

# Cotton Rats (*Sigmodon hispidus*) and Black Rats (*Rattus rattus*) as Possible Reservoirs of *Leishmania* spp. in Lara State, Venezuela

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A total of 519 wild animals belonging to eleven species were collected during a two year study in a cutaneous leishmaniasis endemic area in Venezuela (La Matica, Lara State). The animals were captured in home-made Tomahawk-like traps baited with maize, bananas or other available local fruits, and parasites were isolated from 27 specimens. Two different species were found naturally infected with flagellates, i.e., cotton rats (*Sigmodon hispidus*) and black rats (*Rattus rattus*).

Characterization of the parasites using PCR, kDNA restriction pattern and hybridization with species-specific probes revealed the presence of *Leishmania* (L.) mexicana in three of the black rats and *Leishmania* (V.) braziliensis in two others. The latter species was also identified in the single positive specimen of *S. hispidus*. The results suggested both species of animals as possible reservoirs of *Leishmania* sp.

Key words: *Leishmania* - reservoirs - leishmaniasis - polymerase chain reaction - Venezuela

Cutaneous leishmaniasis is produced by different species and subspecies of *Leishmania* of the subgenera *Viannia* and *Leishmania*, which infect a wide variety of mammalian hosts. In Venezuela, during the last five years, an annual total of 2,500 new cases have been reported by the Ministry of Health. Cutaneous leishmaniasis is most abundant in rural and suburban areas and is commonest in people with scarce economic resources, dedicated to agricultural activities. The disease is widely distributed in the whole country, especially in the Andean region, dealing with 60% of the total cases reported by the National Registry of Health. Although leishmaniasis has been described as a zoonosis, the natural reservoirs in most of the endemic areas are unknown because of the difficulties in isolating and identifying parasites from wild animals.

Previous work carried out by Bonfante-Garrido et al. (1981) demonstrated the presence of *L. braziliensis* in domestic animals but with clinical manifestations similar to human disease. The relationship of this parasite with wild animals has not yet been demonstrated. Other studies mainly restricted to domestic animals and experimental infections have been reported in Venezuela (Torrealba et al. 1972, Aguilar et al. 1984).

Many mammals have been described as hosts of *Leishmania* and some of them are considered to play an important role as reservoirs (Lainson & Shaw 1987). In Brazil, sylvatic rodents as well as edentates and marsupials are reported to be the natural reservoir hosts of dermatotropic leishmaniasis (Arias et al. 1981, Lainson et al. 1981).

In Venezuela, despite the high incidence of cutaneous leishmaniasis in the rural population and efforts to control the disease, there have been few epidemiological studies and in the majority of the foci the natural vectors and reservoirs are unknown.

The studies in wild animals have been limited by the number of animals studied or due to the techniques used in the identification of the parasites isolated from animals.

Finding the natural host of *Leishmania* is crucial to determine the natural cycle of the parasite, in order to understand the epidemiology of the disease.

In this study we used molecular techniques to identify the parasites isolated from wild animals and incriminate wild animals as natural hosts of *Leishmania* spp. in an endemic area in Lara State, Venezuela.

## MATERIALS AND METHODS

**Study area** - La Matica is a small village located between latitudes 9°42'50"N and longitudes 69°41'53"W in the Andres Eloy Blanco district in the southeastern sector of the Venezuelan state of Lara (Fig. 1). The district has a population of 39,842 inhabitants; the village and the surrounding areas which have an estimated population of 1,500 inhabitants and an economy based on agricultural activities, reported 10 cases of cutaneous leishmaniasis during this study. Lara State is located in west central Venezuela with a population of 1,522,043 inhabitants. It is a highly endemic area for cutaneous leishmaniasis with an incidence of 5 new cases/100,000 inhabitants/year, the state being responsible for about 20% of the new cases registered in Venezuela each year.

**Animal trapping** - The animals were captured five nights per month over a period of two years from October 1996 to October 1998, trapped in home-made Tomahawk-like one door metal traps of two different dimensions, 19x5x5 and 26x9x9 inches, with the capacity to capture single small animals or several animals at the same time.

