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## PRELIMINARY STUDY TOWARDS A NOVEL EXPERIMENTAL MODEL TO STUDY LOCALIZED CUTANEOUS LEISHMANIASIS CAUSED BY *Leishmania (Leishmania) mexicana*

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### SUMMARY

There is not an experimental model of localized cutaneous leishmaniasis (LCL) caused by *Leishmania (Leishmania) mexicana*. The aim of the present study was to characterize the clinical and histological features of *Peromyscus yucatanicus* experimentally infected with *L. (L.) mexicana*. A total of 54 *P. yucatanicus* (groups of 18) were inoculated with  $1 \times 10^6$  promastigotes of *L. (L.) mexicana* in the base of the tail. They were euthanized at three and six months post experimental infection. The control group was inoculated with RPMI-1640. The predominant clinical sign observed was a single ulcerated lesion in 27.77% (5/18) and in 11.11% (2/18) *P. yucatanicus* at three and six months respectively. The histological pattern described as chronic granulomatous inflammation with or without necrosis was found in 7/7 (100%) biopsies of euthanized *P. yucatanicus* at three (n = 5) and six (n = 2) months, respectively. These results resembled clinical and histological features caused by *L. (L.) mexicana* in humans, and support the possibility to employ *P. yucatanicus* as a novel experimental model to study LCL caused by this parasite.

**KEYWORDS:** *Peromyscus yucatanicus*; *Leishmania mexicana*; Symptoms; Histology.

### INTRODUCTION

Localized cutaneous leishmaniasis (LCL) known as “chiclero’s ulcer” (collector of natural chewing gum) in southern Mexico, was described by Seidelin in 1912<sup>31</sup>. Since then, the forest region of the Yucatan peninsula has been recognized as an endemic focus of LCL<sup>3,6,32</sup>. *Leishmania (Leishmania) mexicana* Biagi, 1953, *emend.* Garnham, 1962, is the main agent causing CL in this focus; however, *L. (Viannia) braziliensis* has also been isolated in a few cases<sup>9,10</sup>.

The clinical features of “chiclero’s ulcer” have been studied and it is characterized predominantly by cartilage involvement of the pinna of the ear<sup>7,16,39</sup>. The most frequently observed presentation is a single, ulcerated, rounded small lesion, with elevated borders and necrotic center, located on the ear, with an evolution time of less than three months, and with neither cutaneous metastases nor lymphatic nor mucosal involvement<sup>4</sup>. Criteria for scars resulting from healed lesions of humans with CL caused by *Leishmania (Viannia) braziliensis* include: no history of trauma, round or oval shape, smooth surface, depressed, and pigment change, hypopigmentation<sup>38</sup>. No differences have been observed in scars from LCL caused by *L. (L.) mexicana* (unpublished data).

The histopathology of LCL due to *Leishmania (Leishmania) mexicana* in the Yucatan Peninsula, Mexico, has also been described. Type III and IV of Magalhaes’ classification<sup>21</sup> have been found as the

most common patterns in 21/73 (28.7%) and 32/73 (43.8%) respectively of our LCL cases caused by *L. (L.) mexicana*<sup>5</sup>.

The cutaneous leishmaniasis laboratory mouse model using C57BL/6 (resistant) and BALB/c (susceptible) infected with *L. major* is the most employed system for analyzing the regulation of the immune response against this pathogen only contained by a cell-mediated response<sup>19,30</sup>. However, C57BL/6 mice inoculated with *L. (L.) mexicana* developed a chronic disease<sup>2</sup>. Although the *L. major* mouse model is considered by many to be the best small animal immunological model for CL, concern about its relevance suggests other models should be developed. In particular, because laboratory mice are usually reduced to homozygosity, are hybrids of three species (*Mus musculus musculus*, *M. m. domesticus*, *M. m. castaneus*)<sup>37</sup> and are not natural reservoirs of leishmaniasis parasites, the use of other, wild-type species that are naturally infected should be considered as alternative models.

