is of great epidemiological importance for prevalence of disease in people as well.

2. Materials and methods

Data on CanL cases in Slovenia were collected via mail with a short and simple questionnaire that whether veterinarians had ever registered a CanL case that was born in Slovenia and that had never traveled outside the country. Mail was sent through the official mailing of Slovenian Veterinary Chamber in December 2015 and January 2016 to all veterinarians dealing with small animal practice.

Data on human cases in Slovenia were collected through the SURVIVAL program for official surveillance of communicable diseases supported by the Ministry of Health of Republic of Slovenia.

During 2013 and 2015, Phlebotomine flies were collected throughout the period of peak seasonal activity (15/05–31/09) at six sites from two diverse areas of Slovenia, including the coastline as well as the karst region (**Figure 1**).

Outdoors, Phlebotomine flies were collected by Centers for Disease Control (CDC) miniature light traps and sticky papers. CDC traps were operated all night, once per week in animal barns

and backyards and a cave. Two traps were set at each collecting site. The insect caught was checked each morning and the trapped flies were sorted and kept either dry or in 70% ethanol. Sticky traps were set in outbuildings, within walls, in tree holes in olive groves, and in the open surrounding phrygana-type scrubland. The trapped flies were removed with brushes and stored in 70% ethanol. Indoor collection of Phlebotomine flies was carried out using mouth and electric aspirators. The collected flies were mounted on permanent microscope slides for species identification, which was carried out according to keys by [14, 15].

Figure 1. Map of the study area. **(a)** Veterinary clinic in Spodnji Duplek, **(b)** Kočevje, place of the first autochthonous case of canine leishmaniasis in Slovenia, **(c)** Slovenian littoral, and **(d)** Phlebotomine flies collection sites.

3. Results

Slovenian society, named "Hrtji svet" (Hounds world) has been rescuing dogs from Spain via adoption by Slovenian owners. During 2009–2016 they have imported 117 dogs. Although routine leishmaniasis testing on these dogs was suggested to all of the new owners, this could not have had prevented possible infective state of these dogs. In 7 of 117 dogs high antileishmania antibody titer was found and these dogs were treated with allopurinol. One dog died, and one has finished the therapy, but the rest five out of seven dogs were still under treatment at the time of writing this chapter. In Slovenia there is also another society that rescues dogs from Spain, but data on their numbers and epidemiological status are not available.

In **Table 1**, results on additional CanL cases, reported by one single veterinary practice, being settled in the North-East of the country are presented (**Figure 1a**). We can see that 14 seropositive dogs had been presented to the practice in the period 2012–2015. Nine of them had been tested because of clinical signs and five of them were found positive on routine testing.

No.	Gender/breed/age*	Clinical presentation**	IFAT	Electrophoresis
1	F/SG/8y	Routine testing	1:256	+
2	F/SG/3y	Skin changes	1:128	+
3	F/SG/6y	Skin changes, symmetrical lymphadenomegaly	1:256	+
4	F/SG/4y	Routine testing	1:256	+
5	F/SG/2y	Skin changes	1:256	+
6	M/SG/4y	Skin changes, symmetrical lymphadenomegaly gastrointestinal disturbances	1:128	+
7	M/SG/7y	Anorexia, epistaxis	1:128	+
8	M/SG/6y	Skin changes, symmetrical generalized lymphadenomegaly, pale mucous membranes, gastrointestinal disturbances	1:256	+
9	F/SG/5y	Routine testing	1:128	+
10	F/MIX/2y	Pale mucous membranes, skin changes , weight loss, lethargy	1:256	+
11	F/SG/3y	Anorexia, skin changes	1:128	+
12	F/SG/4y	Pale mucous membranes, skin changes	1:256	+
13	F/IH/3y	Routine testing	1:256	+
14	M/SG/4y	Routine testing	1:128	+

Abbreviations: F, female; M, male; SG, Spanish greyhound; IH, Ibizan hound; MIX, mixed breed.

Table 1. Seropositive dogs, adopted from Spain during 2012–2015, all successfully treated, settled mainly in Podravska (North-Eastern) region of Slovenia.

	P. neglectus		***P. perniciosus***		***P. papatasi***		***P. mascitii***		***S. minuta***		**Total m/f**	
2013–2015	m	f	m	f	m	f	m	f	m	f	m	f
	336	93	81	10	5	12	5	22	1	0	428	137
Total	429		91		17		27		1		565	

Abbreviations: m, male; f, female.

Table 2. Phlebotomine flies collected in the coastal and karst region of Slovenia (**Figure 1**) during 2013–2015.

One autochthonous CanL case was reported from South-Eastern region of Slovenia by a private practitioner. It was a female dog living in the South-East of the country (**Figure 1b**). This region has mild microclimate and is close to the Croatian border. The infected female dog regularly slept outdoors and had never left the country. The dog was presented to the local vet when it was 5 years old with signs of skin changes, loss of weight, general malaise, and changes in laboratory parameters, compatible to leishmaniasis (hypoalbuminemia, hypergammaglobulinemia, leukocytosis, monocytosis, and anemia). Immunofluorescence antibody titer (IFAT) test revealed 1:>160 antileishmania antibody titer. Clinical improvement was achieved following therapy with allopurinol.

Five human cases (two women and three men, 24–55 years old) were reported during the period from 1997 to 2016, two of them in 2015, and one of them in 2016.

During the period from 2013 to 2015, five species of Phlebotomine flies were collected and identified (**Table 2**).

4. Discussion

In this chapter, we present data on human leishmaniasis cases, CanL cases, as well as the presence of important leishmania Phlebotomine fly vectors in Slovenia. Among the five collected species that were found in the region, two of them *P. neglectus* and *P. perniciosus* are well known and proven vectors of *L. infantum* in the Mediterranean and two others *P. papatasii* and *P. mascitii* are known vectors of several phleboviruses [16]. Leishmania parasites were not isolated from Phlebotomine flies that were caught and tested in Slovenia. Nevertheless, since one autochthonous CanL case and at least one autochthonous human case were reported, we presume that there is a natural life-cycle of *Leishmania* sp. in the coastal region of Slovenia. Although other infection routes are proved in dogs (placental route, by sexual intercourse, by blood transfusion, and by infected flea bites) [17–20] they seem unlikely in our CanL case. The female dog was born in the region and had never traveled outside the country. It never got blood transfusion. It regularly slept outdoors. Clinical state of the mother and the spouse of the female dog (it got litter once), concerning leishmaniasis, was unknown but it is very likely that the female dog has been infected by Phlebotomine fly bite. The presence of infected vectors in Southeastern part of Slovenia is therefore highly probable.

Concerning the Mediterranean region, Phlebotomine fly's distribution pattern is quite bimodal. Western population was and it still is under direct influence of the African lineages. On the other hand, Eastern population is under the influence of both African and Asian continents. Northern part of the Mediterranean land mass is characterized by two narrow passages which directly influence spreading of Phlebotomine flies species. First one is restricted by Maritime Alps in the Southwestern part of the Alps, on the border between France and Italy. The second one and much more important for the Phlebotomine fly distribution is Slovenian Littoral, the westernmost part of Slovenia, bordering with the Italian region of Friuli-Venezia Giulia, true contact zone between Eastern and Western populations. It is about 13 km wide, karst plateau, a unique geological formation displaying distinctive surface features, containing cenotes, sinkholes, or dolines [21].

As a bridge between East and West, the region of Slovenian Littoral hosts unknown number of Phlebotomine fly species. Until now we have found some of the most important vectors of *Leishmania* parasites in the Mediterranean, including *Phlebotomus neglectus*, *P. perniciosus*, and *P. perfiliewi*, species that already have penetrated this narrow land bridge (**Figure 1**). Nevertheless, due to both environmental transformations and human activities there could be some other, more neglected ones such as *P. kandelaki* proven vector of *Leishmania* parasites in the Middle East.

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Generally, most of the Phlebotomine fly species belonging to the subgenus Larroussius are potential vectors of *L. infantum*, and evidently due to rapid climatic changes, two of these are showing rapid aerial expansion. In 2003, the westernmost point of the range of *P. kandelaki* was in Montenegro [22], while in 2008, it was collected further to the west, at the coast of Croatia in Krk island (V. Ivović, unpublished data). Another fast spreading species and even more important vector, *P. neglectus* regularly situated in the Balkans, was recently found near Budapest (Hungary) [23]. Thanks to the attention recently drawn to the formerly neglected discipline of medical entomology, the latest results show the widening of the Phlebotomine fly distribution range to regions where they have never been found before.

Sixteen symptomatic dogs in Slovenia in the population of 220,700 officially registered dogs at the end of 2015 seem to be a small number. Nevertheless, point of view turns different taking into consideration that majority of the infected dogs never developed clinical symptoms. Incidence rate in endemic regions is usually less than 10% while seroprevalence can be as high as 90% [24]. We can speculate that the number of infected dogs in Slovenia is already higher in the moment. As many as 65% of asymptomatic dogs can harbor circulating parasites in their blood and as high as 93% of asymptomatic dogs are competent to transmit Leishmania to the vector, therefore these dogs allow transmission and spread of the disease [25, 26]. Beside dogs, other animals such as rats, cats, horses, rabbits, foxes, and jackals can be infected and may serve as a reservoir [12, 24, 27, 28]. Import of infected dogs to nonendemic regions creates one of the main risk factors for the spread of the disease. Many infected stray dogs have been brought to the north of Europe by compassionate tourists as well as social rescuing societies. There is an estimate that about 20,000 dogs infected with Leishmania presently live in Germany [29]. From the Crete and Cyprus example we can learn and predict increase of seropositive dogs in the Slovenian population. Seroepidemiological studies in dogs on island Crete, during the last 25 years, showed that the number of seropositive animals has been increasing [30]: from 0.27% in 1990 (data of the Greek Ministry of Agriculture) to 2.9% in 1994 and 19.8% in 2009. Same happened in Cyprus with a ninefold increase of seropositivity in dogs in the last 10 years [8]. This may be explained by the fact that dogs are brought into the island from mainland, especially from Attica, where leishmaniasis is endemic. The number of seropositive dogs in Crete continues to increase every year [30]. Another example is a situation that recently emerged in Australia. Before 2004, Antarctica and Australia were the only continents in the world that were free of leishmaniasis. In 2006, Biosecurity Australia ordered mandatory serological testing of dogs stationed in quarantine prior to importation. Few positive cases have been recognized until now, but there is concern of possibility of higher numbers [31]. Incubation time in leishmaniasis may be quite prolonged and clinical signs are not specific; therefore, veterinarians in nonendemic countries might overlook it.

Seroprevalence in people and dogs in Slovenia has not been estimated yet. High seroprevalence ratios in dogs were recently found in neighboring countries such as Italy and Croatian region of Dalmatia (21% and 42.85%, respectively) [32, 33]. Unfortunately, no information on the prevalence of healthy human inhabitants from Dalmatia is available. Recent data on seropositivity of human population residing in the Istrian region nearby the southern Slovenian border confirm the possibility of spreading the disease toward the north of Europe, including

Slovenia [34]. Moreover, seropositivity of Austrian inhabitants, living nearby the northern Slovenian border [35], indicates that the spread might have already happened. A potential vector *Phlebotomus mascitii* was found in this region [36]. Although *P. mascitii* is only an assumed vector of *Leishmania* spp.—data on its experimental transmission capacity are still lacking—the wide distribution of Phlebotomine flies in Austria, a country thought to be free of these insects, further supports a potential emergence of endemic leishmaniasis in Central Europe. Studies from France showed that *P. perniciosus*, species that was present in Slovenia too, is the most common leishmania vector in regions at low altitudes (less than 600 m above sea level). This suits to the spread of the disease in the Slovenian coastal region. Another species *P. ariasi* was found in France as the main vector at the attitudes between 200 and 1400 m above sea level [6]. If this species would spread to Slovenia, it looks that mountain region would perfectly suit it. Until now the presence of this species in Slovenia has not been proved.

Epidemiological studies show that incidence of human infections are directly related to the number of infective dogs and the presence of suitable vectors in the region. Control of reservoirs by dog culling is, apart from being expensive and time consuming, also not efficient. Because the breeding sites of Phlebotomine flies are unknown, control measures are focused mostly on adults [37]. There are several strategies targeting adult vectors. Nevertheless, applying environmental changes such as trimming trees and shrubs and cleaning and reorganizing in and around human dwellings and animal shelters can prevent favorable conditions for the development of Phlebotomine fly larvae. In some regions with high Phlebotomine flies control efforts have focused on the use of chemical insecticides, mostly on synthetic pyrethroids. Unfortunately, these measures, although initially attractive, are generally not permanent but are still most frequently used in controlling adult vectors. More advanced and sophisticated methods in Phlebotomine control includes planting of different plant species rich in phytochemicals that have a toxic effect against adult insects and larvae and use of bioinsecticides, particularly entomopathogenic against Phlebotomine flies [38].

Visceral leishmaniasis in people and dogs show similar clinical presentation involving intermittent pyrexia, lymphadenopathy, malaise, anemia, cachexia, hypergammaglobulinemia, and hepatosplenomegaly. Beside these dogs develop skin changes, mainly in the form of exfoliative and nodular dermatitis [39]. Hyperglobulinemia is thought to be related to a Th2-dominated immune response resulting in a marked humoral response and increased gamma globulin production [31].

Unfortunately, chemotherapy is not a successful measure in control of canine visceral leishmaniasis. Relapsing cases are common and drugs do not lead to the inhibition of infectivity to Phlebotomine flies [1]. Parasites were proved even from healthy looking skin of infected dogs. Unlike dogs, people cured of leishmania infections usually develop lifelong immunity. Treatment of VL in people is dependent on chemotherapy that should cure the patient and reduce the risk for relapse. The first-line drug for VL treatment should be liposomal amphotericin B or alternatively pentavalent antimonials and amphotericin B deoxycholate [40]. Miltefosine is being used on a compassionate basis in several European AIDS coinfected patients unresponsive to amphotericin B or pentavalent antimonials. Recently, this drug has been launched in the market for canine leishmaniasis treatment in Portugal, Spain, Italy,

Greece, and Cyprus. Because dogs are never cured parasitologically and given the long half-life of the drug, the lack of European policy might contribute to the emergence of parasites resistant to miltefosine. Indeed, while this drug is successfully used in CanL treatment, there are reports on increasing incidences of relapse in humans on this treatment [41]. The same may happen with the use of ambisome in domestic pets that might produce spreading of resistant strains [3]. Like the Leish Vet group (international group of veterinary experts dealing with CanL), human experts conclude that chemotherapy alone would probably not be sufficient to eliminate the disease. Therefore, an effective vaccine should be developed and used in animals as well as in humans [40].

In the past, an effective vaccine against human leishmaniasis has already been used. This involved inoculation with live, virulent parasites in a process called leishmanization. It was practiced successfully in the former Soviet Union, the Middle East, and Israel. However, it was abandoned in most countries because of logistical problems and safety concerns, as some individuals developed nonhealing lesions and immune suppression [40, 42]. According to the authors' knowledge, no vaccine for routine use in people has been produced yet. The reason is that good understanding of immunity generated against pathogens is surely important for developing an effective vaccine. Current understanding of human immune responses generated against Leishmania parasites is mainly based on the studies in animal models and this cannot be simply extrapolated to humans [40]. While interferon-γ seems protective in mice and people, there have been enough of differences to prove that more studies in humans are needed. Patients developing visceral or diffuse cutaneous disease exhibit helper T-cell subtype 2 cytokine profile [13]. Genetically modified Leishmania parasites lacking essential genes such as dihydrofolate reductase, biopterin reductase, or cystine proteases have been shown to stimulate protection against challenge with virulent parasite strains in people. The main problem is the concern relating to safety and feasibility for large-scale use in the field [40]. Veterinary medicine probably gained an advantage in development of vaccines against leishmaniasis. A saponin formulation of fucose mannose ligand that is expressed throughout the lifecycle of a parasite was found to be safe, protective, and immunogenic and has become the Leishmune veterinary vaccine, licensed after a series of canine VL field studies [40]. In Europe, a vaccine based on the secreted-excreted antigen of *L. infantum* (CaniLeish, Virbac Animal Health) has been recently licensed, and has been available in some European countries since 2011. Some studies show good immunogenicity of this vaccine, although large-scale field studies are missing [43]. CanL vaccines proved to be efficacious not only in prevention of the disease but also in prophylactic manner, converting immune status of the infected dogs to more efficacious cell-mediated immunity, that is able to prevent visceral leishmaniasis [43]. We therefore agree with the authors Foroughi-Parvar and Hatam stating in their review article that the only efficacious method for control of CanL might be a vaccine [44].

The issue of notification of leishmaniasis differs in EU countries but even where notification is compulsory (i.e., Italy and Spain), notification of CanL cases is not a common practice. Generally, notification is compulsory in southern EU countries (Bulgaria, Greece, Italy, Portugal, and parts of Spain) but not in the part of EU, traditionally considered as nonendemic (France, Netherlands, etc.) [6, 45]. In Slovenia, notification of human case is mandatory in 3

days after the diagnosis [46] and record of CanL case should be reported to regional veterinary administration as well as to regional public health service as soon as confirmed by laboratory tests. These data are entered in the computer system of the Veterinary Administration of Republic of Slovenia monthly and reported twice annually to the World Organisation for Animal Health (OIE) via World Animal Health Information System (WAHIS). Notification of CanL cases in Slovenia is mandatory since 2014 but no CanL cases have been reported. Our data confirm that underreporting is taking place in Slovenia too, similarly to other border countries. Unrecognizing and underreporting of human and animal leishmaniasis in nonendemic countries can have wide-ranging consequences. Long reporting delay may happen even in endemic region like it was the case during recent community outbreak in Madrid (median of 151 days—41 days for visceral leishmaniasis and 183 days for cutaneous leishmaniasis). The delay arises from a number of factors that may be related to the patient (delay in seeking care) or the healthcare system (delay in diagnosis and reporting) [47].

Number of notified human cases in Slovenia at this moment is low. Interviews of these patients unfortunately were not done therefore no data were collected on clinical presentation nor traveling abroad. We even do not know whether reported cases were VL cases or cutaneous leishmaniasis cases. Nevertheless, having increased number of infected dogs and proven Phlebotomine fly vectors at the same time in climatically suitable region may lead to endemic spread of the disease [9]. That is a situation calling for governmental regulation. According to WHO's recommendations for epidemic-prone areas, basic preparedness and rapid response mechanisms should be in place. Leishmaniasis cases should be detected early and reaction should be quick. In epidemic-prone areas and before the anticipated outbreak season, the responsibilities of the outbreak task force members should be defined; the necessary needs for response, surveillance, and control should be assessed; the surveillance system should be reinforced; criteria for epidemic alert should be set up; and all health facilities should be provided with minimum stocks of basic diagnostic and treatment supplies [2].

Successful preventive measures in Slovenia and Slovenia-like border countries should include regular veterinary checks of all imported dogs and dogs traveling outside the country, vector control, combined to use of effective repellents, and sleeping indoors with nets on the windows, and importantly, antileishmanial vaccination of dogs.

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Chapter 2

Clinical Manifestations of Visceral Leishmaniasis (American Visceral Leishmaniasis)

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Additional information is available at the end of the chapter

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Abstract

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis. It is endemic in tropical and subtropical countries and responsible for about 50,000 deaths annually. It is characterized by irregular fever, progressive pallor, spleen and liver growth, and consequent increase in abdominal volume, lymphadenopathy, anorexia, and weight loss. Some changes in epidermal structures can be observed such as dry, brittle and depigmented hair, while the eyelashes are long and silky, pale skin, and as the disease progresses may arise petechiae, ecchymosis, hemorrhagic suffusion, and sometimes jaundice. Edema appears very often, mainly in lower limbs. Hematologic changes are manifested by the reduction of all blood cells. Hypoalbuminemia is a frequent finding, while globulin increases. The patient suspected of having the disease is the one who has fever and splenomegaly. It is valuable to the diagnosis of epidemiological data, history of irregular fever, hepatomegaly, splenomegaly, and blood disorders such as pancytopenia and hypoalbuminemia. In the course of the disease, bacterial infections are established, especially in the respiratory tract, sometimes responsible for the death. VL is a consumptive disease that requires specific treatment as early as possible.

Keywords: American visceral leishmaniasis, clinical manifestations

1. Introduction

American visceral leishmaniasis (AVL) is a systemic protozoan infection characterized by fever, malaise, adynamia, and weight loss, besides splenomegaly, hepatomegaly, anemia, leukopenia, pancytopenia, and hypergammaglobulinemia. Later, cachexia, hepatic dysfunction with jaundice, hypoalbuminemia, and edema also arises. If untreated, almost always progresses to death [1].

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis, endemic in tropical and subtropical countries. It is estimated that there are 200,000–400,000 cases of VL each year worldwide, 90% of these cases are from Bangladesh, Brazil, Ethiopia, India, Sudan, and South Sudan [2].

American visceral leishmaniasis is similar to that found in the Mediterranean region regarding the etiologic agent, the animals reservoir, domestic and wild canidae, and the concentration of cases in people under 10 years old [3]. The signs and symptoms are also similar whatever the region the disease is found. Some differences are observed such as blackened skin (India and Sudan) and bulky lymph nodes, mainly in Sudan, whose aspiration is used for diagnosis, while the skin of patients in Americas reminds aged wax and the lymph nodes are little bulky. In the Indian subcontinent, there is no animal reservoir; the disease is transmitted from one individual to another (anthroponosis) through sand flies [3].

In Latin America, VL has been found in at least 12 countries, 90% of the cases are from Brazil [4]. The first case described in Americas dates back to 1913 in a patient from Mato Grosso, diagnosed in Asuncion, Paraguay [5]. In Brazil, the first case of visceral leishmaniasis was a male patient, coming from Aracaju [6].

Nowadays, the disease is described from Mexico to northern Argentina. In Brazil, according to the Ministry of Health, 21 of the 27 Brazilian states have reported autochthonous cases. Until the decade of 90, 92.9% of the cases coming from Brazil were concentrated in the Northeast region, expanding to the North, Midwest, and Southeast regions. In 2011, only 47.8% were from the Northeast region [7]. In the State of Alagoas, located in the central-eastern area of the Northeast region, the disease occurs predominantly in the countryside, affecting mostly children. In recent years, VL has occurred in urban areas of several cities in Brazil.

The disease worried health authorities from the 1990s because of the increased incidence, urbanization, and expansion, a phenomenon observed worldwide. It is believed that some factors favor the expansion such as migration from rural to urban and urban to rural areas, as a result of agro-industrial projects, conflicts between people, and natural phenomena.

The disease can present an acute, subacute, or chronic evolution [8]. Acute leishmaniasis appears most often in children under 2 years old. It is manifested by a severe and continuous high fever and moderate spleen growth, leading to death in 3–4 months [9]. Subacute leishmaniasis last between 6–18 months; patients have persistent fever, progressive splenomegaly, anemia, cachexia, and sometimes diarrhea and bronchitis. The course of VL may be modified by the appearance of opportunistic infections. In other patients, the course is chronic, lasting 2 or more years, interspersed with periods of almost complete recovery; during these periods of apparent cure, splenomegaly is maintained [9].

The clinical course is usually divided into four periods. Despite the imprecision between them, it is thus considered for being a better way to comprehend in an extended period of observation [10]. They are incubation period, period of start or invasion, period of state, and final period.

The incubation period is difficult to characterize, patients do not know the time of the infecting bite, because often there is no obvious changes. Generally, it is accepted limits between 3–6 months [11].

The initial period is marked by the spread of the parasite, the manifestations appear, sometimes abruptly, sometimes insidious. The patient has fever, loss of appetite, weight loss, pallor, hair loss, bleeding, and apathy [11]. Fever is often the first symptom [12]. Splenomegaly is observed early in the course of the disease, although sometimes it is discreet. The liver also starts to grow. Other manifestations may dominate such as diarrheal attacks, respiratory distress, and seizures, especially in children. The signs and symptoms are unspecific and can be confused with other conditions.

In the state period, signs and symptoms are the same from the initial period, but more intense. Fever may be continuous, irregular, relapsing with remissions of 1 or more weeks and two or more daily peaks [13]. The anemia presented from the onset of the disease is accentuated. The spleen grows at the same rate of the pregnant uterus, which means 4 cm per month [14]; its consistency is firm and sometimes it is painful. The hepatomegaly is common but hardly ever reaches the size of the spleen. The hair is dry, thinned, brittle, depigmented, dull, and falls easily. The skin is dry, rough, and pale, remembering aged wax. The abdomen is large. Edema of the lower limbs and ascites can emerge as well. Even polyadenia and reduced muscle mass are observed. Bleeding becomes frequent. Jaundice, delayed puberty, and amenorrhea can also occur.

The final period is marked by the exacerbation of changes in the state period. Death may be caused by changes resulting from the disease itself or associated infections [10].

The etiological agent of American visceral leishmaniasis (AVL) is the protozoan *Leishmania infantum/chagasi*. It is transmitted to humans and other animals almost always by the bite of the female sand fly *Lutzomyia longipalpis*. During blood feeding on infected warm-blooded animals, they suck mainly foxes and dogs and become infective from 15 to 24 h. Since then, every new blood meal, they inoculate promastigotes, which are engulfed by macrophages of the monocytic phagocytic system (MPS) and become amastigotes. Inside the macrophages, the amastigotes are housed in phagosomes that fuse to lysosomes, giving rise to phagolysosomes.

Amastigotes survive and multiply in this environment, resisting to toxic substances such as hydrogen peroxide and hydroxyl radicals [15]. In endemic region, it is known that the interaction between the parasite and its host may result in individuals not infected, infected individuals free of the disease (asymptomatic carrier), and individuals who develop the disease. In a research work, from the total of 88 cases suspected of having the disease, 17 (19.3%) were infected, 24 (27.2%) were infected without the disease, and 47 (53.5%) were sick [16].

It is known that 80–90% of all human infections are asymptomatic or subclinical; these individuals have a competent cell-mediated immunity [17]. Researchers initially linked the illness to malnutrition. It is known that malnutrition weakens the defense mechanism, which predisposes to infectious diseases [18]. In an animal model infected with L. *infantum/chagasi*, it was found that low protein levels are associated with disease progression and lead to the severe form. It was also observed depletion of leucocytes, monocytes and granulocytes and CD4+ subpopulation. The inability to perform an efficient hematopoiesis influences the host's ability to combat infection [18].

Later, it was observed that there would be other elements involved apart from malnutrition. It is believed that genetic factors act in determinants of susceptibility to illness and how the disease develops. Research shows an interrelationship between environmental and host features [17].

This interaction between the parasite and its host can lead to equilibrium, and the host becomes asymptomatic carrier. It is believed that the clinical expression of disease is linked to susceptibility of the host, genetics of the parasite, and vector-dependent factors [18]. Some individuals are unable to control the spread and multiplication of parasites, developing clinical manifestations of varying severity [18].

Dr. Celia Pedrosa, the author of this chapter and professor of the Medicine Faculty of Federal University of Alagoas, followed up patients from admission to outcome at the Tropical Diseases Hospital in Maceio, Alagoas/Brazil, a reference Hospital, from 1981 to 1995 [15]. By then, the hospital had the capacity of 80 beds, including general and intensive care, covering a population of 2.514.100. AVL cases were confirmed by clinical history and the identification of the parasite in medullary aspirate. In the cases that parasite was not found, the diagnosis was based on clinical manifestation and favorable response to treatment. The study included 646 patients, 394 (61.0%) were male and 252 (39.0%) were female. The patients' ages ranged from 6 months to 59 years, with a mean of 8.7 ± 9.4 years and a median of 5.0 years of age. Among male patients, the mean age was 9.8 ± 10.4 years and the median of 6.0 years (minimum 6 months and maximum of 59 years), and among female patients, the average age was 6.8 ± 7.0 years with a median of 4.0 years (minimum of 10 months and a maximum of 37 years). **Table 1** shows the distribution of patients by age and sex.

In both genders, the highest percentage of patients consisted of children, peaking between the ages of 1 and 4 years and thereafter decreasing with age.

Age (years)	Male	Female	Total
<1	15	10	25
1–5	140	128	268
5–10	103	54	157
10–15	49	31	80
15–20	21	10	31
20–30	38	12	50
30–40	18	7	25
≥40	10	0	10
Total	**394**	**252**	**646**

Table 1. Patients admitted to the Hospital for Tropical Diseases (Maceió-AL) with American visceral leishmaniasis, distributed by sex and age.

A higher frequency of patients in the younger age groups was observed, especially before the age of five. Similar findings were reported by other researchers [16] who found that 60.9% of the viscerotomies in patients with leishmaniasis were in children under 5 years old. Other researchers [17, 18] found resembling data in Ceará (67%) and Bahia (75%).

The neotropical kala-azar, also known as AVL, is located in an intermediate position between the "Indian," in which 62% of the cases are in people between 5 and 19 years, and the "Mediterranean" types, in which children under 5 account for 93% of the cases [19]. However, since the emergence of the human immunodeficiency virus (HIV), the use of immunosuppressant in transplanted patients, and chemotherapy, half of European cases are in adults [1].

Male patients have a higher incidence of the disease than female ones, whatever the age. Some researchers attribute this to larger male body area usually discovered [17], and consequently more exposed to the bite of the vectors; however, it is unlikely that only this fact explains the difference. Studies suggest that genetic modulation is linked to sex in the susceptibility to visceral leishmaniasis [20].

The most common clinical manifestation was fever. In the course of the disease, the fever is very variable. In general, the fever is highest early in the disease, with two or more peaks in 24 h. As the disease is established, temperature becomes lower. Some patients, even presenting fever, claim not to feel it.

Among the patients admitted, the duration of the disease was obtained in 622 patients (**Table 2**). It was observed that 405 (65.1%) of them arrived at the hospital referring 30–179 days of illness. This time is too long for a disease with marked clinical changes such as fever, weight loss, pallor, and increased abdominal volume. Other authors found similar data [21].

Since the onset of the disease, patients always seek medical care. However, even in endemic region, diagnosis is not considered and patients generally receive antimicrobials. By the irregularity of fever itself, patients may spend days or weeks with normal temperature, causing the false impression of cure of another infectious process, believed to be one of the reasons for the difficulty to know the right onset of the symptoms.

Duration of the disease	**Patients**	
(days)	**Number**	**%**
<30	107	17.2
30–90	238	38.3
90–180	167	26.8
180–360	73	11.8
≥360	37	5.9
Total	**622**	**100.0**

Table 2. Duration of the disease in 622 patients with American visceral leishmaniasis admitted to the Tropical Diseases Hospital, in Maceió-AL.

The clinical manifestations most often found in the admission include hepatomegaly, splenomegaly, fever, and pallor. Hepatomegaly's predominance over splenomegaly draws attention, and this fact stems from a patient who inadvertently has been splenectomized. The sum of the clinical manifestations exceeds 100% because most of the patients had simultaneously more than one clinical manifestation (**Table 3**).

Clinical manifestations	Number of patients	%
Hepatomegaly	633	98.0
Splenomegaly	632	97.8
Fever	628	97.7
Pallor	533	82.5
Increased lymph nodes	500	77.5
Increased abdominal volume	463	71.7
Weight loss	462	71.5
Long eyelashes	454	70.3
Dry hair	450	69.7
Asthenia	447	69.2
Anorexia	416	64.4
lower limbs edema	151	23.3
Cough	104	16.2
Diarrhea	102	14.4
Abdominal pain	80	12.4
Bleeding	67	10.4
Jaundice	90	13.9

Table 3. Clinical manifestations most often observed in patients with American visceral leishmaniasis admitted to the Hospital for Tropical Diseases in Maceió-AL.

Irregular fever, generally high, and pallor were relevant, associated with increased abdominal volume, led patients to seek medical attention. On admission, the contrast between the hair and the eyelashes drew attention, because while the hair was depigmented, dry and dull lashes were long and silky. A Brazilian researcher studied clinical and laboratory features of kala-azar and also found patients with dry, brittle, depigmented hair, and long eyelashes [22]. According to this author, lower hyperthermia is common when the disease has a long duration, observation confirmed by us. However, other manifestations such as jaundice and edema, also observed in our study, did not maintain relation with the duration of illness.

Some researchers have noted that patients with jaundice had worse prognosis [21]. To other authors, not only jaundice, but also the low age, marked pallor, creatinine elevation, and presence of amastigotes in bone marrow aspirate are factors associated with poor outcome [23].

However, in our study, we found no association between low age, presence of parasites in the bone marrow, and poor prognosis.

The spleen has firm consistency, not painful on palpation, except in some cases. Since it is a good parameter for estimating the duration of the disease and response to treatment, it must be carefully examined and its features registered on medical records. Always measure the spleen and liver size with a tape, describe the location of measurement, or use the Hackett scale.

Liver has firm consistency too, sometimes painful on palpation. Its size as well as the spleen accompanied the duration of the disease; liver is always a little lower than spleen (**Table 4**), but after the institution of specific therapy, liver regression occurred more slowly.

Duration of the disease (days)	Number of patients	Size of the liver (cm)		
		Mean ± standard deviation	Minimum	Maximum
<30	105	4.2 ± 1.8	0.0	9.0
30–90	221	4.7 ± 2.2	0.0	12.0
90–180	163	5.6 ± 2.5	0.0	14.0
180–360	68	5.8 ± 3.3	0.0	16.0
≥360	36	7.3 ± 3.6	2.0	20.0

Table 4. Size of the liver in hospital admission for the duration of the disease in 593 patients.

