ARTIGO

USE OF SENTINEL ANIMALS TO DEMONSTRATE ACTIVE LEISHMANIAL TRANSMISSION IN AN AREA WITH LOW FREQUENCY OF HUMAN LESIONS IN WESTERN VENEZUELA

Nestor Añez, [1] *Agustina Rojas,* [1] *Gladys Crisante,* [1] *Palmira Guevara* [2] *and Jose Luis Ramirez* [2]

ABSTRACT

One-hundred healthy animals of different species, including dog (8), fox (1), donkey (1), goat (1), opossum (3), rabbit (8), hamster (33) and guinea pig (45), kept under natural conditions, were used as sentinel animals (SA) to prove active and constant leishmanial transmission, in an area where human cutaneous lesions are rarely observed. The investigation was carried out in a field station located at the Andean region of Western Venezuela, where both sand flies species and *Leishmania*-parasites have been previously reported. The study consisted of a follow-up using serological techniques. Blood samples from the SA were taken monthly and the sera processed to demonstrate seroconversion by detecting anti-*Leishmania* circulating antibodies (Abs). In 56% of the used animals belonging to 8 species of susceptible mammals, seroconversion was detected during the time of observation. To corroborate the serological results, 68 serum samples were selected for a PCR assay with 32 (47%) of them showing positive results. The results indicate that combination of seroconversion and PCR in SA are useful tools to demonstrate constant and active *Leishmania* transmission in areas where clinical manifestation is uncommonly observed in the human population. The potential of using SA as a promising method to investigate leishmanial activity under field conditions is stressed and the epidemiological implications of the present findings is discussed.

KEYWORDS: Sentinel animals. *Leishmania*. Vectorial transmission. Serology. PCR.

INTRODUCTION

Cutaneous leishmaniasis (CL) is a parasitic disease endemic in most Latin-American countries. It is caused by species of *Leishmania* of the

1 Universidad de Los Andes, Facultad de Ciencias, Mérida, 5101, Venezuela.
2 Universidad Central de Venezuela, Facultad de Ciencias, Caracas, Venezuela.

Corresponding author: Néstor Añez, Universidad de Los Andes, Facultad de Ciencias, Departamento de Biología, Mérida, 5101, Venezuela. Tel.: 58 274 240 12 85; Fax: 58 274 240 12 86. E-mail: nanes@ula.ve

Recebido para publicação em 27/11/2002. Revisto em 7/4/2003. Aceito em 5/6/2003.

subgenera *Leishmania* and *Viannia* and transmitted by phlebotomine sand flies of the genus *Lutzomyia.* In Venezuela, CL is endemic and focal in virtually all territorial units with more than 4,000 human cases being recorded each year (5). However, there are regions in the country where CL lesions in humans are rarely observed although the people are living in localities with high-risk conditions where *Leishmania*-parasites have been isolated and anthropophilic sand flies have been identified (2, 7, 14). In order to investigate the *Leishmania*-transmission dynamic and looking for an explanation for the low frequency of human active lesions in one of these areas, we used different species as sentinel animals (SA), which have been previously used in CL epidemiological studies in Latin-America (8, 9). Previous authors have stated that the use of SA appears to be a good method for detecting leishmanial activity in nature, since they can provide information related to time and place better than the examination of wild animals (9). However, information on the use of SA to study the *Leishmania* dynamic transmission is scarce. The present paper deals with the combined use of seroconversion and polymerase chain reaction (PCR) assay as tools to demonstrate active and constant leishmanial transmission in SA kept in an area where human lesions are rarely observed in the human population.

MATERIALS AND METHODS

One hundred specimens of different species (8 dogs, 1 fox, 1 donkey, 1 goat, 3 opossums, 8 rabbits, 33 hamsters and 45 guinea pigs) were used as sentinel animals (SA). The reason to select the species used as SA was that most of them have proven to be susceptible to natural and experimental CL (9, 3, 7). Previous to the experiment, they were serologically and molecularly evaluated to be declared negative for *Leishmania*-infection, and then they were placed either in small yards or animal cages and fed *ad libitum.* The SA were kept up to 6 months under natural conditions in a field station located at 1,300 m. a. s. l. and N 08°31'23'';W 71°30'05'', in Merida state of the Andean region of Western Venezuela. Two seasons are recognized in the area, a dry season from November to April and the wet one from May to October. In the study area, where main activity is agricultural at the coffee plantations, leishmanial clinical manifestations, i.e., skin ulcers due to CL, are rarely observed in local people despite the circulation of *Leishmania*-parasites and anthropophilic sand flies species (1, 2, 4, 7). To support this observation, a sample of 60 people living in close proximity to the field station were evaluated for *Leishmania*-infection. The evaluation was carried out in two groups. One in 35 school children, and the other in 25 individuals, taken at random, from the different families living in the village. The 60 sampled individuals showed a male: female ratio of 1 (50 males and 50 females), and a mean ± SD age of

