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

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

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

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 [³²P]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

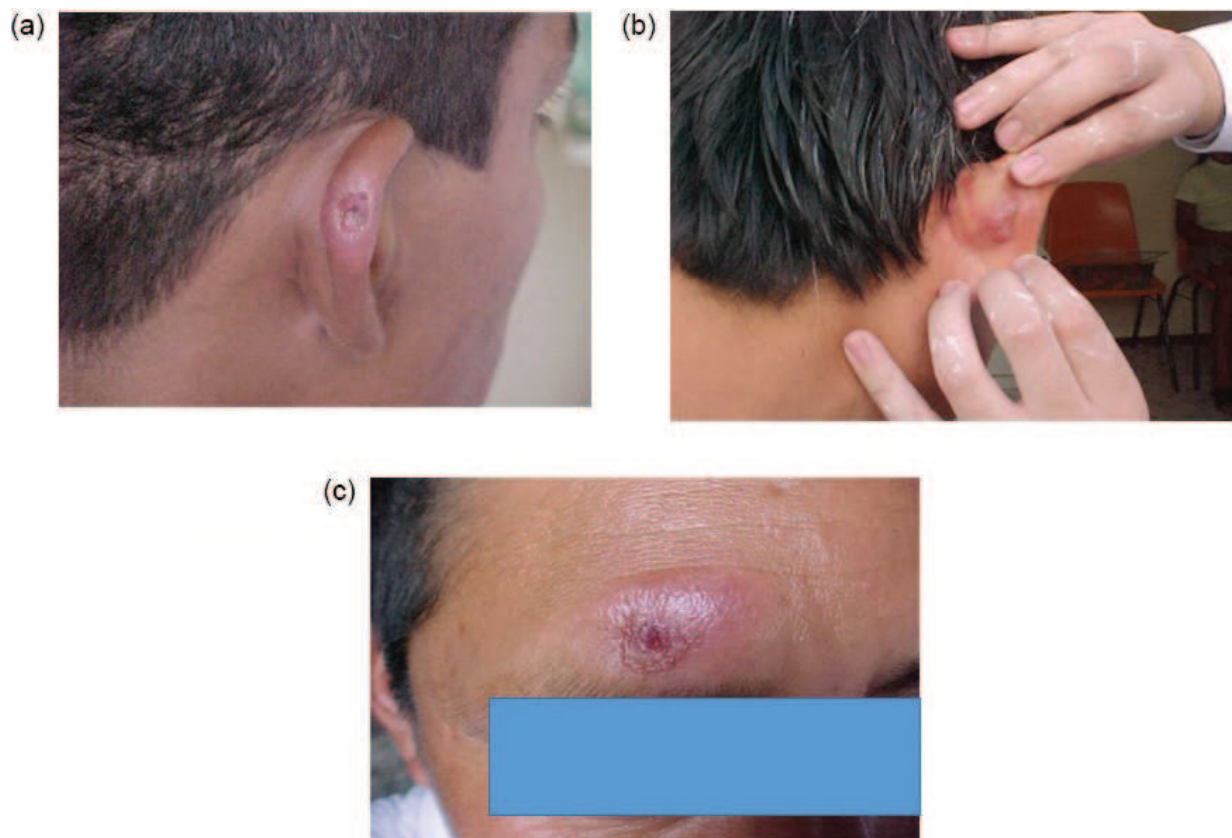


Figure 2. Patients from the endemic Gulf of Mexico region, with skin lesions suffering from cutaneous leishmaniasis.



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 coinfecting 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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