During the follow-up, the duration of the disease compared to spleen size measured at hospital admission (**Table 5**) was observed. The average size of the spleen does not correspond to what is expected of its size for this duration of illness. Since fever becomes continuous later, patients believe this illness is recent. Investigating important data, such as fever and relating them with celebrating days, you get more accurate information about disease's duration.

Duration of the disease (days)	Number of patients	Size of the spleen (cm)		
		Mean ± standard deviation	Minimum	Maximum
<30	102	7.2 ± 3.3	0.0	16.0
30–90	220	8.6 ± 4.0	0.0	33.0
90–180	159	9.8 ± 3.6	0.0	20.0
180–360	66	10.3 ± 4.2	0.0	20.0
≥360	36	12.4 ± 3.8	3.0	20.0

Table 5. Size of the spleen in hospital admission for the duration of the disease in 583 patients.

The normal spleen is rarely palpated, located on the left upper quadrant of the gastric fundus and the diaphragm. In VL, spleen growth takes place since the beginning of the disease. Ancient authors compare its growth to the gravid uterus [24]. Based on this knowledge, for those patients who do not remember since when they were sick, it is possible to estimate the duration of the disease by spleen size. Disease duration is an important factor because the prognosis is directly related to the time they are sick [25].

Liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), measured at the time of admission in 444 patients, showed values above normality (>40 units/l) in 169 (38.1%) and 258 (58, 1%) patients, respectively (**Table 6**). Liver enzymes that were abnormal at the time of admission hold no relation to the duration of the disease. Otherwise, for other researchers, the aminotransferase was more pronounced in cases diagnosed late [26].

Enzymes[a,b]	No of patients (%)	Mean ± standard deviation (units/l)	Maximum-minimum (units/l)
ALT ≤ 40	275 (62.0)	19.5 ± 11.2	2.0–40.0
ALT > 40	169 (38.0)	145.5 ± 210.1	41.0–1750.0
AST ≤ 40	186 (41.9)	23.6 ± 10.2	3.0–40.0
AST > 40	258 (58.1)	126.9 ± 174.5	41.0–1530.0

[a] ALT, alanine aminotransferase; AST, aspartate aminotransferase.

[b] Normal values ≤ 40 units/l.

Table 6. Liver enzymes measured in 444 patients at hospital admission.

After treatment, liver enzymes returned to normal, except in four patients who died with liver failure. Histopathologic findings suggested toxic hepatitis.

Associated infections were diagnosed at admission in 134 (20.7%) patients. Airway infections, especially pneumonia, were most often found. Children under 5 years old were the most affected by associated infections, 104 (60.8%) cases.

During the period they were hospitalized for treatment, 258 patients developed some type of infection; the most frequent was pneumonia, which affected 126 (48.8%) subjects. Regardless of the grievance, the higher incidence of infections acquired during hospitalization occurred in children under 5 years old with 229 (61.4%) occurrences.

In our observation, associated infections were not related to the duration of the disease, but they affected mortality.

In 554 patients, we performed blood tests at hospital admission. The count of red cells ranged from 1.03 to 4.55 million red cells/mm^3, with an average of 2,762,000 ± 620,307 red cells/mm^3, while in 603 patients white blood cell (WBC) counts ranged from 600 to 15,600 leukocytes/mm^3 with an average of 4174 ± 2127 leukocytes/mm^3. In differential counting, the absolute number of lymphocytes ranged from 102 to 11,100 lymphocytes/mm^3 with a mean of 2339 ± 1422 lymphocytes/mm^3. Platelets counted in 279 patients ranged from 1000 to 732,600 platelets/mm^3,

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with an average of 140,020 ± 103,049 platelets/mm^3. Except the leukocytes, all these averages are below normal values. Using analysis of variance, it was observed that except the medium of leukocytes and lymphocytes, which were significantly higher in patients younger than 30 days of illness, all other results showed no significance regarding disease duration (**Table 7**).

Duration of the disease (days)	Hm[a] (×10^3/mm^3)	Ht[b] (%)	Hb[c] (g/dl)	Platelets (×10^5/mm^3)	Leukocytes (mm^3)	Lymphocytes (×10^3/mm^3)
<30	2.770 ± 697	23.6 ± 6.4	7.2 ± 2.2	1.35 ± 0.78	4.872 ± 2.186	2.7 ± 1.6
30–90	2.683 ± 633	23.0 ± 5.5	7.0 ± 1.8	1.36 ± 0.11	4.168 ± 2.183	2.2 ± 1.4
90–180	2.784 ± 599	24.0 ± 5.5	7.4 ± 1.8	1.39 ± 0.99	3.962 ± 2.001	2.2 ± 1.2
180–360	2.797 ± 635	24.0 ± 6.0	7.4 ± 2.0	1.68 ± 0.12	3.763 ± 2.190	2.0 ± 1.3
≥360	2.988 ± 453	26.1 ± 4.1	8.1 ± 1.5	1.40 ± 0.88	3.955 ± 2.141	1.9 ± 1.2
p[d]	0.155	0.062	0.061	0.0653	0.004*	0.004*

[a] Red cells.

[b] Hematocrit.

[c] Hemoglobin.

[d] Comparation of the means (variance analysis, g.l. = 4).

* C.I.: 95%: significant differences ($p < 0.05$).

Table 7. Blood test data (mean ± standard deviation) of patients at admission according to the duration of the disease.

Our patients had varying degrees of anemia associated with leukopenia and thrombocytopenia. Anemia seems to occur when the spleen becomes palpable and progresses with its gradual increase; this opinion is shared by others [27, 28]. Anemia is one of the most remarkable manifestations of visceral leishmaniasis, and the pathogenesis of this anemia, caused in part by the destruction of red blood cells, is multifactorial (splenic phagocytosis of opsonized erythrocytes, hemodilution, and increased destruction of normal red blood cells by hypersplenism) [3]. In addition, there is a bone marrow failure to replenish red blood cells removed from the circulation, which can be attributed to poor nutrition and infection extension.

There are several hypotheses proposed to explain the pathogenesis of anemia in visceral leishmaniasis. Some pointed to the increased blood volume, while others argue that autoimmune mechanisms are responsible for the decreased survival of red blood cells. Their destruction occurs intensely in the spleen. Others emphasize that the reduced erythrocyte survival and iron deficiency are more common in children under 3 years [29, 30].

For others, the severity of hematological changes depends on the duration of the disease and the spleen size, hypersplenism primarily responsible for these changes. The pancytopenia and thrombocytopenia reflect an extended illness, before the diagnosis is made [31].

In 450 patients in which the temperature was measured during the course of treatment, 25 (5.6%) cases showed no fever when treatment was initiated. In 330 (73.3%) cases, the temperature normalization occurred until day 7 after initiation of therapy. In 56 (12.4%), temperature normalized between the 8th and 14th day, and in 39 (8.7%), from 15th to 21st day.

In 630 patients who started treatment, 109 (16.8%) received incomplete treatment (62 because they died and 47 because they left before the treatment was completed) and 521 (80.6%) received full treatment. There was a death after completion of therapy. Five patients (0.7%) had a new treatment cycle.

Regardless of the duration of the disease, there was a decrease in the average size of liver and spleen after treatment. The percentage reduction of these organs at the end of treatment was higher in patients with less disease duration (**Table 8**). The analysis of variance showed that the spleen reduction percentage was higher in patients with less disease duration. However, considering the liver, this difference was not observed.

Duration of the disease	Spleen		Liver	
	Number of patients	% of reduction[a]	Number of patients	% of reduction[a]
<30	73	70.75	60	58.73
30–90	157	60.03	1248	55.41
90–180	115	53.50	86	46.17
180–360	49	54.37	42	45.64
≥360	23	52.47	16	46.61

[a] 100 − (mean of the viscera in the end of the treatment × 100).
Note: mean of the viscera before treatment.

Table 8. Percentage reduction in the average size of the spleen and liver after treatment.

Among the patients who progressed to death, 44 (57.9%) were male and 32 (42.1%) were female. Forty-six of them died during the first or second weeks of treatment. The clinical manifestations most often related to death were bleeding, edema (anasarca and ascites), and pneumonia. In the third and fourth weeks, 17 deaths were registered, with a predominance of pneumonia as a probable cause of death.

Table 9 shows the distribution of deaths by age, including those 13 individuals who died before treatment was started, while **Table 10** shows the distribution of deaths by disease duration. There was no statistically significant difference in the percentage of deaths in relation to age ($\chi^2 = 13.64$, $p = 0.058$, g.1. = 6). However, since p-value is close to convention (0.05), some different interpretation may be important, like the high mortality rates in patients under 1 year old (33.3%). There was no statistical significance regarding disease duration ($\chi^2 = 0.51$, $p = 0.973$, g.1. = 4).

According to observations of Brazilian researchers, the deaths occur by the delay in starting the treatment or lack of response to it. Advanced case can present worsening in the first days of treatment, resulting from Herxheimer reaction. In these patients, death occurs mostly by bacterial complications or bleeding [25]. In a survey of medical records at the "Hospital das Clinicas," in São Paulo (Brazil), about 13% of deaths in 162 patients with visceral leishmaniasis was found and the main causes were pneumonia and sepsis [32].

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Age (years)	Number of deaths/Total number of patients (%)
<1	8/24 (33.3)
1–5	34/256 (13.3)
5–10	18/149 (12.1)
10–15	7/79 (8.9)
15–20	2/28 (7.1)
20–30	4/47 (8.5)
30–40	3/22 (13.6)
≥40	0/10 (0.0)
Total	**76/616 (12.3)**

Table 9. Distribution of deaths by age in 616 patients admitted at the Tropical Diseases Hospital "Constança de Góes Monteiro".

Duration of the disease (days)	Number of deaths/total number of patients (%)
<30	11/102 (10.8)
30–90	25/225 (11.1)
90–180	20/157 (12.7)
180–360	7/71 (9.9)
≥360	4/36 (11.1)
Total	**67/591 (11.3)**

Table 10. Distribution of deaths according to the duration of the disease.

We did not observe an association between disease duration and the number of deaths, as some claim. According to a study conducted by these authors, the death was more frequent in cases with longer disease duration [18–23, 25–31, 33, 34]. Our mortality data are consistent with a study conducted in Sudan which found 12% of deaths [35].

The World Health Organization [36] considered as a factor that worsens prognosis the late start of treatment, especially for younger children as well as associated infections. However, in our study we found no relationship between disease duration or age and mortality. Our data, regarding the causes of death, are comparable to other Brazilian researchers who have found bacterial infections in 59% of their patients; the respiratory tract was the most involved with 48% of the cases. These infections had no relation to the duration of the disease [37].

It is likely that the progress of visceral leishmaniasis, favorable or not, depends on the agent and the genetic constitution of the host, and therefore of the ability of the body in defending itself.

Continuing observation over the years allows us to conclude that the duration of the disease is correlated to the size of the liver and spleen, the temperature, the number of leukocytes and lymphocytes at hospital admission, and spleen size at the end of treatment. However, associated infections and complications are not influenced by disease duration, as well as the normalization of temperature after the start of specific therapy. Finally, we find that death is not associated with age, sex, positive medullary puncture, or disease duration. We also observed that at the end of treatment, there is no full recovery, but considerable improvement in all clinical and laboratory parameters.

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Chapter 3

Application of the Eco-Epidemiological Method in the Study of Leishmaniasis Transmission Foci

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Additional information is available at the end of the chapter

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Abstract

The study of transmission foci involves the clinical characterization of disease within a focus using active surveillance of human cases, characterization of the population group that is most frequently infected or at highest risk of becoming infected, diagnosis and treatment of infected people and identification of the conceptions, attitudes, beliefs and practices related to the disease. An entomological survey is necessary to determine the geographical distribution of species and incrimination of vector species, the ecological characteristics of the zone (macro-focus), times of the year and day of highest biting activity and places in homes in which the most frequent contact between the people and the vectors occurs. A survey of peri-domestic and wild mammals in the area is advisable to try to identify potential reservoir hosts. Using this information, it is feasible to design specific and accurate prevention and effective, rational and economic control measures and define the times of the year and locations in which these measures must be applied. In this chapter, a description of the application of the eco-epidemiological method to the study of leishmaniasis transmission foci is provided. A special emphasis is placed on the methodology, multidisciplinary work and analysis of findings.

Keywords: Leishmaniasis, eco-epidemiology, reservoirs, *Leishmania*, *Lutzomyia*, factor risk

1. Introduction

Leishmaniases are a group of diseases caused by at least 20 species of parasites of the genus *Leishmania* that are transmitted to humans and other mammals by the bite of *Lutzomyia* species (in America) and *Phlebotomus* species (in the Old World). Reservoirs of these parasites include domestic and wild animals and, sometimes, humans; therefore, Leishmaniasis transmission

may be zoonotic (from animals to humans) or anthroponotic (from one human to another). In humans, *Leishmania* infections cause a spectrum of illness that depends on the parasite species involved, inoculum size, and host immune response [1, 2], affecting skin, mucous tissues, or organs of the mononuclear phagocyte system and producing clinical symptoms of cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and visceral leishmaniasis (VL), respectively.

Leishmania spp. infections are acquired when an infected vector bites a mammal to consume its blood. In turn, the vector is infected when it feeds upon the blood of an infected reservoir species and ingests the transmitting parasites (as amastigotes). Transmission cycles of leishmaniasis have been found to have a focal distribution in specific geographic areas. These sites are called the natural foci of infection. The foci of infection are the places where the key elements necessary for transmission are present: vectors and reservoirs. The limits of infection foci are generally determined by the spatial distribution and relative density of the vector species. Hence, conducting entomological surveys and determining the behavior of vectors are important to clarify the epidemiological risk of infection. In turn, the presence of these elements, and especially of vectors, is conditioned by abiotic ecological factors such as climate, humidity, altitude, temperature, and vegetation [3, 4].

The study of the foci of Leishmaniasis transmission is complex due to the following factors: (1) the diversity of phlebotomine species; (2) the variety of *Leishmania* species; (3) the incrimination of a phlebotomine species requires that the vector meet certain criteria, including exhibiting anthropophilic behavior, being infected with the same species of *Leishmania* isolated from humans infected during the outbreak, and demonstrating geographical distribution consistent with the distribution of reported human cases; (4) the variety of methods required to incriminate potential mammalian reservoirs in an area, which involve capturing and analyzing many samples, isolating and identifying the species of *Leishmania* and determining the parasite prevalence and transmissibility; and (5) the challenge of diagnosing human cases with the clinical form of leishmaniasis, *Leishmania* species causing the disease, and locations in dwellings where transmission occurred [5, 6].

On the other hand, to facilitate the design and implementation of specific control measures, recognition of the transmission mode within a focus and determination of the eco-epidemiological risk of infection are required. Factors that must be determined include the following: (1) the geographical area associated with greatest risk of transmission, or "macrofocus"; (2) the population group at increased risk of becoming infected and developing the disease; (3) the time of year during which increased activity of phlebotomine species in the intradomicile environment occurs (nictemeral behavior); and (4) the place within the dwelling where contact between vectors and the population group at highest risk of infection, or "microfoci," are located [2]. Therefore, understanding the transmission of *Leishmania* infection, identifying the foci of transmission, and designing strategies and control measures requires a combination of different disciplines such as the health sciences, epidemiology, social sciences, entomology, cellular and molecular biology, and ecology, giving rise to eco-epidemiology. In addition to the inclusion of transmission (i.e., the vector), parasite, and reservoir dynamics as objects of eco-epidemiological study, this methodology also includes the study of associated ecological factors and human behaviors that affect the transmission of the disease.

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As such, application of the eco-epidemiological methodology allows for determination of the following parameters: (1) identification of the species of *Leishmania* and phlebotomine in a given region, both overall and specifically those that serve as vectors, and determination of the role of domestic or wild reservoirs; (2) definition of the macrofocus (i.e., the geographical areas where the vector is present), its limits, and the environmental characteristics that allow the vector to develop, including ecological markers such as temperature, vegetation type, soil, altitude, and other aspects that characterize the macrofocus; (3) identification of both times during which there is a greater risk of infection (i.e., the times of night during which increased vector insect activity occurs) and locations in which vectors have a higher rate of natural infection with *Leishmania* and more contact with a population at risk using vector bionomics and behavior; (4) definition of the microfocus, which, as previously stated, is the location in a dwelling where contact between the human population and the infected vector occurs, that is, if this location is inside the home (intradomicile), around homes (peridomicile) or outside the home (extradomicile); (5) accurate determination of the population group most affected by the disease or at increased risk of infection, for example, if this population comprises men, children, or people who perform some activity (such as loggers, workers in mines, or some special type of farming or hunting) or if entire families are affected by the disease; and (6) identification of medical systems used by the community, i.e., how the population conceives the disease and its cause, from whom they seek treatment, and how the disease is treated. The identification of these parameters allows for the generation of elaborate risk maps and design of rational, economical, and effective prevention and control measures [7].

2. Activities to develop in a study of leishmaniasis transmission focus

When the study of a focus of transmission is initiated, it is necessary to carry out different activities involving all stakeholders involved in the transmission of infection, that is, the human population living in the area, possible vectors, and populations of mammals serving as potential reservoirs. Similarly, knowing the conceptions, attitudes, and practices of the people about the disease is critical to design appropriate control measures for each community. The following sections describe each of these activities.

2.1. Study of human population

Studies of resident populations are conducted by active case finding, conducting epidemiological surveys, and comparing the knowledge, conceptions, attitudes, and practices within a community against the prevalence of the disease and behaviors that facilitate being bitten by vector species.

Active surveillance for cases is performed to develop a detailed report describing the residents of a population, with a special emphasis on the identification of leishmaniasis lesions on both the skin and mucous membranes through clinical examination of the nasal-oropharyngeal region. Additionally, any signs or symptoms compatible with VL, such as fever and/or hepatosplenomegaly, should be identified, especially in children and adolescents. Examination of naso-oropharyngeal mucosa should also be performed on all people with a history of the

disease or with compatible scars. During active case finding, it is possible to identify patients who have very advanced mucosal lesions causing destruction of the nose, mouth, pharynx, and lip. Because of their disfigurement, these individuals may not seek treatment at health centers or hospitals within the region, as they have lost all hope of being cured and have been isolated from the social environment due to their facial disfigurement. These people should be actively sought out using information provided by the community in order to obtain the necessary samples to make a diagnosis and initiate treatment. Samples may be then collected from the suspected cases of CL or ML, including scraping for smear and aspiration for culture in the NNN (Novy-MacNeal-Nicolle) medium. All samples should also be analyzed using PCR. In cases of ML, scraping for smear has been found to provide a useful sample [8]. In VL cases, a sample of spleen aspirate or bone marrow (for the smear, culture, and blood tests to detect specific antibodies against *Leishmania* spp.) may be collected; all these procedures have been established by the World Health Organization (WHO) [2].

It is very important to examine the largest possible number of residents in a study site to diagnose active cases and include patients presenting injuries in the healing process due to the use of empirical treatment (such as people with compatible scars and a clinical history that gives reason to suspect that the person had leishmaniasis) and record previous cases. In the analysis of this information, new and old cases may be compared by age, sex, profession, or occupation. Thus, population groups at increased risk of leishmaniasis may be individually identified.

It is also important to diagnose leishmaniasis early because it allows for the initiation of specific treatment as soon as possible. When treatment is initiated early, it is possible to control the progression of the disease, relieve the signs and symptoms of the disease and improve quality of life in patients who are exposed to great social stigma due to the physical "marks" (scars) left by leishmaniasis that make it easy to identify those who had or have the disease.

Since clinical and epidemiological findings are not pathognomonic of the disease, and it is necessary to obtain a laboratory diagnosis to verify clinical suspicion of leishmaniasis. This diagnosis is based on visualization of the parasite in spread (smear) or cultures obtained from lesion material (in cases with CL and ML) or material obtained from aspirate, biopsy of bone marrow, or the spleen (in cases with VL).

However, in some VL or ML cases, it is not always possible to visualize or isolate the parasite, and thus clinical diagnosis may be aided by the presence of specific antibodies against *Leishmania* spp. or molecular tests.

In the case of VL, the use of a clinical and epidemiological history with a positive rK-39 has been accepted as the criteria for diagnosis of the disease. For diagnosis of leishmaniasis, the following clinical laboratory tests may be performed:

1. Smear (or spread) is an easy, economical, and rapid procedure; with appropriate sampling and interpretation techniques, the sensitivity of this procedure can reach 90%. The test involves the collection of tissue from the active edge of a lesion and center of an ulcer for CL and ML. If a case has VL, the sample is taken from bone marrow, liver, or the spleen by aspiration, as described later. For cases with skin lesions, it is important to select lesions that are younger and do not display superimposed infection [9].

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2. Cultivation technique consists of aspirating material from a lesion or the mucosa and cultivating the sample in the center of a biphasic NNN culture to encourage growth of the parasite to the promastigote stage. Cultures have been found to provide good diagnostic results, with a sensitivity of approximately 70% [9].

3. Polymerase chain reaction, or PCR, enables detection of the genetic material (DNA or RNA) of parasites in sampled lesions in patients, potential mammalian reservoirs, and phlebotomine vectors [10, 11]. The material analyzed using PCR can be scraped off or aspirated from the injury site; additionally, a blood sample or a small piece of biopsied tissue preserved in absolute alcohol or other media, such as NTE (NaCl, Tris-HCl and EDTA). For detection of parasites in phlebotomines, samples should include a pool of at least five insects or the anterior portion of an insect, including the first abdominal tergite area that includes parasites attached to the stomodeal valve [12, 13]. PCR can also distinguish between parasite species using a technique known as PCR-RFLP, which consists of determining the length of the restriction fragments of the amplified product when it is subjected to digestion by different restriction enzymes [14, 15].

There are variations of PCR, such as real-time PCR, that employ labeled probes to visualize the amplification reaction. This characteristic makes PCRs more sensitive and enables quantitative analysis of the parasites in a sample [16].

2.2. Epidemiological survey using the Montenegro skin test

This activity includes the application (to the general community) of the Montenegro skin test or "leishmanin" antigen test. This test causes an intradermal reaction that measures delayed cellular immunity response, and it has been commonly used in epidemiological studies for the identification of *Leishmania* exposure [17]. This delayed hypersensitivity test should be read 48 hours after being applied, and the pen method should be used to define the area of induration [18]. Although the Montenegro skin test is very sensitive and specific, it does not differentiate between current and past infections. The test is considered positive, or reactive, when the induration is more than 5 mm [2]. It is important to apply the test always on the same forearm (right or left) on each member of the population to facilitate reading, especially when people have negative reactions.

To interpret the results of an epidemiological survey, the common characteristics of people who have a positive test result (which can be a particular age group, profession, or occupation, without discrimination among household members) may be analyzed. For the analysis of these data, it is convenient to divide the population into four age groups: 0 to 4 years (children who usually stay inside the house), 5–14 years (children and adolescents who may have school activities and, in some rural regions, help their parents in agricultural work); 15–60 years (the economically active population); and 61 or more years (the population that most frequently stays at home). Thus, the population group that is at higher risk of infection (the population to which prevention and control measures should be prioritized) may be determined. For analysis purposes, it is helpful to determine the positivity rate, which is the number of positive tests out of the total tests performed in each subpopulation.

2.3. Conceptions, popular attitudes and practices (CAP)

Both rural and sylvan domestic endemic foci of leishmaniasis are characterized by poor and remote communities in urban centers with serious social conflict and little governmental health institution presence. In these areas, the conceptions of the cause of disease vary among population groups and are not necessarily associated with a parasite or the bite of sandflies. Communities have their own medical systems that include their conceptions and practices about the origin of the disease, facilities where they receive care when they are sick and procedures that are available for the diagnosis, treatment, cure, and prevention of the disease [19].

Identifying the conceptions, attitudes, and practices relating to leishmaniasis and medical systems operating in communities is an important component of the study of foci. This information is critical to the design and implementation of primary health education, social assistance programs, and control measures. It is therefore important to determine the locations where residents seek care when they have leishmaniasis, including healers, herbalists, pharmacists, physicians, or other healthcare providers. It is also necessary to determine the variety of treatments used, what residents believe causes disease, how residents refer to the vector and leishmaniasis, and what having leishmaniasis or skin ulcers means within their magical-religious conceptions through a respectful dialogue that enables the sharing of knowledge.

This type of research (the social type) is conducted mainly using qualitative methods but can be supplemented with quantitative methods. Within qualitative studies, techniques such as participant observations may be used. These studies require the constant presence of the researcher in the community, enabling a better approach to obtain this knowledge and facilitating the creation of bonds of trust that allow the collection of more credible information. It also allows researchers to construct an overview of the community, thereby knowing, for example, how people interrelate within the community itself and how these relationships (in one way or another) influence the CAP that have developed related to the disease. Other techniques include interviews with key stakeholders in the community, including herbalists and community leaders. Within the quantitative method exists the application of surveys with closed answers, which are most ideally conducted with all of the community members or with a statistically representative sample.

The use of social science methods allows researchers to characterize the material conditions that, in short, form a simple or complex network of social relations. These conditions influence a large number of people who are consolidated as a community with similar livelihoods and conceptions about disease. These methods also allow for the observance of human behaviors that may predispose individuals towards contact with insect vectors. Hence, there is a need for constant communication throughout the multidisciplinary group to share findings and refocus targeted observations; all together, these processes may help to determine the epidemiological risk of infection, which is the first step to design prevention and control measures that are economical and effective.

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2.4. Study of the phlebotomine population

Leishmania vectors belong to the family *Psychodidae* and subfamily *Phlebotominae*. There has been no general agreement established as to the classification of phlebotomines into genus and more general taxonomic categories, but according to the most widely accepted categorization scheme, there are six genera: *Lutzomyia*, *Brumptomyia,* and *Warileya* in the New World and *Phlebotomus, Sergentomyia, and Chinius* in the Old World. The vector species of *Leishmania* belong to the genera *Phlebotomus* and *Lutzomyia* [2].

Although the biology of each species of phlebotomine is unique and complex, generally they are small nocturnal insects that range from 2 to 5 mm in length. By day, they remain at rest in burrows, caves, and hollow trees. The life cycle and behavior of phlebotomines are conditioned by abiotic factors (temperature, humidity, photoperiod) and biotic factors. Only the female is hematophagous, needing blood to develop eggs and maintain the gonotrophic cycle [20]. Phlebotomines are very fragile insects, and they are considered sedentary species with a short range of flight. Some studies using the techniques of capture, marking, and recapture with fluorescent powders have determined that the majority of recaptures occur within a range of less than 200 meters from the site of release [21].

The identification of phlebotomines is based on morphological characteristics in both male and female insects, especially the genitalia, alar indices, pharynx, and cibarium. Examination of morphological characteristics may help to solve problems associated with identification (usually cases where males can be distinguished but not females, which have the greatest epidemiological importance). Currently, taxonomic identification based on morphological characters may be corroborated by mitochondrial cytochrome c oxidase (COI) DNA gene-based molecular techniques, which can be provided as a barcode-specific marker for each phlebotomine species [22].

There are more than 800 currently described phlebotomine species. Approximately 465 of these species have been identified in the New World, and 375 of these species have been identified in the Old World; however, not all phlebotomine species are considered vectors [23]. A phlebotomine naturally infected with the promastigote forms of *Leishmania* is not necessarily capable of transmitting the parasite. For a species to be incriminated as a vector, certain criteria are required: an anthropophilic nature; contact with both humans and the disease reservoir; and infection by the same Leishmania strain identified in human cases. Additionally, the transmitted parasite must be able to develop in the vector, the vector must be able to be transmitted through the bite of the parasite, and the geographical distribution of the parasite must be compatible with that of the vector [2]. In America, approximately 400 species of *Lutzomyia* have been identified, but only 22 of these species have been implicated as vectors [24]. In Colombia, 153 species of *Lutzomyia* have been identified [25], but only the following species have been found to be naturally infected with *Leishmania* and incriminated as vectors: *Lu. trapidoi* and *Lu. gomezi* with *L. panamensis* [6, 21], *Lu. spinicrassa* and *Lu. gomezi* with *L. braziliensis* [26], *Lu. umbratilis* with *L. guyanensis* [27], *Lu. olmeca* with *L. mexicana* [28] and *Lu. longipalpis a*nd *Lu. evansi* with *L. chagasi* (=*L. infantum*) [29].

Although the incrimination of a species as a vector is a specialized job that requires the support of entomological research laboratories, a list of species incriminated as vectors in different countries has already been developed. Furthermore, by analyzing collected data, it is possible to identify species that potentially transmit the disease, thereby linking different elements of the study of transmission foci.

In the study of foci, one of the objectives is to determine the limits of the macrofocus, and this determination requires a combination of techniques, such as entomological transects, which involve the simultaneous capture of phlebotomines in different geographical locations, for example, along paths and roads and in houses and the extradomicile using sticky traps (which are traps with castor oil-impregnated paper). Five to 10 days later, the traps may be removed and sand flies be recovered from each sticky trap. This information can be used to determine the relative density per square meter of a particular vector species in a given geographical area. It is also possible to establish the area where the phlebotomines are present, thus specifying the limits of the focus.

To determine the risks within a microfocus, simultaneous captures of phlebotomines may be performed inside, around, and outside the home. Presence and relative density of the vector species may be then determined and correlated with other study data, such as Montenegro test positivity and the population group with the highest number of new and old cases of the disease.

To determine high activity hours during which the vector species is most likely to bite, catches may be made overnight from 18:00 in the evening to 6:00 the next day inside the dwelling (nictemeral monitoring). The specimens caught may then be stored for periods of 2 hours using a vial for each capture period (each properly labeled, indicating the hour of the capture and the place and date to be stored until identification). Additionally, captures should be performed during different seasons (rainy–summer) because the species composition and density may vary during different seasons.

To capture phlebotomines, a series of traps have been designed, among which we will mention the most frequently recommended for the study of foci.

- CDC traps with white light: These traps are installed between 6:00 pm and 6:00 am inside houses and in the peri- and extradomicile. These traps enable the capture of live specimens, which is useful for the isolation of parasites.
- Shannon trap: A Shannon trap is usually installed in the peri- or extradomicile and combines attraction to light with CO_2 emitted by people to capture insects that land on the fabric. Captures are performed using a "mouth aspirator," which consists of a glass or transparent acrylic tube connected to a rubber hose to which mouth suction is applied to suck the insect into the tube. This manual type of capture may be also used to collect insects that are dormant in housing walls, animal pens, rocks, caves, burrows, tree trunk, the buttresses of large trees, etc.
- Capture on protected "human bait": During capture, people are dressed properly and leave exposed only a portion of the lower extremities (legs); in their hands they charge a grabber and a flashlight and stay attentive to the sandflies landing. When a sand fly

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lands, it is captured as soon as possible thus avoid being bitten by the sand fly. This procedure has been approved by the Ethics Committee after reviewing the protocols. This technique allows for the identification of anthropophilic behavior in phlebotomine species that attempt to bite humans. During these captures, information regarding landing rates (# of sandflies/hour/man) can be obtained. Capture on protected human bait can be performed in the intra-, peri-, and extradomicile. When performed on animals, it can be used to infer the zoophilic behavior of a phlebotomine.

- Sticky traps: These traps are considered complementary to captures at rest and require less effort. Sticky traps consist of sheets of 20 by 25 cm bond paper that are fixed on a bamboo stick with a side that is castor oil-impregnated [29, 30]. Phlebotomines that settle on these traps can be used determine the density of each species per square meter trap, considering that a trap has an area of 0.01 square meter for capture. These traps are very useful for transects and to determine the spatial distribution of species and limits of foci of transmission. The traps are placed in the intra-, peri-, or extra-domicile and the number of phlebotomines captured at each point or "station" can vary, as can density because it is determined per square meter trap. When placing these traps, the geographical coordinates of the location and relationship to the domicile should be taken into consideration [6, 29–31].

The community can and should actively participate in entomological studies. The community may recognize *Lutzomyia* species using local popular names, which may vary in different regions of the country, and the community can help guide practitioners regarding the times when they see a greater abundance of phlebotomines. In Colombia, the highest phlebotomine density is frequently recorded after the onset of the rainy season. Thus, the community may inform researchers that they are being bitten more frequently inside or outside the house or at certain peak hours; however, this information must be corroborated by capture of specimens.

Entomological specimens captured using light, bait animal, resting, and human traps should be killed by introducing cotton soaked with ethyl acetate or other reagents in capture containers. Ethyl acetate must be handled carefully because of its toxicity. Subsequently, specimens should be deposited on a plate with a white background, and entomological tweezers should be used to separate the phlebotomines from bycatch species (other insects). Finally, phlebotomines should be deposited in vials, plastic bottles, or glasses with screw caps that are dry or contain 70% alcohol, according to the study objectives. The vials containing the phlebotomines should be marked with the capture source, date, method of collection, and person responsible, among other data.

2.5. Study of reservoirs

The ecology of *Leishmania* spp. is associated with their hosts; therefore, all factors affecting the survival and behavior of a host may affect the transmission cycle of a parasite. Most leishmaniases are zoonoses for which different species of animals may act as reservoirs of the human parasite. The transmission of the disease to humans requires interactions between a human being and the ecological niche of a vector and wild or domestic reservoirs [32].

It is understood that reservoirs provide an ecological system in which the infectious agent survives indefinitely. This system includes hosts, any intermediate host, or vector and any environmental component that is necessary to maintain the agent indefinitely [33]. Depending on the duration that hosts are able to maintain the parasite, they may be classified into primary (maintaining the infection for a long time), secondary, or accidental.

Reservoirs may be domestic, wild, or synanthropic, and for some species of the parasite, humans are the main reservoir. That is, the case of VL caused by *L. donovani donovani* and CL caused by *L. tropica tropica*. In the New World, leishmaniases are zoonoses; however, there is evidence of anthroponotic transmission during outbreaks, previously identified in the Andean region of the country [6, 34].