Traps were baited with local fruits (banana, maize, etc.) set at 20 or 30 m from the houses and in the cornfields

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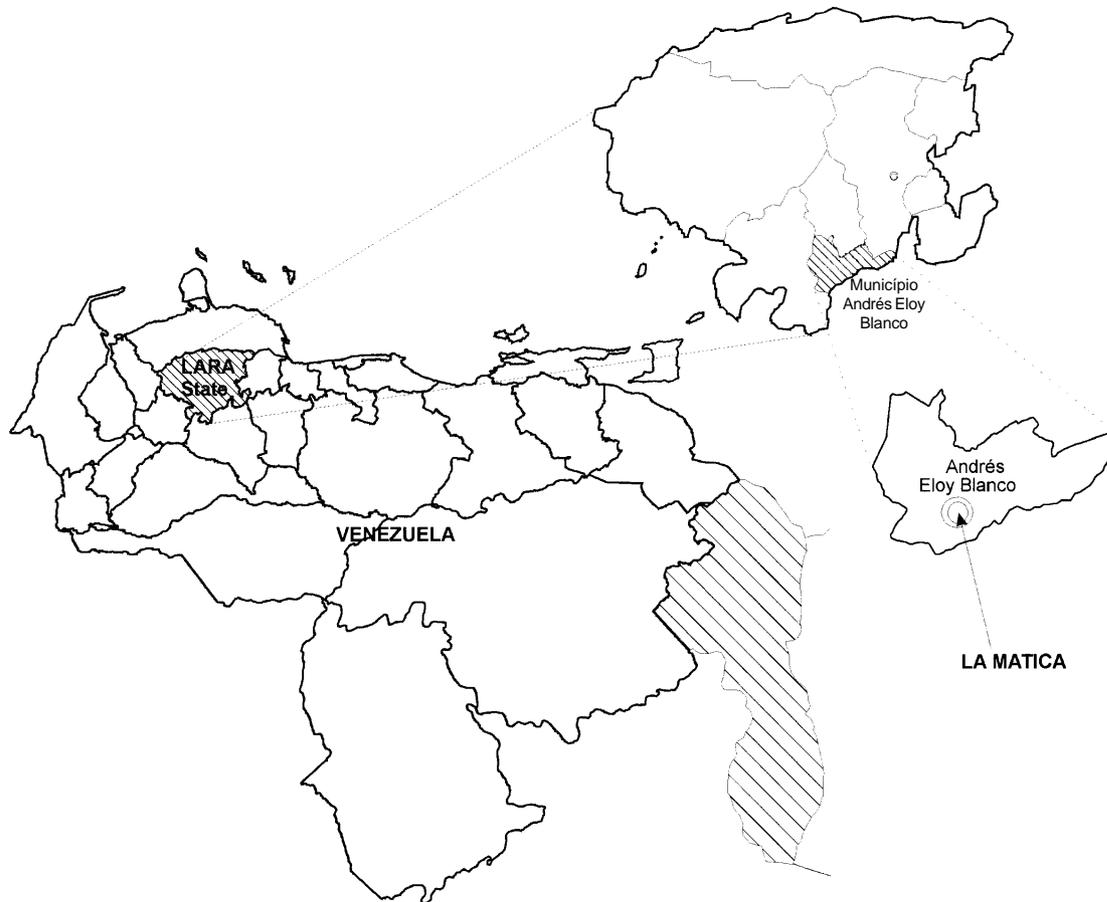


Fig. 1: map showing the study area.

from 6 p.m. to 8 a.m. Twenty traps were used to capture an average of five animals per night; all animals were kept alive until they were studied.

**Diagnostic methods** - The animals were examined, looking for suspicious lesions such as skin ulcers, scars, and alopecia. Blood samples were taken from the heart under anaesthetic conditions. Each sample was used for fresh microscopic examination and culture. If any of the samples resulted positive, the animal was humanely sacrificed and samples from skin, liver and spleen were taken for parasitological studies, microscopic preparation, PCR and culture in blood agar medium.

