

## Susceptibility of Spiny Rats (*Proechimys semispinosus*) to *Leishmania (Viannia) panamensis* and *Leishmania (Leishmania) chagasi*

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*The role of Proechimys semispinosus as reservoir of Leishmania (Viannia) panamensis on the Colombian Pacific coast was experimentally evaluated. The susceptibility to L. chagasi also was assessed to determine the utility of this rodent as a model for studying reservoir characteristics in the laboratory. Wild-caught animals were screened for natural trypanosomatid infections, and negative individuals were inoculated intradermally (ID) in the snout or feet with 10<sup>7</sup> promastigotes of L. panamensis. L. chagasi was inoculated intracardially (10<sup>7</sup> promastigotes) or ID in the ear (10<sup>8</sup> promastigotes). PCR-hybridization showed that 15% of 33 spiny rats were naturally infected with L. Viannia sp. Animals experimentally infected with L. panamensis developed non-ulcerated lesions that disappeared by the 7th week post-infection (p.i.) and became more resistant upon reinfection. Infectivity to sand flies was low (1/20-1/48 infected/fed flies) and transient, and both culture and PCR-hybridization showed that L. panamensis was cleared by the 13th week p.i. Animals inoculated with L. chagasi became subclinically infected and were non-infective to sand flies. Transient infectivity to vectors of spiny rats infected with L. panamensis, combined with population characteristics, e.g., abundance, exploitation of degraded habitats and high reproductive rates, could make them epidemiologically suitable reservoirs.*

Key words: spiny rats - *Leishmania* - reservoir - experimental infection

Spiny rats (*Proechimys* spp.) are parasitized by a variety of *Leishmania* species, including *L. aristidesi* and recognized pathogenic species such as *L. guyanensis*, *L. amazonensis*, and *L. mexicana* s.l. Although these rodents have been incriminated as natural hosts for pathogenic species in several endemic foci in South America, encompassing Brazil, French Guiana, Venezuela and Trinidad (Lainson et al. 1979, WHO 1990), their reservoir role still needs to be established.

In Colombia, information on spiny rats and their relationships with both cutaneous and visceral leishmaniasis is fragmentary. *P. semispinosus* is the most abundant small mammal inhabiting tropical wet forests of the Colombian Pacific coast where cutaneous leishmaniasis is endemic (Weigle et al. 1986, Gonzalez & Alberico 1993). In the Northern region of the country, another species of spiny rat, *P. canicollis*, is infected with *L. chagasi* in tropical dry forests and areas of subsistence agriculture where the original forest has been extensively degraded (Travi et al. 1998).

Incriminating wild animals as *Leishmania* reservoirs is based on several biological parameters, among which natural infection, population dynamics, and interactions

with and infectivity to vectors are of capital importance. In the present study, using an experimental approach, we evaluated the potential role of *P. semispinosus* as a reservoir of cutaneous leishmaniasis caused by *L. (V.) panamensis*. Also, we determined the susceptibility of this species to *L. (L.) chagasi* to evaluate the utility of this rodent as a model of reservoir host that could be utilized in the laboratory.

### MATERIALS AND METHODS

*Collection of spiny rats and quarantine* - Spiny rats from the Pacific lowlands of Colombia (Tumaco, Nariño; 1°48'N, 78°46'W) were captured with National-type traps, after the appropriate permission from the departmental agency for the protection of wild fauna (Corponariño) was obtained. Approximately 100 trapping stations were established in areas of secondary forest, where banana, plantain and cocoa are grown. The traps were baited with plantain, set before dusk, and inspected at dawn. To avoid excessive stress and self-inflicted wounds, the animals were transferred immediately to transport cages of aluminum walls, where access to food and water was provided. Specimens were transported by air to the central laboratory in Cali and quarantined for 8 weeks in the vivarium. An individual coprological analysis was conducted, and an anti-parasitic treatment [ivermectin (Virbamec®), and propoxur (Bolfo®)] was implemented. A blood sample (0.5 ml), drawn by intracardial puncture, was cultured in NNN and Senekjje's media to detect natural infections with *Leishmania* or *Trypanosoma*. Serum was collected for detecting antibodies against the same parasites by means of ELISA. Total IgG antibodies were determined in serum (diluted 1:100) using a soluble *T. cruzi* or *L. panamensis*

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antigen (1 µg per well) and protein A labelled with peroxidase (Kirkegaard & Perry Labs).