Generally, within a focus, there is a primary reservoir for each species of *Leishmani*a; however, other mammals in the same area can become infected, thereby becoming secondary or accidental hosts. Domestic, synanthropic, or wild (marsupial carnivores, rodents, Meshed, insectivores, and primates) reservoir species infected with *Leishmania* may or may not show obvious signs of infection. In general, reservoirs do not exhibit symptoms of the disease [2]. To be a source of transmission is not necessary to be a primary reservoir, and it is possible that different populations of mammals can maintain a continuous cycle of infection and become the source of infection or "primary reservoir" [35].

In America, approximately 310 species of mammals have been incriminated as possible reservoirs. More than half of these species have been incriminated just for having been detected as infected, but the majority of species have been studied and considered as wild reservoirs, such as species in the order Didelphimorphia, *Didelphis marsupialis* and *Didelphis albiventris*, which are important hosts of all Leishmania species, especially *L. amazonensis amazonensis* and *L. braziliensis*. Meanwhile the most important hosts of *L. mexicana* are *Peromyscus yucatanicus* and *Ototylomys phyllotis* rodents. For *L. panamensis* and *L. guyanensis,* an important reservoir has been identified to be *Choloepus hoffmanni* sloths [36].

The most important domestic reservoir is the dog; canine species are mainly a reservoir of *L. infantum,* but cases of infection by *L. braziliensis* and *L. panamensis* have also been observed [37, 38]. Donkeys and horses have also been incriminated as secondary reservoirs [2].

The study of reservoirs has been associated with decreased interest because of the difficulty in establishing incrimination. Briefly, to incriminate a host reservoir, the following criteria must be met: chronic maintenance of populations of parasites in each ecosystem, presentation of a parasitic load sufficient to ensure transmissibility, and identification of an appropriate population density (20% or more of studied wildlife) to provide opportunities for host-vector, host-environment, or host-host interactions depending on the type of transmission [32].

The activities used for identifying reservoir hosts include clinical, serological, and parasitological evaluations of domestic and wild mammals within a given locality. Performing these activities requires the participation of professionals specialized in the capture, identification, and sampling of domestic and wild mammals. For CL, it is very important to examine the snout, ears, genitals, appendages, and areas with less body hair (as *Lutzomyia* bites may occur

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on exposed areas of the skin) of a potential reservoir species and identify early CL lesions, or nodules, from which samples may be taken for direct examination, culture and/or PCR.

Some larger lesions, such as ulcerated or plate-type lesions, may also be identified [39]. In the case of VL, it is important to examine dogs for the previously defined clinical signs of VL such as peri-orbital waxing, increase in popliteal lymph node size, growing nails (onychogryphosis), emaciation (cachexia), peeling, and, sometimes, keratitis. For serology, dog blood should be drawn from the cephalic vein to facilitate collection; however, other blood vessels, including the jugular and saphenous veins, may also be used. It is important to take samples for culture, which can also be obtained by puncturing the popliteal lymph node; this sampling requires, removal of hair from the sampling area and good skin scraping [40].

Skin biopsies can be used for both pathological study and culture and PCR. It is inadvisable to use aspirate samples because pollution levels are usually very high; however, a high percentage of antibiotic and antimycotic can be used on this media [41].

2.6. Determination of the epidemiological risk of infection

Studying the eco-epidemiology of infection risk involves the identification of different segments of the population who are at risk of becoming infected with *Leishmania*. Through this methodology, the spatial, temporal, and population risk may be evaluated. Spatial risk corresponds to the macrofocus, or geographically and ecologically defined area in which the transmission process occurs, as defined by Pavlovsky [42] and developed for use in the study leishmaniasis by Rioux et al. [43]. A macrofocus can be recognized through the identification of ecological indicators such as the presence of certain plants, altitude, or average rainfall [3, 4, 7, 30].

Geographic information systems and spatial analysis of vector-borne diseases are currently very useful tools for the depiction of regions with a high prevalence of vector-borne diseases, allowing for the identification of risk factors for infection in a given area. From the human occupation perspective, establishment of specific schematic (noso-ecological) maps is the last phase in the determination of risk areas; in these maps, research groups may depict the boundaries and internal organization of the foci. These maps are a tool for the study of human ecology but may also provide a practical guide to the health authorities responsible for the implementation and maintenance of programs for the prevention and control of disease.

Maps should have a small scale of one-billionth or 1 in 500,000, and in these maps, large zones of risk may be identified, that is, the macrofoci. The maps can be used to set parameters for vector density, that is, the spatial or geographical distribution of a vector at an appropriate density, or determine the correlation between the bioclimatic or phytoclimatic zones, and living areas or vegetation types in the areas where the vector species inhabits. Within these small-scale maps, the transmission areas for each *Leishmania* species and areas in which each vector inhabits can be determined [44].

Temporal risk corresponds to the period of greatest risk and can be defined in different time scales: daily, seasonal, or one or more years. Generally, it corresponds to the periods during which there is a higher density of vectors and greater risk of parasitic infection.

3. Findings of the application of eco-epidemiological method in some focus studies on Colombia

In Colombia, leishmaniasis has been identified in all its clinical forms. It may occur in the endemic form, causing natural outbreaks throughout almost all the national territory areas with altitudes lower 2000 meters above sea level, including wilderness areas, tropical dry forests, the Andean region where coffee is grown and plains and deserts areas located in the inter-Andean region, eastern region, and Guajira Peninsula.

Our group has studied the natural infection patterns of leishmaniasis in different foci of transmission, and these studies have demonstrated that women and children are affected by leishmaniasis with equal or greater frequency than men. This finding is in opposition with the belief that leishmaniasis is more common in men (previously considered as an occupational disease that affected men during the performance of rural labor activities in forested areas inhabited by the insect vector). However, studies have shown that women (adults and children) have less access to proper diagnosis and treatment, leading to infrequent case reporting. These studies have also shown that due the domestic adaptation of vector species, the occurrence of epidemic peaks of CL in different regions may affect entire families. It was observed that the presence of gender differences depended on the locations in which the consultations took place; however, no changes in the activities performed by women in the field were identified that could explain the increased frequency of contact with the vector observed [45].

In the different studied foci, three major leishmaniasis transmission cycles have been identified: sylvan, domestic/rural, and urban.

- Sylvatic cycle: Humans are infected when they enter into the proximity of forests or jungles and are bitten by the insect vectors; in this case, humans are an accidental host who are not involved in the transmission cycle. In Colombia, military members, miners, loggers, and indigenous communities are the most affected by this transmission cycle.

- Rural/domestic cycle: This cycle occurs mainly when intra-domiciliary transmission of the disease occurs (in which vectors are inside homes and affect the entire family without discrimination by age or sex). In foci, such as Montebello and San Roque (Antioquia) [6, 28, 33] and areas with traditional coffee cultivation [46], children under the age of 5 years have been found to have the highest incidence of leishmaniasis; in these areas, the most frequently incriminated vector has been identified as *Lu. gomezi*, which has increased biting activity during the early hours of the night. When an outbreak occurs in the epidemic form, the data have shown that humans are part of the transmission cycle and act as a reservoir [29, 34].

- Urban cycle: Similar to the rural/domestic cycle, in this cycle, vectors enter the peridomicile or home and may transmit the infection to the entire family; in this cycle, a higher rate of infection in children has been observed. Additionally, in this transmission cycle, humans can be accidental hosts not involved in the transmission cycle or act as reservoirs.

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In Colombia, sylvatic and domestic-rural endemic foci are characterized by locations that are generally in remote and impoverished areas of cities with great social inequities and little state health institution presence. Because the lesions are visible (usually located on the exposed areas of the skin), these lesions may become chronic and disfigure the skin or mucous membranes, and indigenous peoples have developed their own medical systems for the disease.

For the evaluation of medical systems in foci of transmission, qualitative ethnographic methods have been used in which participant observation techniques (requiring the presence of researchers within the community) are applied. Interviews also allow for the establishment of a personal dialogue with the community and key stakeholders such as healers and community leaders. Quantitative methods have also been applied through the use of surveys conducted by the investigator with questions designed in consultation with community members. Comparisons of CL cases seeking care at urban centers with cases identified through active surveillance have also shown that inhabitants in each region have developed their own conceptions about the origin and management of disease. These medical systems were found to vary between indigenous and peasant communities, such as those described below.

3.1. Medical systems in indigenous communities: Zenú, Emberá and Tikuna

The indigenous community Zenú, an endemic focus of VL in Colombia, is located in a tropical dry forest region on the Caribbean coast. The Emberá community, an endemic focus of CL, is located in a tropical wet forest region of the Colombian Pacific coast, while the Tikuna community lies within the Amazon rainforest. Despite differences in their culture and the ecological characteristics of the places they inhabit, the medical systems in these communities are similar. However, two types of disease that differ in these communities have been identified:

The first type of disease is called "bush illnesses," "Indian illnesses" or "*Esperajai*" in Emberá. The second type of disease includes Western diseases or "White diseases" that are treated by Western doctors and a type of facultative medicine called "Kapuniajai" in Emberá.

The "bush illnesses" or "Indian illnesses" are distinguished according to their etiology and include three types of diseases: (i) diseases caused by supernatural beings such as "Jais," which populate the jungle, "charms," "chimpine" or the spirits of ancestors or other evil spirits; (ii) diseases caused by jinxes resulting from an enemy administering a potion, usually in food, obtained from a sorcerer, healer or herbalist; and (iii) diseases caused by natural causes and due to a sudden contact between hot and cold matter.

"Bush illnesses" or "Indian illnesses" that relate to VL and are observed in the Zenú community include the following:

1. Diseases caused by supernatural beings: In this type of disease, it is believed that a "mushroom" or "wind mushroom" is produced by evil winds from the world of the dead. The symptoms of this disease include fever, body aches, headache, and possible unconsciousness or coma. This disease is also known as "wind sickness" or "mountain view" and is thought to be the separation of the "soul" from the body. When afflicted by this disease,

the spirit is believed to wander aimlessly, sometimes resulting in death in sick children. The "soul" is thought to leave the body when a mother leaves the house with a child in her arms without providing any protection to prevent the evil wind that roams the mountains from taking over the child's soul while walking. The hours most conducive to this phenomenon are believed to occur at sunrise and sunset. When the mother takes the sleeping child from home to home, it is thought that the soul stays in the first location. This spirit loss may result in crying, loss of appetite, fever, hair loss, diarrhea, edema, and appearance of a "bun" or ball on the left side of the stomach.

2. This feature is important because 100% of interviewed mothers who had children with VL consulted a healer as their first medical resource, with all cases of diseases diagnosed as "wind illness" or "milkbun illness" [19, 47].

3. Diseases caused by natural causes: These diseases are believed to be the result of sudden contact between hot and cold matter. For example, indigestion is thought to be caused by walking barefoot or bathing in hot weather. These diseases include the "milkbun illness" or "child in *Chime*," which is believed to occur when a pregnant mother breastfeeds another child. Through this process, breast milk is damaged, and the child that drinks the milk suffers from constant fever, loss of appetite, and stomach swelling (liver, spleen). A "bun" or ball is then believed to grow in the navel, and then the child dies. Elderly people say that this disease is very old, and many children have died from fever because they would not "refresh" and had a swollen abdomen.

Given these elements, it can be concluded that the VL is recognized in the Zenú medical system within the context of "bush illnesses" but is explained as originating from two different causes: the first being of supernatural origin and the second being due to natural reasons. For diagnosis, the patient's family may seek care from a "healer" or traditional doctor and provide a urine sample from the patient. Following observation of the urine, the healer diagnoses the patient with a disease and establishes a plant-based treatment that is administered and accompanied by a special diet for the child that includes chicken broth.

Medicinal plants used by a "healer" may be classified as "hot" or "cool." Hot plants include cinnamon (*Cinamomum zeilanicum*), pennyroyal (*Bistropogon mollis*), coriander (*Coriandrum sativum*), and "the happy" (*Lantana* spp.). "Cool" plants include basil (*Ocimum basilicum*), flaxseed (*Linum usitatis simum*), corn (*Zea mays*) and soursop leaves (*Annona muricata*). Some of these plants grow in the reserve, and others may be purchased at market places in nearby cities. The leaves are prepared in an infusion to drink or a concoction of leaves, stems and roots for baths, compresses, and poultices.

The Emberá Indians do not recognize CL as an individual clinical entity but consider it to be part of the skin conditions called "Aidá" in their language [47]. This disease is believed to occur in people who violate the social norms associated with menarche or widowhood. During menarche or widowhood, a person acquires a temporary taboo status that, if violated, is believed to then cause the disease. This disease is also thought to occur due to chance encounters with the "Jais" of the jungle, which are the forces that act on and control human well-being and comprise the spirits of ancestors (tutelary, protective and/or aggressive "Jais")

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and of prey animals, which are almost always aggressive "Jais" and agents of diseases who want to exact revenge on hunters [48].

In the Emberá community, a patient may be taken to the Jaibaná (shaman) who is in charge of controlling both evil and beneficial spiritual forces. Through ritual singing to the "Jai," the Jaibaná is thought to "see" the agent that has possessed the body of the victim and look for a way to exorcise it and heal the person. The Jaibaná also provides answers and defines the disease. According to this diagnosis, the Jaibaná can cure the patient or sends him/her to the herbalist or hospital to receive treatment. The agents causing "Aidá," represented as the "Jais," are thought to be small worms found in the forest. When treatment is provided by an herbalist, he or she may diagnose the disease by examining the urine of the patient. The herbalist may then add a plant to the urine, which may produce the typical signs that result when someone has made a curse on a person. When treatment is provided by a Jaibaná, he or she uses the songs of the "Jai" and plants, purification rituals or daily allowances, depending on what the "Jais" advises. Meanwhile, herbalists also prescribe a special diet without sweet or salty foods.

For Tikunas Indians, CL is thought to be produced by an encounter inside the forest with the sloths (*Choloepus hoffmanni*) that transmit the disease by staring at the person, and this belief is consistent with the epidemiological findings of higher disease prevalence in hunters and the presence of vector insects that live in the forest. For treatment, the doctor uses indigenous macerated bark of a tree, which is highly caustic and helps to heal the lesions.

3.2. Medical systems in rural communities

The first descriptions of CL in Colombia were made during the last century under the name "sows" and attributed to poor hygiene. This view persists in rural eastern Colombia, where the cause of the disease is attributed to the bite of flies from pig pens known as "pigflies." Meanwhile, the peasants of northwestern Colombia believe that the disease is caused by the bite of the "pito," which is the designated name for both CL and for some herbivorous and hematophagous hemipteran, and specifically reduviid, insects that live in logs and decaying timbers found near houses. These insects cause skin lesions by biting and defecating on people. Additionally, this disease is recognized by the name "vine" in these communities due to the bite of small animals that have a thread-like appearance and are called "vines" or "I saw you"; these animals have been found to often bite people while in the forests and in the branches of trees. Peasants in northwestern Colombia say that if a person sees this animal, he or she can shout, "Saw you," and the animal is paralyzed and does not cause itching or disease [46]. In these communities, the diagnosis of this condition is made by examining the lesion or urine.

Other communities believe that the etiology of the disease is due to the action of the "warty louse," a small parasite of *Lachesis muta* snakes, known as rattlesnakes or "warty" snakes. People believe that when they kill these snakes, they are exposed to and bitten by these "lice" thus causing the disease.

Treatment of CL in rural communities varies according to the community's conception of the origin of the disease and the degree of antiquity of the human settlement. Despite sharing

the same geographic area, the CAP of rural populations may be very different. For example, populations that have been settled in this area for a longer period are of African descent. These populations fear the disease and strongly prefer to avoid jobs that involve entering the jungle to prevent infection. People in these communities also believe that leishmaniasis is a "hallmark of the jungle" and that having the disease means that the jungle is welcoming your arrival; therefore, they have no preventive measures against it.

Another population, known as "paisas," is a recent settlement of people from coffee cultivating areas who came to this region over the last 30 years; this region has an endemic focus of CL produced by *L. panamensis*, and people in this population use caustic agents and plants to treat the disease. Treatment with caustic agents consists of the local application of various substances such as silver nitrate, sulfuric acid, hot water, and hot brown sugar and, frequently, cauterization of the lesion with a spoon or the tip of a machete that has been placed in a fire and is applied without anesthesia to the ulcer, leaving a smooth scar [49]. Treatment with plants consists of the local application of macerated leaves or bark of various trees and shrubs and some latex from vines, many with leishmanicidal action previously demonstrated in the laboratory [50]. In recent settlement communities, no preference for a plant or group of plants exists, and a wide range of plants is used; however, moxa is one of the most popular.

Conceptions of the disease are closely associated with not only culture but also social relations. For example, gender relations are represented by the provision of feminine or masculine attributes to ulcers, the papular, silent type of ulcers that are not easily observed and generally isolated and difficult to treat are called "male pito" and the ulcerous lesions that are often numerous, "weep" (produce fluid), and more easily observed and treated are called "female pito" [49].

Nonspecific medications are often used, including antifungal drugs, ointments, salves, local application antibiotics, and veterinary medicines; these medications are generally self-made or prescribed by doctors who have had no possibility to confirm the diagnosis with laboratory tests. Among the most commonly used veterinary drugs is ivermectin, which has been recently approved for human use and has been found to be effective in some studies [51].

In our studies conducted in indigenous communities, it was not possible to verify the use of urine as a healing element, which has been described in black communities in central San Juan [52]; in these communities, in addition to using urine for diagnosis, it has been used for treatment of various skin diseases, rheumatism, and snakebites and smeared on the skin or drank.

It is interesting to compare the observations described here with the Igun model [53], which showed that the selection of a given treatment or system of health care is determined by personal, family, and sociocultural conceptualizations related to the cause, severity, and potential consequences of the diseases as well as the effectiveness and cost and difficulty in obtaining different types of health care (traditional or modern).

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3.3. Characterization of the macro-, meso- and microfoci

Although the first record of leishmaniasis in Colombia was reported in 1872 by Indalecio Camacho, it was not until the early 1980s that the study of the macrofoci of leishmaniasis began. During this decade, leishmaniasis was defined as a sylvan disease affecting men working in affected areas as loggers, soldiers, and hunters. One of the first eco-epidemiologic studies was conducted in Montebello in 1986; in this mesofocus, the risk of infection in homes (microfocus) as opposed to in the extradomiciliary environment was characterized for the first time because the vectors were identified in the intradomicile and much of the affected and at risk populations was identified to be women and children under the age of 5 [6, 54].

In the indigenous community of San Andres de Sotavento-Cordoba on Colombia's Caribbean coast, a microfocus for VL has been identified in homes that were located near the gallery forest. In these cases, *Lu. evansi* species were found to be able to easily access homes and bite entire families, including the population group that is most at risk of developing VL, children under the age of 5 years. In the same region, a high rate of infected dogs with *Leishmania* has been identified, as have vectors, which have even been found to be present in areas with a low incidence of VL; these infected vectors and reservoirs have been found in the extradomicile, but *Lu. evansi* has not been found to enter homes, and therefore VL cases have not been identified. In the evening hours when increased biting activity of *Lu. evansi* has been identified, children are often inside homes and within the reach of infected vectors. However, adults that have been bitten in the extradomicile have not been found to develop the disease [7].

Humans play a modulatory role in facilitating or hindering the risk of transmission. For example, in the Magdalena River valley region, deforestation and rangeland establishment have confined the CL insect vectors to the forest. These species have been found to not reach the home when a paddock belt is more than 100 meters away. However, deforestation, while increasing the distance from some vector species, also creates favorable environments for the establishment of others. Such is the case of *Lu. longipalpis*, a VL vector in the Magdalena River valley region, which has been found to invade new areas and create new risks for populations living in these regions.

Characterization of microfoci has helped the creation of proposals for prevention and control.

3.4. Contributions to the knowledge of phlebotomines

As a result of the study of foci, valuable information about the presence and distribution of *Lutzomyia* species in Colombia has been obtained. Some species have been reported as novel to Colombia, such as *Lu. suapiensis* [55] and *Lu.a Franca* in the Amazon region [56], or described as novel species in general, such as *Lu. velezi* [57]. Others have been newly reported in certain areas, such as *Lu. reburra* in Antioquia [58] and *Lu. ovallesi* in Amazonas (unpublished data).

However, knowledge has not only been generated about the presence of a species in a focus but also on biodistribution, behavior, and location relative to home of these species. Thus, changes in the geographical distribution of some vector species over time have been detected

that require adaptability to both urban environment and novel areas. Such is the case of *Lu. longipalpis,* which had been reported at a maximum altitude of 1100 meters above sea level until 2006, when we identified this species in the Andean region of the country (Caldas) at 1387 meters above sea level; this was the second global report of the prevalence of this species at a higher altitude [59]. *Lu. panamensis*, one of the main vector species of CL in Colombia, has become the predominant species in the most urbanized areas, showing its high capacity for adaptation, unlike other species present in the jungle areas of Darien Colombia (Department Choco) (Carillo LM, unpublished data).

While the biting activity of insect vectors has been found to be crepuscular and nocturnal, these species have variable hours of activity. For example, in Montebello and Antioquia, *Lu. gomezi* has been found to have greater biting activity from 6 to 8 pm. In the municipality of La Guaira, Valle del Cauca, *Lu. youngi* has generally been identified as having increased biting activity between 7:00and 9:00 pm [60]. *Lu. evansi* in San Andres de Sotavento has been found to have increased biting activity from 11:00 pm to 1 am [7]. Specifying the peak hours of biting is important for the selection of the control measure to be used.

Seasonal variations in the density of vectors have been identified. For example, in sub-tropical countries, the period of greatest activity of a vector has been found to correspond to the warmer months of the year. In France, *Ph. ariasi* has been found to be active from June to September, with its highest density identified at the end of July [5, 60, 61]; however, after examining the physiological age of the insects, it was found that the highest proportion of calved females and natural infections with *Leishmania* occurred in August and early September. In tropical regions and notably in Colombia, we have found that the period of greatest risk corresponds to the rainy season. In San Andres de Sotavento (*Lu. evansi*) [27], Montebello (*Lu. gomezi*) [6] and Choco (*Lu. panamensis* and *Lu. trapidoi*) (Carrillo LM, unpublished data) a significant increase in vector density during the rainy season has been identified. At the end of this period, the rate of calved females and natural *Leishmania* infections has also been determined to be significantly higher. In the area of Urabá during the phenomenon "*El Niño*" a tendency towards decreased vectors has been identified, contrary to what was reported by Cardenas et al. [62].

Over longer periods, disease outbreaks (e.g., Montebello, San Roque, and Saiza) have been observed that correspond to the confluence of multiple factors such as the presence of infected reservoirs and vector abundance; in all of these outbreaks, it was found that transmission occurred in the intra- and peridomicile, with children highly affected and no gender difference identified. Additionally, humans were identified as a possible reservoir due to the large number of active lesions present at any given time and high number of bites (see the photo of the girl with bites and photos of extensive lesions). Climatic factors, such as a phenomenon of "*La Niña,"* characterized in Colombia by the rainy season, could favor higher vector densities and outbreaks; however, studies confirming this hypothesis are needed.

4. Conclusions and recommendations

Leishmaniasis is a complex disease of multifactorial origin in which different species of parasites, reservoirs, vectors, and ecological and social factors interact. The focal nature of the

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transmission of leishmaniasis in each endemic area has been found to be associated with the particular environmental conditions in a given area, such as climate, humidity, altitude, temperature, and vegetation, that favor the vector's presence. Like other vector-borne diseases, leishmaniasis has been found to be strongly influenced by global warming and climate variability, which affect the dynamics of the vectors, reservoirs and human populations. The use of eco-epidemiological assessments provides a multidisciplinary approach to understanding heterogeneities and dynamics that occur in the foci of leishmaniasis transmission.

The application of the eco-epidemiological method then enables the following processes to occur in the natural foci of transmission: (a) identification of the species of *Leishmania* and role of phlebotomines and wild and domestic reservoirs; (b) definition of the geographical areas or macrofoci and description of the environmental characteristics that may become ecological markers of the presence of a vector, such as temperature, vegetation, soil type, and altitude; (c) establishment of the time of the year (spatio-temporal) associated with increased risk of transmission based on the rates of natural infection and biting activity; (d) establishment, with respect to housing (microfocus), of the risk of infection for inhabitants in the intra-, peri- and extradomicile; (e) determination of the different age groups in a community that are most affected or at risk of becoming ill; and (f) identification of the conceptions, attitudes, and practices in communities related to the disease. The improvement of eco-epidemiological surveillance for leishmaniasis should be based on the identification of these parameters to facilitate the design and implementation of cost-effective prevention and control measures. In addition, the sustainability of these measures is dependent on intersector collaboration and support (municipal/Department of Health) and active participation of the community.

The results of different studies have shown that in a focus, transmission may be intra-, peri- and extradomiciliary, and while women and children may be as substantially affected by leishmaniasis as men, women have less access to healthcare. These data have also shown that official statistics are not a good reflection of the true epidemiology of leishmaniasis in the rural areas where a greater number of cases are recorded.

The fact that women less frequently seek care causes the prevalence in this population to be underestimated, and therefore there are inaccurate estimates of the gender-specific rate of infection. Implementation of better governmental strategies will be required to correct the inequality in access to the treatment for women, and strategies for controlling the disease that are oriented towards preventing intra- and peridomiciliary transmission are also required. There is also a need to increase the focus within health departments towards correcting the unequal access to health services in women (regarding leishmaniasis), as this disparity can reflect other problematic situations in the rural health setting.

Based on these different findings, it is suggested that the design of control strategies take into account the following considerations:

When it is determined (through the study of a focus) that transmission is occurring in the interior of a domicile, then measures for vector control and the prevention of leishmaniasis can be used to prevent people from becoming infected with the parasite by preventing bites from the infected vectors.

Control measure should be applied before the period of greatest risk and aimed at the protecting people at higher risk in the geographical area in which there has been transmission, whether that area is intradomiciliary or extradomiciliary. Additionally, the hours of greatest risk should be considered. The rainy season has been identified as the time when there is a greater risk transmission in foci and a higher density of vectors; therefore, control measures should be implemented before the rainy season begins. However, these findings must be validated in each foci.

Various vector control measures are available, including the use of bed nets or curtains impregnated with insecticides (even hammocks) and spraying of insecticides inside houses (in those foci where transmission is intra- and peridomiciliary).

In the case of bed nets, it is necessary to take into account the type of material and size of the holes and educate the community so they will not wash the mosquito net and damage the residual effect and nature of the material (regardless of whether it is impregnated). For example, nylon tulle or polyester bed nets are most effective because the residual effects of the insecticide are much higher; additionally, in these bed nets, the holes are very small and prevent *Lutzomyia* passing through the mosquito net. While bed nets are generally well accepted by most communities, acceptance must be considered individually in each community because it can vary from one community to another. For example, color has been identified as one factor that may affect the degree of acceptance of bed nets. If a mosquito net is not impregnated with insecticide, *Lutzomyia* species may get through even very small holes. However, when it is impregnated, vectors will not go through the bed net.

In foci where transmission occurs in an intra- and/or peridomicile, the infection of pets (and people with lesions) has been hypothesized to demonstrate that in these foci, the disease affects both women and men. Proper treatment of people may serve as a control measure of transmission in foci where humans are reservoirs and provide support for the implementation of better methods of control.

Finally, implementing primary health education to inform the community about issues related to the disease will increase knowledge about why the disease occurs, what causes it, what transmits it and how it heals. Recognizing the parasite that causes the disease (causative agent), transmitting insect, its behavioral habits, clinical manifestations of the disease, and mucosal compromise will help to promote disease control.

Individuals in these communities need to understand that it is necessary to perform some diagnostic procedures before initiating treatment and that there are options for treating the disease. These treatments are provided free of charge by the Ministry of Health through the sectional health services for all people with a diagnosis of leishmaniasis with any of its clinical presentations. Additionally, explaining the need to complete treatment to prevent the emergence of drug resistance, relapse, and complications in the mucosae and educating community members about some important measures that can contribute to avoiding contact with insect vectors are crucial.

It is important to emphasize during primary education that control measures (mosquito nets) should be provided to the populations at greatest risk. Specifically, children should be pro-

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tected, as they are the population at highest risk of visceral leishmaniasis. It is also necessary to protect the family unit in general in the intra-domicile through the use of mosquito nets every night.

Through the use of primary health education, the community itself may help to guide control efforts by reporting increases in the intradomiciliary density of *Lutzomyia* species. Informing health providers to recognize early disease (leishmaniasis) is also important. A provider may then diagnose the patient or refer the patient for diagnosis (in early cases). Through community participation, it is possible for this disease become a priority issue for community activities.

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Section 2

Regional Epidemiology of Leishmaniasis

Chapter 4

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

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Additional information is available at the end of the chapter

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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**).

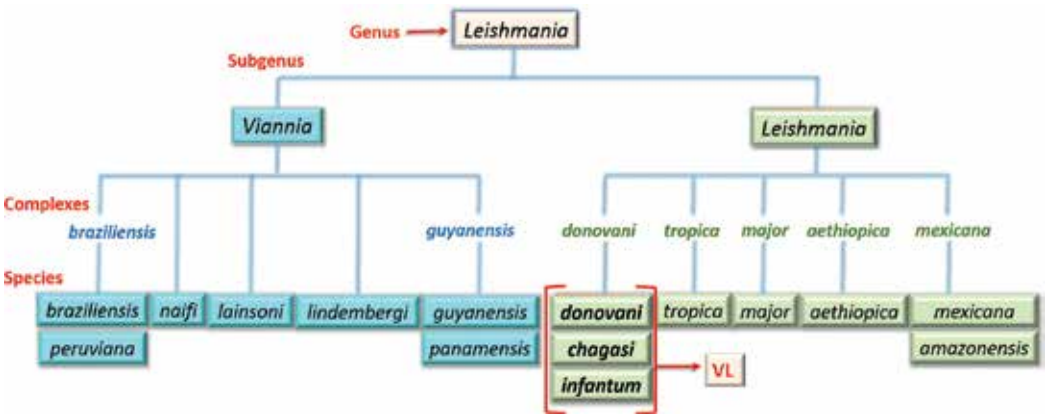

Figure 1. Main species of the *Leishmania* genus. Those causing visceral leishmaniasis (VL): *Leishmania (L.) donovani* in Asia, *Leishmania (L.) chagasi* in the Americas and *Leishmania (L.) infantum* in Asia, Europe and Africa. Note: *Leishmania (L.) infantum chagasi* nomenclature was proposed by Marcili 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**).

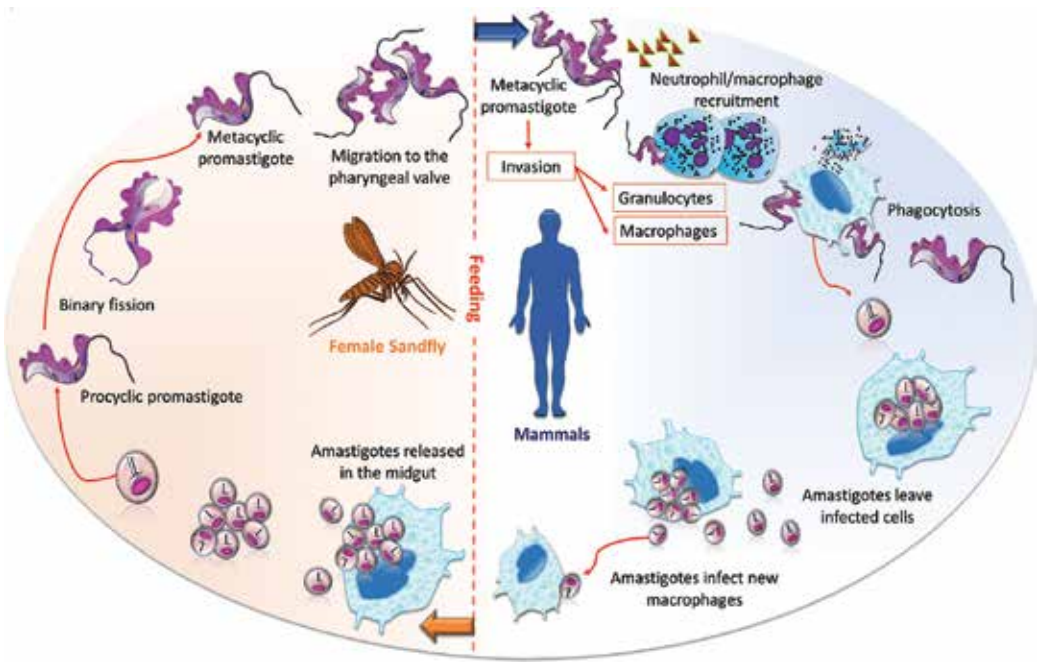

Figure 2. Life cycle of *Leishmania* spp. *Leishmania* parasites are transmitted by the bites of infected female sandflies during their blood meals. The vector injects the metacyclic promastigote forms, which are engulfed by phagocytic cells at the bite site. Inside the cells, promastigotes transform 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].

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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**).

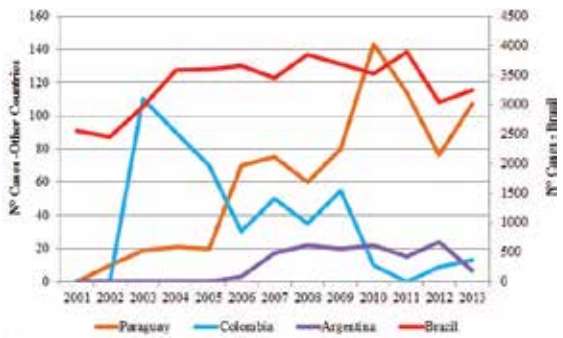

Figure 3. Visceral leishmaniasis cases in four Latin American countries: Brazil, Paraguay, Colombia and Argentina (2001–2013). Source: PAHO/WHO [21, 25].

