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Report of autochthonous cases of localized cutaneous leishmaniasis caused by *Leishmania (Leishmania) mexicana* in vulnerable, susceptible areas of Southeastern Mexico

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ABSTRACT

Localized cutaneous leishmaniasis (LCL) is an endemic disease in several Mexican States with the main endemic areas located in the South-Southeast region of the country, where 90% of *Leishmania (Leishmania) mexicana* cases are registered. The Southeast region is located in the Yucatan Peninsula, including Campeche, Quintana Roo and Yucatan States. Campeche and Quintana Roo register more than 60% of the cases in the country each year, while in Yucatan the reports are of imported cases due to residents traveling to endemic areas. However, since 2015, autochthonous cases have been diagnosed by health authorities in municipalities with no previous transmission records. We aimed to identify *Leishmania* parasite species involved in autochthonous cases by means of the PCR technique. The present study included 13 autochthonous cases of LCL with clinical and parasitological diagnoses during 2018 and 2019 by health authorities, without specific identification of the causal agent. Tissue samples were taken by scraping the margins of active lesions and then they were spotted onto an FTA™ Elute Microcard. Next, DNA was eluted and used for PCR amplification of specific *Leishmania* genus and *L. (L.) mexicana* species-specific fragments. Molecular analysis showed evidence that *L. (L.) mexicana* was the causal agent of LCL in 12 of the 13 patients; in one patient, PCR was not performed due to the patient's refusal to participate in the study. Identifying *Leishmania* species that cause LCL is necessary to define efficient treatment schemes and control strategies for the disease in vulnerable and susceptible areas of the Yucatan State's municipalities.

KEYWORDS: Leishmaniasis. *Leishmania (Leishmania) mexicana.* Autochthonous cases. Emergence. Neglected tropical diseases. Species-specific PCR.

INTRODUCTION

Leishmaniasis is a vector-borne zoonotic disease that is widespread in tropical and subtropical areas of the world. It is caused by obligate intracellular protozoan parasites of the genus *Leishmania* and is transmitted to humans and other mammals through the bite of female sandflies of the family Psychodidae (Phlebotominae). Globally, leishmaniasis is among the top 10 neglected tropical diseases, responsible for 12 million people infected, 0.9 to 1.6 million new cases each year, about 20,000 and 30,000 deaths and 350 million people at risk of infection[1].

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There are four main clinical manifestations of the disease: visceral leishmaniasis (VL), post-kala-azar dermal leishmaniasis (PKDL), mucocutaneous leishmaniasis (MCL), also called mucosal leishmaniasis (ML), and cutaneous leishmaniasis (CL). VL causes irregular episodes of fever, weight loss, hepatosplenomegaly and anemia, which, if not treated, causes death in more than 90% of the cases. PKDL is a sequel of VL that appears as a macular, papular, or nodular rash, usually on the face, upper arms, trunks and other parts of the body. MCL can lead to the partial or complete destruction of the mucous membranes in the nose and mouth and may cause severe disability[1]. CL is the most frequent clinical form of the disease, causing mainly ulcerative lesions that leave scars on exposed body parts, severe disability and stigma. About 95% of CL cases occur in the Americas, the Mediterranean Basin, the Middle East and Central Asia[2].

In the American continent, 1,028,054 cases of CL and MCL were reported from 2001 to 2019 by 17 endemic countries, with an average of 54,108 cases per year[3]. From 2015 to 2019, there was a decline in cases in 12 of these countries, while five had a significant increase, highlighting Mexico with a 76% cases increase[3]. In 2019, Mexico had remarkable growth in the incidence rate compared to 2018 (13.27/100,000 habitants), with a 110% increase, suggesting that leishmaniasis might be emerging or reemerging in some areas[3,4].