Lack of *Leishmania* infection was confirmed by PCR using 3-mm<sup>2</sup> skin samples from the ear. Primers specific for *L. Viannia* kDNA (B1/B2) developed by de Bruijn and Barker (1992) and for *L. chagasi* kDNA (DB8/AJS3) developed by Smyth (1992) were used in these assays. PCR products were hybridized with biotinylated probes.

Maintenance cages were furnished with small removable metal boxes for shelter during the day. Due to the fact that spiny rats would not adapt to commercial rodent food, animals received assorted vegetables and fruits (guava, kidney-beans, peanuts, citron, potatoes, carrots, etc.), and water ad libitum throughout the study period.

*Experimental infection and follow-up* - To prepare the inocula, *L. panamensis* and *L. chagasi* with no more than 3 passages after being isolated from hamsters were grown at 25°C in Senekjie's and Schneider's culture medium, respectively. Promastigotes were harvested during the stationary phase of growth (6 days of culture) and suspended at the corresponding concentration in phosphate-buffered saline.

Young spiny rats of both sexes, weighing more than 130 g and with no apparent clinical alterations, were used. The rats were anaesthetized with a combination of ketamine hydrochloride: xylazine (10:1) at a dosage of 30-40 mg/kg ketamine, i.m. A group of four spiny rats was intradermally inoculated with 10<sup>7</sup> promastigotes of *L. panamensis* in both the snout and hind feet. A similar inoculation strategy was used for reinfesting these animals with *L. panamensis* on the 7th and 14th week post-infection (p.i.). Two groups of 5 animals each were infected with *L. chagasi* by the intracardial (10<sup>7</sup> promastigotes) or intradermal route (10<sup>8</sup> promastigotes) at the base of the ear; 3 uninfected individuals were used as controls.

Spiny rats infected with *L. chagasi* were evaluated on a monthly basis by searching for clinical signs of infection: weight loss, deterioration of fur condition, dehydration, and decrease in food consumption. Blood was collected from anesthetized rats for hematocrit determination, and serum was preserved for estimating the humoral response to *Leishmania* by ELISA. Liver aspirates were cultured in NNN medium, and samples of skin from the ear were preserved for subsequent detection of parasite DNA by PCR (Travi et al. 1998). All spiny rats infected with *L. chagasi* were sacrificed on the 7th month p.i. using a CO<sub>2</sub> chamber and previous anaesthesia with ketamine hydrochloride-xylazine. The clinical, serological, and parasitological status as previously described was evaluated. Also, samples from the spleen were seeded in NNN medium and smeared for direct microscopic examination after Giemsa staining.

Animals inoculated with *L. panamensis* were inspected every week for visible signs of cutaneous leishmaniasis (skin redness, swelling, depilation, and skin necrosis). Lesion size was determined with a caliper by measuring the area of swollen or depilated skin of the snout or by measuring the diameter of the inoculated and contra-lateral foot. The evolution index was obtained with the following formula:

observed measurement – initial measurement = initial measurement

At the end of the experimental period (7 mo), animals were sacrificed as previously described. Persistence and dissemination of parasites were evaluated by PCR and/or by culturing in Senekjie's medium samples of spleen, liver, and regional and distant lymph nodes.

According to the availability of sand flies, 2 or 3 xenodiagnoses per animal were conducted at different time intervals beginning on the first month post-infection. Spiny rats inoculated with *L. chagasi* were anaesthetized, introduced into tulle cages, and exposed for 30-45 min to laboratory-reared *Lutzomyia longipalpis*. In the case of *L. panamensis*, either wild-caught *L. youngi* [suspected vector of cutaneous leishmaniasis due to *L. (V.) braziliensis*, and *L. (V.) panamensis*; Rojas & Scorza 1990, Alexander et al. 1995] or colonized *L. longipalpis*, were allowed to feed only on the lesions of anaesthetized individuals. Engorged sand flies were maintained for 5-6 days at 26°C and 80% relative humidity under a sugar-water diet. Flies were individually dissected in a drop of phosphate-buffered saline, and the presence of promastigotes in the midgut was established microscopically at 400X.