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**).

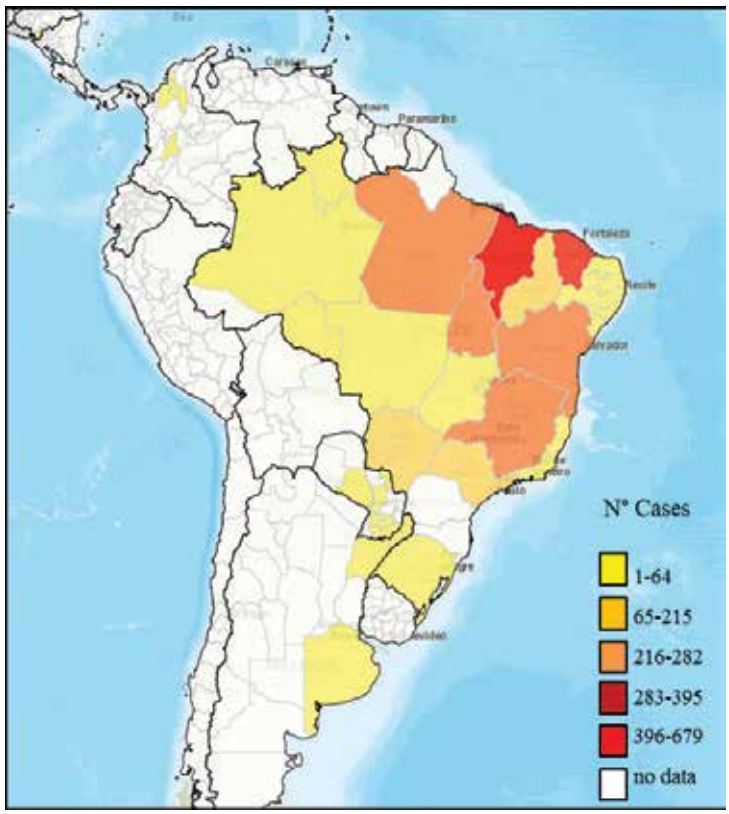

Figure 4. Distribution of visceral leishmaniasis cases in Latin America countries in 2013. Source: PAHO/WHO [97].

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

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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.

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Chapter 5

Epidemiology of Cutaneous Leishmaniasis in Tunisia

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Additional information is available at the end of the chapter

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Abstract

In Tunisia, Zoonotic cutaneous leishmaniasis (ZCL) represents the most significant leishmaniasis form. The epidemic of ZCL emerged in Central Tunisia in 1982 and expanded to the whole central and southern parts of the country. Tunisian ZCL is caused by *Leishmania (L). major* zymodeme MON-25 and transmitted by *Phlebotomus papatasi*. Rodents constitute the reservoir for ZCL. They include *Psammomys obesus, Meriones shawi* and *Meriones libycus*. ZCL occurs as seasonal epidemics and the annual incidence ranges from 2 to 10 thousand cases. Transmission of L. major by the phlebotomine sandfly vector occurs during the summer months, and active lesions in humans tend to emerge during the autumn and winter months. The symptoms of the disease are rather polymorphic, ranging from benign self-limited cutaneous sores to more protracted and extensive lesions that may cause severe disfigurement. Asymptomatic infection occurs frequently in endemic areas indicating a high level of immunity of the residents in these regions. The transmission of ZCL, its drastic increase and its spread are influenced by environmental changes affecting the reservoir and vector geographic distributions and by the lack of efficacy of the control tools available.

Keywords: Epidemiology, zoonotic cutaneous leishmaniasis, Tunisia

1. Introduction

Tunisia is the northernmost country in Africa, covering 165,000 km^2. It is bordered by Algeria to the west, Libya to the southeast and the Mediterranean Sea to the north and east. Tunisia's population was estimated to be just fewer than 11 million in 2014 (National census, 2014). It includes a contrasted relief with mountainous regions in the north where the Atlas range continues from Algeria, coastal plains along Tunisia's eastern Mediterranean coast and the desert in the southern region.

In this country, zoonotic cutaneous leishmaniasis (ZCL) is the most frequent form of cutaneous leishmaniasis (CL) and is caused by the parasitic protozoan *Leishmania major* (*L. major*) [1]. It is mainly transmitted by sandfly vectors, and rodents constitute the reservoir of the parasite.

The transmission dynamic of this zoonotic vector-borne disease is complex: the seasonal activity of the vector species directly impacts on the transmission process of the parasite, whereas the length of the activity period and sandfly vectors abundances are affected by the environmental conditions that influence their life cycle [2].

Since its emergence as an epidemic in Kairouan in 1982 [1], the disease has spread in several parts of Tunisia, particularly in the central and southern parts [3]. The epidemics are cyclic, and annually, 2000–3000 cases are reported. Although ZCL is usually self-curing and not life-threatening, individual cases may be psychologically and socially damaging, especially women with indelible scars that skin lesions leave on their faces. For these reasons, the epidemics are considered as a major public health priority, which remains an unresolved problem until now in Tunisia.

Disease prevention and control are difficult because of the complexity of CL epizoology, and the few options available for effective vector control [4]. Furthermore, ZCL dynamic is influenced by environmental, demographic and human behavioral factors. In fact, in the recent decade, several new foci have been reported indicating the potential spread of the disease in Tunisia. Understanding the epidemiology, the distribution and the ecological structure of the Tunisian ZCL is a crucial prerequisite for applying efficacious control measures.

2. Recall in the history of CL in Tunisia

"*In Tunisia, CL was historically confined to the oasis of Gafsa and its surroundings (South-West Tunisia) where the disease was typically sporadic and occasionally epidemic, particularly in French soldiers that camped in the Gafsa region in the late 19th century. This cutaneous affection was named 'clou de Gafsa'. In 1982, a CL outbreak occurred near the dam of Sidi Saad (Kairouan governorate, Central Tunisia) that had been just finished*" [2]. Over the next few years, the disease continued its contiguous spread to the western, eastern and southern parts of the country, leading to the emergence of several new foci every year [3]. Therefore, a notable increase in the incidence of CL cases was reported, ranging from 1 to 10 thousand new cases recorded annually depending on environmental changes and the pool of susceptible humans. This form of CL was identified to have a zoonotic transmission cycle.

To date, ZCL represents the dominant Tunisian CL form in terms of burden of disease, and it is mainly distributed over the central and southern arid and semiarid regions of the country, where is responsible for seasonal epidemics and regional outbreaks [4].

3. Epidemiology and ecology

3.1. Disease burden and distribution

In 2002, 15 governorates from 23 were considered as endemic in Tunisia. Between 1998 and 2007, the total number of CL cases reported to health authorities during this period was 57,591. The global yearly incidence of CL is almost 20–30 per 100,000 persons [5].

As many CL forms are symptomless and misdiagnosed, the global burden of Tunisian CL is likely to be underestimated.

Most of cases are concentrated in rural area where public health human resources and infrastructure are limited. Sidi Bouzid, Gafsa and Kairouan governorates are the areas where ZCL is most endemic in Tunisia. Sidi Bouzid has the highest population infected by CL (18,508 cases between 1998 and 2007).

Over the past decade, the epidemiological situation of CL has changed significantly. The latest study conducted in 2009 for evaluating the prevalence and the determinants of *L. major* human infection in central Tunisia demonstrated the hyperendemicity of this region. In fact, the ZCL prevalence infection rate found was quite higher to those reported so far in previous surveys conducted elsewhere in the country reflecting the putative high ZCL transmission in Tunisia across space and time. The authors of the study concluded that the control program was not effective enough to stop transmission in endemic regions [6].

It is acquiring an increasingly epidemic status with geographic expansion to previously free areas and the emergence of new foci in several regions of Tunisia. In fact, the ecological niche model elaborated by Chalghaf et al. [7] predicts that CL will extend, within few years, to many new regions in Tunisia (the northwestern side of Sfax, the western part of Mahdia, the southern part of Zaghouan and the western side of Gabès), which will be at high risk for CL emergence.

The recorded increment in the distribution and number of cases is principally a response to environmental changes, either anthropogenic or natural, which favor the rise and establishment of vector species' populations in proximity to human settlements [8].

3.2. Epidemiology

Tunisian CL epidemiology is characterized by an autumn-summer seasonality with an incidence peak from October to December. Transmission of *L. major* by the phlebotomine sandfly vector occurs during the summer months, and active lesions in humans tend to emerge during the autumn and winter months.

A previous study conducted by Toumi and colleagues in Sidi Bouzid showed the seasonality of the incidence of ZCL during the same cycle with an inter-epidemic period ranging from 4 to 7 years [9].

A recent study on the epidemiology of CL was conducted in five neighboring villages in central Tunisia, which were classified into one old and four emergent foci [6]. The prevalence and the incidence findings of this study illustrated an interesting phenomenon long known about CL; two foci as close as a few kilometers apart can have disparate prevalence and incidence statistics. The major risk factor for CL infection found in this study was the past history of transmission in a given geographic area. These findings provide additional evidence that the prevalence of the infection increases with length of residence in endemic areas, as an indicator of time exposure to the parasite [10]. This study suggests that people who resided in the old focus acquired a relative protection due to the presence of continuous boost of the immune

system by exposure to infectious sandfly bites. Moreover, the higher rate of infection in the old focus may be due to a higher density and infection rate of rodent reservoirs and, consequently, a higher infection rate of vector sandflies.

They also demonstrate in this survey that CL prevalence typically increases with age, presumably because of the acquisition of immunity. Furthermore, in established endemic areas of CL in Tunisia, the risk of infection was found to be strongly associated with the presence of family history of disease and increases significantly with the number of past ZCL cases among other persons in the same household. This finding indicates a significant clustering of ZCL transmission within households.

It has been traditionally believed that Leishmania infection is associated with agricultural activities in Tunisia [6]. Environmental changes, whether natural or man-made, in land use and cover, urbanization and unplanned settlements, have probably created suitable conditions for domestic transmission cycle that shifted the risk of infection from sylvatic environment to rural settlements [11].

3.3. Parasitology

3.3.1. Leishmania parasite

The majority of *L. major* strains isolated in Tunisia belong to the MON-25 zymodeme [3, 12]. It is largely recognized that the population structure of pathogens is influenced by different evolutionary factors, particularly during invasion of new ecosystems [13].

Over the past few decades, we reported the emergence of newly evolved *L. major* species that are genetically best adapted to the environment in Tunisia. The result of this selective process may explain the increasing chances of parasitic transmission to humans and the rise of ZCL cases in endemic regions over the last years.

Indeed, a recent study conducted in Sidi Bouzid to evaluate the temporal organization of *L. major* genetic diversity in Tunisia [14] showed that the historical *L. major* population was genetically less diverse than the current one with a significant genetic differentiation across time. In 20 years, the genetic drift in *L. major* population has played a major role in the increase in species diversity.

The same study suggested also that the parasite transmission process does not follow a vertical south-north gradient as presumed from results of other research. In fact, the disease seems to have spread from Gafsa to Kairouan and then to Sidi Bouzid. Human settlement strategies and rodent population dynamics could lead to this nongradual spatial spread.

3.3.2. Vector

Leishmania parasites are transmitted from a vertebrate host to another vertebrate host by a tiny 2- to 3-mm-long insect vector, the phlebotomine sandfly. Only the female sandfly bites vertebrates can therefore transmit the parasite.

Phlebotomus papatasi Scopoli (Diptera: Psychodidae) is the main vector of *L. major* in Tunisia.

The geographical distribution of this vector was assessed by Chelbi I. et al. in September 2006 by setting CDC light traps placed in peridomestic areas in a transect from the north to the south of Tunisia [15]. Their data verify the remarkably spatial correlation between the sandfly vector density and ZCL cases. Both of them were abundant in the arid and Saharan bioclimatic zones and rare in the humid, subhumid and semiarid bioclimatic zones.

In the same period, another study was conducted in central Tunisia to assess the population density of *P. papatasi* using sticky traps (ST) [16]. Based on the sticky traps capture data, *P. papatasi* showed a peak of density in early spring and again in the autumn, while the lowest densities were recorded in the late summer. However, the peak incidence of ZCL cases in the governorate of Sidi Bouzid took place in December, three months after the fall of sandfly density, indicating a close temporal relationship with the abundance of *P. papatasi* [16].

In Tunisia, the percentage of *P. papatasi* females naturally infected with *L. major* likely increases over the summer and peaks to 7.9% in the fall [17], corresponding to seasonal prevalence peak in *P. obesus* of 70% [18].

3.3.3. Reservoir

Many ecological studies to investigate Tunisian CL reservoir hosts were realized. Three rodent species carrying *L. major* in Tunisia were identified: *Psammomys obesus* Cretzschmar 1828 (*P. Obesus*) with a major part in amplifying the transmission, *Meriones shawi* Duvernoy 1842 (*M. shawi*) and *Meriones libycus* Lichtenstein 1823 (*M. libycus*).

Psammomys Obesus is the main reservoir host of *L. major* and the source of epidemics in the central Tunisia [19]. It was naturally infected in Tunisia [18, 20]. Its local distribution is governed by that of the halophytic Chenopodiaceae on which it depends for food [21–22]. *Meriones libycus* was suggested to have a role to propagate the parasite between *P. obesus* colonies because of their common migration, thus increasing the distribution of the parasite [23].

In the other hand, *M. shawi* is the reservoir host of *L. major* in some parts of Tunisia [5, 24]. This rodent has a particular ability to tolerate long period of water deprivation that allow survival in clay and sandy deserts, arid steppes and mountain valleys. It may also be found in cultivated fields [25].

It is a terrestrial rodent, mainly active at night, that nests in deep and complex underground burrows with several food storage cavities [26]. These saline ecological biotopes are discontinuous in distribution in the center and south of Tunisia, thus leading to a fragmentation of the populations of sand rat.

Furthermore, natural infection by *L. major* has been identified in a specimen of least weasel (*Mustela nivalis*) in Sidi Bouzid [27]. This finding might imply just an incidental infection of the *Mustela nivalis* by the *L. major* parasite. However, further research on larger samples of this animal is needed to verify its role as a potential reservoir host for CL caused by *L. major*.

3.3.4. Transmission of CL

ZCL is transmitted to humans by sandflies vectors when they are in close contact with infected reservoir hosts, as a result of activities including agricultural practices, housing and residence in close proximity to active colonies of rodents [28].

Meriones species, even though a minor reservoir of Leishmania in Tunisia, is thought to contribute to the dispersal of Leishmania because of their large range compared to Psammomys which is more restricted. In addition, Meriones species of rodents tend to live in close proximity to human settlements, and their main food source is gramineae.

Human activities that interfere with the ecologic niche of reservoirs such as deforestation and destruction of natural habitats can change the epidemiology of ZCL. Emergence of ZCL epidemics can take place when humans invade the territory of Psammomys [29] or the incidence can be reduced when burrows of rodents and chenopods are properly destroyed.

Epidemics of CL in Tunisia may be associated with migration and the introduction of nonimmune people into lands with existing transmission [30]. Prediction of such outbreaks depends on the availability of ecological information and one valuation of development areas before implementation of projects or population movements. Noticeable increase in the number of CL cases has been observed when susceptible population migrate to formerly unsettled areas located near *L. major* reservoir host biotopes [5].

Poverty and CL transmission risk are tied closely together. Poor hygiene and inadequate sanitation facilities (e.g., lack of wastewater treatment and disposal, open sewerage) may favor the proliferation of sandflies which increase human-vector exposure. Crowding and proximity of people play also a role in attracting sandflies.

CL is a climate-sensitive disease, occupying a characteristic "climate space" that is strongly affected by changes in rainfall, atmospheric temperature and humidity [31]. Climate conditions affect the leishmaniasis complex components (parasite-reservoir-vector) and their ability to interact, persist and establish in new ecosystems.

In Tunisia, there are two climate types. It is typically Mediterranean in the north where the terrain is mountainous, with hot, dry summers and mild winters, whereas the southern part close to the Sahara experiences a hot desert climate with high humidity. Annual average precipitation in the northern region reaches a high of 1500 mm, while rainfall in the extreme south averages less than 200 mm [7].

A previous Tunisian study [31] indicated that the occurrence of significant environmental changes driven by agricultural development projects created suitable conditions that did not previously exist for the emergence of ZCL. Toumi and colleagues confirmed in their study [9] that the risk of disease in Sidi Bouzid is mainly influenced by the humidity related to the months of July to September during the same season and mean rainfall lagged by 12–14 months.

Another Tunisian study [7] showed that the most important climate risk factors explaining the variability of CL incidence over time are precipitation and temperature. The decadal increase in the number of ZCL occurrence in the region suggests that changes in climate increased minimum temperatures sufficiently and created conditions suitable for endemicity that did

not previously exist. The gradual warming trend in Tunisia resulted in the extension of the hot season, and as a consequence, there was an elongation of the transmission period and the exposure to the parasite bites [7].

Climate change may influence geographical distribution of both sandflies and rodent densities. Ambient temperature is one of the most important factors affecting developmental times and survival of sand flies [32].

Low and high temperatures are key in limiting the distribution of *P. papatasi* and its activity [33, 34]. It cannot tolerate the extreme conditions of temperature and low humidity. Temperatures above a critical range suppress ZCL incidence by limiting the vectors' reproductive activity. The highest densities of *P. papatasi* are associated with temperatures between 25 and 28°C [16]. In fact, sand flies have adaptations to help them live in thermal preference conditions. They spend most of their lives in protected refuges, such as rodents burrows, animal shelters, wells, cracks and crevices in walls and floors [35, 36], to avoid prolonged exposure to extreme weather events.

In the other hand, higher rainfall in endemic area in Tunisia would generally increase the vegetation abundance such as chenopods, a halophytic plant that represents the strict diet of *P. obesus*. These environmental conditions are suitable for both rodents and sandflies to reproduce in large numbers and survive in abundance throughout the winter diapause to the following cycle. Therefore, following an extension of the sand rat and sandflies populations, the pool of the parasite transmissible from the reservoir to the vector could lead to a higher human exposure risk to Leishmania-infected sandfly bites over the next season [9].

4. Disease presentation: clinical symptoms

ZCL is characterized by a painless skin ulceration, which is an erythematous papule that becomes darker and develops a crust in the center over the course of several weeks. The lesions at the site of inoculation are usually situated on exposed areas such as the face and extremities, ranging from benign self-limited cutaneous sores to more protracted and extensive lesions that may cause severe disfigurement. Generally, these lesions are self-healing within 4–6 months.

It has been shown by several Tunisian epidemiological studies that the majority of human infections by Leishmania parasites remain asymptomatic, especially in endemic areas, indicating a high level of immunity of the residents in these regions [30]. Asymptomatic *L. major* infection constitutes a relatively frequent mode of natural immunization, and the ratio of asymptomatic infection to patent ZCL may reach approximately one-third, especially in the context of low transmission rates [30].

5. Disease control

Since the onset of ZCL epidemic in 1992 in the town of Sidi Bouzid, many control interventions were planned and evaluated by Pasteur Institute of Tunis with the cooperation of local health authorities. The control program was based essentially on manual pulling of chenopods

around the town where *P. Obesus* was very dense, deep plowings of colonies of the rodent and their poisoning. These interventions led to a significant reduction in the incidence among humans with a prevention fraction of disease exceeding 90%.

Consequently, in 2000, the Tunisian National Control of Parasitic Diseases Program (PDP) introduced ecological surveillance of areas at risk for ZCL before the occurrence of the epidemics based on the surveillance of the emergence of rodent colonies, such as *P. obesus*. Despite this significant effort, and the analysis of transmission dynamics of the disease in other regions, control strategies remain unsatisfactory, as indicated by the number of annual cases [5]. In fact, such actions are demanding and expensive, and consequently are often partially and intermittently performed. As such, it was therefore not possible to reduce the temporal and spatial spread of the disease [4].

Thereby, the national strategy for Leishmaniasis prevention and control has mainly focused on passive case detection and free diagnosis and treatment rather than on the reservoir and sandfly control.

Since ZCL is polymorph in terms of disease severity (number and size of lesions, duration for healing), we can hypothesize that some immune factors, depending on their intensity, will not protect against the development of the disease but rather against the severe forms. Their identification could help the development of a vaccine that protect against a severe disease, which could constitute an interesting option. The rationale for vaccine development is provided by the evidence that most individuals that had leishmaniasis or symptomless infection are resistant to subsequent clinical infections. The only proven CL vaccine (practiced for centuries) is the deliberate inoculation of virulent Leishmania parasites, so-called leishmanization [37].

In order to decrease the CL incidence in Tunisia during future years, some points are recommended and should be considered such as health education and awareness about disease, traffic control of immigrants and travelers to endemic regions, personal protection from sandfly biting by curtains and bed nets, eliminating and destroying habitats of the reservoir rodent and spraying insecticides in habitats of sandflies [38]. Therefore, the high awareness among the community, health decision and policy makers were key elements for sustainability of surveillance and control measures in Tunisia. Constructing a risk map of geographical spread of ZCL across Tunisian regions is also important to guide such programs [39]. Indeed finding areas with high probability of presence for both vectors and reservoirs of ZCL will be beneficial to prevent human infection by planning relevant activities [28].

6. Conclusions

The prevalence and the incidence of ZCL infection are increasing, which may reflect the higher endemicity of ZCL transmission in Tunisia over time and across geographic space. This suggests that the control strategy was not effective enough to reduce man vector contacts in endemic regions. The lack of efficacy of the control tools available is partly explained by the complexity of the transmission cycle and the insufficient knowledge of the epidemiology and the natural history of the disease. Due to its limited health resources, prioritization of

successful public health interventions and identification of populations likely to be exposed to sandflies are essential in disease management in Tunisia.

In the absence of a safe and efficacious vaccine, control measures should be adapted according to the epidemiological characteristics of the foci concerned. More researches are needed to study the diversity, the dynamics and the ecology of infectious agents and endemic foci in the country. There is also a need to develop more appropriate and effective treatment.

Finally, collaboration among medical physicians, veterinarians, researchers and public health authorities is critical to find a suitable platform and strategy for the control and prevention of CL in Tunisia.

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Chapter 6

Ecoepidemiology of American Visceral Leishmaniasis in Tocantins State, Brazil: Factors Associated with the Occurrence and Spreading of the Vector *Lutzomyia (Lutzomyia) longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae)

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Additional information is available at the end of the chapter

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Abstract

Leishmaniases are considered serious public health problems, and their geographical expansion has enabled their establishment in urban areas of medium and large cities in Brazil. Continuous processes of deforestation, construction of dams, and hydroelectric plants, among others, cause environmental impact and may favor the increase in the number of human cases of leishmaniases, as well as the establishment of epidemic outbreaks. This scenario reflects the reality of some regions of Brazil, such as Tocantins State, which in recent years has recorded high levels of American visceral leishmaniasis (AVL). This study is aimed to analyze environmental and epidemiological factors related with the spatial and temporal distribution of AVL and with the occurrence of *Lutzomyia (Lutzomyia) longipalpis*, the main vector of AVL, in the state of Tocantins. The results indicate that the vector is adapted to all environments, especially the ones under human influence, and that anthropogenic environmental impacts can support the development and adaptation of AVL in Brazil. Such information could be applied in control strategies aimed at decreasing AVL incidence.

Keywords: *Lutzomyia (L.) longipalpis*, American visceral leishmaniasis, urbanization and expansion, deforestation, land use, Tocantins

1. Introduction

Leishmaniases are zoonosis caused by heteroxenous flagellate protozoa of genus *Leishmania* (Ross, 1903), order Kinetoplastida and family Trypanosomatidae. The infection is transmitted by the bite of infected female sand flies, dipteran insects of family Psychodidae, and subfamily Phlebotominae, genus *Lutzomyia* (New World) and *Phlebotomus* (Old World) [1]. These diseases are major public health problems, affecting indiscriminately men, women, and children, and are the ninth leading cause of infectious diseases in the world, despite remaining within the framework of neglected diseases [2–4]. Leishmaniases manifest in different clinical forms, mainly due to the variety of parasites that affect the human population [5]. They are endemic in 98 countries, reaching America, Europe, Asia, Africa, and Australia, with about 350 million people living in risk areas [2, 6, 7].

American visceral leishmaniasis (AVL) has become one of the most important tropical diseases, due to its high incidence, high mortality rates in untreated individuals, and malnourished children, and it can also progress to death [3]. In Latin America, AVL has been recorded in 12 countries, with 90% of cases occurring in Brazil. The geographical expansion of AVL in Brazil has enabled its establishment in urban areas of medium and large cities [3]. Autochthonous human cases are recorded in most Brazilian states, except Acre, Amapá, Amazonas, Paraná, Rondônia, and Santa Catarina [3, 8].

The etiologic agent of AVL in the Americas is *Leishmania (Leishmania) infantum chagasi* (Cunha and Chagas, 1937), whose main vector is *Lutzomyia (Lutzomyia) longipalpis* (Lutz and Neiva, 1912), a species with strong evidence of vector competence, and closely linked to the expansion process of the disease, as revealed by its wide geographical distribution in the Americas [9]. In Brazil, it is currently considered the main vector of AVL in all regions; however, its presence was not yet detected in the states of Acre, Amazonas, Rondônia, and Santa Catarina. The ability of *L. (L.) longipalpis* to often feed on domestic and synanthropic animals, as well as its remarkable anthropophily favored its adaptation to changing environments, favoring the maintenance of the transmission cycle in the rural environment and, at the same time, the spread of the disease into urban areas [9–12].

A variant epidemiological situation is observed in the central region of Brazil, in Corumbá and Ladário (Mato Grosso do Sul State), and Jaciara (Mato Grosso State), where *L. (L.) longipalpis* is absent and *Lutzomyia (Lutzomyia) cruzi* (Mangabeira, 1938) has been incriminated as a vector because of its high abundance, anthropophily, and natural infection with *L. (L.) infantum chagasi* [13–15].

Leishmaniases produce major impacts on human health as a consequence of environmental change, mainly through the possible expansion of transmission areas. Continuous environmental change processes, such as deforestation, fires, agriculture, mining, construction of dams and hydroelectric power plants, migration, unplanned urbanization, and lack of urban infrastructure are examples of situations that have led to an increase in people at risk of infection, and fostered the emergence of outbreaks of leishmaniasis in a new ecoepidemiological pattern [10, 16].

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Geographic information systems (GIS) have generated valuable contributions to the control and prediction of vector-borne diseases [17–19], and to evaluate the influence of environmental factors on the habitats of vectors and hosts, and the risk of transmission to humans [20]. Such studies aim to characterize and analyze the spatial and temporal dynamics of the diseases and consequently identify epidemiological patterns, generating information that can be valuable tools when planning control actions.

The AVL shows a persistent scenario in Brazil, with most of the factors contributing for its endemicity residing in processes that are external to the health sector. This makes the strengthening of new strategies necessary. Thus, this study is aimed to analyze the spatial and temporal distribution of AVL in Tocantins State, through evaluation of epidemiological and environmental factors that are potentially related to its expansion: disease incidence, presence of the vector *L. (L.) longipalpis*, type of land use, and deforestation. In the current scenario, where environmental changes impact public health, it is essential to intensify research in diseases related to the environment, especially vector-borne diseases such as AVL.

2. American visceral leishmaniasis and *Lutzomyia (Lutzomyia) longipalpis* in Tocantins

Tocantins is the newest of the 26 Brazilian states, its territory has 277,720.567 square kilometers, and it is located in the geographical center of the country, in the North Region (**Figure 1**). Most of the state is made up of plains and plateaus, and it has the largest river basin in the entire country, the Tocantins-Araguaia basin. Tocantins is one of nine states that form the Amazon region, its vegetation consists of 88% of the Cerrado biome and 12% of the Amazon biome (**Figure 1**). It has a semihumid tropical climate with two seasons: wet and dry. Its annual average temperature varies between 25°C and 29°C and average rainfall is around 1200–2100 mm [21, 22]. Among the phytoecological regions found in the state, the savannah (52%) and pasture (27%) are the majority [21, 23].

In the last decade, the state of Tocantins has suffered environmental changes from agricultural activities and construction of hydroelectric plants. In such scenarios, high numbers of human cases of leishmaniasis were recorded [24]. To quantify and describe the spatial and temporal distribution of AVL in Tocantins, the number of human cases recorded from 2000 to 2015 was provided by the Health Department of Tocantins State [personal communication]. All maps and spatial analysis were performed in ArcGIS (version 10.4).

In the analyzed period, Tocantins State had records of 4476 human cases of AVL in 124 of its 139 municipalities. From 2001 to 2013, 212 deaths were reported, and the municipalities Araguaína and Palmas (the state capital) reported 37% of the human cases of the state [24]. The spatiotemporal map (**Figure 2**) shows that human cases of AVL in the state are concentrated in the cities of Araguaína, Palmas, Porto Nacional, Paraíso do Tocantins, Araguatins, and Tocantinópolis, comprising 66% of the state's human cases. The municipality

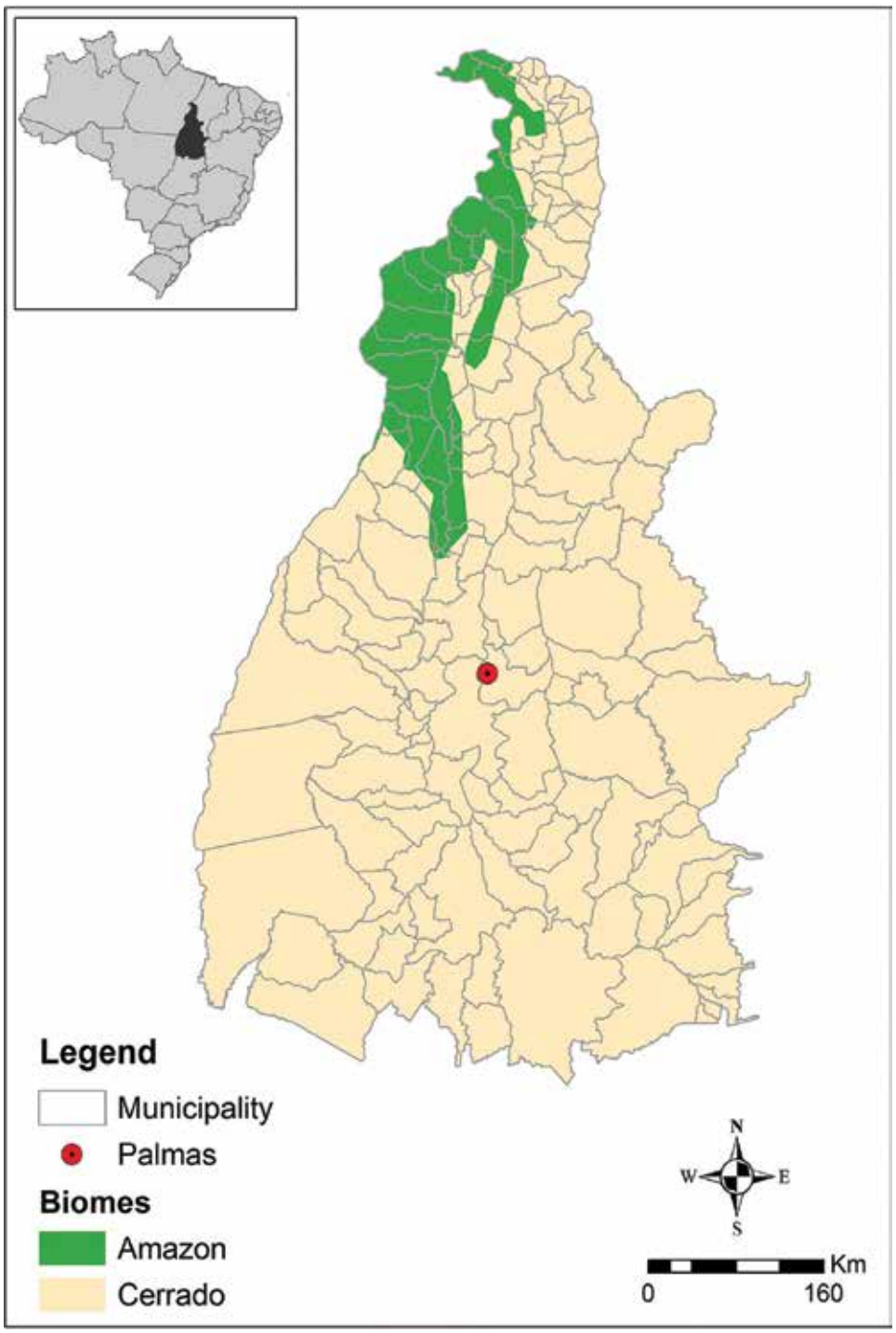

Figure 1. Biomes of Tocantins State, Brazil. Source: IBGE. Map design: Laboratório Interdisciplinar de Vigilância Entomológica em Diptera e Hemiptera LIVEDIH/IOC/FIOCRUZ.