18±14 years with a range from 7 to 82 years. Details on the age composition and gender proportion of the two groups are given in Table 1. In all cases search for leishmanial infection was carried out using leishmanin skin test (LST), serological methods and clinical examination for skin active lesions. Most of the sampled people declared that they had never abandoned the village, could recognize phlebotomine sand flies and had been bitten by the flies with a high frequency principally during dry season. In addition, the chosen area is considered to be free for *Trypanosma cruzi* infection. No cases of Chagas disease have been reported from the village during the last 30 years and sampled people do not recognize triatomine bugs.

Table 1. Detection of Leishmania- infection in people living close to the field station where sentinel animals were studied

Place of Sampling	N° of sampled individuals	Age in years X± SD (Range)	Gender F	N° (%) M	Detection of Leishmania infection N° (%) +ve LST seropositive active lesions		
Village	25	27±18 (7-82)	10 (40)	15 (60)	8 (32)	19 (76)	1 (4)
School	35	11±1.7 (9-16)	20 (57)	15 (43)	6 (17)	11 (31)	NLS
TOTAL	60	18±14 (7-82)	30 (50)	30 (50)	14 (23)	30 (50)	1 (1.6)

LST: Leishmanin skin test; NLS: No lesions seen.

The sampling methodology for SA consisted of monthly follow-up collecting blood samples by veno-puncture or cardio-puncture according to the animal size. The obtained blood was divided into two parts. One was used for hemoculture in NNN medium and the other to obtain sera which was stored at -20°C until needed. Serological methods were used to detect circulating anti-*Leishmania* antibodies (Abs). These included a direct agglutination test (DAT) pretreated with 2-mercaptoethanol and an ELISA following standard procedures, and adapted for detection of *Leishmania* Abs in our laboratory (4, 11). In all cases a negative control serum for each of the considered species was used for the performed serological techniques. The equivalent dilution value for both techniques to be considered as seropositive (cut-off titers) was that above a 1:64 dilution for human samples; over 1:128 for dog, rabbit, hamster and guinea pig, and from 1:256 for opossum, fox, donkey and goat. As diagnostic criteria, seroconversion was established comparing the differential reactivity to specific *Leishmania* antigen between the serum of SA and its respective negative control. A SA was only declared as seroconverted when the two techniques were coincident, at least for the above indicated minimal dilutions or Abs titers. Animals that seroconverted during the first sampling were equally re-examined until the end of the experiment. For molecular procedures, 68 sera samples were selected from *all the included animal species. They were 45 seropositives specimens* representative of the 8 species of mammals used, and 23 guinea pigs that showed negative results for serology. Sera samples were processed for PCR using a *Viannià* subgenus specific primer set for the non-transcribed

ribosomal gene spacer DNA sequences (6). The DNA from the frozen sera samples was isolated according to a previous procedure (7). A repetitive sequence of 126 basepairs of ribosomal DNA of *L. braziliensis* was used as a PCR amplification target using the forward primer 5'GCAGCACAGGGAA AG3'; reverse primer, 5'TACCTCTCTCCGTGATCG3'. The reaction mixture (final volume, 25µl) was prepared as previously described (7). DNA amplifications were carried out in a Perkin-Elmer thermal cycler (Gene Amp PCR System 2400) programmed as follows: The PCR had an initial denaturation at 94°C for 5min, followed by 35 cycles of 94°C, 50°C and 72°C of 1min each, with a final extension at 72°C for 5 min. Reactions were performed as duplicates, and accordingly, each set of reactions included a positive control. Additionally, positive controls were run for the reaction by adding 10 and 100 fg of purified DNA of *L. braziliensis* and a negative control without the addition of DNA. The PCR product was visualized by electrophoresis in a 2% agarose gel stained with ethidium bromide. All the samples were hybridized with a *L. braziliensis* DNA probe labeled with digoxigenin (Roche Diagnostics Gmbh).