**Parasite isolation and culture** - The parasites were isolated from blood and cultured in blood agar base medium (Difco), with 10% defibrinated rabbit blood plus 200 units of penicillin. Within seven days, samples of the cultures were taken for microscopic examination. Negative cultures without contamination were kept for one month before discarding. When the culture was positive, rapid growth of the parasites was obtained in petri dishes in the same medium. Parasites in the stationary phase were harvested by centrifugation at 5,000 rpm for 10 min at 4°C and washed three times with sterile phosphate buffered saline (pH 7.4)

**kDNA isolation and digestion** - Kinetoplast DNA was isolated as previously described (Rodríguez et al. 1994). For schizodeme analysis, 5 µg of kDNA were digested

with five units of Msp I endonuclease (Gibco-BRL). After digestion to completion, the digested products were separated by electrophoresis in 4.5-10% linear gradient polyacrylamide gels, run at 7 mA overnight. The gels were stained with silver nitrate and photographed. Dot blot hybridization was carried out as was previously described (Barrios et al. 1994). Pre-hybridization and hybridization were performed overnight at 42°C in 50% formamide containing buffer. Filters were developed using Genius Systems (Boehringer-Mannheim) according to the instructions of the manufacturer.

**PCR reaction** - For the PCR, DNA was extracted from biological samples after digestion in lysis buffer (10mM Tris-HCl pH 8, 10mM EDTA, 1 µg/ml Proteinase K) incubated for 2 h at 56°C followed by phenol/chloroform extraction and ethanol precipitation. The DNA was precipitated from the aqueous phase and resuspended in TE buffer (10 mM Tris HCl, pH 8, 1 mM EDTA). PCR was carried out with 5 µl of total DNA using the same conditions and primers previously described (Rodríguez et al. 1997).

## RESULTS

**Live trapping** - The species and number of wild animals trapped are shown in Table I. Animals were captured during five days periods each month for two years and identified using taxonomic criteria.

Most of the animals captured were rodents (482) or opossums (37), cotton rats (*S. hispidus*) accounted for 391 animals followed by black rats (*R. rattus*) with 68 animals. Others species found were *Akodon* sp. (10), *Rhipidomis* sp. (2), *Oryzomys* sp. (3), *Sciurus* sp. (3), *Zygodontomys* sp. (1), *Mus musculus* (1) and *Proechimys* sp. (3). Opossums were all *Didelphis marsupialis* (37/37).

*Clinical and parasitological diagnosis* - The results obtained using different diagnostic criteria are shown in Table II. Comparison between microscopic observation and culture indicated that culturing of the parasites was more sensitive than smear and microscopic observation after Giemsa staining in the detection of infected *S. hispidus*. Microscopic examination of fresh blood was

TABLE I  
Total animals captured and parasite identification

Common name	Scientific name	Numbers		Identified species
		Clinical exam	Culture	
Rodents				
Cotton rat	<i>Sigmodon hispidus</i>	391	5	<i>Leishmania (V.) braziliensis</i> (1) Unidentified (4) <sup>b</sup>
Black rat	<i>Rattus rattus</i>	68	17	<i>L. (L.) mexicana</i> (2) <i>L. (V.) braziliensis</i> (3) <i>Trypanosoma</i> sp. (3) <sup>a</sup> Unidentified (9) <sup>b</sup>
Marsupials				
South American Opossum	<i>Didelphis marsupialis</i>	37	5	<i>Trypanosoma</i> sp. (2) <sup>a</sup> Unidentified (3) <sup>b</sup>
Other species	<i>Akodons</i> sp., <i>Rhipidomis</i> sp., <i>Oryzomys</i> sp., <i>Sciurus</i> sp., <i>Zygodontomys</i> sp., <i>Musmusculus</i> , <i>Proechimys</i>	23	0	
Total		519	27	

a: specific identification in progress; b: unidentified because of insufficient growth in artificial medium

TABLE II  
Positive wild animals infected with flagellates

Species name	Animals captured	Fresh blood		Stained smear		Culture	
		Positive	%	Positive	%	Positive	%
<i>Sigmodon hispidus</i>	391	0/107	0	0/391	0	5/391	1.2
<i>Rattus rattus</i>	68	15/30	50	28/68	41.1	17/58	29.3
<i>Didelphis marsupialis</i>	37	5/18	27.7	6/31	19.3	5/31	16.1

