Frequency of Infection of *Lutzomyia* Phlebotomines with *Leishmania braziliensis* in a Brazilian Endemic Area as Assessed by Pinpoint Capture and Polymerase Chain Reaction

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Leishmania *infected of* Lutzomyia *spp. are rare in endemic areas. We tested the hypothesis that there is clustering of infected vectors by combining pinpoint capture with sensitive* L. braziliensis *kDNA minicircle specific PCR/dot blot in an endemic area in the State of Bahia. Thirty out of 335 samples (10 to 20 sand flies/sample; total of 4,027 female sand flies) were positive by PCR analysis and dot blot leading to a underestimated overall rate of 0.4% positive phlebotomines. However, 83.3% of the positive samples were contributed by a single sector out of four sectors of the whole studied area. This resulted in a rate of 1.5%* Leishmania *positive phlebotomines for this sector, far above rates of other sectors. Incidence of American cutaneous leishmaniasis cases for this sector was about twice that for other sectors. Our results show that there is a non-homogeneous distribution of* Leishmania-*infected vectors. Such a clustering may have implications in control strategies against leishmaniasis, and reinforces the necessity of understanding the ecological and geographical factors involved in leishmanial transmission.*

Key words: *Lutzomyia* spp. - phlebotomines - *Leishmania braziliensis* - kDNA PCR - Brazil

The incidence of American cutaneous leishmaniasis (ACL) has markedly increased in Latin America, particularly in Brazil during the last 20 years. The disease is distributed countrywide but 45% of the cases occur in the Northeastern region (Deane & Grimaldi Jr 1985). *Leishmania braziliensis* is the most commonly involved pathogen (Marsden et al. 1984, Jones et al. 1987) and sand flies of the genus *Lutzomyia* are their vectors (Lainson 1983). However, the rate of infection of *Lutzomyia* with *Leishmania* has not been established so far for this region. Additionally, cases are not distributed homogeneously, which make difficult the identification of infected sand flies. This knowledge would help further understanding of the ecogeogra-phy of New World leishmaniasis and improve the current view on the efficiency of disease transmission from sand flies to human beings.

Natural infection of *Lutzomyia* spp. with *Leishmania* in endemic areas is very low. In the highly endemic area of Corte de Pedra (southeast of Bahia in Northeastern Brazil), previous studies carried out by dissection of phlebotomines captured at random have shown absence of infection in 1,832 females of various species (Vexenat et al. 1986). Furthermore, only one sand fly out of 193 caught around the houses of infected individuals was found to be infected (Ryan et al. 1990). Such a low rate of detection may be due to a non-homogeneous distribution of infected vectors. Spatial distribution diversity has been reported with vectors of malaria and virus (Smith et al. 1995, Nasci & Mitchell 1996, Ribeiro et al. 1996, Hii et al. 1997, Thompson et al. 1997). Herein we have tested the hypothesis that there is a clustering of infected vectors by combining a spatial stratification of sample harvesting and analysis in pools of vectors with a very sensitive *L. braziliensis* kDNA minicircle specific PCR and a dot blot hybridization procedure in different sectors of the Corte de Pedra area.

MATERIALS AND METHODS

Study area - The endemic region covered by this study spans approximately 2,800 km^2, located within the cartographic boundaries of 13°15' latitude south (LatS) to 13°45'LatS and 39°15'longitude east (LonE) to 39°45'LonE. Forty-five recently diagnosed ACL cases, dwelling in 25 different locations in this region, served as indexes for the sand fly searches performed in the corresponding domiciliary and peridomiciliary areas. We defined peridomiciliary area as that contained within a radius of up to 20 m from the houses of the actual ACL, which corresponded to the domiciliary areas.

Phlebotomine capture and evaluation - Sand flies were captured using Shannon traps, brought to the lab in special cages and frozen at -20°C. Then, male and female specimens were distinguished under magnification, sorted and stored differently: females were pooled in sets of 10 to 20 sand flies and stored at -70°C until DNA extractions and PCRs were performed. Males were stored in 70% ethanol until taxonomic evaluation. This decision of separating specimens by gender was taken in order to maximize

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sample usage, and took into account that females are the actual hematophagous among *Lutzomyia* sand flies while males are likely to reflect their local fauna.