In Mexico, 99% of the cases registered each year correspond to localized cutaneous leishmaniasis (LCL) caused mainly by *Leishmania (Leishmania) mexicana*[5,6]. This clinical form presents with ulcers in areas of the skin exposed to the vector bite, with a chronic evolution, leading to mutilating and stigmatizing lesions when it affects the cartilage of the nose and ears. Meglumine antimoniate (Glucantime®) is the drug of choice used to treat and heal lesions[5]. LCL, also known as "chiclero's ulcer," was first registered in 1912 by Seidelin in gum tree harvesters (known as "chicleros" in the Spanish language) that used to work in sylvatic areas of the Yucatan Peninsula[7]. Since then, 87.5% of the country's States have reported the presence of the disease, even though the areas with the highest transmission rates are located in the South-Southeast region of the country, including Tabasco State, Chiapas State and the Yucatan Peninsula, which includes the Campeche, Quintana Roo, and Yucatan States[8].

According to the Pan American Health Organization's (PAHO) and the World Health Organization's (WHO) criteria, the Yucatan State is classified as a vulnerable, susceptible area for transmission because it neighbors Campeche and Quintana Roo's highly endemic areas and has historical records of the vector distribution in several municipalities[9]. Annually, the Yucatan Health Services (SSY, by its acronym in Spanish) reports several imported cases due to residents of the Yucatan State traveling to an endemic State in the country. However, in 2015, the SSY reported autochthonous cases in five municipalities with no local transmission records or history of patients traveling outside of their municipalities. Subsequently, autochthonous cases continued to be registered by SSY in 2017, 2018 and in 2019 in eight different municipalities.

The noticeable increase in autochthonous clinical case reports in the last decade and the probable emergence of LCL transmission foci in the Yucatan State indicated the need to identify the etiologic agent involved in autochthonous cases occurring in vulnerable and susceptible areas and to start gathering epidemiological data to implement proper control and prevention strategies.

MATERIALS AND METHODS

Study area

The Yucatan State is located in the Northern portion of the Yucatan Peninsula in Southeastern Mexico, between 21° 36' and 19° 32' latitude North, and 87° 32' and 90° 25' longitud West. It has a tropical warm sub-humid climate regime, and its rainy season is from June to October. The mean annual temperature varies between 24 ºC and 26 ºC, with cumulative annual precipitation between 500 mm and 1,500 mm in Southern areas[10]. The vegetation consists mainly of tropical deciduous forest on the mainland and shrub-mangrove vegetation in coastal areas[11].

Patients

The patients were volunteers undergoing routine diagnosis and treatment programs promoted by SSY and the Mexican Ministry of Health[12]. Through these programs, trained personnel continuously conduct active case detection to locate suspected patients based on epidemiological characteristics and clinical presentation compatible with LCL, collect clinical samples, perform diagnosis by parasitological testing with Giemsa-stained smears and provide medical treatment. They apply a demographic questionnaire and an informed consent form after explaining the diagnostic process and before any sample collection. Forty-five patients were diagnosed through this program from October 2018 to December 2019. Of these, 13 cases (29%) were classified as autochthonous LCL cases as there was no history of the patients traveling outside of their municipalities in the prior 12 months, and 32 cases (71%) were classified as potentially imported LCL as the patients had a history of traveling to endemic areas in Quintana Roo State[9,13,14].

Sampling and diagnostic procedure

For parasitological diagnosis, the lesion and the adjacent normal-looking skin were cleaned with topical antiseptic. Then, tissue scrapings from the lesions were collected using a sterile lancet and spread on a glass slide, or, depending on the lesion area, the glass slide was gently pressed onto the moist ulcer to allow the tissue fluid to imprint on its surface. The slides were stained by Giemsa for optical microscopic examination performed by the technical personnel of the Yucatan State Public Health Laboratory (LESP, by its acronym in Spanish), SSY[15,16].

For the molecular diagnosis, tissue samples were taken by scraping the margins of active lesions and then spotted onto an FTA™ Elute Micro Card (Whatman™ GE Healthcare Biosciences, USA), air-dried, and then stored at room temperature until its use for parasite species determination in the Regional Research Center "Dr. Hideyo Noguchi", Autonomous University of Yucatan[17,18].