RESULTS

Detection of *Leishmania*-infection in people in close proximity to the field station where sentinel animals were studied

Examination for *Leishmania*-infection of 60 individuals representative of the human population living in close proximity to the field station where SA were studied revealed an average of 50% seropositives. The observed seropositivity ranged from 31% in school children to 76% in other people from the village. In addition, application of LST revealed 23% (14/60) of positive results, with a range from 17% in school children to 32% in the rest of sampled people. The high prevalence of subclinical or inapparent leishmanial infection demonstrated with serological methods and/or the reaction to LST, contrast with the low frequency of active lesion detected in the study area. From the total examined individuals only one of them (1.6%) showed active leishmanial lesion. This clinical case according to the information obtained from people of the village appears to be the unique CL case observed during the last 5 years. A detailed account about the people studied is given in Table 1.

Seroconversion and PCR assay in sentinel animals to demonstrate active and constant leishmanial infection under field conditions

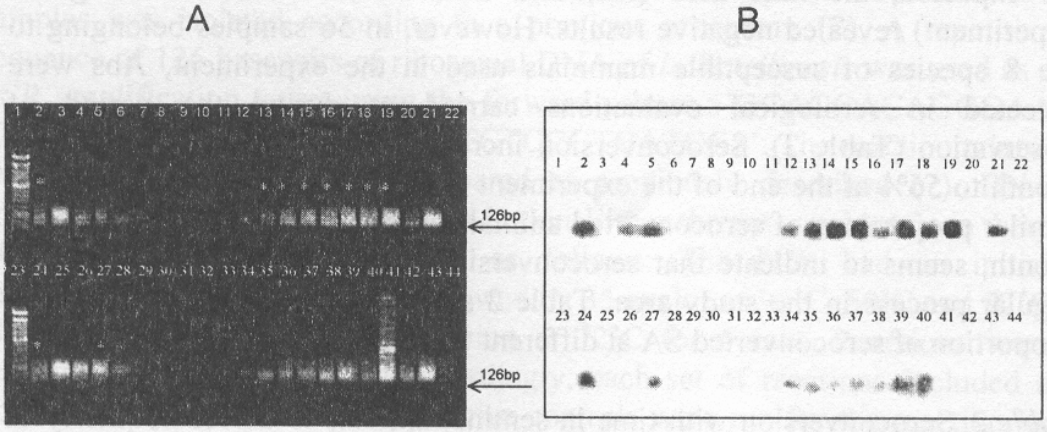
Samples collected from a 100 initially healthy animals used as sentinels, were serologically processed to demonstrate anti-*Leishmania* Abs.

As expected, the time zero (i.e., sera from animals before starting the experiment) revealed negative results. However, in 56 samples belonging to the 8 species of susceptible mammals used in the experiment, Abs were detected in serological evaluations carried-out during the period of observation (Table 2). Seroconversion increased from 30% during the first month to 56% at the end of the experiment in the sixth month. However, the *similar proportions of seroconverted animals observed from the* 2^{nd} to the 6^{th} month, seems to indicate that seroconversion appears to be a constant and regular process in the study area. Table 2 shows details on the number and proportion of seroconverted SA at different times of the experiment .

Table 2. Seroconversion with time in sentinel animals to detect frequency of transmission of *Leishmania*

Sentinel animal	N° of used sentinel animals	N° of seroconverted during time of observation (months)						Total N° (%) of seroconverted
		1	2	3	4	5	6	
Dog	8	4	1	2	-	-	-	7 (87.5)
Fox	1	1	-	-	-	-	-	1 (100.0)
Donkey	1	1	-	-	-	-	-	1 (100.0)
Opossum	3	1	1	-	-	-	-	2 (66.6)
Rabbit	8	2	-	1	2	1	1	7 (87.5)
Hamster	33	10	2	2	2	4	4	24 (72.7)
Guinea pig	45	10	2	-	1	-	-	13 (28.8)
Goat	1	1	-	-	-	-	-	1 (100.0)
Total	100	30	6	5	5	5	5	56 (56.0)