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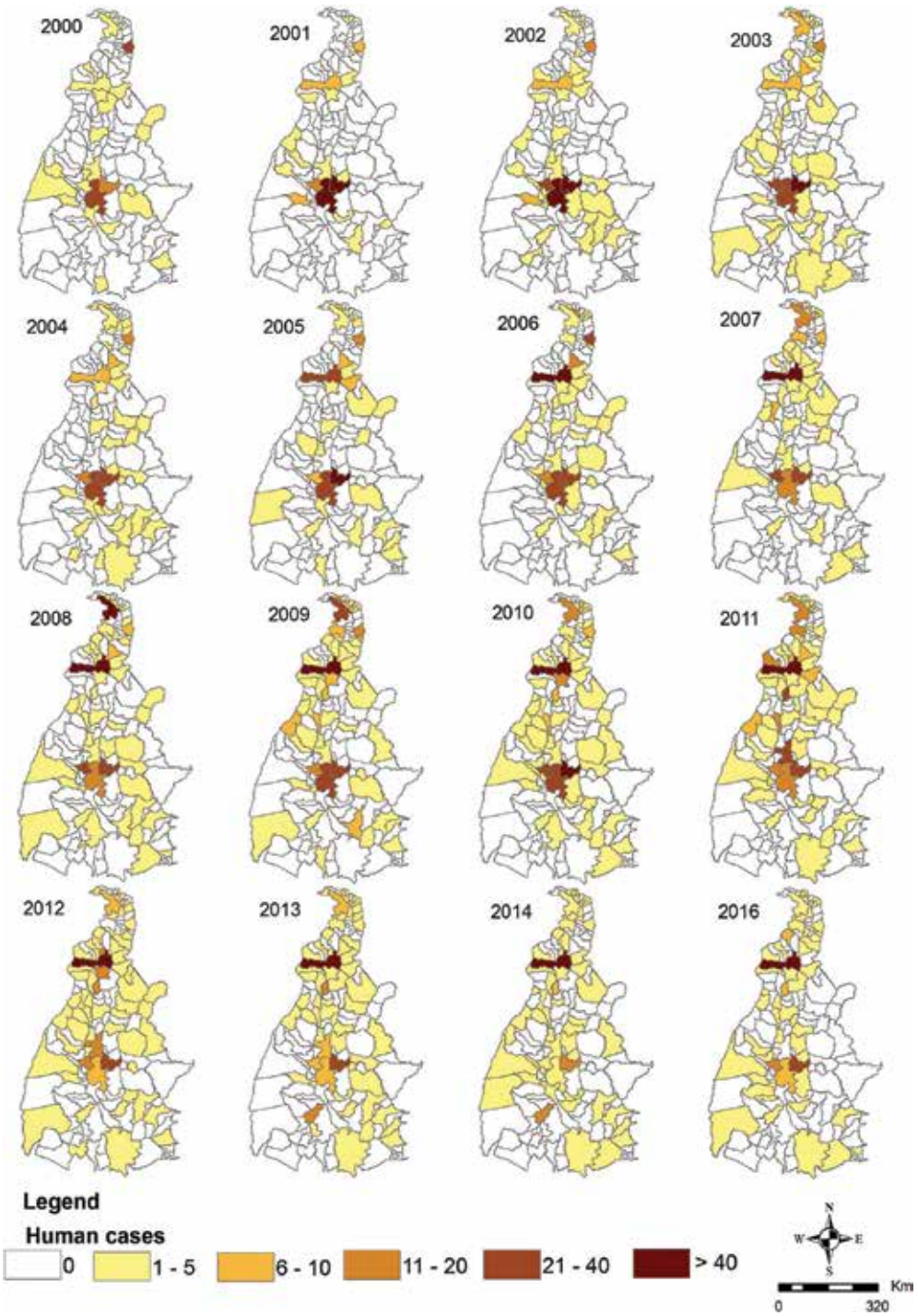

Figure 2. Spatiotemporal profile of American visceral leishmaniasis human cases in the State of Tocantins, 2000–2015. Source: Health Department of Tocantins State. Map design: Núcleo de Geoprocessamento LIS/ICICT/FIOCRUZ.

of Araguaína is mainly responsible for the increased production of human cases in the state, with the temporal pattern of human cases matching the state's pattern (**Figure 3**).

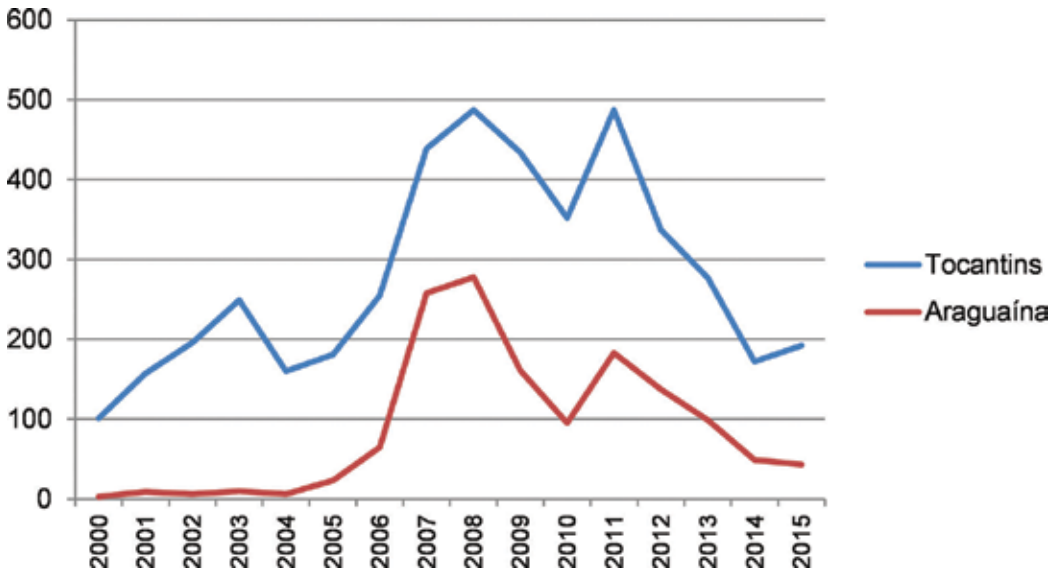

Figure 3. Number of American visceral leishmaniasis human cases recorded in Araguaína municipality and in Tocantins State, 2000 –2015. Source: Health Department of Tocantins State.

Epidemiological surveillance is a major component of the Brazilian Control Program of Visceral Leishmaniasis (CPVL). The program has guidelines for stratifying municipalities under different categories, as areas with or without transmission of AVL. Through epidemiological analysis, health professionals and managers can thus classify the municipalities then adopt the adequate actions for monitoring, surveillance, and control of AVL [3].

According to the methodology proposed by the CPVL, municipalities with transmission are stratified according to the average of human cases reported in the last 3 years and then are categorized as sporadic, moderate, or intense transmission [3]. In Tocantins State, from 2004 to 2015, some municipalities remained classified as intense transmission, especially in the northern and central region, showing that the number of human cases remained high and constant throughout the years, especially in areas where there is high environmental impact (**Figure 4**).

From 2004 to 2015, intense transmission remained in the municipalities of Araguaína, Paraíso do Tocantins, Porto Nacional, and Palmas. However, municipalities such as Gurupi, Miracema do Tocantins, Nova Olinda, Araguatins, Carmolândia, Colinas do Tocantins, and Sampaio, in recent years, have moved from the sporadic or moderate category to the intense category, possibly due to environmental impacts. Other 57 municipalities were now classified as of sporadic transmission, originally being municipalities without transmission, comprising 50% of the municipalities with expansion of transmission. Only 15 municipalities, equivalent to 11% of the state, do not have transmission, and 34% of the municipalities have decreased the number of cases during the study period.

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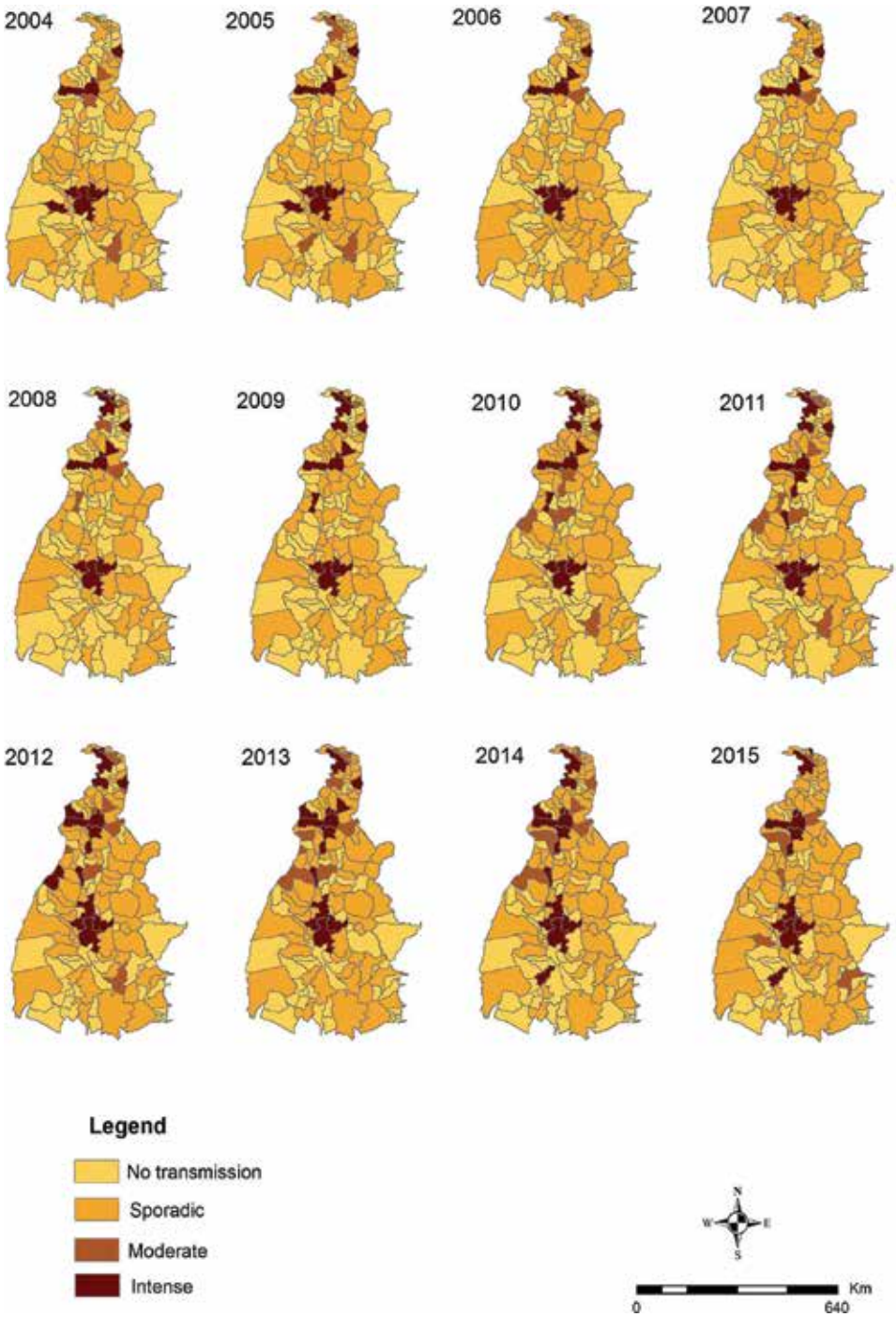

Figure 4. Spatiotemporal profile of the stratification of municipalities for American visceral leishmaniasis in Tocantins State, 2004–2015. Source: Health Department of Tocantins State. Map design: Núcleo de Geoprocessamento LIS/ICICT/FIOCRUZ.

To characterize the municipalities with the presence of the vector *L. (L.) longipalpis*, a literature search was held in the following databases: LILACS [25], MEDLINE [26], and SciELO [27]; using the keywords [28]: *Lutzomyia*, American visceral leishmaniasis, *L. (L.) longipalpis*, and Tocantins. Searches were also performed for conference abstracts, theses, dissertations, and monographs, and unpublished information was provided by the Health Department of Tocantins State [personal communication].

For the municipalities without information on the vector, it was assumed that *L. (L.) longipalpis* occurs where there are human cases of AVL, because so far *L. (L.) longipalpis* is the only vector species associated with the disease in the state of Tocantins and there is no record of *L. (L.) cruzi* in the state [personal communication Health Department of Tocantins State]. While this assumption does introduce a minor uncertainty in the analyses, not considering the presence of the vector in areas where AVL transmission is well-known would compromise the results.

From the scarce bibliographic records that were found (9), the vector was recorded in only 22 municipalities of Tocantins [29–37]. In contrast, there are records of human cases in 124 municipalities, which demonstrate the lack of entomological studies in the state (**Figure 5**).

3. Environmental factors

In order to evaluate the association between AVL, *L. (L.) longipalpis*, and different environmental factors (land use and deforestation), nonparametric Spearman correlation tests were applied to the data. Analyses were performed in the software SPSS (version 22) and correlations were considered significant at levels 0.95–0.99.

3.1. Land use

Data on land use for the Cerrado biome was provided by the Federal University of Goiás [23]; the data for the Amazon biome was obtained at the Web site of the Brazilian Institute of Geography and Statistics [21]. The land use maps and municipal boundaries were integrated in ArcGIS, which enabled the calculation of the percentage of land use class in each municipality (**Figure 5**).

Significant positive correlation was identified between the cumulative incidence of human cases (2000–2015) and area with urban influence, ombrophilous forest, and ecological tension areas (**Table 1**). The land use classes that had positive correlation with the yearly incidence were secondary vegetation, urban area, ombrophilous forest, ecological tension areas, pioneer vegetation areas, and agriculture. Savannah had negative correlation with the yearly AVL incidence (**Table 1**).

The presence of *L. (L.) longipalpis* was significantly correlated with secondary vegetation and with ombrophilous forest (**Table 1**). Considering the occurrence of *L. (L.) longipalpis* in all municipalities where there are records of autochthonous human cases of AVL, in addition to the information from the literature [29–37], it is suggested that the vector is present in all classes of land use (**Figure 5**, **Table 1**).

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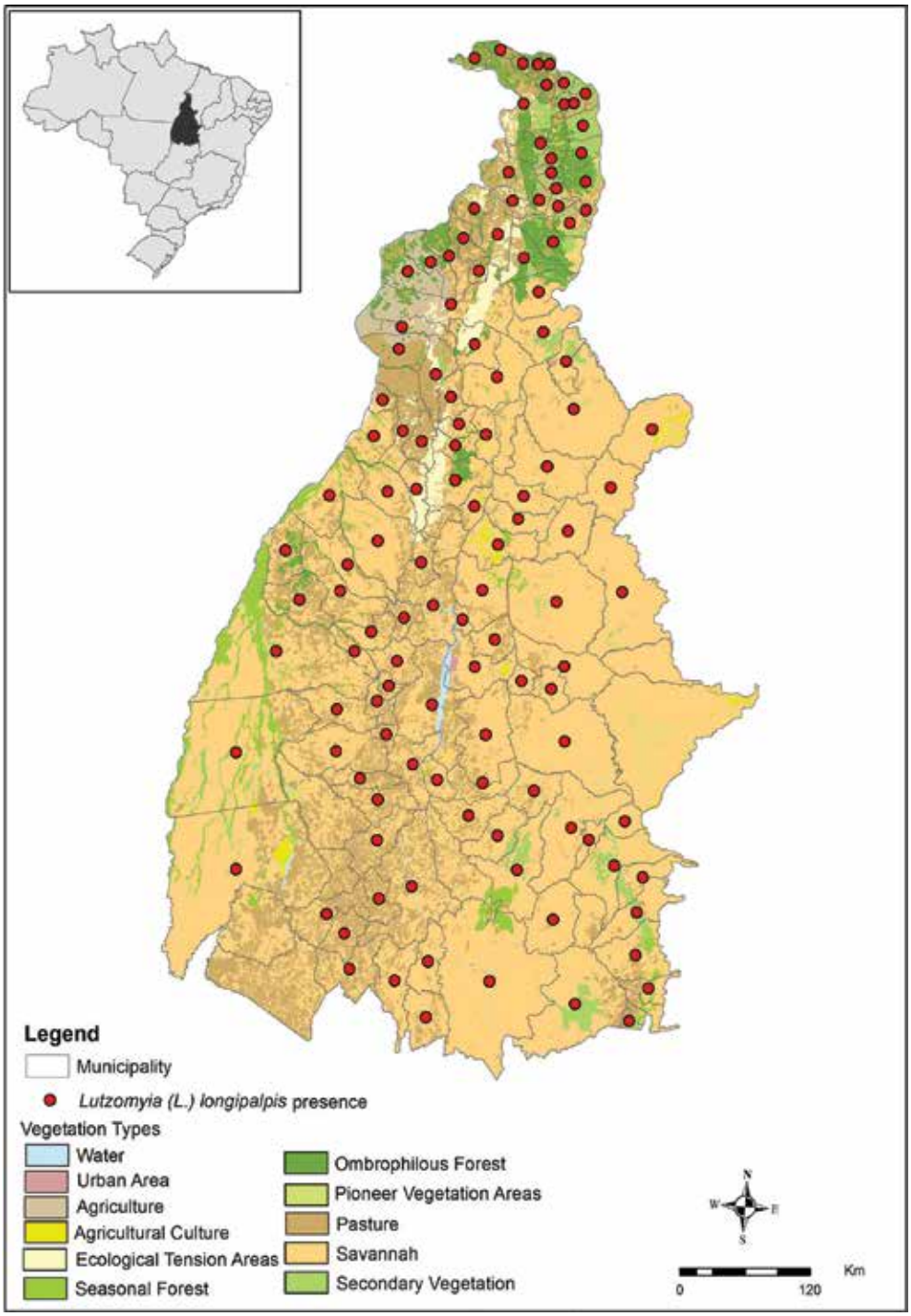

Figure 5. Map of Tocantins State with land use classes in association with the presence of *Lutzomyia (Lutzomyia) longipalpis*. Source: UFG, IBGE. Map design: Núcleo de Geoprocessamento LIS/ICICT/FIOCRUZ.

AVL incidence	Pasture	Secondary vegetarian	Urban area	Ombrophilous forest	Savannah	Pioneer vegetation areas	Agriculture culture	Ecological tension areas	Agriculture	Seasonal forest
	r	r	r	r	r	r	r	r	r	r
2000	−0.072	−0.010	0.067	0.129	0.027	−0.049	−0.002	−0.088	−0.052	−0.032
	0.399	0.910	0.431	0.132	0.749	0.570	0.977	0.304	0.544	0.707
2001	−0.022	−0.033	0.144	−0.080	0.068	0.254**	−0.024	−0.060	−0.039	0.019
	0.800	0.698	0.090	0.348	0.423	0.003	0.775	0.480	0.646	0.824
2002	0.000	−0.053	0.163	−0.038	0.068	−0.048	−0.055	−0.094	−0.020	−0.001
	0.999	0.538	0.055	0.655	0.426	0.574	0.520	0.269	0.813	0.986
2003	0.042	0.026	0.247**	0.209*	−0.144	−0.063	−0.049	0.084	−0.034	−0.080
	0.624	0.758	0.003	0.014	0.092	0.461	0.563	0.324	0.690	0.352
2004	−0.136	0.044	0.062	0.202*	0.011	−0.062	−0.040	−0.003	−0.081	−0.105
	0.112	0.610	0.466	0.017	0.899	0.467	0.642	0.968	0.345	0.219
2005	−0.045	0.025	0.130	0.155	−0.051	−0.042	0.006	0.030	−0.038	−0.081
	0.595	0.768	0.126	0.068	0.553	0.625	0.944	0.726	0.655	0.344
2006	−0.018	−0.007	0.085	0.134	−0.047	−0.075	−0.055	0.073	−0.033	−0.054
	0.835	0.937	0.320	0.115	0.582	0.379	0.524	0.394	0.703	0.529
2007	0.117	−0.006	0.094	0.152	−0.263**	−0.062	−0.080	0.232**	0.232**	−0.034
	0.169	0.942	0.269	0.074	0.002	0.469	0.352	0.006	0.006	0.690
2008	0.061	0.050	0.141	0.156	−0.220**	−0.067	−0.064	0.251**	0.117	−0.042
	0.478	0.557	0.099	0.067	0.009	0.437	0.455	0.003	0.172	0.622
2009	0.099	0.039	0.046	0.110	−0.231**	−0.055	−0.091	0.282**	0.071	0.048
	0.244	0.647	0.588	0.197	0.006	0.523	0.286	0.001	0.407	0.574

Table 1. Correlation between classes of land use and incidence of American visceral leishmaniasis; correlation between classes of land use and the vector, *Lutzomyia (Lutzomyia) longipalpis*, from 2000 to 2015, in Tocantins State.

AVL incidence	Pasture	Secondary vegetarian	Urban area	Ombrophilous forest	Savannah	Pioneer vegetation areas	Agriculture culture	Ecological tension areas	Agriculture	Seasonal forest
	r	r	r	r	r	r	r	r	r	r
2010	0.020	0.169*	0.084	0.044	−0.165	−0.097	−0.114	0.280**	−0.014	−0.050
	0.813	0.047	0.326	0.604	0.052	0.254	0.182	0.001	0.866	0.558
2011	0.104	−0.044	0.046	0.151	−0.246**	−0.078	−0.084	0.290**	0.212*	−0.087
	0.225	0.608	0.588	0.076	0.004	0.361	0.324	0.001	0.012	0.310
2012	0.103	−0.031	0.039	0.038	−0.139	−0.059	−0.055	0.247**	0.061	−0.063
	0.226	0.717	0.649	0.654	0.102	0.493	0.524	0.003	0.473	0.459
2013	−0.006	0.120	−0.023	0.231**	−0.198*	−0.086	−0.022	0.153	0.116	−0.142
	0.944	0.160	0.792	0.006	0.020	0.314	0.793	0.073	0.174	0.095
2014	0.005	−0.014	0.080	0.170*	−0.148	−0.050	−0.069	0.066	0.205*	−0.043
	0.952	0.869	0.349	0.046	0.082	0.556	0.423	0.441	0.016	0.617
2015	−0.082	−0.003	0.051	0.140	0.027	−0.047	−0.008	−0.083	−0.050	−0.043
	0.335	0.968	0.552	0.101	0.754	0.585	0.925	0.331	0.557	0.618
2000–2015	0.104	0.029	0.171*	0.216*	−0.270**	−0.100	−0.091**	0.273**	0.104	−0.090
	0.222	0.737	0.044	0.011	0.001	0.243	0.027	0.001	0.222	0.292
Presence of the vector	−0.116	0.203*	0.104	0.213*	−0.044	−0.011	−0.077	0.070	0.091	−0.080
	0.173	0.017	0.222	0.012	0.605	0.896	0.370	0.412	0.284	0.352

*Significant correlation at level 0.05.
**Significant correlation at level 0.01.

Table 1. Correlation between classes of land use and incidence of American visceral leishmaniasis; correlation between classes of land use and the vector, *Lutzomyia (Lutzomyia) longipalpis*, from 2000 to 2015, in Tocantins State (Continued).

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3.2. Deforestation

To assess deforestation in each municipality, the rates of the yearly increase of deforestation for the period 2001–2014 were produced through digital classification of satellite imagery and provided by the PRODES project [38].

In the state of Tocantins, the increase of deforested areas remained constant throughout the years in some municipalities, mainly in the north and west of the state (**Figure 6**).

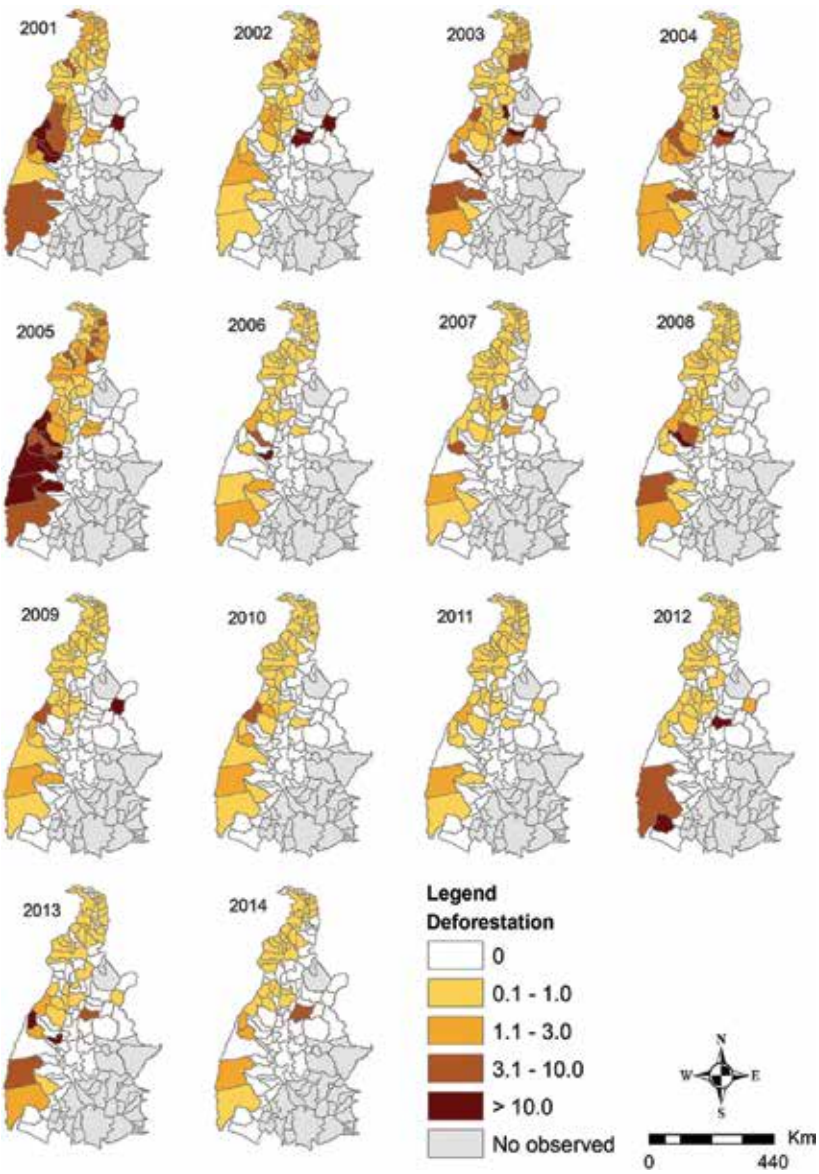

Figure 6. Spatiotemporal profile of the increase of deforestation in Tocantins State, 2001–2014. Source: PRODES. Map design: Núcleo de Geoprocessamento LIS/ICICT/FIOCRUZ.

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Deforested area	*r*	*p*-value	Deforested area	*r*	*p*-value
AVL cases 2000	−0.017	0.845	AVL incidence 2000	−0.070	0.475
AVL cases 2001	−0.103	0.294	AVL incidence 2001	−0.107	0.279
AVL cases 2002	−0.067	0.496	AVL incidence 2002	−0.130	0.187
AVL cases 2003	0.098	0.322	AVL incidence 2003	−0.004	0.969
AVL cases 2004	−0.067	0.498	AVL incidence 2004	−0.132	0.178
AVL cases 2005	0.054	0.582	AVL incidence 2005	−0.030	0.763
AVL cases 2006	0.005	0.957	AVL incidence 2006	0.021	0.835
AVL cases 2007	0.247*	0.011	AVL incidence 2007	0.506**	0.000
AVL cases 2008	0.220*	0.024	AVL incidence 2008	0.512**	0.000
AVL cases 2009	0.337**	0.000	AVL incidence 2009	0.358**	0.000
AVL cases 2010	0.207*	0.034	AVL incidence 2010	0.195*	0.047
AVL cases 2011	0.266**	0.006	AVL incidence 2011	0.322**	0.001
AVL cases 2012	0.286**	0.003	AVL incidence 2012	0.203*	0.038
AVL cases 2013	0.246*	0.012	AVL incidence 2013	0.262**	0.007
Casos de LVA ano 2014	0.111	0.260	AVL incidence 2014	0.085	0.391

*Significant correlation at level 0.05.
**Significant correlation at level 0.01.

Table 2. Correlation between deforested areas and human cases of American visceral leishmaniasis; correlation between deforested area and incidence of American visceral leishmaniasis from 2000 to 2014, in Tocantins State.

Deforested area	*r*	*p*-value
Stratification 2004	−0.033	0.742
Stratification 2005	−0.022	0.824
Stratification 2006	0.035	0.721
Stratification 2007	−0.011	0.914
Stratification 2008	0.149	0.129
Stratification 2009	0.192*	0.050
Stratification 2010	0.337**	0.000
Stratification 2011	0.299**	0.002
Stratification 2012	0.307**	0.001
Stratification 2013	0.337**	0.000
Stratification 2014	0.327**	0.001

*Significant correlation at level 0.05.
**Significant correlation at level 0.01.

Table 3. Correlation between deforested area and stratification for American visceral leishmaniasis; correlation between the increase of deforestation and stratification of AVL, from 2004 to 2014, in Tocantins State.

The municipalities that had higher increase of deforestation were Aragominas, Araguatins, Lagoa da Confusão, Araguaína, Ceará, Xambioá, Santa Fé do Araguaia, Pequizeiro, and Piraquê. Araguaína was the municipality that showed the most deforested area in the state.

As it was with the incidence of AVL and land use, deforested areas were positively correlated with human cases of AVL from 2007 to 2014 (**Table 2**).

Deforested areas and stratification of AVL were positively correlated in the last years, from 2009 to 2014 (**Table 3**).

4. Discussion

American visceral leishmaniasis is a disease that has been showing significant geographic expansion in Brazil. Decades ago, it was mainly present in the states of the Northeast and North regions. Currently, it has gained importance spreading into the Southeast and Midwest regions, and recently in the South region [3, 39].

Lutzomyia (L.) longipalpis is present in most of the states of Brazil, except in Santa Catarina, Acre, and Amazonas, demonstrating the high adaptability to different types of vegetation, climate, habitats, and feeding sources [3, 9, 11, 40]. The state of Tocantins lacks entomological studies with records of *L. (L.) longipalpis* in 16% of its municipalities [29–37], while there are reported humans cases in 89% of the municipalities [24]. These facts show the need for further entomological studies, given the wide distribution of the disease in the state. The assumption of the presence of *L. (L.) longipalpis* in municipalities with AVL records does introduce some minor uncertainty in the analyses. The vector could be absent from an AVL focus if another sand fly species acts locally as a competent vector. So far, the only species that gathers enough evidence in the literature that plays an important role in the transmission of *L. (L.) infantum chagasi* is *L. cruzi* [13–15], and its distribution is restricted to Mid-West Brazil, in municipalities as far as 600 km from Tocantins State. Its presence in Tocantins is unlikely, then. The presence of *L. (L.) longipalpis* was assumed only in municipalities where the information was missing, so given the wide distribution of *L. longipalpis* in Brazil [12] and, more specifically, in Tocantins State [29–37], the chances of its capture in a new sand fly survey are high.

It is noteworthy that the 13 municipalities that have no record of human cases can be classified as vulnerable areas to AVL, because they border other municipalities that have records of human cases. Such municipalities deserve special attention, because according to the guidelines of the Manual of Surveillance and Control of AVL, conducting entomological survey is recommended in order to verify the presence or absence of the vector, and to check its spread in the city, in order to classify the vulnerable municipality as receptive (with the presence of vector) or unreceptive [3].

The application of remote sensing products and techniques in epidemiological studies began in the 1970s [41], and in conjunction with the use of GIS, it has facilitated the integration of environmental parameters and health data to develop models that can be used

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for understanding the AVL epidemiology [42]. The analysis of the stratification of the municipalities showed that Araguaína, Paraíso do Tocantins, Porto Nacional, and Palmas retained the intense transmission through the years, while 50% of the municipalities had expansion of the transmission of AVL, demonstrating its importance as a public health issue in the state.

Some studies used GIS as an important analysis tool of the distribution of leishmaniasis vectors. In Belo Horizonte, an area that has one of the highest rates of human and canine AVL of Brazil, it was possible to correlate peridomestic environmental features and the vectors. *Lutzomyia (L.) longipalpis* showed higher abundance in areas of animal sheds with poor hygiene conditions, which favor the development of sand flies. In contrast, the proximity of areas with vegetation exerted little influence on the incidence of AVL, corroborating its urban profile [43].

In studies in Maranhão State, *L. (L.) longipalpis* was the most abundant sand fly species found in the Cerrado biome; in Bahia State, specimens were captured in areas of Caatinga and Atlantic Forest, demonstrating its adaptation to different environments [44–46].

Considering the assumption of *L. (L.) longipalpis* being present in municipalities where there are autochthonous records of human cases of AVL in Tocantins, the vector occurs in all classes of land use, being adapted to all environments including disturbed areas, corroborating studies that discuss its adaptation to changing environments [9, 34, 40, 47, 48]. A positive correlation was observed between the presence of *L. (L.) longipalpis* and areas of secondary vegetation [49] and ombrophilous forest in Tocantins, indicating that the vector is adapted to different environments, especially in areas recovering from human interventions or from natural causes.

In the analysis of the cumulative incidence of AVL and classes of land use, there was a negative correlation with agricultural areas, which can be explained by the increasing use of chemicals in plantations that reduce the number of insects [50–53], including sand flies [54, 55], and consequently reduce the number of human cases.

In the state of Mato Grosso, *L. (L.) longipalpis* occurs in the Cerrado biome, in forests and transition zones, which were suggested by some authors as potential breeding sites for this sand fly [56]. *Lutzomyia (L.) longipalpis* is also present in areas of different climatic conditions, such as semiarid areas (in the Caatinga biome), and wetter areas, with high adaptability to different habitats and environmental conditions [57–59]. Considering that the state of Tocantins covers two distinct biomes, Cerrado and Amazon, the occurrence of *L. (L.) longipalpis* in both biomes confirms its generalist behavior, being associated with diverse habitats [14].