Treatment

All diagnosed patients were treated by SSY personnel with meglumine antimoniate (Glucantime® Sanofi Aventis, Brazil; supplied in 5 ml ampules) by intralesional injection twice a week until ulcer resolution. Patients with multiple lesions, inaccessible lesions, or those with intralesional treatment failure were given an intramuscular meglumine antimoniate injection. The mean volume of Glucantime® administered per patient was 33.7 mL, with 6.74 ampules (ranging of 7 mL to 163 mL; 1.4 to 32.6 ampules)[5].

The sample collection, parasitological diagnosis and treatment scheme administration were performed according to the Guidelines for Leishmaniasis Laboratory Surveillance; Guidelines for the Collection, Handling and Samples transportation for Diagnosis to the National Network of Public Health Laboratories by the Institute of Epidemiological Diagnosis and Reference "Dr. Manuel Martinez Baez" (InDRE, by its acronym in Spanish), and the Guidelines for diagnosis, treatment, and control of Leishmaniasis by the National Center of Preventive Programs and Diseases Control (CENAPRECE, by its acronym in Spanish), all from the Mexican Ministry of Health[5,15,16].

Parasite species determination

For DNA extraction, the FTA™ Elute Micro Card protocol was used. Three 4-mm-diameter disks were punched out of each FTA™ Elute Micro Card (Whatman™ GE Healthcare Biosciences, UK) and transferred into a 1.5 mL microcentrifuge tube. Then, 500 µL of sterile water were added and the tube was vortexed five times. The tube was briefly centrifuged and the rinse water was removed. The disks were transferred to a clean 0.5 mL microcentrifuge tube, 30 µL of sterile water were added and the solution was incubated in a heating block at 95 °C for 30 min. Then, the tube was vortexed for 1 minute by pulsing 60 times, then centrifuged to recover the condensation from the top and the disks were withdrawn. The eluted DNA was quantified by a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and used for PCR amplification. The remainder of the DNA elution was kept at -20 °C.

Primers for the *Leishmania* genus were used: forward sequence LM9 (5'-GGA CGA GCT CAT GGC GCC-3') and reverse sequence LM12 (5'-CTG GCA CAC CTC CAC GTA C-3') amplify a 680 base pairs (bp) fragment of the gp63 gene, which is conserved across all *Leishmania* species[19]. To determine *L. (L.) mexicana* species, the forward sequence of the primer IR1 (5'-GCT GTA GGT GAA CCT GCA GCA GCT GGA TCA TT-3'), which corresponds to the 32 final nucleotides of the conserved sequences located at the 3' region of the small ribosomal subunit gene, was used[20]. The primer LM17 (5'-CCC CTC TCC TCC TCC CC-3') was used for the reverse sequence, which corresponds to the internal transcribed spacer (ITS) of the ribosomal RNA (rRNA) gene[19]. These primers amplify a 790 bp DNA fragment specific to *L. (L.) mexicana*.

The amplification was performed using 50 µL of the following mixture: 25 µL of 2× Thermo Scientific™ DreamTaq™ Green PCR Master Mix [DreamTaq DNA polymerase, 2× DreamTaq Green Buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4 mM $MgCl_2$] (Thermo Fisher Scientific, USA), 100 ng of the corresponding oligonucleotides, 10 µL (50–100 ng) of eluted DNA and nuclease-free water to 50 µL. The amplification was carried out in a 2720 Thermal Cycler (Applied Biosystems®, Thermo Fisher Scientific, Waltham, Massachussets, USA) under various conditions based on the oligonucleotides applied. For the *Leishmania* genus (LM9/LM12), we used 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. For *L.* (*L.*) *mexicana* (IR1/LM17), we used 30 cycles of 1 min at 94 °C, 1 min at 65 °C, and 1 min at 72 °C. In addition, the cycles were preceded by a 5-minute denaturation cycle at 94 °C and ended by a a 7-minute final extension cycle at 72 °C.