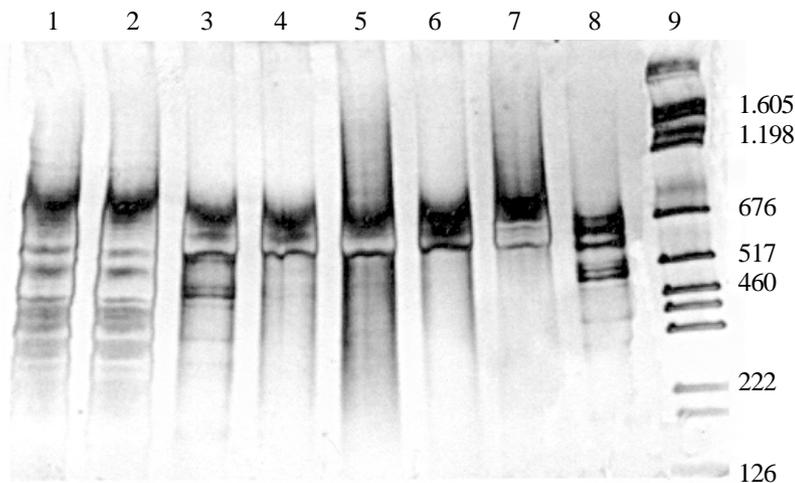


Fig. 2: restriction pattern after digestion with Msp I restriction enzyme. Order of the samples: lane 1: 2B94; lane 2: 4E94; lane 3: 3E94; lane 4: 2E94; lane 5: 259A; lane 6: 25B; lane 7: *Leishmania braziliensis* (M2903) kDNA; lane 8: *L. amazonensis* (PH8) kDNA. Samples identified with letters "B" and "E" correspond to *Rattus* sp., while those identified with "A" correspond to *Sigmodon* sp.; lane 9: molecular weigh markers (pGEM DNA Markers, Promega)

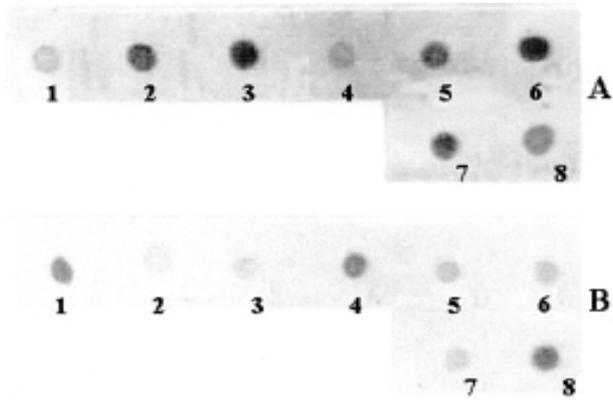


Fig. 3-A: hybridization with species specific probes (M2903 kDNA) for *Leishmania* (*V.*) *braziliensis*; B: hybridization with *L. (L.) amazonensis* probe (PH8 kDNA). Order of the samples: 1: 2B94; 2: 2E94; 3: 3E94; 4: 4E94; 5: 259A; 6: 25B; 7: *L.(V.) braziliensis* (M2903); 8: positive control (PH8 kDNA).

more sensitive than other methods in the identification of parasites in blood samples from *Didelphis* sp. and *R. rattus*, but was not able to detect infected *S. hispidus*.

Smears from lymph node, liver, spleen and skin were negative for amastigotes. Blood cultures were successfully used to obtain 27 isolates (Table II), from three different species of mammals; *S. hispidus* (5/391), *R. rattus* (17/58) and *Didelphis* sp. (5/31). Only eleven cultures grew in artificial culture medium, to be characterized using molecular techniques.

**Taxonomic identification of parasites** - The restriction patterns of six isolates after digestion with *Msp* I restriction enzyme are shown in Fig. 2. Samples 1 and 2 have similar restriction patterns with *L. (L.) amazonensis* (lane 8, IFLA/BR/68/PH8), while isolates in lanes 3, 4, 5 and 6, have high homology with *L. (V.) braziliensis* (MHOM/BR/68/M2903) in lane 7. These results were confirmed after hybridization with probes for *L.(V.) braziliensis* and *L.(L.) mexicana* respectively. The results after hybridization with *L.(V.) braziliensis* probe (M2903 kDNA) are shown in Fig. 3A. A high hybridization signal was observed in samples 2, 3, 5 and 6, corresponding to the same samples in Fig. 2. In Fig. 3B the high hybridization signals correspond to 2B94 and 4E94 (samples 1 and 4), after hybridization with a *L. (L.) amazonensis* (PH8) kDNA probe. Both samples were isolated from black rats.