*Ethical considerations* - All the field and experimental work carried out in this study conformed with the international Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences, and with the Standards for Human Care and Use of Laboratory Animals. Cideim also complies with all applicable provisions of the Colombian law 84 of 1989 in the "Estatuto Nacional de Protección de los Animales".

## RESULTS

*Natural trypanosomatid infections* - Thirty-three spiny rats were captured and transported to the laboratory, of which 5 individuals (15%) were positive for *Leishmania (Viannia)* spp. as determined by PCR. Three additional specimens also were positive by serology, probably due to infection with *Trypanosoma* sp. because PCR for *L. (Viannia)* spp. and *L. chagasi* were negative. Although *Trypanosoma*-infected individuals were excluded from the study, one of the rats putatively infected with *L. (Viannia)* was experimentally infected with *L. panamensis* because the PCR result became available after its inoculation was accomplished, and the initial culture for detecting *Leishmania* was negative. Nevertheless, this animal behaved clinically and parasitologically as the other naïve animals. In 3 animals, serological cross-reactions between *Trypanosoma* and *Leishmania* occurred, and differences in the optical density, using freeze-thaw *L. panamensis*, *T. rangeli* and *T. cruzi* as antigens, did not distinguish between the 3 infections.

*Infection with L. panamensis* - Inoculation of *L. panamensis* caused nodular, non-ulcerated lesions in both foot and snout. Maximum lesion size was observed at 3 weeks p.i. in the foot and 4 weeks p.i. in the snout, followed by a sustained decrease until its clinical disappearance by the 7th week p.i. (Fig. 1). The body weight of infected animals was similar to that of uninfected controls

throughout the observation period, indicating that *L. panamensis* did not affect the general condition of spiny rats. Reinfection at seven weeks after the primary infection resulted in the development of lesions that were equal or smaller in size and of shorter duration. A second reinfection on the 14th week p.i. had no clinical consequences, i.e., no lesion development was observed.

Based on sand fly availability, 3 of 5 infected spiny rats were subjected to xenodiagnosis with *L. youngi* on the 4th week p.i. Approximately 65% (range 32-100%) of the 50 sand flies exposed to the lesions of each animal took a blood meal. Two individuals were infective to the vector, but the capacity to transmit the parasite was very low as determined by the proportion of infected flies over those that were blood-fed (1/20 and 1/48). One of the infective animals died of causes unrelated to leishmaniasis. On the 7th week p.i., when all the animals had no macroscopic lesions, no *Leishmania* transmission occurred when a second xenodiagnosis using *L. longipalpis* was performed on the 4 remaining individuals. Likewise, additional xenodiagnoses performed after re-inoculating the spiny rats, using either *L. youngi* or *L. longipalpis*, failed to demonstrate transmission to sand flies.

Isolation of *L. panamensis* by aspirate-culture was possible only from the lesion of 2 of the 4 animals when the first xenodiagnosis was performed. Only one of the animals was positive by both xenodiagnosis and culture. No parasites could be recovered from any of the animals by culturing tissue homogenates of skin, lymph nodes, liver, and spleen at the end of the study period, 13 weeks post-infection. Moreover, at this time no parasite DNA could be detected by PCR-hybridization in skin samples from the snout, foot, and ear.

**Infection with *L. chagasi***

*Clinical evolution* - Although there was considerable variation in body weight within all 3 groups of rats, repeated measures analysis of variance revealed an increase in weight towards the end of the study period ( $F = 16.81$ ,  $df = 6$ ,  $P < 0.0001$ ). There was no diminution in food consumption, and no statistical differences in body weight were found among the three treatment groups ( $F = 1.90$ ,  $df = 2$ ,  $P = 0.2043$ ).