PCR products were analyzed by electrophoresis in 1.2% agarose gels in Tris Acetate-EDTA (TAE) buffer 1× at 80 V. The gels were stained with SYBR® safe (Invitrogen, Thermo Scientific, Waltham, Massachusetts, USA) at 1 µL/10 mL TAE 1× and visualized in a high-performance

UV transilluminator Gel Doc™ XR+ System (Bio-Rad Laboratories Inc, Hercules, California, USA).

Ethical considerations

This study was part of a larger research project to analyze the molecular and cellular profile of *Leishmania* infection in humans (CB-2015-253641) and was evaluated and approved by the Ethical Committee of Regional Research Center "Dr. Hideyo Noguchi" of the Autonomous University of Yucatan (CEI-21-15), in agreement with the Regulations of the General Law of Health in Research for Health, Mexico[21].

RESULTS

Thirteen LCL autochthonous cases were diagnosed from October 2018 to December 2019 by clinical and parasitological criteria. The patients were from seven municipalities. Four of the infected patients were from Peto municipality (30.7%), two were from Tekom municipality (15.4%), two were from Espita municipality (15.4%), two were from Tinum municipality (15.4%), one was from Chichimila municipality (7.7%), one was from Rio Lagartos municipality (7.7%) and one was from Ticul municipality (7.7%) (Figure 1).

The median age of the patients was 52 years (ranging from 14–70 years), and all the cases were males. The main occupational activities were agriculture (92%) and hunting in the surrounding forests (8%). The most common signs were single (92%) and paired ulcers (8%), located primarily in the ear (50%). Other frequent areas were the face (21.4%) and upper limbs (28.6%).

Samples for molecular analysis were collected from 12 patients; one patient did not agree to donate samples for PCR. In all samples analyzed, we identified the *Leishmania* genus (Figure 2) and all showed molecular evidence of *L. (L.) mexicana* species as the causative agent (Figure 3).

DISCUSSION

Leishmaniasis is an emerging and reemerging disease in many areas of the world[22]. In Mexico, the number of cases and incidence rate of LCL are increasing, and outbreaks are arising in areas in which autochthonous cases have not been reported[3,4]. In the Yucatan State, there have been sporadic reports of human cases of LCL close to Campeche and Quintana Roo, but more frequently, the reports have been of imported cases due to the travel of residents to endemic areas[23]. However, from 2015 to 2019, autochthonous cases of LCL were detected in 13 municipalities without previous transmission records (Tinum, Kaua, Chankom, Kinchil, Tekax, Valladolid, Chemax, Peto, Espita, Tekom, Chichimila, Rio Lagartos, and Ticul), suggesting the emergence of transmission foci.