The taxonomic identification of the male sand flies was performed according to Young and Duncan (1994) taking into consideration the following morphological characters: size and color of insect, measures of palps, antennal ascoids, setae and spines of the male reproductive apparatus and leg.

PCR procedure - For the *L. braziliensis* kDNA specific PCR/dot blot hybridization procedure, total DNA was extracted by incubating the pooled sand flies in 80 µl of 10 mM Tris-HCl pH 9.2, 10 mM EDTA buffer, containing 100 µg/ml proteinase K (Gibco BRL-Life Technologies) for 3 h at 56°C. Then, the mixtures were centrifuged at 12,000 rpm, 4°C for 15 min and the supernatant collected and heated to 95°C for 15 min to inactivate proteinase K. The resulting samples were centrifuged once more for 40 sec as above and the supernatant was collected and stored at -20°C. Previously described primers that anneal to the boundaries of the conserved region of the minicircle, one component of the kinetoplast DNA were employed for the amplification of that segment. That was performed by a hot start PCR. Each reaction mix contained 10 mM Tris-HCl pH 9.2, 75 mM KCl, 1.5 mM $MgCl_2$, 100 µg/ml BSA (Gibco BRL-Life Technologies), 0.2 mM of each dNTP (Gibco BRL-Life Technologies), 0.25 pmoles of each primer, 2.5 U Taq DNA polymerase (Gibco BRL-Life Technologies), 2 µl DNA and distilled water to 50 µl. The reactions were thermocycled in a Perkin-Elmer 9600 programmed as follows: (1) one cycle at 94°C for 4 min; (2) 35 cycles at 94°C for 30 sec, 50°C for 30 sec and at 72°C for 30 sec; and (3) one cycle at 72°C for 10 min. After thermocycling, the reactions were resolved in 2% agarose gel electrophoresis in 1 X TAE as described elsewhere (Schubach et al. 1998).

Since the above primers recognize *Leishmania* sp. minicircles, a dot blot using a *L. (Viannia)* specific probe was performed. Briefly, the amplified products (10 ml) were denatured in 0.4 N NaOH before application to nylon membranes (Zetaprobe, Biorad, Richmond, CA), using a manifold vaccum device (Schleicher & Schuell, Keene, NH). The *L. (Viannia)* specific hybridization probe (5': TAA TTG TGC ACG GGG AGG CCA) corresponding to a cloned minicircle from *L. panamensis* IPAN V, was radiolabeled with a a ^{32}P-dATP by random-hexamer priming. Radioactive probes were purified by P-10 (Biorad, Richmond, CA) spin-column chromatography. Hybridization reactions were performed at 60^{o}C in BLOTTO Johnson 84. Filters were washed in 0.5 X SSC/0.1% SDS at 60^{o}C for 1 h. After hybridization, membranes were washed with 0.5X SSC, 0.1% SDS at 60°C and autoradiography carried out overnight at -70°C with intensifying screen.

RESULTS

Fifty phlebotomine searches were positive totaling a collection of 5,269 sand flies. Peridomiciliary searches rendered 5,238 phlebotomines (i.e. 99.4% total), while domiciliary searches resulted in just 31 captures (i.e. 0.6% total). It was also found that, overall, there were over three times more female than male sand flies in areas surrounding ACL cases (i.e. 4,027 versus 1,242 respectively). Curiously, however, if we compute only those phlebotomines captured inside the domiciliary area, this difference drops to about twice as many female versus male sand flies (i.e. 20 females and 11 males).