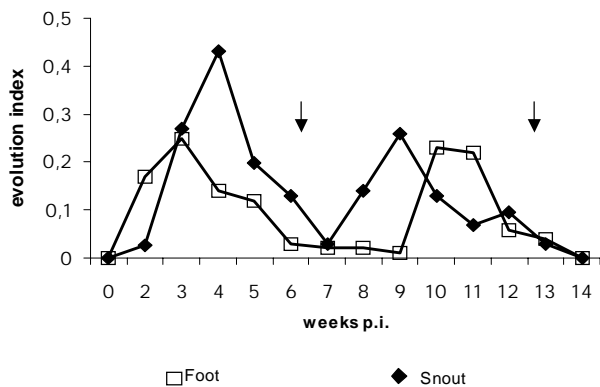


Fig. 1: lesion evolution of spiny rats infected and reinfected with *Leishmania panamensis* either in the snout or foot. The arrows indicate when re-infections were done (no lesions were observed beyond the 14th week p.i.).

Despite the fact that animals were quarantined, dewormed, and subjected to the same nutritional regime, hematocrit values prior to infection varied between 29 and 42% in the different groups (Fig. 2). Repeated measures analysis of variance revealed no significant differences in hematocrit values among the 3 treatment groups ( $F = 2.64$ ,  $df = 2$ ,  $P = 0.1504$ ).

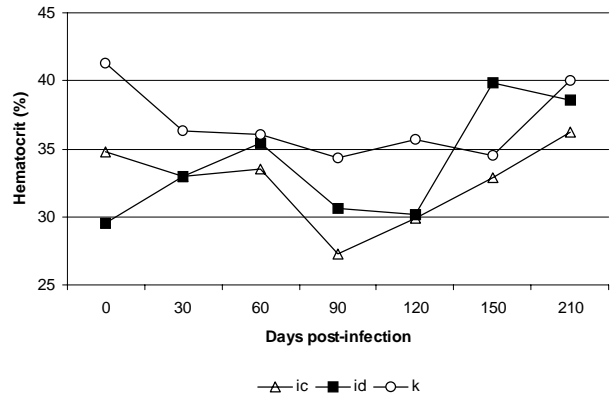


Fig. 2: evolution of hematocrit of *Proechimys semispinosus* intradermally (id) or intracardially (ic) inoculated with *Leishmania chagasi* (k = uninfected controls).

*Parasitological evolution* - No parasites could be recovered by culturing liver aspirates at different times or tissue homogenates of this organ at the end of the study. Upon sacrifice, parasites were detected by means of culture in the spleen of 2/5 intracardially-infected rats and 3/5 intradermally-infected rats. Burden of *L. chagasi* in the spleen and antibody titers were not consistently associated, and the number of parasites found through serial dilutions was highly variable regardless of the inoculation route (Table). The failure to detect amastigotes in smears indicated that the spleen was not heavily colonized by *L. chagasi*.

None of the infected rats was capable of infecting sand flies after 2 or 3 xenodiagnoses were performed at different times (1-6 mo). Dermal colonization (ear samples) by *L. chagasi* could not be demonstrated by means of PCR-hybridization in samples obtained at 0, 90, and 210 days p.i.

TABLE

Parasite burden in the spleen of spiny rats inoculated either intracardially (ic) or intradermally (id) with *Leishmania chagasi*, that resulted positive by culture or serology

Animal code	Inoculation route	Maximum positive dilution	ELISA (cut-off point: 0.78)
4022	ic	1:256	0.97
4099	ic	1:1024	1.83
4098	ic	0	0.93
4048	id	1:16	0.38
4135	id	1:16	1.72
4392	id	1:512	1.39

**Humoral response** - Anti-*Leishmania* antibodies were found in 6/10 experimentally-infected rats, and in the majority of animals antibody titers peaked between the 60 and 120 day p.i. and tended to decrease thereafter (Fig. 3). In one individual, no humoral immune response was detected despite having parasites in the spleen.