Environmental changes, such as deforestation, impact the distribution of tropical diseases [60–63], potentially affecting the spatial distribution of the vectors of leishmaniasis [64]. The state of Tocantins presented constant increase of deforestation, especially in northern and western regions, areas with occurrence of human cases of AVL. In this scenario, Araguaína (municipality with the highest deforested area from 2001 to 2014) has become a priority for the Ministry of Health, for surveillance and control of AVL. In recent years, Araguaína has been producing high records of AVL. From 2007 to 2014, it was the second Brazilian municipality

with the highest production of AVL, while in the years 2007 and 2008 it had the highest number of human cases in Brazil [24].

It is known that AVL transmission remains active in areas with environmental changes, such as deforestation [65]. Thus, it is argued that continuous deforestation processes increase the number of people exposed to infection, creating conditions for the emergence of epidemic outbreaks [10, 16, 48], because it alters the natural conditions and habitats of some species of mammals, hosts of *leishmania*, that become closer to areas inhabited by the human population. This fact enables sand fly vectors with feeding plasticity, such as *L. (L.) longipalpis*, to transmit the parasite to humans [9, 10, 66, 67].

Studies conducted in Mato Grosso do Sul (area that has experienced a loss of native vegetation), demonstrated the presence of *L. (L.) longipalpis* in regions with little vegetation and low humidity, suggesting that the species would be adapted to different environmental conditions, and that it has been associated with human dwellings (captured inside houses) [68]. This study showed positive correlation between deforested areas and human cases, as well as with the incidence of AVL, from 2007, showing that deforested areas have higher incidence rates for AVL, and that deforestation would maintain transmission.

In the state of Tocantins, *L. (L.) longipalpis* was present in all classes of land use, and its dispersion into new areas clearly demonstrates that it is a species adapted to impacted environments, such as large areas of deforestation. In recent years, 57 municipalities, equivalent to 47% of the total, were originally classified as municipalities without transmission of AVL, and they are currently classified as sporadic transmission. According to the Manual of Surveillance and Control of Visceral Leishmaniasis [3], in municipalities with sporadic transmission, the actions related to the vector are limited to the knowledge of the species and dispersion of the sand fly population, besides the canine survey. Such evidence suggests that the actions of surveillance and control, in Tocantins, are not planned in a satisfactory manner, i.e., without considering the loss of large areas of vegetation and as a result people live in risk areas, becoming exposed to the infection of AVL.

The current aims of the CPVL [3] highlight silent municipalities (those with no human or canine cases), suggesting that they must be incorporated into surveillance and control actions of AVL, in order to avoid or minimize the spread of the disease into new areas. For example, in Tocantins, the analysis of stratification in the state has shown that nearly half of the municipalities have gained transmission of AVL through the years. The results presented here show that in the last 12 years, the cities of Araguaína, Paraíso do Tocantins, Porto Nacional, and Palmas remained as intense transmission areas, which demonstrates the continued production of new human cases. The positive correlation between deforested areas and stratification since 2009 clearly shows the influence of growing deforestation on the disease in the state. These observations, coupled with the fact that there was an increase in transmission in 50% of municipalities, possibly due to changes in the environment, demonstrates the real need for an evaluation of the control and surveillance actions being carried

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out, and that in the future the AVL can surprise health managers with a high number of human cases.

In a recent study conducted in the city of Porto Nacional (TO), *L. (L.) longipalpis* was more abundant in urban areas compared to rural areas, confirming its adaptation to these environments. In addition, its anthropophilic behavior and feeding plasticity might have contributed to the installation of the AVL transmission cycle in urban areas and to its maintenance in rural areas [34].

The correlation analysis of the incidence of AVL with the classes of land use showed distinct correlations over the years, demonstrating again that the vector of AVL, *L. (L.) longipalpis* is adapted to different types of vegetation. The positive correlation with urban areas shows that human cases are present in the urbanized environment, and that *L. (L.) longipalpis* was near the urban areas of the state of Tocantins municipalities, corroborating studies that indicate its adaptation to this modified environment [3, 9, 69, 70].

In the urban area of Campo Grande (MS), correlation was found between the abundance of *L. (L.) longipalpis*, percentage of vegetation cover, and the average vegetation index. However, there was no significant association between the diversity of habitats and abundance of the vector; the authors suggest that large trees can offer better microenvironmental conditions favoring the reproduction of the sand fly [71].

Urbanization changes the microclimate in cities and nearby locations, creating heat islands that result in warmer average temperatures when compared to less disturbed areas [72, 73]. Temperature changes might increase vectorial capacity and reshape epidemic curves that determine the receptivity of areas for the pathogen [73].

A study conducted in northeast Brazil showed no significant correlation of *L. (L.) longipalpis* with the average monthly temperature, relative humidity, or precipitation, demonstrating its adaptation to different climatic conditions [74]. In Barra do Garças (MT), a priority municipality to the Ministry of Health, it was found that *L. (L.) longipalpis* was the most abundant sand fly species with abundance peaks occurring during the rainy season, and correlation with relative humidity in urban areas [75].

The diversity of environments where AVL should be considered as determinant factors in the maintenance of the disease added to the biological, geographical, and social factors [12, 48]. This evidence is related with epidemiological data from last decades, which reveals the suburbanization and urbanization of the disease, with outbreaks in major cities and capitals [3].

Most of Tocantins State (88% of its area) is covered by the Cerrado biome, which had 49% of its forest cover cleared due to anthropogenic environmental interventions in the period 2002–2011. The state of Tocantins lost 0.45% of the biome between 2010 and 2011. It is noteworthy that the Cerrado is the second Brazilian biome that suffered the most changes with human occupation, following the Atlantic Forest [49].

5. Concluding remarks

In the current scenario, where environmental changes enhance implications on public health, it is critical that the studies of environmental-related diseases are intensified, especially for vector-borne diseases, such as AVL. *Lutzomyia (L.) longipalpis* is the most important link in the transmission chain of AVL and it is undoubtedly a major biological risk factor, essential for the transition between different epidemiological profiles and for the increasing urbanization of the disease. Clearly, the urbanization of the vector has been the main challenge for the surveillance and control of the disease.

The results presented here demonstrate a correlation between deforestation and the possible emergence of outbreaks, since AVL persists in areas with environmental changes. The increase of deforested areas remained constant in the state of Tocantins, and also showed expansion in the record of human cases, especially in the municipality of Araguaína.

The vector *L. (L.) longipalpis* was present in all land use classes, being adapted to all environments, including impacted areas. This information coupled with the correlation between the incidence of the disease and urban areas demonstrate once again the vector's adaptation to anthropic environments.

Brazil faces geographic expansion and urbanization of AVL and the Manual of Control and Surveillance of AVL from the Ministry of Health has as one of its goals to decrease the vector population and/or the minimization of vector contact with man (reducing the risk of transmission); the manual also has as a challenge to evaluate the vector behavior in the urban area and the factors of its adaptation to new habitats and environmental changes in a way to better understand the spatial dynamics of the disease. It is also hypothesized that vector populations are already resistant to insecticides applied against adult sand flies. As a result, recommended control actions for AVL are focused on early diagnosis and treatment of human cases, reduction of the sand fly vector population, elimination of domestic reservoirs, and additionally, health and education activities aimed in particular at patients and populations at risk of contracting the disease. However, despite well-defined guidelines, the actions are not always successful in controlling the vector.

In this context, despite the efforts made by the local Health Department, the state of Tocantins has a persistent scenario of AVL transmission. It is also important to note that the state has suffered over the last few years through major environmental impacts, especially through large enterprises. Such evidence points to a disturbing scenario regarding the transmission of AVL, one of expansion and urbanization; therefore, the determinants of such impacts must be constantly evaluated.

In conclusion, planning and implementation of public policies are necessary to minimize the impacts of anthropogenic environmental change. The results demonstrate the need to incorporate integrated actions, since AVL is expanding as a result of environmental impacts and the adaptation of the vector *L. (L.) longipalpis* to various habitats.

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Chapter 7

Leishmaniasis in Tunisia: History and New Insights into the Epidemiology of a Neglected Disease

Najoua Haouas and Hamouda Babba

Additional information is available at the end of the chapter

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Abstract

In Tunisia, both cutaneous (CL) and visceral leishmaniases (VL) are historical diseases that have been described since the nineteenth century. Cutaneous form is more prevalent than the visceral one. It is caused by three taxa (*Leishmania major, Leishmania infantum*, and *Leishmania killicki* synonymous *Leishmania tropica*) and six zymodemes (MON-1, MON-8, MON-24, MON-25, MON-80, and MON-317). Among these dermotropic zymodemes, sand flies vectors and reservoir hosts were identified for only three ones. Transmission cycles of *L. infantum* MON-24 and MON-80 and L. *killicki* MON-317 are still unknown. Zoonotic CL is largely distributed and covers mainly the sub-arid and arid bioclimatic stages. Nevertheless, it has recently spread to the humid and sub-humid stages in northern Tunisia. Sporadic and chronic CL are less prevalent with limited geographical distribution. Visceral leishmaniasis (VL) is mainly infantile that affects children of <13 years. It is caused by the single taxon *L. infantum*. Transmission cycle of this parasite is zoonotic but not well elucidated. Three zymodemes are responsible for the genesis of VL (MON-1, MON-24 and MON-80). Only the transmission cycle of *L. infantum* MON-1 is identified. Geographically, VL is mainly distributed in the humid, sub-humid, and semi-arid bioclimatic stages of the country. Despite the large progress of knowledge in the ecoepidemiology of leishmaniases in Tunisia, many parameters of the transmission cycles of these taxa are still unknown and need further investigations to identify them.

Keywords: *Leishmania*, cutaneous leishmaniasis, visceral leishmaniasis, epidemiology, Tunisia

1. Introduction

In the Mediterranean basin region, both cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) are well established diseases with an estimated annual incidence ranges from 239,500

to 393,600 and from 1200 to 2000 cases, respectively. In Tunisia (North Africa, South Mediterranean basin), leishmaniases are largely spread causing a serious public health problem. Clinically, both CL and VL are encountered in this region. Nevertheless, the cutaneous form is most prevalent and largely distributed. Visceral leishmaniasis is less prevalent in this region with a zoonotic transmission of the causative agent.

In Tunisia, leishmaniases are historical. Indeed, the first documented cutaneous case was reported in 1884, while the first VL case was in 1904. Nevertheless, these infectious diseases were stayed neglected for a long period and the epidemiological investigations were scarce. Indeed, an analysis of the published research on leishmaniases in Tunisia over about a century (1884–1980) showed around 20 published items. From the beginning of 1980s of the last century, the number of publications has increased from 5 publications (1981–1985), to 14 publications (1991–1995), 41 publications (2001–2005), and 85 publications (2011–2015).

The aim of this chapter is to review the history of leishmaniases in Tunisia and to present the new insights into the epidemiological features of this disease. This includes clinical forms, transmission cycles, and geographical distribution.

2. Body

As many other regions of the Mediterranean basin, Tunisia is an endemic focus for both cutaneous and visceral leishmaniases. Each of these two clinical forms has its own epidemiological profile.

2.1. Cutaneous leishmaniases

Cutaneous leishmaniasis refers to a dermal lesion which appears at the site of the infected sand fly bite. The lesion is usually painless and characterized by a gradual evolution. It firstly appears as a tiny erythema, which then develops into a papule and nodule that can ulcerate within 2 weeks to 6 months. It heals gradually over months or years. Although CL is mild and not life threatening, its disfiguring lesions and scars with altered pigmentation severely affect the social and psychological functioning of the affected individuals causing anxiety, depression, decrease in body satisfaction, and low quality of life [1–3]. The clinical form of CL lesions varies between patients, reflecting different species of parasite, different virulence degree inside the same species or a difference in the immunological status of patients.

The first real documented case of CL in Tunisia date from 1884 in the region of Gafsa, south Tunisia [4, 5]. Indigenous people named it "Habb El Seneh" (sore of a year) or "Bess El Tmeur" (evil of the dates) related to their supposition that the disease is the result of the consumption of dates, sting of palms or the drinking of the water [6]. In 1882, Achard, military physician in Gafsa, gave the infection the name of "Clou de Gafsa" (boil of Gafsa). It was only in 1905 that Nicolle and Cathoire made microbiological analysis of the sore scraping and reported the presence of small oval bodies sized of 4 μm similar to those already described by Wright in 1903 in the oriental sore [7–9]. While Wright proposed the name *Helcosomatropicum* to the

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reported protozoa, Nicolle as well as other authors named them *Leishmania tropica* [6]. In 1908, Nicolle made the first isolation of the parasite by using the Novy-MacNeal media and inoculated it to the monkey *Macacus sinicus* in order to reproduce the boil of Gafsa [10].

Given that the Gafsa boil was almost observed on the uncovered parts of the body, that the infection was restricted to some cities of Gafsa near water sources, and that patients reported the bite of insects few days before the onset of the lesion, Billet supposed that the infection is transmitted by the bite of the mosquito Pyretophorus chaudoyei [11]. It was only in 1921 that the brothers Sergent proved the transmission of CL by the female sand fly *Phlebotomus papatasi* [12]. Since that date, some studies were conducted on the phlebotomine fauna in some Tunisian regions such Tunis, Zaghouan, Kebili, El Kef, and Makhtar [13–18].

Nevertheless, few were the data available on the incidence of the disease, its geographical distribution and the causative species. Since the 1980s of the last century and by the introduction of both biochemical and molecular tools, many research teams have investigated the epidemiology of CL in many regions of the country focusing on the characterization of the parasite and the identification of both reservoirs and phlebotomine sand fly hosts.

The precise characterization of the parasite circulating in Tunisia started in 1981 using the gold standard method (isoenzymatic analysis) [19]. Since then, many research teams have been involved in the isoenzymatic analysis of *Leishmania* strains. Three taxa were identified to be responsible for the genesis of CL: *Leishmania major, Leishmania infantum*, and *Leishmania killicki*.

2.1.1. Cutaneous leishmaniasis due to Leishmania major

2.1.1.1. History

Zoonotic cutaneous leishmaniasis (ZCL) due to *L. major* was the first described form of leishmaniasis in Tunisia in 1884 by Deperet and Boinet in the region of Gafsa, southwest Tunisia. Between 1882 and 1893 outbreaks affected different regions of Gafsa and thereby autochthonous cases were recorded each year in this region [6].

In 1908, an extension of the Gafsa boil occurred from the southwest (Gafsa) to the west (Feriana, Kasserine) and southeast (Aioun, Tataouine) regions. Since then, the endemic area did not go beyond Kasserine (Sbeitla) [20]. The Tunisian Centre has been free from ZCL until a major outbreak in 1982 in the Sidi Saad Region (Kairouan) [21, 22]. Then, ZCL has spread to many foci in centre and south of Tunisia [23, 24]. Ruiz Postigo 2010 [25] reported an annual incidence of 2750 new case of ZCL. Nevertheless, the true incidence of this noso-geographical form of CL is underestimated due to multiple factors including the increasing prevalence of the disease, the unrecorded cases, and the expanding areas of endemicity.

2.1.1.2. Clinical forms

L. major is responsible for localized cutaneous lesions. It is always "wet" with a deep ulceration in the center and covered by a crust. It heals slowly leaving ugly scars that severely affect the social and psychological functioning of the affected individuals.

Although *L. major* is the main species responsible for CL in Tunisia, a very limited number of studies have investigated and explored the clinical spectrum of CL caused by this *Leishmania* species. Indeed, the published studies have described the clinical polymorphism of cutaneous leishmaniasis without any isoenzymatic or molecular identification of the causative species. Masmoudi et al. have studied the different clinical aspects of CL in some zoonotic CL foci of the centre and the south of the country. Eleven different clinical forms of ZCL (vegetative, impetiginoid, erysipeloid, necrotic, warty, erythematosquamous, lupoid, sporotrichoid, papulous, eczematoid, and recidivans) were reported but without any identification of the parasite. The ulcerocrusted form was the most predominant form (54.9%) followed by the sporotrichoid and lupoid aspects with 18.6 and 15.7%, respectively [26, 27]. A recent study conducted in 2012 in our laboratory has analyzed the clinical polymorphism of CL due to *L. major* based on the identification of the parasite by PCR sequencing. Thus, 12 clinical forms were noticed. The most common type was the ulcerocrusted form (38.66%) followed by the papulonodular form (16%) and the impetiginous form (13.33%). The ulcerated, mucocutaneous, lupoid, and sporotrichoid forms were less common. The eczematiform, erysipeloid, verrucous, psoriasiform, and pseudotumoral types were represented by a single CL case (1.33%) (unpublished data) (**Figure 1**).

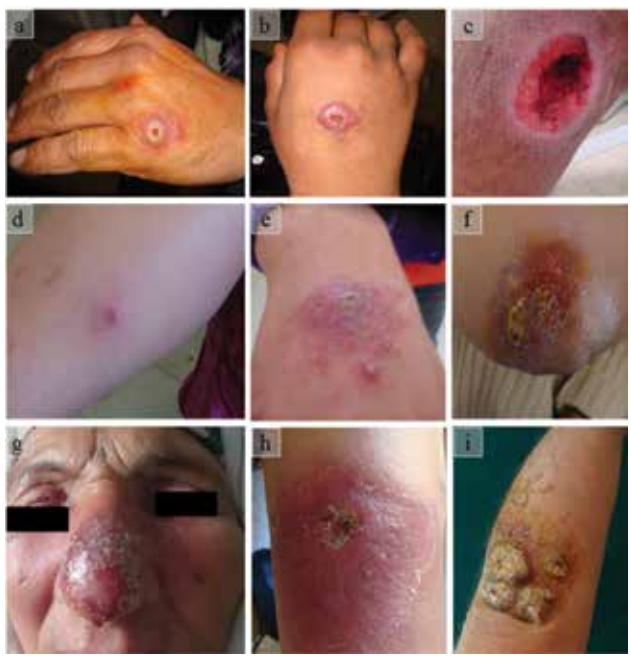

Figure 1. Clinical forms of CL lesions caused by *L. major*: (a, b) ulcerocrusted, (c) ulcered, (d) nodular, (e) papulonodular, (f) warty, (g) lupoid, (h) erysipeloid, and (i) vegetative. (Laboratory of Parasitology-Mycology, Faculty of Pharmacy, University of Monastir, Tunisia).

This clinical polymorphism seems to be rather high, which could reflect the complexity of the disease involving several factors related to the parasite (virulence, parasitic load, and the

presence of other pathogens), the type and duration of clinical lesion, the geographic location, the disease reservoir, and the host immune status [28, 29].

2.1.1.3. Causative species

The precise characterization of the parasite circulating in Tunisia foci started only in 1981 [19]. Since then, many research teams have been involved in the isoenzymatic analysis of *Leishmania* strains from ZCL foci. All the strains were identified as *L. major* with the single zymodeme MON-25. This genetic homogeneity gathered with the wide distribution of MON-25 suggests the rapid diffusion of this zymodeme in many Tunisian provinces [23, 24, 30, 31].

2.1.1.4. Transmission cycle

L. major has a zoonotic transmission cycle. The fat sand rat *Psammomys obesus* and the gerbils *Meriones shawi* and *Meriones libycus* are reservoir hosts [32, 33]. Recently, a natural infection with *L. major* zymodeme MON-25 has been reported in a specimen of least weasel (*Mustela nivalis*) suggesting its potential role as reservoir host of ZCL [34].

In 1987, Ben Ismail et al. [33] proved that *Phlebotomus* (*P.*) *papatasi* is the vector of *L. major* in Tunisia. Indeed, the isoenzymatic identification of isolated promastigotes from infected females of *P. papatasi* revealed the zymodeme MON-25 already identified in human CL cases (**Figure 2**). In Tunisia, This sand fly species is essentially spread in semi-arid, arid, and Saharan bioclimatic stages [35, 36].

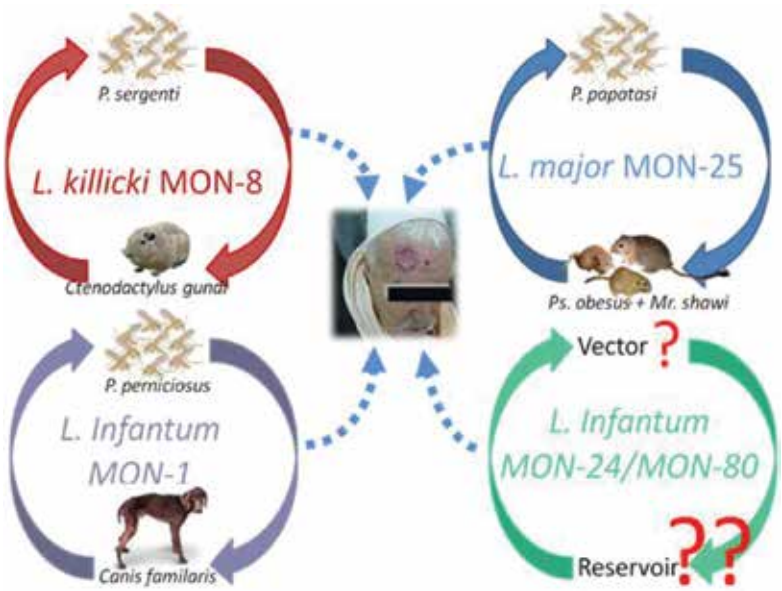

Figure 2. Transmission cycles of the dermotropic *Leishmania* zymodemes in Tunisia. (Laboratory of Parasitology-Mycology, Faculty of Pharmacy, University of Monastir, Tunisia).

2.1.1.5. Geographical distribution

Zoonotic CL due to *L. major* is the main noso-geographic form widespread in whole central and southern part of Tunisia. In 2002, 15 of 23 Tunisian provinces were considered as endemic for ZCL with two to three thousand cases annually [37]. Since 2012, *L. major* has been reported in 19 provinces. Out of them, seven were in the north of the country. Key factors leading to the spread of this disease are presently unknown. Dynamics of rodent populations, vector dispersal, and climate change may be involved in the spatiotemporal dynamics of the disease [24] (**Figure 3**).

Figure 3. Geographical distribution of dermotropic *Leishmania* taxa [24].

2.1.2. Cutaneous leishmaniasis due to Leishmania infantum

2.1.2.1. History

L. infantum is primary known as a viscerotropic species responsible for the genesis of visceral leishmaniasis. Nevertheless, the first case of CL due to this species in Tunisia was described in

1916 in Sakiet Sidi Yousef, El Kef (north of Tunisia) by Nicolle and Blanc (1918) [20]. This disease has been sporadically reported with an annual incidence of approximately 20 or 30 cases per year [38]. The geographical distribution of this sporadic form overlaps with that of VL.

2.1.2.2. Clinical forms

Unfortunately, the low prevalence of sporadic CL (SCL) as well as the absence of published data concerning the clinical polymorphism of this noso-geographical form of CL prevents us to make a define description of the lesion. Previous studies reported that in over 80% of cases, CL caused by *L. infantum* is characterized by a single small lesion on the face. The ulcerocrusted form is the most common [38, 39]. Nevertheless, lupoid with a striking infiltrated patch, erythematous and squamous forms were also reported.

2.1.2.3. Causative species

The precise identification of the causative agent of SCL using the golden standard method has been started in the beginning of the 1990s of the last century. Thus, three zymodemes of the *L. infantum* taxon were identified as responsible of SCL. *L. infantum* MON-24 was the first identified dermotropic zymodeme [40]. Currently, it is the most isolated one from SCL lesion in Tunisia [24, 39, 41]. Its represents between 90.56 and 92.85% of all SCL cases in Tunisia [24, 41]. The second identified dermotropic zymodeme is MON-1. The first case of SCL caused by *L. infantum* MON-1 was reported by Aoun et al. in 2000 [38]. This dermoviscerotropic zymodeme is less frequent than *L. infantum* MON-24. Indeed, it represents between 7.14 and 8.5% of the SCL cases in Tunisia [24, 41]. The last one is the zymodeme MON-80. Only a single sporadic case of CL due to this zymodeme was reported in 2012 in Zaghouan, north Tunisia [24] (**Figure 3**).

2.1.2.4. Transmission cycle

The transmission cycle of *L. infantum* is not completely elucidated. While *L. infantum* MON-1 was isolated from the domestic dog *Canis familiaris* suggesting that this animal is the reservoir of this zymodeme, the reservoir hosts of the other zymodemes MON-24 and MON-80 are still unknown (**Figure 2**). In 2009, Benikhlef et al. [42] reported three cases of canine VL in Tunisia due to *L. infantum* MON-80; nevertheless, its role as reservoir for this zymodeme is still discussed. The zymodeme MON-24 was also isolated from dogs in Morocco and Algeria but never in Tunisia [43, 44].

Phlebotomus perniciosus was described as the vector of *L. infantum* MON-1. However, the vector species of MON-24 and MON-80 are still unidentified. In Tunisia, *P. perfiliewi* is abundant in *L. infantum* CL foci and was thereby suspected to be the vector of *L. infantum* [35]. Nevertheless, no *L. infantum* strain has been isolated from this phlebotomine sand fly species yet. Also, *L. infantum* DNA was detected from a *P. langeroni, P. longicuspis, P. perfiliewi, P. papatasi*, and *Sergentomyia minuta* using molecular tools [45, 46]. However, neither parasite isolation from these sand flies species nor *L. infantum* isoenzymatic identification were carried out in Tunisia yet to confirm their role as vector of *L. infantum* taxon.

2.1.2.5. Geographical distribution

Geographical distribution of SCL is apparently restricted to the humid and sub-humid bioclimatic areas. Its distribution overlaps with that of VL in north and central Tunisia. Indeed, Haouas et al. reported that 95.3% of dermotropic *L. infantum* strains were isolated from the north of Tunisia, 2.83% from the centre (Kairouan and Sidi Bouzid) and 1.9% from the south (Sfax) [24]. The sporadic cases reported in the south of the country suggested an extension of SCL to the arid bioclimatic areas [24, 41]. Also, *L. infantum* MON-24 was unevenly distributed from the northern areas of the country: It was mainly isolated in Siliana, Manouba, Béja, Bizerte, and Jendouba provinces. The dermoviscerotropic zymodeme MON-1 was isolated in northern Tunisia, mainly in Siliana province, and the single CL *L. infantum* MON-80 strain was isolated in Zaghouan province (**Figure 3**).

2.1.3. Cutaneous leishmaniasis due to Leishmania killicki (synonymous L. tropica)

2.1.3.1. History

Chronic cutaneous leishmaniasis (CCL) due to *L. killicki* was discovered for the first time on the basis of 30 strains isolated in 1980 when an outbreak of cutaneous leishmaniasis occurred in the microfocus of Tataouine in southeast Tunisia [32, 47]. The annual incidence of this disease was estimated to 10 cases per year [48].

The taxonomic status of *L. killicki* is not well defined yet. Indeed, it was initially characterized within the *L. tropica* complex [49]. By the revision of the *Leishmania* genus classification, *L. killicki* was considered as a separate phylogenetic complex [50]. In 2009, an update study by Pratlong et al. confirmed the inclusion of *L. killicki* within the *L. tropica* complex [51]. Phenetic and phylogenetic studies using multilocus microsatellite typing [52], PCR sequencing [53], and multilocus sequence typing (MLST) [54] also classified *L. killicki* within the *L. tropica* complex. A recent study on the evolutionary history of *L. killicki* relative to *L. tropica* suggested that *L. killicki* emerged from a single founder event and evolved independently from *L. tropica*. Thereby, they suggested naming this taxon *L. killicki* (synonymous *L. tropica*) [55, 56].

2.1.3.2. Clinical forms

Clinically, the lesion is frequently unique, ulcerous with a scab of 1–3 cm in diameter located on the face, with chronic evolution that can last for 4 years [57, 58] (**Figure 4**).

2.1.3.3. Causative species

The first description of CL due to *L. killicki* in 1980 was based on the isoenzymatic identification of about 30 strains from southeastern Tunisia. All of them were characterized as *L. killicki* zymodeme MON-8 [32]. Since this date and over a period of 36 years, only about 90 *L. killicki* strains were identified using the golden standard method [24, 30, 32, 51, 56]. The isoenzymatic analysis showed the presence of two zymodeme inside *L. killicki* taxon. Zymodeme MON-8 is the most frequent one. However, a new zymodeme MON-317 has been recently identified from two patients in Metlaoui, southwestern Tunisia [56].

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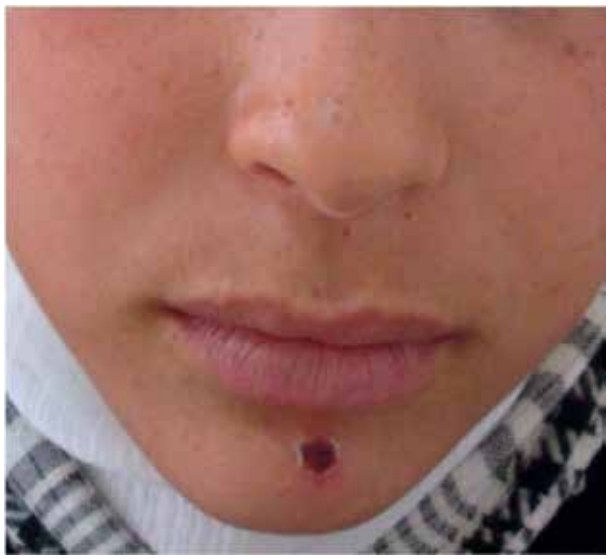

Figure 4. Ulcerocrusted lesion of the neck caused by *L. killicki*. (Laboratory of Parasitology-Mycology, Faculty of Pharmacy, University of Monastir, Tunisia).

2.1.3.4. Transmission cycle

In the last century, the transmission cycle of *L. killicki* in Tunisia was considered as anthroponotic. However, in 2011, two epidemiological studies realized in the southwest and southeastern of Tunisia described the natural infection of *Ctenodactylus gundi* with *L. killicki* using molecular techniques suggestion a zoonotic transmission of this taxon [59, 60]. By the detection of *L. killicki* in its mid gut, *P. sergenti* was suspected to be the probable vector of this taxon [36, 61].

2.1.3.5. Geographical distribution

L. killicki was firstly described in the focus of Tataouine (southeast of Tunisia) [47]. For more than 20 years, no case was described outside this focus. Since 2004, some cases have been reported in Kairouan and Sidi Bouzid (centre of Tunisia), in Gafsa in the southwest and in Siliana in the north of the country [31, 62, 63]. A recent study focusing on the evolutionary history of this parasite using the microsatellite typing has supported the hypothesis of a zoonotic transmission cycle for *L. killicki* (syn. *L. tropica*) and suggested that Gafsa could be the historical focus of this parasite [56].

2.2. Visceral leishmaniases

Visceral leishmaniasis refers to the dissemination of the parasite *Leishmania* to the spleen, liver, lymphatic nodes, and bone morrow of the patient. A multitude of clinical features of the disease ensue gradually, the most important being splenomegaly, recurring and irregular fever, anemia, pancytopenia, weight loss, and weakness.

2.2.1. History

The first case of VL in Tunisia was reported by Laveran and Cathoire in 1904 [64] in the region of La Goulette, north of the country. Between 1904 and 1908, Charles Nicolle reported two new VL cases of children living in Tunis (north of the country). Since this date and till 1935, Charles Nicolle and collaborators reported 120 new VL cases mainly distributed in the north (Tunis, Bizerte, Zaghouan, Grombalia, Beja, and El Kef), the centre (Sousse and Kairouan) with one case in Tozeur (south of Tunisia) [65]. Some outbreaks of VL were reported in centre Tunisia mainly Kairouan where 247 cases were reported between 1984 and 1996 [66].

Since the description of VL in Tunisia, the annual incidence has increased progressively going from three cases in beginning of the twentieth century to 57 in the 1980s of the same century [66]. Currently, VL in Tunisia shows a stable incidence of about 100 cases per year [67, 68].

2.2.2. Clinical forms

As many other Mediterranean basin countries, LV in Tunisia has an infantile form affecting mainly children under 5 years. Indeed, the age of infected children ranged from 2 months to 13 years with a median, 18 months. The most common clinical symptoms at admission were splenomegaly, fever, and hepatomegaly. The principal biological disturbances were anemia, thrombocytopenia, and leucopenia [69].

While infantile VL is the most common form in Tunisia, cases of VL in both immunocompetent and immunocompromised (with HIV infection) adults were also reported in Tunisia [24]. Twenty-two (22) cases of adult VL (including six patients infected with HIV virus) were recorded over a period of 20 years [70]. Within this group, the triad of VL symptoms (fever, anemia, and splenomegaly) was less stable.

2.2.3. Causative species

The isoenzymatic identification of the isolated parasite have revealed three zymodemes of a single taxon *L. infantum*: The zymodeme MON-1 is the most identified one (89.12% of the VL cases). It was isolated in both infantile and adult VL cases [24]. The second zymodeme responsible for VL is *L. infantum* MON-24. The first case of VL due to this zymodeme was reported in 2000 [71]. Currently, *L. infantum* MON-24 is responsible for 8.08% of VL cases in Tunisia [24]. Finally, *L. infantum* MON-80 was identified in some sporadic VL cases in centre Tunisia (Zaghouan and Kairouan). It is responsible for 2.07% of VL cases [24].

2.2.4. Transmission cycle

The domestic dog has been incriminated in the transmission of VL since the first report of canine leishmaniasis in 1908 [72]. By the introduction of the isoenzymatic analysis, all strains isolated from infected dogs throughout the country were identified as *L. infantum* MON-1 [24, 30]. This result confirms the dog as the reservoir host of zoonotic VL caused by the zymodeme MON-1. In 2009, Benikhlef reported three cases of canine VL in Tunisia due to *L. infantum* MON-80; nevertheless, their role as reservoir for this zymodeme is still discussed [42].