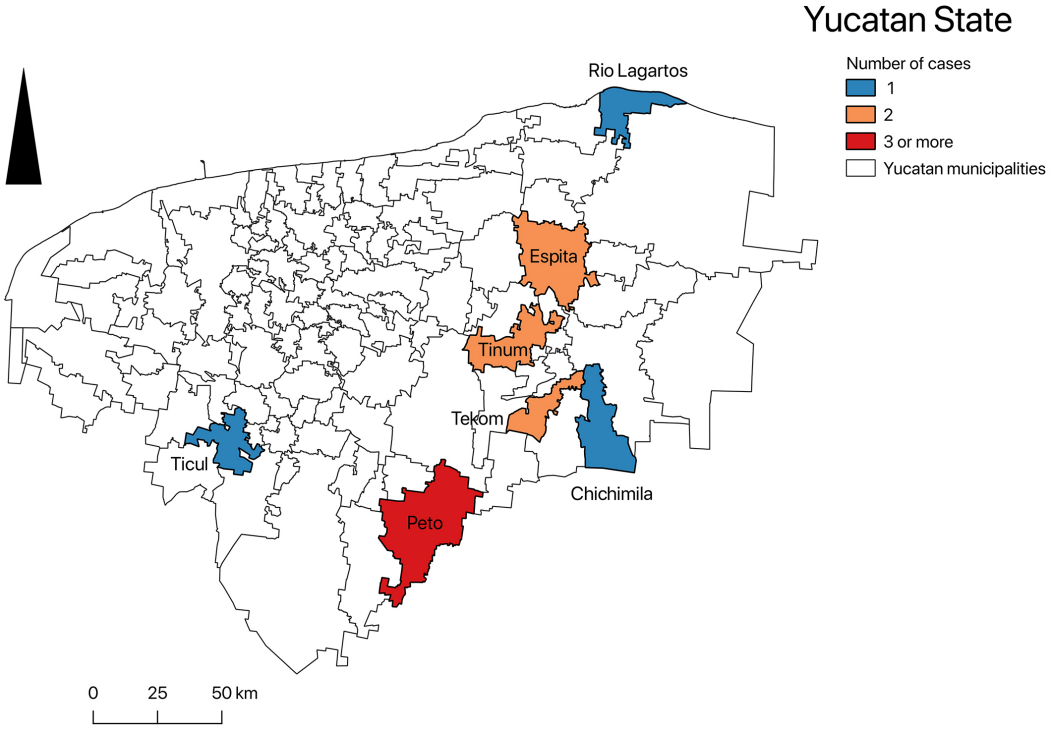

Figure 1 - The geographic distribution of LCL autochthonous cases in municipalities of the Yucatan State.

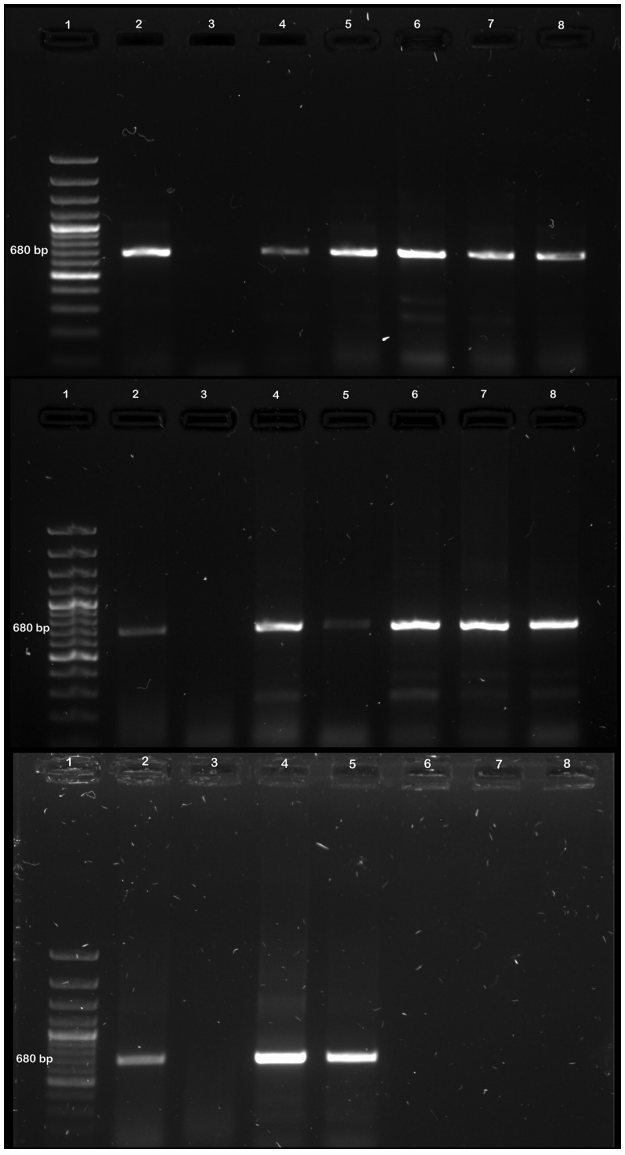

Figure 2 - Detection of *Leishmania* genus in LCL autochthonous cases by PCR analysis. *Leishmania* genus (LM9/LM12 primers, 680 pb). Line 1: 100 bp DNA ladder; Line 2: Positive control *L. (L.) mexicana* DNA from a clinical isolate; Line 3: Negative control (no DNA); Lines 4-8: Positive amplificafion of LCL autochthonous cases.