### DISCUSSION

Spiny rats have been considered to be reservoir hosts of cutaneous leishmaniasis in different countries because in leishmaniasis-endemic areas they are infected with *L. amazonensis* (Dedet et al. 1989) and *L. guyanensis* (Lainson & Shaw 1979). Although these mammals have not been linked to the transmission of *Leishmania* in Colombia, their natural infection with other trypanosomatids, including *T. cruzi*, previously was reported (Travi et al. 1994). During the present screening of spiny rats from the Pacific coast, as a preliminary step to the experimental work, we found that 15% (5/33) of individuals were positive for *L. (Viannia)* sp. as determined by PCR. Although limited in number, this survey provided data that, for the first time, incriminated spiny rats in the transmission cycle of *L. (Viannia)* sp. in this country.

The clinical evolution of infection with *L. panamensis*, which was characterized by nodular, non-ulcerated lesions, showed that spiny rats are susceptible to this species of *Leishmania*. Nevertheless, this susceptibility is lower than in other rodents (e.g., hamsters) as indicated by the failure to isolate *L. panamensis* from the inoculation site at the end of the study and the lack of dissemina-

tion to lymphoid organs. Although no necrosis was observed, lesions were more conspicuous on the snout than on the foot, a site-related disease pattern similar to that found in hamsters (Osorio et al. unpublished observations). The decrease in size and duration of lesions or absence of overt disease upon the first and second reinfection, respectively, suggest that spiny rats mount an effective immune response. Protection in *P. semispinosus*, as opposed to other rodents in which lesion development is still found after secondary challenge (Osorio et al. 1998), is robust and appears to clear *Leishmania*. This parasite clearance was suggested by the failure to detect *L. panamensis* in any tissues or organs, either by culture or the highly-sensitive PCR-hybridization method.

The transitory infectivity of spiny rats, as indicated by the transmission of *L. panamensis* to a small proportion of vectors at the peak of lesion development (4 weeks p.i.), suggests that in nature only recently-infected individuals would act as reservoirs. The inability to consistently infect *L. youngi*, an efficient experimental vector of *L. (Viannia)* spp., including *L. panamensis* (Travi et al. unpublished observations, Rojas & Scorza 1990, 1991, Warburg et al. 1991), throughout the study period, indicates that several xenodiagnoses were carried out after the animals lost their infectivity to sand flies. An early study by Lainson and Shaw (1968) suggested that Amazonian spiny rats do not transmit *Leishmania* at high rates because only 0.4% (8/1,996) of *L. flaviscutellata* harbored *Leishmania* after feeding on infected individuals.