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At the middle of the twentieth century, *P. perniciosus* has been reported to be the vector of *L. infantum* MON-1 and the complete life cycle was demonstrated [73]. However, the vector hosts of the two other zymodemes MON-24 and MON-80 are still unknown (**Figure 5**). Recently, *L. infantum* DNA was detected in the sand flies mid gut of the *Larroussius* (*P. langeroni*, *P. longicuspis*, *P. perfiliewi*) and *Phlebotomus* (*P. papatasi*) subgenera as well as the *Sergentomyia* genus (*S. minuta*) [45, 46]. However, neither parasite isolation from these sand flies species nor *L. infantum* isoenzymatic identification were carried out in Tunisia yet to confirm their role as vector of *L. infantum* zymodemes.

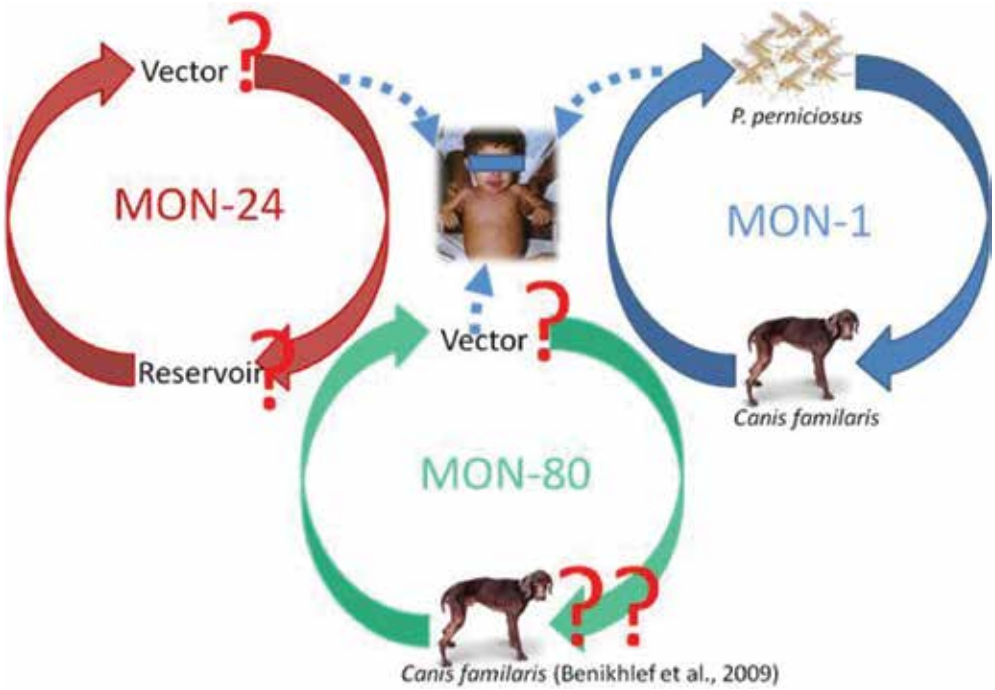

Figure 5. Transmission cycles of the viscerotropic *Leishmania infantum* zymodemes in Tunisia. (Laboratory of Parasitology-Mycology, Faculty of Pharmacy, University of Monastir, Tunisia).

2.2.5. Geographical distribution

Until 1980s, geographical distribution of VL in Tunisia was limited to the humid, sub-humid, and semi-arid bioclimatic stages. The main endemic foci were localized in the north of the country including Zaghouan, Kef, Jendouba, Seliana, Nabeul, Beja, and Tunis [24, 74, 75]. However, more recently, VL has extended to the arid areas in central and southern Tunisia including Kairouan, Monastir, Kasserine, Sfax, Gabes, Sidi Bouzid, and Tozeur [24, 41, 66, 75–77] (**Figure 6**). Such extension could be the result of many factors including the travel of the reservoir host and the environmental changes sustaining sand flies populations.

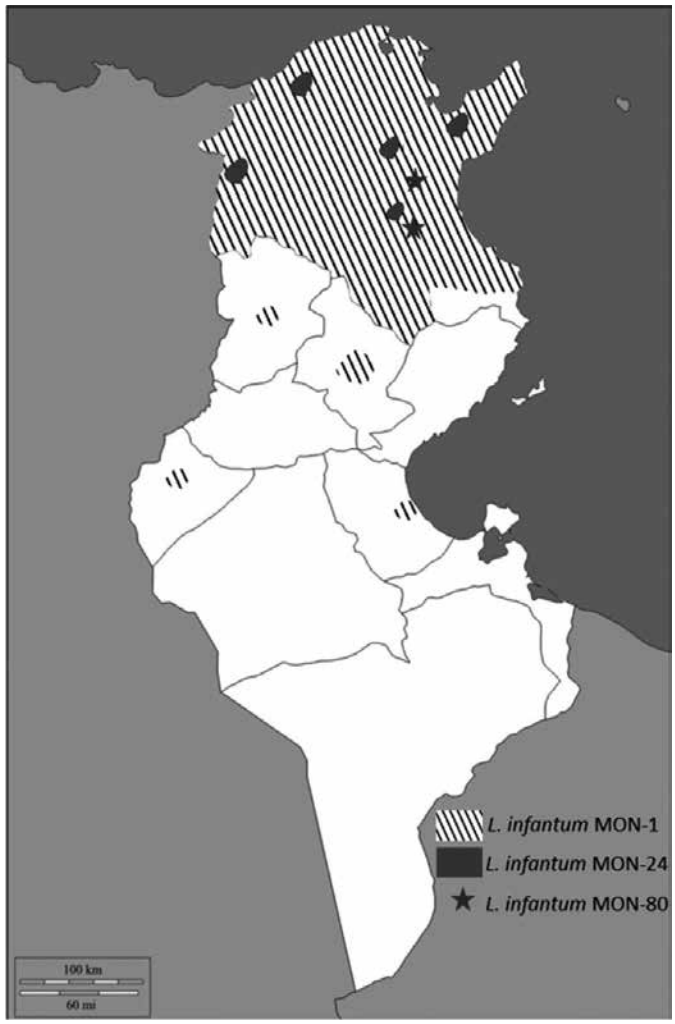

Figure 6. Geographical distribution of viscerotropic *Leishmania infantum* zymodemes [24].

3. Conclusion

Both cutaneous and visceral leishmaniases are old infectious diseases in Tunisia. Over more than a century since the discovery of the disease, we have witnessed an extraordinary progress in the knowledge of the epidemiology of *Leishmania* infection. At least six transmission cycles are present in this geographical area. Unfortunately only the life cycle of *L. major* MON-25, *L. infantum* MON-1, and *L. killicki* MON-8 are elucidated. Many other investigations are urgently needed to understand the dynamic of the different zymodemes in the Tunisian foci. The absence of data on the vector and the reservoir hosts of some zymodemes prevent us to follow

and predict the spatiotemporal evolution of the disease and consequently to establish effective control strategies.

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Chapter 8

Eco-Epidemiological and Immunological Features of Localized Cutaneous Leishmaniasis in Southeastern Mexico: Thirty Years of Study

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Additional information is available at the end of the chapter

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Abstract

The Yucatan Peninsula is considered an important endemic area of localized cutaneous leishmaniasis (LCL) caused by *Leishmania (Leishmania) mexicana* and mainly the states of Campeche and Quintana Roo where 41.5% of all new cases in Mexico were reported in 2015. People were affected due to the lack of the resources for early diagnosis and treatment and although many aspects of the disease are known, control of LCL is absent in this region. Thus, better case detection and epidemiological surveillance are required. The presence of emerging focus and changes in the clinical form suggest the importance of continuing the eco-epidemiological studies, which could lead to the implementation of a sustainable control on the disease. In this review, we focus on the results of our multi-disciplinary studies carried out in the southeastern Mexico, including LCL burden, clinical aspects, causal agents, vectors, reservoirs and the host immune response to *Leishmania (L.) mexicana* infection.

Keywords: ecology, epidemiology, immune response, localized cutaneous leishmaniasis, southeastern Mexico

1. Introduction

American cutaneous leishmaniasis (ACL) is a vector-borne protozoan zoonotic disease widely spread in Latin America. At least 12 different *Leishmania* species cause ACL. The disease occurs

in countries from the United States to Argentina, except in Uruguay and Chile. ACL is an important health problem affecting the poorer population and is thus one of the most neglected diseases. Depending mainly on the *Leishmania* species and the host immune response, the spectrum of clinical forms includes localized cutaneous leishmaniasis (LCL), disseminated and diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL). The former is the most commonly occurring disease with approximately 0.7–1.2 million new cases every year [1, 2].

In south-eastern Mexico, the Yucatan Peninsula is an important endemic area of LCL, locally known as the "chiclero´s (gum collectors) ulcer". The LCL was first described by Seidelin in 1912, who classified the agent as morphologically indistinguishable from *Leishmania tropica* [3]. Since then, the humid forest of the Yucatan Peninsula has been documented as an endemic focus of LCL [4–6].

The purpose of this chapter is to review the most relevant studies performed in the last 30 years in the Laboratory of Immunology of the Autonomous University of Yucatan. This research has covered the characterization of the "chiclero´s ulcer", its diagnosis and treatment, and the identification of risk factors as well as the *Leishmania* vectors and reservoir species that are important to be known in order to develop control strategies.

2. Epidemiology of LCL in southern Mexico

2.1. Incidence and prevalence

Leishmaniasis control is usually hampered by ignorance of the true incidence/prevalence of infection, thus underestimating human suffering and disability caused by the disease. After parasites are inoculated by a sand fly, the infection outcomes might be either an asymptomatic infection or a clinically manifested infection. Most studies done in Latin America with reference to ACL have been focused on incidence/prevalence of the disease (clinical infection).

In the first approach and after diagnostic tools were implemented, a total of 63 cases of LCL were recorded in the state of Campeche between 1982 and 1987. The most common clinical presentation was a chronic ulcerated lesion (with an evolution time longer than 10 years), located predominantly on the ear (39%). Single lesions were found in 49/63 (78%) cases affecting men working in the field [7].

Based on these data, a program for the study and surveillance of LCL in collaboration with health services from the state of Campeche was established. First of all, a study to determine the incidence of LCL was carried out in seven rural health communities of the state of Campeche from January to December, 1987. Montenegro skin test (MST) was carried out on a sample batch of 449 persons randomly selected from men aged 15–45 years. Risk factors including age (15–45 years old), sex (male) and exposure (working in the field) had been identified previously [8]. MST-positive response ranged from 24 to 90% among the communities studied. These wide-range results could reflect differences in endemicity of LCL in the state of Campeche. A total of 56 new LCL cases with both a positive parasitological diagnosis

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(smear, isolation-culture and/or biopsy) and MST-positive response were recorded during 1987. In summary, an annual incidence rate of 0.5 per 1000 inhabitants was reported [9].

Asymptomatic infection is the term used to refer to those individuals living in endemic areas of LCL, exposed to sand fly bites, presenting a MST-positive response but without signs and symptoms of the disease. Based on the criteria given above, a study to determine the prevalence of asymptomatic infection was performed in four rural communities from Campeche. From January to December 1999, a total of 22/116 (18.9%) men of 15–45 years of age and working in the field showed a MST-positive response in the absence of signs and symptoms [10]. Asymptomatic infection by *Leishmania* is the most common outcome after parasite inoculation. It must be highlighted that studies of the biological, immunological and epidemiological significances of the asymptomatic infection have been neglected in Latin America. Therefore, this important challenge should be addressed.

In the state of Yucatan, cases of LCL were restricted to villages located in the South, near to the characterized endemic areas from Campeche and Quintana Roo. Recently, a new outbreak of LCL was reported in the municipality of Tinum, Yucatán. This village is located in the West of the state and no cases had been reported before. In 2015, 17 new cases were recorded by the health services of the Yucatan State in comparison with the only case reported in 2014. From those cases, 11 were from Tinum. This increased incidence is alarming and suggests possible changes in the epidemiological patterns of leishmaniasis in the Yucatan Peninsula that need to be studied [11].

2.2. Clinical picture

The clinical picture of LCL in the Yucatan Peninsula was characterized through a study performed between January 1990 and December 1995 [12]. A total of 683 patients with cutaneous lesions suggestive of LCL were examined. Parasite demonstration by smear, biopsy and/or isolation-culture was positive in 445 cases (65.1%). From these samples, *Leishmania (Leishmania) mexicana* was successfully isolated, cultured and identified by either isoenzyme characterization and/or monoclonal antibodies in 136/445 cases (30.5%). The LCL clinical picture was limited to these 136 cases in which *L. (L.) mexicana* was identified. Males (94.1%) between 10 and 40 years of age (85.3%) were mainly affected. The most common lesions were single (84.5%), rounded (52.6%), ulcerated (72.5%) and located on the ear (39.9%). A total of 72.8% cases detected were classified as acute with less than 3 months of evolution. Since in those years an active surveillance program for LCL was implemented in the state of Campeche most cases were acute. In summary, the clinical picture of LCL caused by *L. (L.) mexicana* is characterized by a commonly single, rounded, painless ulcerated lesion, without lymphangitis and/or adenopathy; with the absence of mucosal involvement, and when located on the ear (the most common location) tends to become chronic if left untreated, causing the destruction of the pinna and disfiguration.

The importance of the active surveillance program was highlighted by the observation of changes in the clinical form with time. The manifestation of LCL has evolved, during the last years, from the typical single, rounded, small and ulcerated lesion worldwide recognized as "benign" (**Figure 1A–C**), to nodular lesions with an increased diameter as well as the

appearance of multiple lesions (**Figure 1D–F**). Those findings are suggestive of changes in pathogenicity of the parasite that need to be studied.

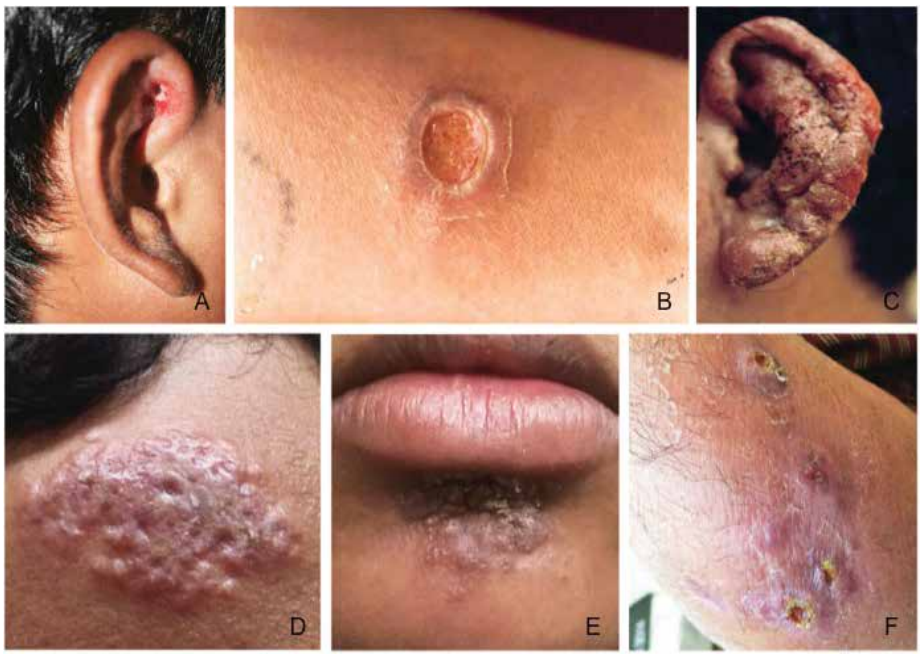

Figure 1. Clinical spectrum of the LCL in southeastern Mexico. (**A)** Acute ulcerated lesion on ear; (**B)** typical small, rounded, ulcerated lesion in forearm; (**C)** chronic lesion on ear; **(D and E)** nodular infiltrated lesions; (**F)** multiple ulcerated lesions in forearm.

2.3. Histopathological picture

From the previous clinical study, 73 biopsies were taken to characterize the histopathology of LCL caused by *L. (L.) mexicana*. The histopathological picture observed varied widely impairing classification into a meaningful pattern. Magalhães classification [13] identified a total of five histopathological patterns: type I) exudative-cellular reaction due to infiltration of histiocytes, lymphocytes and plasma cells, without granuloma; type II) exudative-necrotic reaction, characterized by cellular infiltration, necrosis and no granulomatous response; type III) exudative and necrotic-granulomatous reaction (unorganized granuloma) corresponding to pattern described as chronic granulomatous inflammation with necrosis; type IV) exudative granulomatous reaction (unorganized granuloma) without necrosis characterized by the presence of an unorganized granulomatous reaction; type V) exudative tuberculoid reaction in which a typical tuberculoid granuloma (organized) is formed. According to those histopathological patterns, 28.7% of type III and 43% of type IV were most commonly found. Parasite identification was positive in 68.5% of the biopsies. The size of the lesion was directly correlated with the time of evolution; however, an inverse correlation between the lesion size and abundancy of amastigotes was detected [14]. Therefore, the histopathology of LCL caused by

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L. (L.) mexicana as in other leishmaniasis is characterized by a chronic granulomatous inflammatory response to this obligated intracellular protozoan infecting the macrophages.

2.4. Treatment

An investigation on the response of LCL to treatment with pentavalent antimonials ($Sb5^+$) was carried out between January 1990 and December 1994 [15]. This study was not designed to be a controlled clinical trial, but rather to evaluate the response of the chiclero´s ulcer to treatment with meglumine antimoniate. Patient eligibility for the study included a confirmed diagnosis of acute LCL (time of evolution lesser than 12 months) based on both clinical diagnosis and parasite visualization by smear, biopsy and/or isolation-culture, as well as no previous treatment with any antileishmanial drug, absence of any serious concomitant disease, and to be available for a 12-month follow-up. In all the 105 cases presented, at the end of the treatment, a complete re-epithelialization of all lesions occurred without both residual erythema and relapse during a 1-year monthly follow-up. The mean number of injections required for complete re-epithelialization of chiclero's ulcer was 25.1 (range = 5–60), with a daily dose of one ampule. Since then LCL caused by *L. (L.) mexicana* in the Yucatan Peninsula has been successfully treated with a daily dose of meglumine antimoniate for 20 days, average 10 mg/kg/day. The dose seems lower dose compared to international recommendation of 20 mg/kg/day but it is effective. Moreover, the lack of report on resistance to the treatment is important to point out.

3. Ecology of the endemic area

The Yucatan Peninsula is a discrete biotic province of approximately 143,500 km^2. The region is a broad, flat shelf of marine limestone of geologically recent formation (Paleocene to Recent). The peninsula includes the states of Yucatan, Campeche, Quintana Roo and a portion of Tabasco east of the Rio Usumacinta and north of the Sierra del Norte de Chiapas. The peninsula is surrounded on three sides by water and bounded on the south by highlands that isolate this region from the rest of Central America. An interesting observation is that because of its geographic isolation, the Yucatan Peninsula is an area of mammalian endemism with fauna that differs markedly from the rest of Mexico. The climate is subtropical with a relative humidity of 80%, an unpredictable rainy season (annual rainfall over 1401 mm) mostly during the summer, and an average temperature of 27 ± 5°C [16].

3.1. Parasites

Epidemiological studies and molecular characterization of the New World leishmaniases have revealed that the genus *Leishmania* Ross, 1903 (Protozoa: Trypanosomatidae) is by far much more complex than originally thought. The genus is comprised of 30 species infecting a wide variety of mammalian hosts (wild or domestic) and vectors. Each of the New World species of *Leishmania* has unique ecological and geographical distributions [17]. From an epidemiological point of view and disease-control stand-point, to know whether an organism, causing the

disease in a given area, is of the same species as that found in suspected mammalian reservoirs and insect vectors, is very important [18].

Based on both the clinical and epidemiological features of the disease, as well as on the biological characteristics of the parasite in laboratory animals [19–21], *L. (L.) mexicana* Biagi, 1953 emend. Garnham, 1962, was considered the main agent of LCL in the Yucatan Peninsula. Nevertheless, its characterization at the genus and sub-genus level was not done in those studies.

Therefore, from January 1990 to July 1992, 153 patients with LCL determined by both clinical diagnosis and parasite visualization (smear, biopsy and/or isolation-culture) were studied. All of them were infected in the state of Campeche. Parasite isolation by needle aspirates taken from the edge of the lesions was positive in 49%. Isolates were characterized by isoenzyme markers (glucose phosphate isomerase, mannose phospate isomerase, nucleoside hydrolase, phosphoglucomutase, 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase). Seventy (93.3%) were identified as *L. (L.) mexicana* Biagi, 1953 emend. Garham, 1962 and 5 (6.7%) as *Leishmania (Viannia) braziliensis* Viannia 1911 emend. Matta, 1916 [22].

Later on, a study to identify *Leishmania* parasites isolated from humans and wild rodents from the state of Campeche, using IFA with Mabs was carried out. The main purpose was to determine if the parasites of both types of hosts were of the same biotype. All the isolates obtained from wild rodents reacted with monoclonal antibodies M7 and M8 and were thus identified as *L. (L.) mexicana*. No differences in reactivity patterns were found among the different strains of *L. (L.) mexicana* from humans and wild rodents [22].

Finally, to assure that *L. (L.) mexicana* is the main agent causing LCL in the state of Campeche, a study leading to the identification by PCR of *L. (L.) mexicana* in the potential vectors *Lutzomyia olmeca* and *Lutzomyia cruciata* was performed [23].

3.2. Vectors

Phlebotomine sand flies (Diptera: Psychodidae: Phlebotominae) are insects of medical and veterinary importance since they are involved in the transmission of diverse pathogens [24]. The blood-feeding females are usually considered as the only natural vectors of protozoan *Leishmania* species. Among the phlebotomine sand flies recorded in the New World, 56 species, all belonging to the genus *Lutzomyia*, are involved in the transmission of the *Leishmania* spp. [25].

To determine the transmission of leishmaniasis in south-eastern Mexico, vectors were captured near La Libertad, municipality of Escárcega, Campeche (<200 m) and in a medium-sized subperennial forest located 8 km southeast of this village. Near La Libertad, nine sand flies species were collected using CDC light traps, a Shannon trap and a mouth aspirator; the most abundant species were *Lutzomyia deleoni* (61.3%) and *Lu. cruciata* (13.7%). The highest number of sand flies was obtained with CDC traps (46.5%) followed by direct searching in natural shelters (37.0%) and in the Shannon trap (16.5%). The highest peak of abundance of *Lu. cruciata* was obtained in March. The population peak of *Lu. cruciata* was related to the low temperature (21–22°C), high levels of humidity (≥80%) and low rainfall. The anthropophilic species like

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Lu.cruciata, Lu. ovallesi, Lu. panamensis, Lu. shannoni had the lowest densities around the village. In contrast, at 8 km southeast of La Libertad in the forest, 16 species were caught using CDC light traps and a Shannon trap. The most abundant species were *Lu. olmeca* (21.7%), *Lu. cruciata* (19.2%), and *Lu. ovallesi* (14.1%). *Lu. olmeca* had the highest leishmania infection rate of all sand flies collected in the forest. In both study areas, more females than males were captured. The low densities of anthropophilic sand flies species captured near the village and the abundance of *Lu. olmeca* females (zoophilic species) suggested the sylvatic transmission of *Leishmania* in the Yucatan peninsula [26, 27].

Further entomological studies were carried out at 150 km, east of La Libertad in the village of La Guadalupe and the nearby village of Dos Naciones, municipality of Calakmul, Campeche. Using Shannon traps, Disney traps and CDC light traps, 15 sand fly species (*Brumptomyia* and *Lutzomyia*) were caught. In both study locations, the number of sand fly species caught was very similar but the predominant species differed. In the Dos Naciones village, *Lu. panamensis* (1682), *Lu. ovallesi* (504), *Lu. cruciata* (332), and *Lu. olmeca* (329) had the highest abundance; while in La Guadalupe *Lu. cruciata* (754) and *Lu. olmeca* (244) were most abundant. In both locations, the numbers of sand flies attracted to Shannon traps peaked between 18.00 and 22.00 hours. Given the abundance *of Lu. olmeca* in the collections made with Shannon and Disney traps (it was the only species caught in the latter), this species was confirmed as the primary vector of *L. (L.) mexicana* in Calakmul county. Dos Naciones and La Guadalupe differ in terms of vegetation structure, with much more severe and extensive deforestation around La Guadalupe than around Dos Naciones. Destruction of habitats by humans has led to the decrease in sand fly diversity and abundance. However, some species of medical importance have adapted to deforested habitats becoming closely associated with human settlements. Moreover, there is evidence that the deforestation could increase domestic transmission of *Leishmania* parasites [28].

Although the abundance of sand fly vectors was determined in three foci, the species of *Leishmania* in vectors had yet to be determined. Using the PCR method, kDNA of the parasite was amplified from sand flies collected in the forest. Because only two species of *Leishmania* have been reported in the Yucatan Peninsula, specific primer for *Leishmania* genus, namely 13 A (GTGGGGGAGGGGCGTTCT) and 13 B (ATTTTACACCAACCCCCAGTT), B-4 (TCGTACTCCCCGACATGCCTC) for the subgenus *Viannia*, and M1.1 (CCAGTTTC-GACCGCCGGAGC) for the subgenus *Leishmania* were used. Both *Lu. olmeca* and *Lu. cruciata* were found infected by *L. (L.) mexicana* [23].

3.3. Reservoirs

Leishmaniases is a complex of zoonotic diseases, which are infections transmitted from animals to humans. Identifying a reservoir of such zoonosis requires extensive ecological, entomological, mammalian, parasitological and epidemiological studies. The World Health Organization enumerated five criteria to incriminate a primary reservoir [17]. Thus, a step-by-step method is needed to investigate a primary reservoir of a zoonosis.

The first step is to identify animals that harbour the parasite. The first attempt to find leishmanial hosts close to the Yucatan Peninsula was carried out in Belize in the early 1960s

[29–31]. However, the south of Belize has to be considered as a different endemic area of the disease since this area is not part of the Biotic Province of the Peninsula of Yucatán [32]. In the Yucatan Peninsula, *L. (L.) mexicana* was identified by PCR in the base of tails of two heteromid rodents *Heteromys desmarestianus* Desmarest, 1817 and *Heteromys gaumeri* J.A. Allen and Chapman, 1897; and five cricetid rodents *Ototylomys phyllotis* Merriam, 1901, *Peromyscus yucatanicus* J.A. Allen and Chapman, 1897, *Oryzomys melanotis* Thomas 1893, *Sigmodon hispidus* Say and Ord, 1825, *Reithrodontomys gracilis* J.A. Allen and Chapman, 1897; and one marsupial *Marmosa mexicana* Merriam, 1897 [18, 33, 34]. Thus, many small terrestrial mammals of the Peninsula seemed to be able to harbour the parasite. However, the sand-fly vector is absent from the driest and unstable habitats such as corn- and bean-fields [27]. Due to *Sigmodon*'s preference for those disturbed areas, this species is not a potential reservoir of *L. (L.) mexicana* in the Yucatan Peninsula. The endemic area of *L. (L.) mexicana* is limited to the humid medium-size forest (height < 12 m), which characterizes southern Campeche and Quintana Roo states and the low-stature forest (height < 8 m) from eastern and southern Yucatan state and northern Campeche. This area occupies 19,839 km^2 [16].

The second step to identify a primary reservoir is that the species, which is relatively abundant in the focus to provide a food source for sand flies. In southern Campeche, *H. gaumeri* was the most abundant rodent in four of the five studied foci (total 46%), followed by *O. phyllotis* (19%) and *P. yucatanicus* (13%). Hence, the least abundant species such as *H. desmarestianus*, *Oryzomys* and *Reithrodontomys* as well as the marsupial *Marmosa* cannot be primary reservoirs of *L. (L.) mexicana* in the Yucatan Peninsula [34]. Because of the geographic isolation of the Yucatan Peninsula, two species of rodents exist only in the Yucatán Peninsula, the Gaumer's spiny pocket-mouse, *H. gaumeri* and the Yucatán deer-mouse, *P. yucatanicus*; two of the three potential reservoirs of *L. (L.) mexicana* in the area [32].

In the Yucatan Peninsula, a well-defined transmission season has been demonstrated, which is limited to the coolest months of the year, from November to March [35]. Thus, an important step to demonstrate a primary reservoir in an area of seasonal transmission is that the individuals of the reservoir species survive the infection and keep the parasite until the next transmission season, which is more than 7 months. In the field, *S. hispidus* and *O. melanotis* do not live for more than 6 months while *Heteromys* spp., *O. phyllotis* and *P. yucatanicus* can live for more than 2 years [34]. However, in order to survive such a long time, the course of infection has to be relatively non-pathogenic. In other words, the immune system of the reservoir must react to the presence of the parasite in such a way that while preventing it from doing any irrevocable damage, the parasite is not eliminated. The infection by *L. (L.) mexicana* in small rodents is mild and painless lesions situated at a non-vital part of the animal, the tail, and thus do not weaken their survival in the wild [33]. Subclinical infections, detected by PCR in the absence of visible lesion, are common [34]. Moreover, the existence of cryptic parasites in *P. yucatanicus* during the warmest months and the presence of a low-temperature trigger of clinical infection placed the Yucatán deer-mice in the best position as a primary reservoir of *L. (L.) mexicana* [36]. This type of infection either asymptomatic or with a mild pathology results from an ancient parasitic association in a well-balanced host-parasite relationship [37].

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The next step to implicate a primary reservoir is that the proportion of animals infected is high enough (20%) to infect the vector during the transmission season (OMS 1984). In the state of Campeche, the seasonal prevalence of infection of *H. gaumeri* range from 88% (n = 32) to 29% (n = 7), *O. phyllotis* from 100% (n = 33) to 27% (n = 17), and *P. yucatanicus* from 18% (n = 17) to 50% (n = 14). Those alarming prevalence in the municipality of Calakmul, Campeche, indicate the need to study each focus individually in order to assess their consequences on human health [34].

The last step to identify the primary reservoir is that the species of parasite is identical in all hosts (reservoir, vector and human) thus, the geographic and temporal distributions of humans and the transient micro-habitat of reservoir and vector need to overlap. Leishmaniasis existed first among wild animals and sand flies and the forest was not very populated by humans. However, with the human displacements due to overpopulation of some areas, new settlements appear constantly deep in the forest. A deforested ring around village limits the contact with the reservoir species and vectors. However, due to the Mayan slash-and-burn agriculture human enters deep into the forest exposing themselves to the bites of infected sand flies. Moreover, subsistence hunting takes place during the night mainly when the agricultural season is over [38]. Moreover, with the ecological protection of large forests, such as the Calakmul Biosphere Reserve in the State of Campeche, the incidence of wild zoonotic diseases might increase.

In conclusion, *O. phyllotis* and the two endemic rodents *H. gaumeri* and *P. yucatanicus* have been incriminated as *L. (L.) mexicana* reservoirs based on their geographic and temporal distributions and the overlap of reservoir, vector and human habitats. All three rodent species are long-lived and the course of the infection is relatively non-pathogenic. Human encroachment into wild areas increases their contact with the infected vector.

3.4. Seasonal transmission

To know the timing of the transmission cycle in each focus of leishmaniasis is very important because high-risk seasons might be restricted. Thus, intervention measures such as prevention through medical education could be conducted before the high-risk period. Moreover, epidemiological and ecological studies could be limited to that season and consequently the cost of research could be diminished.

Based on this rationale, all the results of previous research were analysed focusing on the timing of transmission of *L. (L.) mexicana* in the state of Campeche, Peninsula of Yucatan, Mexico [35]. The study included the timing of incidence of LCL in humans during 1993–1994, as well as the rate and time of infection in rodents and sand flies between February 1993 and March 1995. Rodents and sand flies were found infected between November and March, when men carried out their field activities and were exposed.

In summary, the median-size humid forests of the Yucatan Peninsula have ideal ecological conditions for *L. (L.) mexicana* transmission, particularly in the winter season when high humidity and low temperature, support the growth of sand fly population and trigger the appearance of lesion in the reservoir. This transmission cycle occurs when men enter the forest

for agriculture, hunting and gum collecting. Although the number of human cases of LCL reported each year has peaked from March to July, if the incubation period is considered, there is a strong correlation with the abundance, rates and timing of infection of both reservoirs and vectors (**Figure 2**). Based on these results, for the first time in the world, a seasonal transmission (from November to March) of LCL caused by *L. (L.) mexicana* in the sylvatic region of the state of Campeche was determined.