In Campeche, three rodents have been identified as primary reservoirs of *L. (L.) mexicana*: *Ototylomys phyllotis* Merriam, 1901; *Peromyscus yucatanicus* J.A. Allen and Chapman, 1897; and *Heteromys gaureri* J.A. Allen and Chapman, 1897<sup>10,11,35,36</sup>. The two latter species are endemic in the Yucatan Peninsula. *O. phyllotis* and *P. yucatanicus* have been adapted to laboratory and a colony for experimental studies has been established at our institution, however the former has been rejected to be employed as an experimental model to study CL caused by *L. (L.) mexicana*<sup>14</sup>. The

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use of local mammalian fauna as a model host to study the development of experimental *Leishmania* spp. infection could give a more approximate whole picture of what probably occurs in natural conditions<sup>12</sup>.

In order to develop a novel experimental model of LCL caused by *L. (L.) mexicana* we sought to characterize clinical and histological features of *P. yucatanicus* experimentally infected with *L. (L.) mexicana* as a fundamental first step for developing an infection model.

## MATERIALS AND METHODS

**Animals:** Six month old *P. yucatanicus* of both sexes were selected from the colony. The animals were maintained in the animal care facility of the Centro de Investigaciones Regionales, Universidad Autónoma de Yucatán (CIR, UADY). The mice were housed individually in small cages (19 x 29 x 12 cm) lined with wood shavings and fed *ad libitum* rodent chow (2018S Harlan, Madison, Wisconsin, USA). For enrichment and food balance, once a week the mice were given fresh fruit or vegetables. During the experiment, the animals were kept at 22 °C with a 12/12h light cycle. Physical enrichment was provided weekly in the form of cardboard tubes for hiding and paper for nesting material. All animals were euthanized with sodium pentobarbital 100 mg/kg, according to the Mexican Law for the use of laboratory animals, and the Guide for the Care and Use of Laboratory Animals<sup>13,29</sup>. The proposal was approved by the bioethics committee of CIR, UADY.

**Parasite:** Although the dose of *Leishmania* promastigotes delivered by the sand fly vector to the skin of mammalian host has never been directly determined, using a real time-PCR approach it has been possible to reveal a remarkable range of 10 to 100,000 in the dose of parasites transmitted to mice exposed to a single infected sand fly<sup>15</sup>. More recently, another study determined infection parameters in the sand fly vector that predict transmission of *L. (L.) major*: it was estimated in the range of 10<sup>3</sup> to 10<sup>6</sup> total parasites per fly<sup>34</sup>. Taking into account these studies we chose to inoculate 1x10<sup>6</sup> promastigotes to increase the possibility of inducing CL clinical signs.

Local strain MPERO/MX/97/Py4, isolated from a *P. yucatanicus* with clinical signs (ulcerated lesion), was identified by isoenzymes and monoclonal antibodies as *L. (L.) mexicana* and was selected for use in this study<sup>9,10</sup>. Its infectivity was restored by a passage in Syrian golden hamsters (*Mesocricetus auratus*). Promastigotes were grown in Senekjic's medium for seven days at 23 °C. Stationary-phase promastigotes were washed three times in RPMI-1640 medium (Gibco) before being counted and adjusted to a 1x10<sup>6</sup> concentration for inoculation at a final volume of 40 µL. All animals were inoculated in the base of the tail.

**Parasite demonstration:** Based on previous studies in which local signs of infection by *L. (L.) mexicana* were always located at the base of the tail<sup>10,11,35,36</sup>, smears were made from samples collected at the edge of the lesion (avoiding ulcerated and/or heavily crusted parts) or from the inoculation site in absence of local signs. Moreover, smears were made from the liver and spleen samples. Furthermore, aspirates and cultures from the skin of the base of the tail in each animal were prepared. Liquid culture medium (0.1-0.2 mL RPMI 1640) with antibiotics were inoculated and aspirated repeatedly with an insulin needle (1 mL, 27G x 13mm) subcutaneously into base of the tail or at the margin of the lesion when it existed. The aspirates were cultured into tubes containing modified

Senekjic's medium, incubated at 23.5 °C and examined weekly by light microscope for parasite growth. A culture was considered positive when at least one promastigote was observed and negative if no parasites were found. If negative, the procedure was repeated until three consecutive aspirates were found with one week intervals.

**Experimental infection:** A total of 54 *P. yucatanicus* in groups of 18 animals were inoculated in the base of the tail and were euthanized at three (group I) and six (group II) months post experimental inoculation. Control group (III) was inoculated with RPMI-1640 and euthanized at three months.