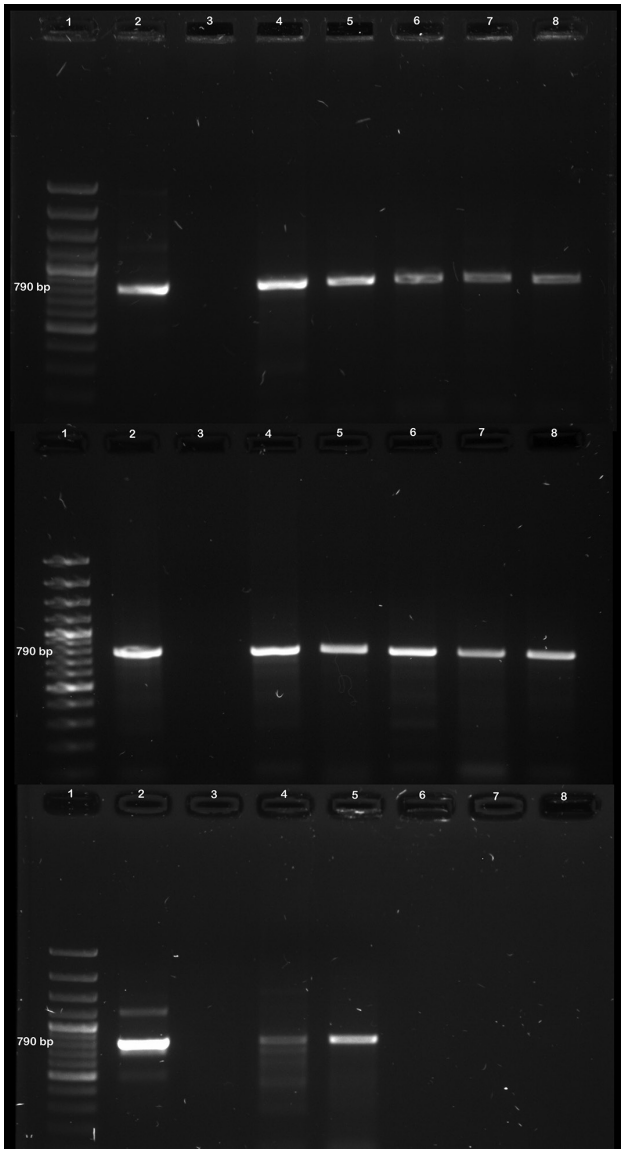

Figure 3 - Detection of *Leishmania L. (L.) mexicana* species DNA in LCL autochthonous cases by PCR analysis. *L. (L.) mexicana* (IR1/LM17 primers, 790 pb). Line 1: 100 bp DNA Ladder, Line 2: Positive control *L. (L.) mexicana* clinical isolate DNA, Line 3: Negative control (no DNA), Lines 4–8: LCL autochthonous cases DNA.

Identifying *Leishmania* species in human cases of LCL is an important eco-epidemiological parameter required to characterize new zoonotic transmission foci and allow the design of appropriate treatment schemes and prevention[24,25]. In this study, we were able to identify *L. (L.) mexicana* in autochthonous cases recently diagnosed in seven municipalities in the Yucatan State. Our findings reveal molecular evidence of this parasite species as the causative agent in 12 of 13 analyzed samples. Moreover, the most frequent risk activity associated with parasite acquisition was agricultural and forestry duties, strongly suggesting a close contact with the sylvatic transmission cycle. All the infected people were males, ranging from 14 to 70 years of age, who predominantly developed a single lesion on the ear, face, or upper limbs. These findings are consistent with transmission foci described for Campeche State and Quintana Roo State, where *L. (L.) mexicana* has its natural foci[26].