The taxonomic classification of male phlebotomines showed that the studied fauna was comprised of only two different *Lutzomyia* species: *L. (Nyssomyia) whitmani* and *L. (Nyssomyia) intermedia* (Table I). *L. (N.) whitmani* greatly predominated with an overall frequency of 92.8% against only 6.9% of *L. (N.) intermedia. L. (N.) whitmani* predominated in both peridomiciliary and domiciliary compartments (Table I). Although both species were frequently found in peridomiciliary areas, differences were detected in their relative frequencies in either environment (Table I). While there was over 10 times more *L. (N.) whitmani* then *L. (N.) intermedia* in the peridomiciliary areas, this difference in relative frequencies dropped to only about twice in the domiciliary areas (Table I). A comparison of domiciliary and peridomiciliary relative frequencies within each species showed that 4.7% of the of *L. (N.) intermedia* were captured in the domicilary area in comparison to a frequency of only 0.06% for *L. (N.) whitmani*. These differences, however, did not reach a statistically significant level.

In order to perform a more representative evaluation of the frequency of *Lutzomyia* spp. infection with *L. braziliensis* in the area, we divided the studied region in four sectors, namely: northwest (NW), 13°15'LatS to

TABLE I

Frequency distribution of different species of *Lutzomyia* sand flies captured in domiciliary and peridomiciliary areas of American tegumentary leishmaniasis cases in the endemic region of Corte de Pedra in Northeastern Brazil

Species	Number of captured phlebotomines (% total) [a]		Total
	Domiciliary area	Peridomiciliary area	
Lutzomyia (N.) whitmani	7 (63.7%)	1,142 (92.8%)	1,149
Lutzomyia (N.) intermedia	4 (36.3%)	85 (6.9%)	89
Lutzomyia (N.) spp.[b]	0 (0%)	4 (0.3%)	4
Total	11 (100%)	1,231 (100%)	1,242

a: numbers correspond to the frequency observed among male sand flies only; *b*: phlebotomines with damaged characters that could not be indisputably classified in either species.

TABLE II

Frequency of *Leishmania braziliensis* infected *Lutzomyia* spp. in the four sectors of the American tegumentary leishmaniasis endemic area of Northeastern Brazil

Sector [a]	Places searched	Samples tested [b] (% total)	Positive samples (% total)	Frequency of *Lutzomyia* spp. infection [c]
NW	7	85 (25.4%)	25 (83.3%)	1.5%
NE	7	72 (21.5%)	3 (10%)	0.2%
SW	5	112 (33.4%)	0 (0%)	0%
SE	6	66 (19.7%)	2 (6.7%)	0.1%
Total	25	335 (100%)	30 (100%)	0.4% [d]

a: Northwest (NW), 13°15'LatS to 13°30'LatS and 39°30'LonE to 39°45'LonE; Northeast (NE), 13°15'LatS to 13°30'LatS and 39°15'LonE to 39°30'LonE; Southwest (SW), 13°30'LatS to 13°45'LatS and 39°30'LonE to 39°45'LonE; and Southeast (SE), 13°30'LatS to 13°45'LatS and 39°15'LonE to 39°30'LonE; *b*: samples correspond to pooled DNA of 10 to 20 sand flies captured at the same place during the same search; *c*: minimum frequency of *L. braziliensis* infected *Lutzomyia* spp. calculated considering all samples consisting of 20 sand flies and only one *Leishmania* positive phlebotomine per sample; *d*: overall minimum frequency for the entire region (i.e. all four sectors)

13°30'LatS and 39°30'LonE to 39°45'LonE; northeast (NE), 13°15'LatS to 13°30'LatS and 39°15'LonE to 39°30'LonE; southwest (SW), 13°30'LatS to 13°45'LatS and 39°30'LonE to 39°45'LonE; and southeast (SE), 13°30'LatS to 13°45'LatS and 39°15'LonE to 39°30'LonE. This criterion of sectored analysis was taken due to the already suspected differences in incidence of ACL cases to each of these subregions. For each sector, 5 to 7 different places evenly distributed in the respective areas, corresponding to the 45 index ACL cases, were searched for phlebotomines (Table II). The 4,027 female sand flies captured were grouped in 10 to 20 specimen samples, which frequency distribution per sector is displayed in Table II. A total of 335 samples were PCR analyzed for *L. braziliensis* with 30 (8.9%) of them testing positive. If a minimum amount of one phlebotomine is to be considered *Leishmania* positive per sample, then a likely underestimated overall rate of 0.4% positive phlebotomines may be calculated for the region. However, as suspected, the sectored analysis proved more revealing (Table II). Of the 30 positive samples, 25 (i.e. 83.3%) were contributed by the NW sector, which provided approximately one fourth of the total amount of analyzed samples. This resulted in a rate of 1.5% *Leishmania* positive phlebotomines (calculated as above) for this sector, markedly contrasting to the 0.2%, 0%, and 0.15% NE, SW and SE rates respectively. These differences were statistically significant (Chi-square = 59.51; $p < 0.001$).