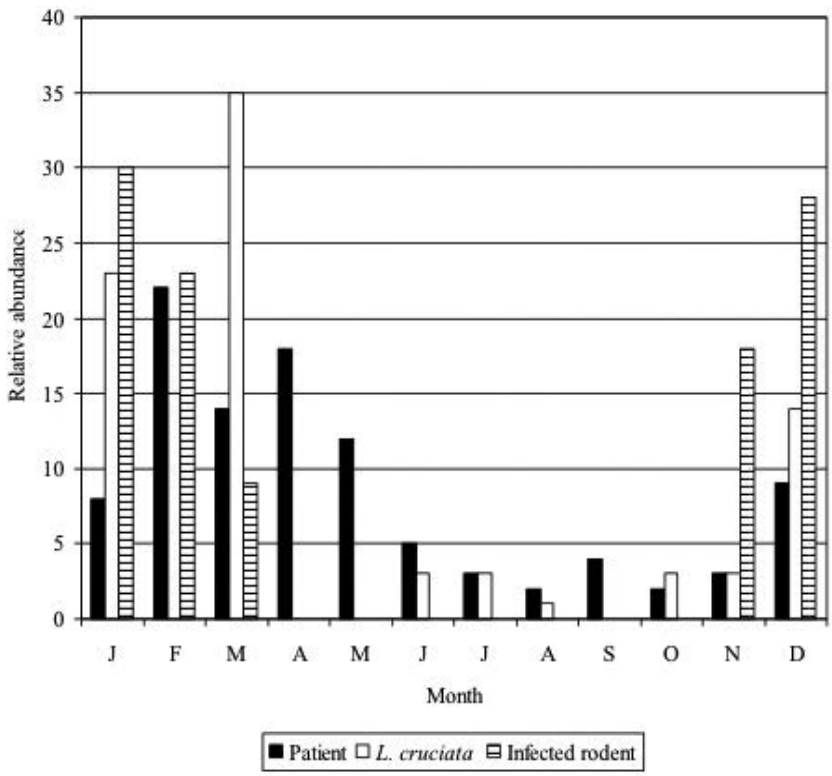

Figure 2. Monthly percentage of patients and rodents infected by *Leishmania (Leishmania) mexicana*, and relative abundance of *Lutzomyia cruciata* in a forest at 8 km from the village of La Libertad, Campeche, Mexico.

4. Immune response to *L. (L.) mexicana*

The skin is the first immune barrier against *Leishmania* promastigotes inoculated by the sand fly vector. The immune response against *Leishmania* spp. is highly complex. Macrophages are the main host cells of *Leishmania* and also responsible for parasite elimination. The activation of the macrophage microbicidal mechanisms depends on the cell-mediated immune response [39]. Thus, the nature and intensity of cellular immune response and their mediators (i.e. cytokines and chemokines) in the lesions are of primary importance for the disease outcome.

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Based on this rationale, the characterization of the cytokine expression profile was studied in 13 LCL lesions caused by *L. (L.) mexicana* from the Yucatan Peninsula [40]. Age of the patient ranged from 10 to 29 years and the lesion evolution time from 8 days to 18 months. Lesions were classified as of early (≤2-month duration) and late (≥4-month duration) evolution. Skin biopsies were taken from the border of the lesion to analyse cytokine gene expression by RT-PCR. *Leishmania* amastigotes were present in 8 of the 11 histological sections. Intra-lesional cellular infiltrate was made up of equal proportion of macrophages and plasma cells. Lymphocytes represented 50% of the cellular infiltrate in three of four of the early lesion evolution but only in one of seven of the late-lesion evolution. The analysis of the *in situ* cytokine gene expressions revealed a concomitant presence of Th1 (IL-1α, IFN-γ, TNF-α) and Th2 (IL-6, IL-10, TGF-β) cytokines in all biopsies. The high expression of IFN-γ, cytokine related with macrophage activation, in both early and late evolution suggested that the presence of this cytokine was not sufficient for parasite elimination and control of the disease. A significant increase in IL-1α, TNF-α, Il-10, and TGF-β expressions was observed in late-lesion evolution compared with that in early lesions suggesting the role of these cytokines in the chronicity of LCL caused by *L. (L.) mexicana*. Both IL-10 and TGF-β down-regulate macrophage functions [41, 42]. Thus, the intra-lesional expressions of these cytokines could promote the persistence of the intracellular parasites in the skin. On the other hand, the presence of TNF-α in cutaneous lesions caused by New World *Leishmania* species has been related with lesion formation and loss of integrity of the infected tissue [43]. Further studies to confirm the role of TNF-α in the immunopathogenesis of LCL caused by *L. (L.) mexicana* are needed.

Another study of 20 LCL patients was carried out to analyse the role of IL-12 in the protective immune response to *L. (L.) mexicana* infection. The correlation of IL-12 with its counter-regulatory cytokine, IL-10, was also evaluated [44]. The patients were 10–48 years old and their lesions ranged from 10 days to 20 months of evolution. Cytokine expressions were evaluated by RT-PCR. Intra-lesional expression of both IL-10 and IL-12 was present in most of the 20 patients. The more chronic, non-healing lesions had higher levels of IL-12 mRNA indicating that the expression of this cytokine alone was not sufficient to induce healing. The IL-10 expression correlated with both IL-12 and IFN-γ, suggesting that IL-10 promotes disease persistence by a direct inhibition of macrophage activation rather than by suppression of the Th1 response.

Epidemiological studies detected many individuals from the endemic area of LCL without suggestive signs of the disease but with a delayed hypersensitivity skin test (DHT) positive to *Leishmania* antigens. Asymptomatic infection is the most common outcome to the infection in the Yucatan Peninsula [10, 45]. From the immunological point of view, asymptomatic infection is explained as the elicitation of an appropriate immune response capable of controlling parasite replication and maintaining tissue integrity [46]. Therefore, the characterization of this protective immune response in asymptomatic individuals becomes imperative for vaccine designs. Thus, the *in situ* cytokine (IL-4, IL-10, IL-12, IFN-γ) and chemokine (MCP-1, MIP-1α) mRNA expressions were analysed in biopsies of the DHT area of asymptomatic individuals (n = 6) and subjects with healed lesions (n = 9) and compared with biopsies from active lesions (n = 11) [47]. The expression was highly variable. Neither IL-4 nor MIP-1 α was

detected in any biopsy. IL-12 was detected in all three groups without significant differences in the median. MCP-1, chemokine that stimulates oxidative burst activity in macrophages thus killing intracellular amastigotes, was expressed in all three groups being significantly higher in active lesions. The most surprising finding was the absence of IFN-γ in both healed lesions and asymptomatic infection. Taken together, these results suggested that IL-12 and MCP-1 in the absence of IFN-γ might be playing a crucial role in the infection outcome at the skin level. Further studies are needed to identify the cytokine and chemokine network and their cell sources in asymptomatic infection. This knowledge is primordial to understand mechanisms involved in immune protection against *L. (L.) mexicana* and to develop better preventive and therapeutic strategies.

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Chapter 9

Survey of Cutaneous Leishmaniasis in Mexico: *Leishmania* Species, Clinical Expressions and Risk Factors

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Additional information is available at the end of the chapter

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Abstract

Leishmaniasis is caused by *Leishmania* sp., which is transmitted to human beings and reservoirs by phlebotomine sand flies, with worldwide prevalence of approximately 12 million cases with population at risk of approximately 350 million. Cutaneous leishmaniasis (CL) is the most widespread form, causing localized skin lesions (LCL), mucocutaneous leishmaniasis (MCL), or nodular lesions in diffused cutaneous leishmaniasis (DCL). American CL includes LCL and DCL caused by *Leishmania mexicana* complex and MCL caused by the *Leishmania braziliensis* complex. In Mexico, CL is distributed in three endemic areas, Gulf of Mexico, Pacific of Mexico, and Central Mexico. In order to monitor clinical outcome and adequately target treatment as well as epidemiologic studies, diagnostic kinetoplast DNA (kDNA), polymerase chain reaction (PCR), Southern and dot blotting, and ITS1 PCR-RFLP of *Leishmania* DNA were evaluated in samples and *Leishmania* isolates from patients with cutaneous ulcers from several endemic areas. In Mexico, LCL can be caused by the *L. mexicana*, *L. braziliensis*, or both complexes. DCL is caused by *L.* (*L.*) *mexicana* or *Leishmania* (*L.*) *amazonensis* and visceral leishmaniasis (VL) by *Leishmania* (*L.*) *chagasi* and *L.* (*L.*) *mexicana* in immunocompromised patients. The geographic range in which CL is endemic has increased due to urbanization, new settlements, and ecological, social, and educative conditions, which favors its permanence and transmission.

Keywords: *Leishmania*, cutaneous leishmaniasis, Mexico, epidemiology, ecology

1. Introduction

Leishmaniasis is a group of clinical entities present in 79 countries at a rate of 400,000 cases per year. The World Health Organization estimates a worldwide prevalence of approximately 12 million cases with population at risk of approximately 350 million. It is caused by a parasitic

protozoan, which belongs to the *Leishmania* genus that is transmitted to human beings and animal reservoirs by phlebotomine sand flies [1].

Cutaneous leishmaniasis (CL) is the most widespread form, causing primary localized skin lesions from which parasites can disseminate to the nasopharyngeal mucosa and cause mucocutaneous leishmaniasis (MCL) or disseminate to the entire body as nodular lesions in diffused cutaneous leishmaniasis (DCL). Visceral leishmaniasis (VL) is the most severe form of the disease; according to the WHO in areas endemic for VL, many people have asymptomatic infection and a concomitant HIV infection increases the risk of developing active VL by between 100 and 2320 times [1].

VL is characterized by irregular fever, weight loss, swelling of the liver and spleen, and anemia. After recovery, patients sometime develop chronic DCL [2, 3].

American cutaneous leishmaniasis is characterized by a spectrum of clinical presentations caused by *Leishmania* species grouped in complexes; these include LCL caused by *Leishmania* (*L.*) *mexicana*; DCL caused by *Leishmania amazonensis*, *Leishmania venezuelensis*, and *Leishmania pifanoi*, all of them belonging to the *L. mexicana* complex; and MCL caused by members of the *L. braziliensis* complex. VL is caused by *L.* (*L.*) *chagasi* belonging to the *L. donovani* complex. Symptomatic diagnosis confuses CL with unrelated disorders such as tropical ulcers, sporotrichosis, leprosy, and skin cancer, among others [4].

In Mexico, Seidelin first recorded LCL caused by *L.* (*L.*) *mexicana* in 1912, who called it "chiclero's ulcer," because he found the disease in rubber workers. CL is distributed in three main endemic areas: Gulf of Mexico, Pacific of Mexico, and Central Mexico. In these regions, multiple species of *Leishmania* may coexist and several species can cause both LCL and MCL [5–7]. Several methods of detection of *Leishmania* based on deoxyribonucleic acid (DNA) have been described. The polymerase chain reaction (PCR) has been employed for selective amplification of *Leishmania* DNA. Several molecular targets for a diagnostic PCR have been evaluated including the minicircle kinetoplast DNA (kDNA), the miniexon (spliced leader RNA) gene, and the internal transcribed spacer (ITS) [8–10], among others.

2. Materials and methods

In order to find a diagnostic method for leishmaniasis that combines high sensitivity with species differentiation in the field, rapid diagnosis, and low cost, several molecular targets for a diagnostic PCR were evaluated from patients with cutaneous ulcers suspected of having LC from several endemic areas. The target was minicircle kinetoplast DNA (kDNA) using specific primers or probes with the PCR and Southern or dot blotting [11] and PCR-RFLP of the internal transcribed spacer 1 (ITS1) [10, 12].

Distribution of CL or VL in social, educative, and ecological conditions was recorded. The patients diagnosed with CL were treated with meglumine antimoniate (Glucantime®).

2.1. Patient population

In these studies, we evaluated samples from patients with clinical symptoms and skin lesions suggestive of CL, MCL, and DCL from several endemic areas of Mexico—Campeche, Tabasco,

Veracruz, Nayarit and Chiapas, and Quintana Roo—or samples from VL patients from Chiapas and Tabasco states. The clinical samples were taken on filter papers or smears, needle aspirates, and tissue biopsy samples (1–2 mm) from the edge of cutaneous or bone marrow aspirates (**Figure 1**).

Figure 1. Map of Mexico, showing the Ocean Pacific, Gulf of Mexico, and Central Leishmaniasis endemic regions.

2.2. Ethical considerations

For bleeding human beings for diagnosis and therapeutics, informed consent was obtained from all the adults who participated in the study. Consent for including young children was obtained from their parents or guardians. The ethics committee of the corresponding health authorities, in agreement with International Ethical Guidelines for Biomedical Research involving human subjects (Norma Oficial Mexicana de Salud: NOM-003-SSA 2-1993), reviewed and approved the protocols of the present studies.

2.3. Leishmania reference strains and Mexican isolate culture conditions

Reference *Leishmania* strains (**Table 1**), used as control and Mexican isolates of *Leishmania* from Tabasco, Veracruz, Campeche, and Quintana Roo states (**Table 2** and **Figure 1**), were cultured in Roswell Park Memorial Institute medium 1640 (RPMI medium 1640) supplemented with 10% fetal calf serum at 26°C. DNAs of *Trypanosoma cruzi* and *Mycobacterium tuberculosis* were used as negative controls.

Number	Strain	Code	*Leishmania* species
1	MHOM/BZ/82/BEL21	BEL21	*L.* (*L.*) *mexicana*
2	MHOM/BZ/62/M379	M379	*L.* (*L.*) *mexicana*
3	IFLA/BR/67/PH8	PH8	*L.* (*L.*) *amazonensis*
4	MHOM/BR/73/M2269	M2269	*L.* (*L.*) *amazonensis*
5	MHOM/PE/84/LC53	LC53	*L.* (*V.*) *braziliensis*
6	MHOM/BR/84/LTB300	LTB300	*L.* (*V.*) *braziliensis*
7	MHOM/BR/75/M2903	M2903	*L.* (*V.*) *braziliensis*
8	MHOM/BR/75/M2904	M2904	*L.* (*V.*) *braziliensis*
9	MHOM/BR/75/M4147	M4147	*L.* (*V.*) *guyanensis*
10	MHOM/PE/84/LC26	LC26	*L.* (*V.*) *peruviana*
11	MHOM/CR/87/NEL3	NEL3	*L.* (*V.*) *panamensis*
12	MHOM/PA/72/LS94	LS94	*L.* (*V.*) *panamensis*
13	MHOM/IN/80/DD8	DD8	*L.* (*L.*) *donovani*
14	MHOM/BR/74/PP75	PP75	*L.* (*L.*) *infantum/chagasi*

Table 1. Reference strains used in this study.

2.4. Isolation of DNA

Clinical specimens cut from the filter paper or eluted from the smear, bone marrow aspirates, skin aspirates, and tissue biopsy samples (1–2 mm) were incubated in 250 μL of cell lysis buffer for 1 h at 56°C. DNA from *Leishmania* cultures was prepared by centrifuging 10^9 parasites in the exponential phase of growth at 2000 × *g* for 10 min at 4°C. The DNA was extracted from the pellet using the High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's instructions. The DNA was stored at −20°C until used.

2.5. Polymerase chain reaction

PCR analysis of kDNA for subgenus *Leishmania* was carried out by using the AJS1 and DeB8 primers [13]. PCR of the *L. mexicana* complex was carried out using the M1 and M2 primers [14] and the LMO1 and LMO2 primers specific for minicircles of Mexican *L.* (*L.*) *mexicana* strains [15]). PCR of the *L. braziliensis* complex was done with the B1 and B2 primers [8]. PCR for *L. donovani* complex was done with the D1 and D2 primers [16]. PCR amplification conditions were performed as described previously [8, 13, 14, 16, 17].

2.6. PCR analysis of genomic DNA of *L.* (*V.*) *braziliensis*

PCR species specific for nuclear DNA from variants of *L.* (*V.*) *braziliensis* was carried out by using the primers 3J1 and 3J2. Amplification conditions were as described elsewhere [18].

2.7. PCR analysis of the internal transcribed spacer 1 (ITS1)

Some samples were analyzed for ITS1 PCR using the primers: LITSR and L5.8S [10]. Amplification conditions were as described [12]. PCR products were digested with *HaeIII* enzyme,

Number	Code	Origin	Clinical expression	*Leishmania* species
1	MHOM/MX/88/HRC JS	Tabasco	DCL	*L. am + L. mex*
2	MHOM/MX/88/HRC MC	Tabasco	LCL	*L.* (*L.*) *mexicana*
3	MHOM/MX/84/ISET GS	Tabasco	DCL	*L. am + L. mex*
4	MHOM:MX:83:UAVY CV	Yucatan	LCL	*L.* (*L.*) *mexicana*
5	MHOM/MX/85/ISET HF	Veracruz	DCL	*L. am + L. mex*
6	LVER	Veracruz	DCL	*L. am + L. mex*
7	REP	Campeche	LCL	*L. am + L. mex*
8	MHM/MX/06/ENCB/MIC	Campeche	LCL	*L.* (*L.*) *mexicana*
9	MHM/MX/06/ENCB CDL	Campeche	LCL	*L.* (*L.*) *mexicana*
10	MHM/MX/06/ENCB FDL	Campeche	LCL	*L.* (*L.*) *mexicana*
11	CR	Campeche	LCL	*L.* (*V.*) *braziliensis*
12	PVS	Campeche	LCL	*Mx. L. mexicana*
13	RGL	Campeche	LCL	*L. b + L. mx*
14	FJJ	Campeche	LCL	*L. b + L. mx*
15	ESP	Campeche	LCL	*Mx. L. mexicana*

L. am + L. mex, *L.* (*L.*) *amazonensis + L.* (*L.*) *mexicana*; *L. b + L. mx*, *L. braziliensis + L. mexicana*; *Mx. L. mexicana*, Mexican variant of *L.* (*L.*) *mexicana*

Table 2. Mexican isolates of *Leishmania* analyzed in this study.

according to the manufacturer's instructions. The amplicons and restriction products were analyzed as described elsewhere [12].

2.8. Southern or dot blot hybridization of kDNA PCR products of biopsies, isolates and *Leishmania* reference strains

The kDNA PCR products of clinical samples, Mexican isolates and reference strains, were Southern or dot blotted onto nylon membranes and were hybridized with the cloned fragments of kDNA used as probes: B4Rsa, which hybridizes specifically to members of the *L. donovani* complex; 9.2 and 9.3, specific for the *L. mexicana* complex; and B18, specific for members of the *L. braziliensis* complex. The probes were labeled with DIG Random Primer DNA labeling kit (Boehringer Mannheim) and either visualized colorimetrically with NBT and BCIP (Boehringer Mannheim) or labeled with [^{32}P]d ATP, using the Prime-it™ Random Primer DNA labeling kit (Stratagene). The hybridization conditions were described elsewhere [14, 17].

2.9. Administration of meglumine antimoniate (Glucantime®)

Patients diagnosed with CL accepted treatment with meglumine antimoniate (Glucantime®). Glucantime is marketed in 5 mL ampules containing 1.5 g of N-methyl-glucamine antimoniate, which corresponds to 425 mg of Sb51. Treatment consisted in one ampule by intramuscular injection per day until healing [19].

3. Results

Primers DeB8 and AJS1, specific for the *Leishmania* (*L.*) subgenus [13], amplified the kDNA of *L.* (*L.*) *mexicana* Bel 21, *L.* (*L.*) *mexicana* M379, *L.* (*L.*) *amazonensis* PH8, *L.* (*L.*) *amazonensis* M2269, *L.* (*L.*) *donovani* DD8, *L.* (*L.*) *infantum/chagasi* PP75, 10 Mexican strains of *Leishmania*, and many clinical samples from patients with skin lesion from Campeche, Tabasco, Veracruz, and Quintana Roo (**Tables 1** and **2**, **Figure 1**) [17].

PCR with the primers M1 and M2 specific for the *L. mexicana* complex [14] resulted in the amplification of kDNA of *L.* (*L.*) *amazonensis* PH8 and M2269 with a band size of 700 bp and *L.* (*L.*) *mexicana* BEL21 with a band size of 800–820 bp. This difference can be used diagnostically to distinguish between *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana* isolates. The size of the kDNA amplicons of the Mexican strains is more similar to the size of the amplicons of *L.* (*L.*) *amazonensis* group than the amplicons of *L.* (*L.*) *mexicana*. Negative controls, *T. cruzi* and *M. tuberculosis,* did not amplify [17].

PCR specific for the *L. braziliensis* complex carried out with B1 and B2 primers [8] produced a kDNA amplification band of 750 bp of *L.* (*V.*) *braziliensis* LTB300, LC53, *L.* (*V.*) *braziliensis* M2903, *L.* (*V.*) *braziliensis* M2904, *L.* (*V.*) *braziliensis* reference strains, and some skin biopsies from Nayarit and several skin samples from Campeche state.

In order to have a more accurate identification of the *Leishmania* species in Nayarit, the skin biopsies were PCR analyzed with primers 3J1 and 3J2 specific for DNA genomic of *L.* (*V.*) *braziliensis.* Most of the samples amplified giving a band of 617 bp. The PCR products hybridized positively with the LbJ38 probe, which is species specific for *L. braziliensis* complex [18, 20].

PCR with specific primers D1 and D2 for the *L. donovani* complex resulted in the amplification of kDNA of the *L.* (*L.*) *donovani* DD8 and *L.* (*L.*) *infantum/chagasi* PP75 reference strains, and bone marrow and liver biopsy from a patient from Chiapas with VL were amplified [16, 21].

PCR products of the kDNA of Mexican strains of *Leishmania mexicana* and clinical samples amplified with primers AJS1 and DeB8, specific for the subgenus *Leishmania*, were dot blotted and tested with probe 9.2, specific for the *L. mexicana* complex. The probe hybridized with high affinity to *L.* (*L.*) *mexicana* BEL21, the 10 Mexican strains of *Leishmania mexicana*, several samples and biopsies from Campeche state, and DNA from a bone marrow aspirate, from a patient from Tabasco, with VL; kDNA from the reference strains other than *L. mexicana* that did not hybridize.

PCR products amplified with primers B1 and B2, specific for the *L. braziliensis* complex, were Southern blotted and tested with probe B18, specific for the *L. braziliensis* complex. This probe hybridized to *L.* (*V.*) *braziliensis* LTB300 and to DNA from skin biopsies from patients from Campeche and some from Nayarit states (**Figure 1**) [20].

PCR with specific primers for ITS1 resulted in the amplification of the *Leishmania* reference strains, the Mexican strains and isolates of *L. mexicana*, and the clinical samples from Campeche giving 300–350 bp amplification bands. Restriction of the ITS1 gene amplicons of *L.* (*V.*) *panamensis*, *L.* (*V.*) *guyanensis*, and *L.* (*L.*) *braziliensis* reference strains with the endonuclease

HaeIII generated patterns with two bands of 170 and 150 bp; *L.* (*L.*) *amazonensis* generated two bands of 220 and 140 bp; and *L. mexicana* generated three bands of 200, 80, and 40 bp.

Most of the Mexican strains and isolates of *Leishmania* displayed a restriction pattern similar to that of *L.* (*L.*) *mexicana* reference strain; nine of these were obtained from LCL patients from Campeche. Some showed a mixed pattern compatible with *L.* (*L.*) *mexicana* and *L.* (*V.*) *braziliensis*; some others showed a mixed pattern compatible with *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana* (**Table 2**) [11].

In relation to the clinical samples from Campeche, most of them amplified a restriction pattern similar to the *L.* (*L.*) *mexicana* reference strain. In some samples, extra bands of 50 and 25 bp were observed, suggesting a coinfection, as it was found in a previous study with kDNA PCR analysis of clinical samples that DNA from both *L.* (*L.*) *mexicana* and *L.* (*V.*) *braziliensis* was identified (**Table 2**) [11, 15].

4. Discussion

In Mexico since 1985, cases of LCL, DCL, MCL, and VL clinical expressions were reported in 15 states; the species involved were *L.* (*L.*) *mexicana*, *L.* (*V.*) *braziliensis*, and *L.* (*L.*) *chagasi*. LCL was the most common, and all cases were considered caused by *L.* (*L.*) *mexicana* [6, 22]. The five major foci of *Leishmania* transmission were in rain forest of southern Campeche, La Chontalpa (the cocoa-producing district of Tabasco), and the southern coffee producing of Nayarit, southern Quintana Roo, and Chiapas (**Figure 1**).

In Nayarit, state of the Pacific endemic region, LCL was recorded in Caleras de Cofrados since 1987 [22], a district near Tepic, the state capital city (**Figure 1**). The etiological agent was thought to be *L.* (*L.*) *mexicana*. In our studies using kDNA PCR and hybridization techniques, we have demonstrated that the *L. braziliensis* complex is present in Nayarit, and we were able to distinguish between two variants or two different species of *L.* (*V.*) *braziliensis*. We believe this was the first report of *L.* (*V.*) *braziliensis* in Nayarit, Mexico [20]. The population affected with skin lesion were 5–65 years old; males were the most affected and their main activity was the harvesting and/or growing coffee. The possible vectors are *Lutzomyia cruciata*, *Lutzomyia diabolica*, and *Lutzomyia shannoni*, which were captured and identified at the plantation. In relation with the animal reservoirs, no studies have been reported [20].

Biopsies, clinical samples, and isolates from LCL patients from several districts of Campeche state, mainly from Calakmul, were PCR amplified with specific primers for kDNA of *L. braziliensis* and *L. mexicana* complex members and primers specific for Mexican strains of *L. mexicana* [19] and also were analyzed by ITS1 PCR-RFLP [12]. We detected in Northern Calakmul 43% of cases infected with *L. mexicana*, 25% of cases with *L. braziliensis* complex members, 62% of mixed infection of Mx *L. mexicana* + *L.* (*L.*) *mexicana*, and 25% of cases infected with *L. braziliensis* complex + *L.* (*L.*) *mexicana*. The most affected community of this area was La Mancolona, with a 6.5% of prevalence; this village is located 3–4 km away from the crops and is more urbanized due to deforestation (**Figure 3a**). The most affected population in this village were adult males (66%) [19].

In central Calakmul 15% of the cases were infected with *L.* (*L.*) *mexicana*, 25% of the cases infected with *L. braziliensis* complex members, and 37% of the cases infected with Mx *L. mexicana L.* (*L.*) *mexicana*. La Guadalupe village had the highest prevalence rate (2.2%) and children were the most affected (67%) [19].

In southern Calakmul 25% of the cases were infected with *L.* (*L.*) *mexicana*, 62% with *L. braziliensis* complex members, and 75% with both *L.* (*L.*) *mexicana* and *L. braziliensis* complex members. Dos Lagunas Sur was the most affected community, located close to the border with Belize, with 12% prevalence (**Figure 2c**). People in this village farm chili crops around their houses, which are located very close to the forest, and the population affected were children (50%), women, and men (50%) (**Figures 2a–c** and **3a–c**) [19]. In relation to the vectors, *L. mexicana* infections in two sand fly species, *Lu. shannoni* and *Lutzomyia ylephiletor*, were found in Dos Lagunas Sur, whereas in La Mancolona, *L.* (*L.*) *mexicana* infections were found in *Lu. shannoni*, *Lu. cruciata*, *Lu. o. olmeca,* and *Lu. Panamensis* [23].

Regarding to the animal reservoirs, *L.* (*L.*) *mexicana* was identified in four species of wild rodents: the black-eared rice rat, *Oryzomys melanotis*; the hispid cotton-rat, *Sigmodon hispidus*; the big-eared climbing rat, *Ototylomys phyllotis;* and the Yucatan deer mouse, *Peromyscus yucatanicus* [24].

We found most of the cases of DCL in the states of Tabasco and Veracruz (**Figure 1**). These states have a common border in the endemic region of the Gulf of Mexico and are

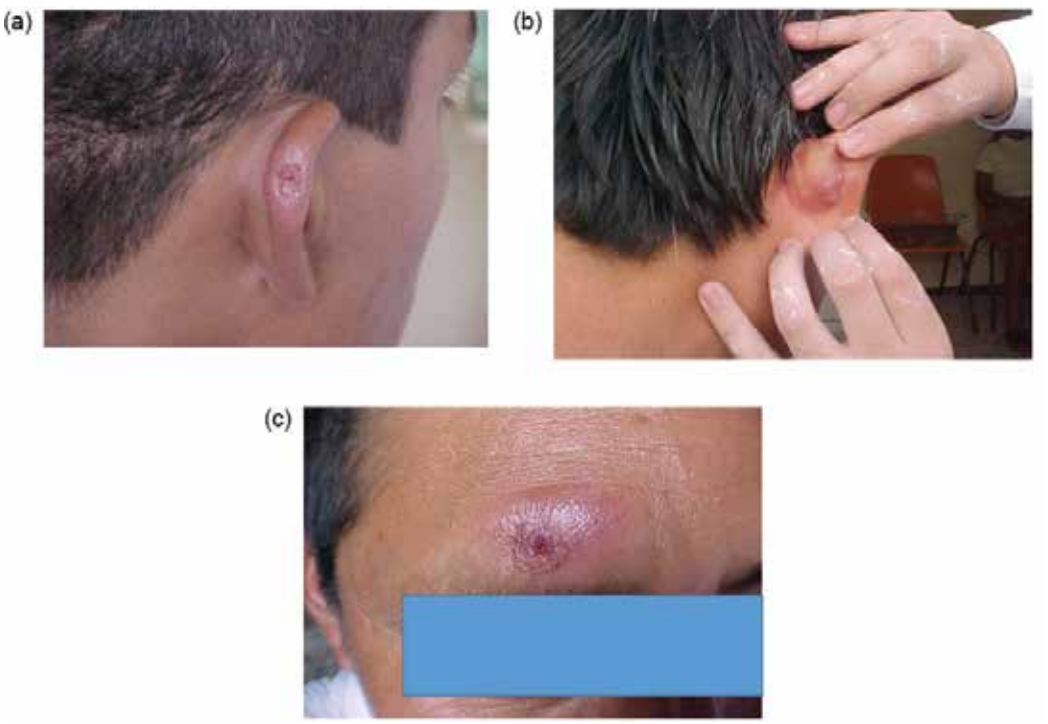

Figure 2. Patients from the endemic Gulf of Mexico region, with skin lesions suffering from cutaneous leishmaniasis.

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Figure 3. Communities situated in the leishmaniasis endemic region of Gulf of Mexico. People in these villages farm chili crops around their houses, located very near the forest close to the border of Belize and Guatemala.

characteristically tropical rain forest, with considerable rainfall and important agricultural activities, including the production of cocoa, sugar cane, and rubber. We collected isolates from patients with DCL or LCL in these states and some from Campeche. Their DNA was amplified with primers M1 and M2 [17] specific for kDNA of *L. mexicana* complex. The size of PCR products (680–720 bp) of the Mexican isolates is more similar to the size of the PCR products (700 bp) of *L.* (*L.*) *amazonensis* group than the PCR products (800–820 bp) of *L.* (*L.*) *mexicana* BEL21. The isolate PCR products hybridized with probe 9.2 specific for the *L. mexicana* complex. Their DNA was also analyzed using ITS1 PCR-RFLP, and we confirmed the presence of both DNA of *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana* in the same isolate (**Table 2**) [12, 17].

In Mexico, it has been reported that VL was caused by *L.* (*L.*) *chagasi* and confined to Central endemic region [22]. Subsequently, in the Pacific endemic region states of Chiapas, Guerrero VL was detected. In Tabasco, only cases of LCL and DCL caused by *L.* (*L.*) *mexicana* have previously been reported [25]. In our studies by kDNA analysis, we have found VL cases in Tabasco (a 6-month-old immunosuppressed male) [21] and in Chiapas (a 36-year-old male coinfected with HIV and *Pneumocystis carinii*) to be caused by *L.* (*L.*) *mexicana* [26]. These findings are important because it indicates that these species, typically cutaneous, can visceralize in immunocompromised patient, and in Mexico, MCL, LCL, and VL coexist in

some endemic areas. This is the first case reported in Mexico of coinfection by *L.* (*L.*) *mexicana* and HIV, which was manifested as VL. Our results agree with those found in Hernandez [26], who reported in Venezuelan patient displaying the symptoms of VL, a coinfection with HIV and a *Leishmania* variant strain sharing kDNA sequences with *L. braziliensis* and *L. mexicana* [27].

Treatment of CL patients with *Glucantime*® was successful in 96% of cases, regardless of the number and location of lesions. To obtain complete healing of lesions, the doses needed were in children from 2 to 20 and in adults from 2 to 67 ampules, although some patients cure spontaneously [19].

In the endemic areas evaluated in the present studies, the risk factors associated with CL were identified as the human colonization of large areas of previously untouched rain forests, where CL is endemic. The urbanization and deforestation are important factors because the *Leishmania* transmission cycles are adapting to peridomestic environments and are spreading to previously no endemic areas with domestic animals as potential reservoirs and spending nocturnal periods in the forest for cultivation of agricultural crops (e.g., chili and coffee) (**Figure 3a–d**) [11, 19, 20].

5. Conclusion

In conclusion, our findings are interesting because we have shown that in the typical endemic regions of Gulf of Mexico and Ocean Pacific of Mexico, CL can be caused by several species of the *L. mexicana* and *L. braziliensis* complexes and in some clinical samples, we found DNA of both complexes. Furthermore, we found DCL caused by a mix infection with strains of *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana* [12], both belonging to the *L. mexicana* complex. VL can be caused by *L.* (*L.*) *chagasi* and in immunocompromised patients by *L.* (*L.*) *mexicana.* Diagnosis of leishmaniasis by PCR and hybridization of kDNA and ITS1 PCR-RFLP analysis of *Leishmania* DNA must be combined for the reliable characterization of *Leishmania* species mainly in endemic areas where the presence of multiple species of *Leishmania* overlap clinical pictures demands simultaneous species identification [12]. In Mexico, the geographic range in which CL is endemic has increased in size due to urbanization, new settlements, and ecological, social, and educative conditions, which favors its permanence and transmission, as it has occurred in Calakmul.

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Leishmaniasis is a vector-borne, parasitic disease with tremendous variety in presentation, biology, and epidemiology. Any book on this disease must acknowledge the nearly impossible task of providing an exhaustive account of leishmaniasis simply because the epidemiology of the disease is so very complex. This book addresses some of this variety with chapters on the epidemiology of leishmaniasis in North Africa, Central America, and South America. The purpose of the book is not to specifically address diagnosis and treatment of the disease, but rather to provide a sample of the differing epidemiologies of leishmaniasis that occur due to variations in local habitats; the presence of different vectors, reservoirs, and agents; and the wide variety of cultures in which this disease occurs.

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