**PCR results** - The results obtained after 35 amplification cycles are shown in Fig. 4. The expected diagnostic band of 506 bp for *L. (V.) braziliensis* was observed in three samples from black rats (lanes 1, 2 and 3) and one sample from *S. hispidus* (lane 7). Two other samples from black rats (4, 5) resulted negative for *L. braziliensis*, but PCR with *L. mexicana* primers was positive for those samples (result not shown). Although the diagnostic band was observed with a high intensity signal, other bands were also observed, probably due to genetic variability within the *L. (V.) braziliensis* species as we previously described (Rodriguez et al. 1998). Hybridization of the positive PCR products to LbJ38 *L. braziliensis* species-specific probe resulted positive (results not shown).

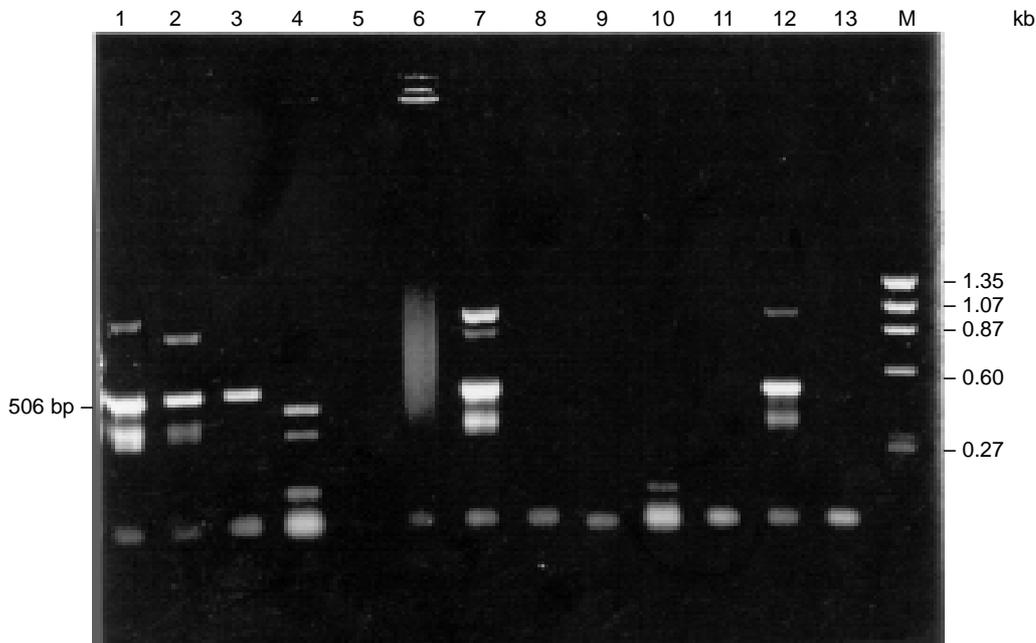


Fig. 4: gel electrophoresis of the PCR products after 35 amplification cycles. Samples 1, 2, 3 and 7 correspond to 2E94; 3E94; 259A and 25B DNA from parasites isolated from *Rattus rattus* and *Sigmodon hispidus*; lane 4: artificial mix *Leishmania braziliensis/L. mexicana*; lane 5: *T. cruzi*; lane 6: *T. rangeli*; lane 8: *L. enrietti* (L88); lane 9: *L. guyanensis* (M4147); lane 10: *L. amazonensis* (PH8); lane 11: *L. mexicana* (Bel 21); lane 12: *L.(V.) braziliensis* (M2903); lane 13: negative control (reaction mix with no DNA) M: molecular weight markers (ØX174, Gibco-BRL)

## DISCUSSION

Many species of wild animals have been suggested as reservoirs of New World *Leishmania* spp. (Lainson & Shaw 1987). Amastigotes of *Leishmania* have been isolated from mammals of many species and several orders. The reservoirs for *L. (V.) guyanensis* include the two-toed sloth, *Choloepus didactylus* and the lesser anteater, *Tamandua tetradactyla*; secondary hosts include marsupials (*Didelphis*) and more rarely rodents (*Proechimys*) (Lainson et al. 1994), while *L. (V.) braziliensis* has been isolated from the rodents *Proechimys*, *Rhipidomys*, *Oryzomys*, *Akodon* and *Rattus* as well as *Didelphis*.