**Clinical features:** All animals were examined weekly for the following local clinical signs of infection including ulcerated lesion, crust and scar, which are the main documented symptoms in humans with LCL caused by *L. (L.) mexicana*<sup>4</sup>.

**Histological features:** Skin biopsies were taken from the edge of the lesion (avoiding ulcerated and/or heavily crusted parts) or from the inoculation site in absence of local signs with a 4-mm disposable punch and 2% xylocaine as an anesthetic just before euthanizing the animals. The material was fixed in neutral formalin, routinely processed and embedded in paraffin. Sections were stained with hematoxylin and eosin. Histopathological findings were grouped in patterns according to a classification proposed by MAGALHAES *et al.*<sup>21</sup>. The following five histopathological patterns were defined as having occurred and it has been done in CL patients infected with *L. (L.) mexicana*: 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, and necrosis and no granulomatous response; type III) exudative and necrotic-granulomatous reaction corresponding to the pattern described as chronic granulomatous inflammation with or without 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.

## RESULTS

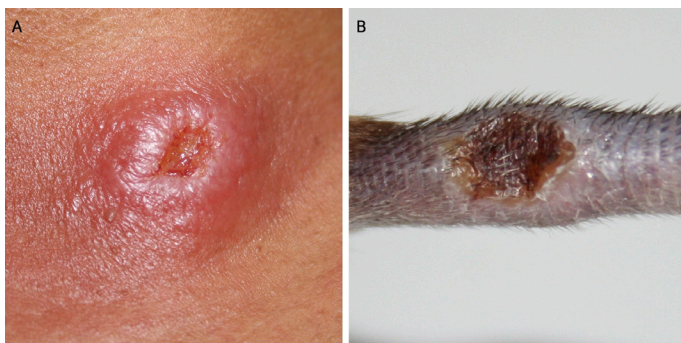
**Clinical features:** Local clinical signs were recorded for each one of the experimental groups inoculated with 1x10<sup>6</sup> promastigotes of *L. (L.) mexicana*. None of the *P. yucatanicus* inoculated with RPMI-1640 developed clinical or histological features of *Leishmania* infection. Absence of local clinical signs was observed in a total of 29/36 (80.55%) of the *P. yucatanicus* experimentally inoculated including both groups I and II. At three months, Group I, the most common clinical sign was a single ulcerated lesion observed in 27.77% (5/18) *P. yucatanicus* that completed the study, within a range of eight to 10 weeks post experimental infection and persisting without healing during the whole period of survey. In *P. yucatanicus* followed for six months, Group II, only in 2/18 (11.11%) ulcerated lesions and crusts were observed, and healing was recorded at week 17 and 18 respectively. Results regarding clinical features are shown in Table 1 and Figure 1. There was no difference in the course of the infection linked to *P. yucatanicus* gender.

**Histological features:** At three months the histological picture of *Leishmania* infection was characterized by pattern type III, i.e. exudative

**Table 1**

Local clinical signs developed by *P. yucatanicus* experimentally infected with  $1 \times 10^6$  promastigotes of *Leishmania (Leishmania) mexicana*

<i>P. yucatanicus</i>	Group	Ulcer	Crust	Scar
Py-9	3 months	Weeks 8-12	Weeks 9-12	
Py-10	3 months	Weeks 9-12	Weeks 9-12	
Py-11	3 months	Weeks 10-12	Weeks 9-12	
Py-12	3 months	-	Weeks 9-12	
Py-15	3 months	Weeks 8-12	-	
Py-16	3 months	Weeks 10-12	Weeks 10-12	
Py-42	6 months	Weeks 8-10	Weeks 11-17	Weeks 18-24
Py-43	6 months	Weeks 16-17	Weeks 16-17	Weeks 17-24



**Fig. 1 - A:** typical patient localized cutaneous nodular ulcerated lesion caused by *L. (L.) mexicana*; **B:** representative nodular ulcerated lesion from a *P. yucatanicus* experimentally infected with  $1 \times 10^6$  promastigotes of *L. (L.) mexicana*.

and necrotic-granulomatous reactions corresponding to the pattern described as chronic granulomatous inflammation with or without necrosis were found in 7/7 (100%) biopsies of euthanized *P. yucatanicus* at three (n = 5) and six (n = 2) months respectively. Parasites were observed in all *P. yucatanicus* with local signs of infection.