In relation to the molecular technique used to corroborate leishmanial transmission in SA, from the 68 sera samples that were selected at the end of the experiment for a PCR to detect the presence of *Leishmania* specific DNA, 32 (47%) showed positive results. These results include specimens representative of all the 8 species of mammals used as SA. From the 32 SA that showed positive PCR, 20 (62.5%) were seroconverted, while 12 (37.5%) belonged to the group declared as seronegative animals. In all cases this assay amplified 126 bp within the ribosomal spacer of the parasite. Figure1 shows some of the positive samples which showed a strong PCR signal with the expected band size, whereas, the negative control showed no signal at all. Statistical analysis revealed a correspondence of 44% between seroconversion and the detection of circulating *Leishmania* DNA by PCR. Neither ulcerated lesions nor nodules attributable to *Leishmania* infection were detected during the period of observation. The microscopic observation of the hemoculture revealed no circulating parasites at any time.



*: indicates samples with seronegative results, but positive for PCR assay.

Figure 1. *Leishmania (Viannia)* specific PCR assay in sera samples from sentinel animals. **A.** Ethidium bromide-stained gel showing PCR amplification products. Lanes 1 and 23: Molecular weight markers; lanes 2 – 20: guinea pig sera; lane 21: *L. braziliensis* purified DNA used as positive control; lanes 24 – 28: guinea pig sera; lanes 29 – 31: rabbit sera; lanes 32 – 34: guinea pig sera; lanes 35 – 37: rabbit sera; lanes 38 – 39: dog sera; lane 40: goat serum; lanes 41 – 43: Negative controls; lane 44: Water. **B.** Hybridization of the gel shown in A. Hybridization was performed with a *L. braziliensis* – specific probe.

DISCUSSION

In the present paper we demonstrate the usefulness of SA to detect constant and active transmission of *Leishmania* in an area of the Andean region of Western Venezuela where cutaneous lesions are rarely observed in the human population. The fact that 56% of the SA showed the presence of specific anti-*Leishmania* Abs and that CL lesions was detected only in 1.6% of the human population of the study area, appears to support the statement above. As an evidence of the occurrence of leishmanial transmission in the area we show that 50% of a sample of 60 people living in close proximity to SA resulted seropositive to *Leishmania.* In this case seroconversion reached levels up to 76% in people aged 7 to 82 years old. In addition, in the same sampled people a 23% reactivity to LST was detected.

These results indicate that, although the frequency of *Leishmania* transmission appears to be high judging for the observed level of seroconversion and reactivity to LST, the appearance of clinical lesions is relatively infrequent in the population. The present results contrast with previous reports which indicate that near 75% of the CL detected in

Venezuela occur in the Andean region (2, 12), and more investigations should be done to understand this behavior.

The choice of SA to precise the frequency of occurrence of leishmanial transmission instead of the evaluation of animals caught in the same area finds support in previous report by Herrer and co-workers (9). These authors stated that SA studies have a definite advantage over searches for natural infections in forest animals, in being able to demonstrate relatively precise times and areas of leishmanial activity. The same authors also stated that even when natural leishmanial infections are demonstrated the wild circulating animals give little idea of where and when the infection was originally contracted. This is particularly so in the case of wide-ranging animals with terrestrial and arboreal habits. In the present work we demonstrate an initial 30% infection in SA, which was measured by seroconversion detected during the first month. The fact that all the specimens used as SA were serologically examined before being placed at the field station revealing negative results, give us confidence on the detected figure. This indicates that the first 30 SA were infected by the bites of infected sand flies within the first 30 days of permanence at the station. This fact allowed us to precise the beginning of the infection, demonstrating that the phenomenon actually happened in the chosen area. After this episode occurred, a constant and regular frequency of infection of about 5% was detected from the 2^{nd} to the 6^{th} month in the group of SA. This observation leads us to conclude that leishmanial transmission is a constant and active process in the study area, which is responsible for the large amount of seropositives detected both in the group of SA and the people living in close proximity. The obtained results also appear to indicate that SA used in the present work, as susceptible animals for CL infection, reacted against the parasite establishing the humoral immune response characteristic in individuals suffering from leishmaniasis. Apart from hamsters, the species chosen as SA were those that are normally hosts of *Leishmania* infecting wild sand flies in the study area (1, 2). This fact, gives the results a realistic value for estimating the frequency of transmission. Indeed, when initially seronegative animals including dog, fox, donkey, opossum, goat, rabbit, hamster, and guinea pig used as sentinels were serologically examined during the following six months, we noted that all of them became seropositive with values ranging from 28% to 100% and with Ab titres from 1:128 to 1:2048. The serological results indicate an active vectorial transmission of *Leishmania* to most species of mammals circulating the area to maintain the zoonosis. In addition, the results appear to suggest that local people exposed to risk conditions, although submitted to infection by the same dynamic transmission than SA, appears to be able to develop an asymptomatic infection from the clinical point of view, being seropositive and LST reactive, representing those people identified as healthy carriers. In the