In Venezuela, an early report suggested *Zymodontomys* and *Heteromys* to be the reservoirs for *L. pifanoi*, based on experimental infections in hamsters after injection with blood isolated from both animals (Kerdel-Vegas & Essenfield-Yahr 1966). Domestic animals such as equines and cats have been reported infected with *L. (V.) braziliensis* (Bonfante et al. 1981).

Here we report the results obtained after a field study carried out in an endemic area of cutaneous leishmaniasis in Lara State, Venezuela.

A total of 519 mammals was trapped including 11 different species. The most abundant were cotton rats (*S. hispidus*) accounting for 391 specimens and black rats (*R. rattus*), with 68 specimens. However, the presence of parasites was only demonstrated in 27 mammals after culturing blood samples from the most frequently captured species. The kDNA restriction pattern together with the hybridization results indicate that four samples were infected with *L. (V.) braziliensis* (one cotton rat and three black rats). *L. (L.) mexicana* was demonstrated in two samples from black rats. The PCR and hybridization results using species-specific primers and probes also confirm the presence of both of the *Leishmania* and *Viannia* subgenera in wild animals. The utility of the mentioned molecular tools in the specific identification of *L. (V.) braziliensis* was previously demonstrated (Rodríguez et al. 1997).

kDNA restriction pattern and hybridization are techniques widely used to differentiate between species and sub-species of parasites (Barker 1989, Rodríguez et al. 1994, Pacheco et al. 1997). Here we demonstrated once again the utility of both techniques to identify *Leishmania* in samples isolated from wild mammals.

Black rats (*R. rattus*) were previously identified as the natural reservoir for *Leishmania* in the State of Ceará, Brazil (Alencar et al. 1960, Vasconcelos et al. 1987). More recently the same species was identified as a natural reservoir for *L. (V.) braziliensis* in Baturite, Brazil (Vasconcelos et al. 1994) and in Colombia (Alexander et al. 1998), using PCR and hybridization techniques. In this study, the results suggest that cotton rats and black rats are putative reservoirs of *L. (V.) braziliensis* and *L. (L.) mexicana*. Both species meet at least three of the five criteria used to define a reservoir: (1) overlap between the geographic distribution of wild animals and human cases. Cutaneous leishmaniasis was reported in the study area and the parasites isolated from the human cases were identified as *L. (V.) braziliensis* (Rodríguez et al. 1997); (2) proportion of wild animals infected: it may be higher than 20%. In this study we found that 17/68 black rats were infected, representing

25% of the specimens; (3) the identification of *L. braziliensis* in both species of animals. Additionally, xenodiagnosis using *Lu. ovallesi* reveals 1/500 sandflies with parasites in the digestive tract after biting an infected *S. hispidus*.

Chable Santos et al. (1995) demonstrated the role of cotton rats in the transmission of *L. mexicana* in Mexico; our findings suggest the same animal as a possible reservoir of *Leishmania* spp. The results presented here offer additional evidence about the role of black rats in the transmission and maintenance of cutaneous leishmaniasis as a zoonosis.

We believe that this is the first demonstration of both species of mammals as natural hosts of *Leishmania* of both of the subgenera *Viannia* and *Leishmania* in Venezuela.

Finding the same species (*R. rattus*) infected with *L. (L.) mexicana* or *L. (V.) braziliensis* is very interesting from the epidemiological point of view, since it has been previously demonstrated (Barrios et al. 1994) that the same sandfly can be the vector for both subgenera. It is also possible that the same animal is bitten by two different species of vector. However, more studies have to be done to confirm the role of both species as natural reservoirs of *Leishmania* spp. in Venezuela.

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