**Parasite demonstration:** Smears from ulcerated lesions were all positive at three months post experimental inoculation and negative at six months (n = 6 and 2 respectively). Smears from the liver were positive in 11/18 (61.11%) at three months, and in 16/18 (88.88%) at six months. Smears from the spleen were 17/18 (94.44%) positive at three months and 12/18 (66.66%) positive at six months. Isolations and cultures from *P. yucatanicus* were positive in 61.11% (11/18) and 66.66% (12/18) at three and six months respectively.

## DISCUSSION

This is the first study that examines *P. yucatanicus* clinical and histological features of experimental infection with *L. (L.) mexicana*. As a preliminary study towards the development of a novel experimental model to study LCL caused by *L. (L.) mexicana*, a first and fundamental criterion was to document that both clinical and histological features resembled those described previously in humans with LCL caused by *L. (L.) mexicana*<sup>17</sup>.

In the present study the most common clinical sign, as reported in patients with LCL<sup>4</sup>, was a single, ulcerated, rounded small lesion, with elevated borders and necrotic center, observed in 7/36 (19.44%) of the *P. yucatanicus* experimentally infected with  $1 \times 10^6$  *L. (L.) mexicana* promastigotes. Moreover, scars developed in *P. yucatanicus* gathered the criteria for healed lesions of humans with CL, i.e. round or oval shape, smooth surface, depressed, and hypopigmentation<sup>37</sup>.

It must be remarked that absence of local clinical signs was observed in a total of 29/36 (80.55%) *P. yucatanicus* experimentally infected with  $1 \times 10^6$  *L. (L.) mexicana* promastigotes. As described previously a criterion to incriminate a primary reservoir of *Leishmania* stated that the infection in a reservoir species should be relatively nonpathogenic, that is, the infection should not lower the survival possibility of the animal since it has to live until the next transmission season, which is more than seven months in the Peninsula. In other words, the immune system of the reservoir must react to the presence of the parasite in such a manner that while preventing irrevocable damage, the parasite evades sterilizing immunity<sup>36</sup>.

There was no difference in the course of the infection linked to *P. yucatanicus* gender. However we do not consider this finding to be conclusive regarding the possible role of sex as demonstrated in the mouse model<sup>33</sup>. More studies will be done regarding the role of sex in susceptibility and/or resistance to the infection by *L. (L.) mexicana* in *P. yucatanicus*.

The histological picture observed in *P. yucatanicus* experimentally infected with *L. (L.) mexicana* was predominantly type III with reference to Magalhaes' classification whereas in patients with CL caused by the same *Leishmania* species it was type III and IV. Although a possible correlation between clinical and histological features has been examined to understand the immunological response in humans infected with *L. (L.) mexicana*<sup>5</sup>, it has been difficult to establish because such examination depends on a wide range of variables such as the host, the *Leishmania* species, the time of evolution at the moment of performing the histological study, the inoculation route and the dose of the parasite.

Parasite isolation and culture from the skin of the base of the tail of *P. yucatanicus* was positive in 61.11% (11/18) and 66.66% (12/18) at three and six months, and in smears from the liver and the spleen. With reference to this finding *L. major* DNA was detected by PCR in the lymph nodes, spleens, bone marrow, and livers of C57BL/6 one year after recovery from infection<sup>1</sup>. Moreover DNA specific for *L. (V) braziliensis* was detected by PCR in scars after clinical cure of CL<sup>24</sup>. We have reported in a previous study the retention of *L. (L.) mexicana* in 29 naturally infected rodents including six *P. yucatanicus*. These cricetids lived in captivity for up to two years and were tested monthly for the presence of the parasite by cultures of needle aspirates from the base of the tail<sup>35</sup>. These data support the persistence of the parasite in *P. yucatanicus* experimentally infected, as in the human treated or spontaneously healed and in the mouse model of CL.