Finally, this imbalance observed in the frequency of *Leishmania* positive phlebotomines was paralleled by an incidence of ACL cases for the NW sector that was about twice the observed for the other sectors for the six months of the study (i.e. 38.5, 14.0, 19.5 and 15.3 new ACL cases per 1,000 inhabitants for NW, NE, SW and SE respectively).

DISCUSSION

The present results clearly show that there is heterogeneity in the spatial distribution of *Leishmania*-infected phlebotomines. There is a statistically significant concentration of infected vectors in a single sector of the study area. The sector with the highest frequency of positive phlebotomines also presented the highest number of new ACL cases, suggesting a possible effect of the rates of *Lutzomyia* spp. infection in human disease. A study from Venezuela reported a statistically significant relationship between the abundance of *Lu. ovallesi* and new cutaneous leishmaniasis cases (Feliciangeli & Rabinovich 1998) in an area with a high rate (1.2%) of infection of this insect by *L. braziliensis*.

This study also confirms the large predominance of *L. (N.) whitmani* over other phlebotomine species in this area. In a previous study in Corte de Pedra, 30 phlebotomine species had been identified but *L. (Nyssomyia) whitmani* represented 99% of flies in the peridomiciliary area (Vexenat et al. 1986). In the present study the phlebotomine fauna was much less diverse and basically two species were identified. Although this aspect can be attributed to ecological changes, it reflects more likely the decision of the present sampling to focus in domiciliary and peridomiciliary areas, without a capture in the forest.

It seems to exist a bias of *L. (N.) intermedia*, when compared to *L. (N.) whitmani*, for the domestic environment in our data, and such a behavior has been reported previously (Rangel et al. 1984). The small number of insects captured intradomiciliary in our study precludes any firm conclusion on this aspect. Additionally, there is no indication so far that intra-domiciliary sand flies are more likely to transmit leishmaniasis. Since we have examined phlebotomine infection in samples of female specimens without separating the two species we do not know the relative rate of infection of *L. intermedia* and *L. whitmani*.

Previous studies in Brazil have stressed the low frequency of infection in *L. (N.) whitmani*. In central Brazil the infection rate was 0.16% (Galati et al. 1996) and in the Northeastern area it ranged from zero when captured at random (Vexenat et al. 1986) to 0.52 when capture was directed near houses of very recent leishmaniasis cases (Ryan et al. 1990). PCR has been used in different areas in order to increase the chance of detecting leishmanial infection in phlebotomines, with variable success (Perez et al. 1994, Feliciangeli & Rabinovich 1998). Our present

results show that a combination of directed capture and PCR did not increase the detection of infected sand flies in a large area. However, the use of these two approaches combined with a sectored analysis revealed sectors with a high incidence.

Although the PCR technique did not lead to increased detection if infected sand flies, such a technique allows for the examination if a huge humbers of sand flies with a considerable reduction if time and effort when compared to manual dissection and microscopic examination. Additionally, using PCR it is possible to identify *Leishmania* at coast at a subgenus level.

The indication that small sectors inside a larger area considered to be endemic for leishmaniasis may have a much larger frequency of infected phlebotomines, and its association with human disease is indicative of a spatial uneven distribution of the disease, and must be considered in planning of control strategies. Determining the micro-regions where leishmaniasis occurs in high frequency is fundamental for the comprehension of ecological and geographical factors which may influence leishmanial transmission.

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