Transmission cycles of leishmaniasis have a focal distribution in specific geographic areas known as natural foci of infection. These areas house populations of parasites, vectors, vertebrate reservoirs and abiotic ecosystems due to factors such as humidity, altitude, temperature and vegetation suitable for the maintenance of transmission cycle. Under this scenario, transmission foci have

spatiotemporal limits determined by the vector sandfly species' local distribution and relative density[25].

The primary vector of *L. (L.) mexicana* in Mexico is the sandfly *Bichromomyia olmeca olmeca*. However, other sandfly species such as *Lutzomyia cruciata* (Coquillett), *Psathyromyia shannoni* (Dyar) and *Psychodopygus panamensis* (Shannon) may also act as vectors due to their abundance at specific transmission sites and because of the relative composition of species in collections[27].

In the Yucatan State, *Bi. olmeca olmeca* was recorded from Becanchen (Tekax), *Lu. cruciata* (Coquillet) was recorded from Becanchen (Tekax), Opichen, Xmatkuil (Merida), Sudzal Chico (Tzucacab), Ixil and Seye, and *Pa. shannoni* (Dyar) was recorded from Becanchen (Tekax) and Opichen[28-30]. However, their role in transmission is unknown, showing the need to address a complete study of transmission foci in new areas susceptible to disease outbreaks.

In Mexico, rodents of the *Heteromys*, *Nyctomys*, *Ototylomys*, *Sigmodon*, and *Peromyscus* species had been associated as hosts of *Leishmania* parasites in transmission foci[5,9]. Their relevance in the transmission of the disease in municipalities of the Yucatan State with LCL outbreaks also needs to be addressed because *H. gaumeri*, *O. phyllotis*, *S. toltecus* and *P. yucatanicus* are common species in forested and fragmented habitats of the Yucatan State[31,32].

Another aspect of the transmission cycle could be attributed to domestic animals. Longoni *et al*.[33] and Lopez-Cespedes *et al*.[34] found serological evidence of *L. (L.) mexicana*, *L. (V.) braziliensis* and *L. (L.) infantum* in exposed dog and cat populations from Molas and Xmatkuil in the Merida municipality, Yucatan State.

This finding suggests the circulation of other *Leishmania* species, probably implicating different vectors or reservoirs, as well as a peridomicile transmission since pets have been proved to be players in the transmission cycle. An additional remark on these findings is that antibodies to *L. (L.) infantum* reveal the need to underpin the study of dogs as a source or acting as a maintenance hosts for *Leishmania* species that cause VL.

The analysis of records on species that fit the natural cycle of *Leishmania* and the documentation of recent LCL cases shows the urgency of addressing eco-epidemiological approaches to describe natural transmission foci and implement adequate prevention and control strategies in these vulnerable and susceptible areas.

CONCLUSION

In conclusion**,** leishmaniasis continues to be a public health problem worldwide. Therefore, given its transmission cycle complexity, which involves several parasite species, reservoirs and hosts, it is necessary to adapt prevention and control strategies according to each specific epidemiological scenario. Our findings show molecular evidence that *L. (L.) mexicana* is the main causative agent of LCL autochthonous cases detected in municipalities with no previous evidence of transmission to humans in the Yucatan State and highlight the need to define further eco-epidemiological studies on the transmission of leishmaniasis to improve prevention and control measures of the disease.

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AUTHORS' CONTRIBUTIONS

EBCP: conceptualization, investigation, methodology, supervision, formal analysis, writing-original draft, project administration, funding, and acquisition; DMCH and MAVM: investigation, methodology, writing-review, and editing; RTM: investigation, methodology, data curation, supervision, writing-review, and editing; ERN: conceptualization, data curation, formal analysis, writing-original draft; LHCP and FJEO: investigation, writing-review, and editing; HARP: Conceptualization, writing-review, and editing; JRTC, JAPV, and CDC: investigation, methodology, supervision, resources, writing-review, and editing; JCE: resources, writing-review, and editing.

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