Since the 1970s parasitic protozoa of the genus *Leishmania* have provided a useful perspective for immunologists in terms of host defense mechanisms critical for the resolution of infection caused by intracellular pathogens in experimental studies done in the mouse model. The paradigm for the CD4<sup>+</sup> T-helper 1 (Th1)/Th2 dichotomy is largely based,

respectively, to the *Leishmania major* infection in a susceptible/resistant mouse model. Different virulence factors have been identified for distinct *Leishmania* species, and there are profound differences in the immune mechanisms that mediate susceptibility/resistance to infection and in the pathology associated with disease. These variations not only point to interesting features of the host-pathogen interaction and immunobiology of this genus of parasitic protozoa, but also have important implications for immunotherapy and vaccine development<sup>22</sup>.

A view of leishmaniasis that only considers mouse model infection with *L. major* misses a wealth of interesting immunobiology associated with other species of *Leishmania*. *L. (L.) amazonensis* and *L. (L.) mexicana* are associated with quite different patterns of disease following infection of mice when compared with *L. major*. For example, C57BL/6 or C3H mice, which heal from the *L. major* infection, develop chronic disease when infected with either *L. (L.) amazonensis* or *L. (L.) mexicana*<sup>2</sup>. Conflicting data may arise in part because different parasite strains or species are being examined, different tissue target (the mice's footpad, the ear, or base of the tail) are being infected, and different doses ("low"  $1 \times 10^2$  and "high"  $1 \times 10^6$ ) of metacyclic promastigotes have been inoculated<sup>20,27,28</sup>. In addition immunological studies carried out in humans did not reproduce the Th1/Th2 dichotomy described in the *L. major*, C57BL/6 and the BALB/c mouse model of CL<sup>18,23,24</sup>. In summary there is not an experimental model to study LCL caused by *L. (L.) mexicana*.

Although classical laboratory mice have advantages, such as colony maintenance, breed all year round, and a short generation time of 10-12 weeks, they have serious limitations when compared with human populations, e.g. they do not exhibit a great variety of natural genetic polymorphisms<sup>12</sup>.

A new approach to study host-parasite relationships bring out because no animal model was available to study the Sin Nombre virus (SNV), a Hantavirus which is a rodent-borne pathogen with a worldwide distribution and that can cause hantavirus cardiopulmonary syndrome (HCPS) in North America. This situation led to the experimental infection of the deer mouse *Peromyscus maniculatus*, a primary reservoir of the SNV<sup>8</sup>. However as in the golden hamster experimental model to study visceral leishmaniasis research is hindered by the lack of commercially available immunological and molecular reagents to investigate mechanisms of disease<sup>25</sup>.

In summary, we have found that both clinical and histological features of *P. yucatanicus* infected experimentally with *L. (L.) mexicana* resembled our previous studies done in patients with LCL caused by this parasite. Moreover parasite persistence was recorded as it has been described in patients where CL healed spontaneously after treatment<sup>1</sup>. These results support the utility of *P. yucatanicus* as a novel experimental model to study LCL caused by *L. (L.) mexicana*.

## RESUMEN

### Estudio preliminar sobre un nuevo y original modelo experimental de leishmaniosis cutánea localizada causada por *Leishmania (Leishmania) mexicana*

No existe un modelo experimental de la leishmaniosis cutánea localizada (LCL) causada por *Leishmania (Leishmania) mexicana*. El

objetivo del presente estudio fue el de caracterizar los cuadros clínico e histológico de *Peromyscus yucatanicus* infectados experimentalmente con *L. (L.) mexicana*. Un total de 54 *P. yucatanicus* (grupos de 18) fueron inoculados en la base de la cola con  $1 \times 10^6$  promastigotes de *L. (L.) mexicana*. A los 3 y 6 meses posteriores a la infección experimental fueron sacrificados. El grupo control fue inoculado con RPMI-1640. El signo clínico predominante fue una lesión única ulcerada en 27.77% (5/18) y en 11.11% (2/18) *P. yucatanicus* a los 3 y 6 meses respectivamente. El patrón histológico descrito como inflamación crónica granulomatosa con o sin necrosis se observó en 7/7 (100%) biopsias de los *P. yucatanicus* a los 3 (n=5) y 6 (n=2) meses respectivamente. Los resultados son similares a los cuadros clínico e histológico de la infección por *L. (L.) mexicana* en humanos, y apoyan la posibilidad de utilizar *P. yucatanicus* como un nuevo y original modelo para el estudio de la LCL causada por *L. (L.) mexicana*.

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