present work we demonstrate that from individuals with this characteristic only 1.6% of them developed CL lesions, which in general are rarely observed in local people, supporting previous findings for other neotropical regions (10, 13). From the above we suggest that further investigation must be done to explain whether resistance and susceptibility to *Leishmania* infection in people from the study area has any genetic base.

To corroborate *Leishmania*-infection in seroconverted SA, and to look for recent infections not yet detected by serological analysis, we processed 68 selected samples for PCR assay. The samples were made up by 45 seropositives from the 8 species used, and 23 guinea pigs with consistent negative results, which were declared as seronegatives. The PCR assay performed during the study was proven to be sensitive and specific enough to detect circulating *Leishmania* or part of its genome in sera samples as previously reported (7).

From the total selected samples, 32 (47%) exhibited positive results for PCR; 20 samples (62.5%) belonging to seropositive animals and 12 (37.5%) to those sentinels with negative serology. Statistical analysis revealed a correspondence value between PCR and seroconversion of 44%. This value appears to be lower than expected considering that PCR was done in 45 sera of seroconverted SA. Although we have not at present a strong argument to explain this low correspondence, it is possible to consider that this fact may be due to a short lasting time of circulating DNA in seroconverted SA. A subject that is being investigated and will be showed elsewhere. However, what appears to be more interesting is the fact that 52% (12/23) of the selected seronegative samples showed positive results for PCR. This indicates that, although SA may have been bitten by infected sand flies, they did not develop the humoral response characteristic to *Leishmania*-infection. One more argument to be considered is the possibility that SA were recently infected, in which case serological methods were unable to detect any signal of the immune response, being detected by PCR.

Whatever the reasons to explain the low frequency of CL lesions observed in people living in the study area are, our results strongly demonstrate an active and frequent leishmanial-transmission taking into consideration the infection rate detected in SA combining serological and molecular (PCR) methods.

Finally, the present results strongly support the recommendation of the use of SA as a potentially promising method to investigate leishmanial activity under field conditions. With no doubt the combination of classical techniques with much more sensitive and specific methodologies as product of the new biotechnology will make it possible to use SA as a very useful tool in epidemiological studies on leishmaniasis.

RESUMO

A utilização de animais-sentinela na demonstração de transmissão ativa de leishmaniose em uma área da Venezuela com baixa prevalência de lesões humanas

Foram utilizados 100 animais-sentinela de diferentes espécies (8 cães, 1 raposa, 1 burro, 1 cabrito, 3 gambás, 8 coelhos, 33 *hamsters* e 45 cobaias), mantidos em condições naturais, para estudar a existência de transmissão ativa e constante de leishmaniose, em área onde raramente são observadas lesões humanas. A pesquisa foi realizada em um campo experimental na região andina da Venezuela oeste, onde foram encontrados flebótomos e parasitos do gênero Leishmania. O estudo foi realizado por seguimento sorológico, para o qual foram colhidas mensalmente amostras de sangue de cada animal no intuito de observar a conversão sorológica pela detecção de anticorpos circulantes antileishmania. Em 56% dos mamíferos susceptíveis utilizados houve soroconversão. Para confirmar os resultados sorológicos foram selecionadas 68 amostras de soro para PCR, dentre as quais 32 (47%) foram positivas. Esses resultados indicam que a combinação de soroconversão e PCR em animais-sentinela é uma ferramenta útil para demonstrar a transmissão ativa de leishmaniose em áreas onde a doença na população humana é pouco observada. São discutidos o potencial desses animais para investigar a transmissão ativa em condições de campo e as inferências epidemiológicas correspondentes.