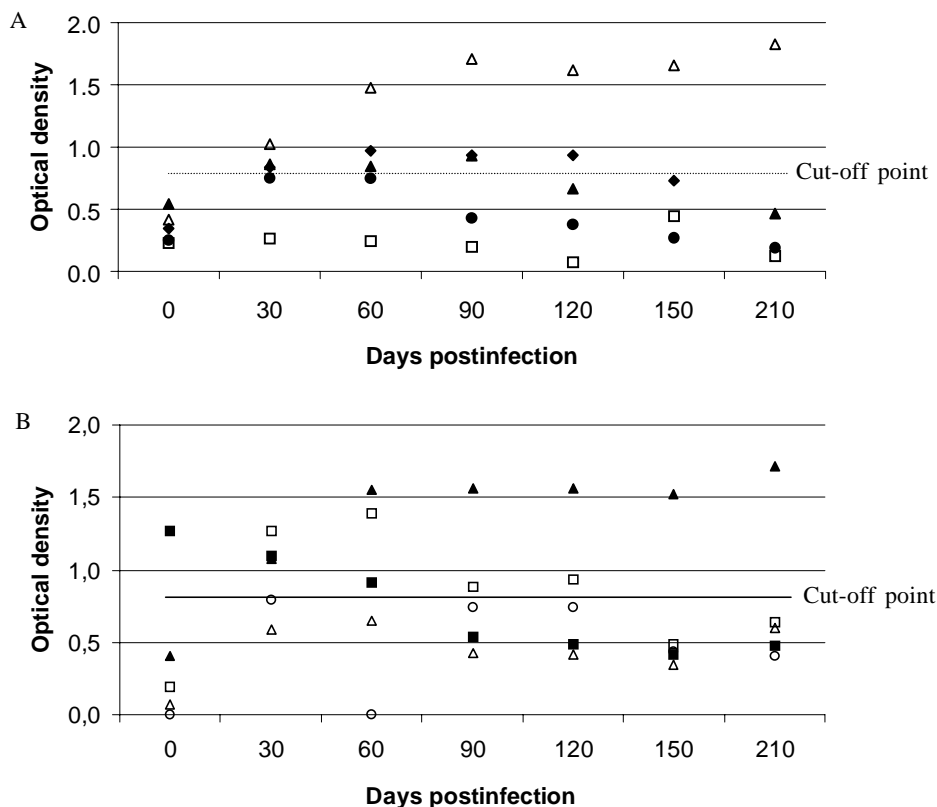


Fig. 3: evolution of the specific humoral immune response (ELISA) in *Proechimys semispinosus* intracardially (A) (n = 5) or intradermally (B) (n = 5) infected with *Leishmania chagasi*.

However, negative results obtained in experimental studies in which a limited number of individuals is used could be misleading. For example, xenodiagnosis as a means of determining host infectivity could be biased by the small number of sand flies (30-50) employed in this type of study, in contrast to the potentially enormous number feeding during the lifetime of free-ranging animals. Therefore, a positive xenodiagnosis could indicate high reservoir capacity, while the opposite result has little biological significance.

To our knowledge, a systematic, longitudinal study of reservoir potential in a large number of wild Neotropical mammals has never been conducted, and therefore it is difficult to ascertain the characteristics of a true reservoir. It is likely that both low parasite burden and low infectivity due to a subclinical state are the common traits of reservoir hosts. In the New World, high transmission rates pertain to canids (Lainson et al. 1990, Travi et al 2000) and more recently to humans (Costa et al. 2000) infected with *L. chagasi*, suggesting that reservoir infectivity may depend both on *Leishmania* species and progression toward overt disease. Therefore, high mammal-vector transmission rates of cutaneous *L. (Viannia)* spp. could be the exception rather than the rule.

The inability to infect *P. semispinosus* experimentally with *L. chagasi* indicated that it is not highly susceptible to this *Leishmania* species and therefore may not be considered a suitable model to study reservoir capacity and host-parasite interactions in the laboratory. Our study suggested that spiny rats experienced a transitory (not significant) decrease in hematocrit and subsequent recovery by the 5th mo p.i. This recovery included a decrease in antibody titers, which in other animals is associated with disease resolution (Mancianti et al. 1988, Moreno et al. 1999). The negativity of the PCR-hybridization method suggests that *L. chagasi* infection in *P. semispinosus* is contained and compartmentalized. It is possible that *P. canicollis*, which is naturally infected with *L. chagasi* in Northern Colombia, may be a more capable reservoir host than *P. semispinosus*. This hypothesis is based on the fact that the genetic background at the inter- and intra-species level can influence susceptibility to particular parasites, as has been well established in murine models of *Leishmania* (Blackwell 1996), and primate models of malaria (Espinal et al. 1984, Cooper 1995).

Based on the experimental results, we suggest that *P. semispinosus* infected with *L. panamensis* are only marginally infective to sand flies under natural conditions. However, the role of *P. semispinosus* as a reservoir of *L. panamensis* could be underestimated if its life history is not considered. Spiny rats are often the most abundant rodents in lowland Neotropical forests, have high reproductive rates, and are able to exploit a wide variety of forested habitats (Fleming 1971, Adler 1996, Adler & Beatty 1997, Tomblin & Adler 1998, Lambert & Adler 2000, Adler 2000). They also exhibit a flexible demography, which apparently enables them to respond to favorable environmental conditions and thereby quickly increase in abundance (Adler 1996). Because they are habitat generalists, they are able to thrive in highly-disturbed forests (Lam-

bert & Adler 2000, Adler 2000), often in close proximity to humans. Thus, *P. semispinosus* could exhibit considerable reservoir potential when summed over an entire population or region, despite their relatively weak infectivity to feeding sand flies. Their potential as reservoirs may be enhanced further by their ability to reproduce year-round if environmental conditions are favorable (Fleming 1971, Adler & Beatty 1997), thereby providing a constant source of naïve individuals (primarily juveniles) that are susceptible to infection.

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