DESCRITORES: Animais-sentinela. Leishmania. Transmissão vectorial. Sorologia. PCR.

ACKNOWLEDGEMENTS

We are indebted to Prof. I. Woodward and Miss Kristen Bunch for careful review of the manuscript.

Financial support: this work was supported by Consejo de Desarrollo Científico, Humanístico y Tecnológico, Universidad de Los Andes, Mérida, Venezuela, grant CDCHT-ULA-C-1016-00-07-AA (NA), and FONACIT- G-99000036 (NA).

REFERENCES

1. Añez N, Cazorla D, Nieves E. Registro de especies flebotominas en focos endémicos para leishmaniasis en el estado Mérida, Venezuela. *Bol Dir Malariol San Amb 29*:12-34, 1989.
2. Añez N, Nieves E, Cazorla D, Oviedo M, Lugo A, Valera M. Epidemiology of cutaneous leishmaniasis in Merida, Venezuela. III. Altitudinal distribution, age structure, natural infection and feeding behavior of sand flies and their relation to the risk of transmission. *Ann Trop Med Parasitol 88*:279-287, 1994.

3. Añez N. Aspectos zoogeográficos de la leishmaniasis en Venezuela. IN: Reservorios y vectores de enfermedades metaxénicas, ed. Scorza, JV. Pp:77-87, Mérida, Venezuela, Universidad de Los Andes, 1992.
4. Añez N, González C, Lugo de Yarbuh A, Rojas A, Crisante G, González N, Premoli de Percoco G, Guevara P, Ramírez J L. Association of trauma with *Leishmania* parasite recall. Clinical and experimental evidences. *Bol Dir Malariol San Amb 40*:13-20, 2000.
5. Convit J, Castellano P, Rondon A, Pinardi M, Urlich M, Castes M, Bloom B, Garcia L. Immunotherapy versus chemiotherapy in localized cutaneous leishmaniasis. *The Lancet 21*:400-404, 1987.
6. Guevara P, Alonso G, Da Silveira J F, De Mello M, Scorza J V, Añez N, Ramirez JL. Identification of new World *Leishmania* using ribosomal gene spacer probes. *Mol Biochem Parasitol 56*:15-26, 1992.
7. Guevara P, Rojas E, González N, Scorza J V, Añez N, Valera M, Ramírez J L. Presence of *Leishmania braziliensis* in blood samples from cured patients or at different stages of immunotherapy. *Clin Diag Lab Immunol 1*:385-389, 1994.
8. Herrer A, Christensen HA, Beumer RJ. Use of sentinel animals in epidemiological studies of cutaneous leishmaniasis. *Trans Roy Soc Trop Med Hyg 65*:538-539, 1971.
9. Herrer A, Christensen HA, Beumer RJ.Detection of leishmanial activity in nature by means of sentinel animals.Trans *Roy Soc Trop Med Hyg 67*:870-879, 1973.
10. Lainson R, Shaw JJ. Evolution, classification and geographical distribution. Peters W, Killick-Kendrick R, Eds. The leishmaniasis in Biology and Medicine. Academic Press, London, Orlando, San Diego, New York, Vol 1:1-120, 1987.
11. Lugo de Yarbuh A, Premoli de Percoco G. Detection and identification of *Leishmania braziliensis* in cutaneous lesions using conventional techniques and PCR amplification probe. *Rev Ecol Lat Am 6*:1-7, 1999.
12. Scorza JV, Valera M, Moreno E, Jaimes R. Encuesta epidemiológica sobre la leishmaniasis cutánea. Un estudio en Mérida, Venezuela. *Bol Of San Panamerican 95*:118-133, 1983.
13. Shaw JJ, Lainson R. Ecology and epidemiology: New World. Peters W, Killick-Kendrick R, Eds. The leishmaniasis in Biology and Medicine. Academic Press, London, Orlando, San Diego, New York, Vol 1: 292-363, 1987.
14. Valenta D T, Añez N, Tang Y, Killick-Kendrick R. The genital atrium as a good taxonomic character to distinguish between species of phlebotomine sand flies (Diptera : Psychodidae) from Venezuela. *Ann Trop Med Parasitol 93*:389-399, 1999.