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MAN–VECTOR CONTACT OF PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE) IN NORTH-CENTRAL VENEZUELA AS ASSESSED BY BLOOD MEAL IDENTIFICATION USING DOT-ELISA

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ABSTRACT. Human bait is traditionally used to assess man–vector contact, which is a key point in the study of the epidemiology of vector-borne diseases. However, in highly endemic foci, where this method should be avoided, this information could be obtained by blood meal analysis of engorged insects. In the village El Ingenio, Miranda State, Venezuela, *Lutzomyia ovallesi* and *Lutzomyia gomezi* are vectors of cutaneous leishmaniasis (CL). From June 1994 to March 1995, sand flies were collected inside houses on 974 CDC trap nights from 1900 to 0700 h. A total of 7,281 female sand flies were caught: 68.7% of them were identified as *L. ovallesi*, and 3.3% were identified as *L. gomezi*. Almost all of the blood-engorged flies (233 of 237) were dissected and identified, and gut contents were examined by dot enzyme-linked immunosorbant assay (dot-ELISA) using antisera against humans and common household animals including the domestic mouse. The Human Blood Index was 0.817 for *L. ovallesi*. These results suggest that intradomiciliary transmission may occur and account for the cases of CL frequently observed in newborn children in El Ingenio.

KEY WORDS Blood-meal analysis, dot-ELISA, *Lutzomyia ovallesi, Lutzomyia gomezi*, cutaneous leishmaniasis, intradomestic transmission, Venezuela

INTRODUCTION

Man–vector contact is an important behavioral trait of hematophagous insects that incriminates them as putative vectors of a disease. Catches with human bait have traditionally been used to assess man-biting rates. Although protection is normally recommended, and different methods have been employed (Perez et al. 1988, Rozendaal 1990), it is generally acknowledged that a tropical hot and humid climate is not ideal for their application. Catches with human bait are to some extent artificial and may not represent the host choice of the vector. Replacing this technique with the use of indirect methods for the identification of natural blood meal sources in engorged females would overcome many of these problems.

Different techniques have been developed for the identification of natural blood sources of hematophagous insects (World Health Organization 1987). For a better understanding of the epidemiology of leishmaniases, bartonellosis, and phleboviruses, there is a need for more information on blood meal sources of phlebotomine sand flies. In the New World, studies on the blood meal sources of phlebotomine sand flies have been carried out in Panama (Tesh et al. 1971, 1972; Christensen and de Vasquez 1982), Brazil (Christensen et al. 1982), Colombia (Morrison et al. 1993), and Peru (Oguzuku et al. 1994) using a precipitin test. In western Venezuela, agarose gel immunodiffusion (Scorza et al. 1968, Añez et al. 1994) and ELISA tests (Bendezu et al. 1995) have been used for that purpose.

This work is part of an epidemiological study at an endemic focus in north-central Venezuela (El Ingenio, Miranda State). In this focus, *Lutzomyia ovallesi* was found to be naturally infected with promastigotes of *Leishmania braziliensis* and *L. mexicana* (Barrios et al. 1994, Feliciangeli et al. 1994). *Lutzomyia gomezi* was found to be infected with *L. braziliensis*, and these parasites were comparable with strains isolated from human cases in the same area (Feliciangeli et al. 1994).

Since epidemiological observations have led to the suspicion that intradomestic transmission may occur in El Ingenio (O'Daly et al. 1995), the purpose of this work was to identify the blood meal sources of engorged sand flies collected indoors. The technique used was a dot enzyme-linked immunosorbent assay (dot-ELISA).

MATERIALS AND METHODS

Study area: The village of El Ingenio represents a hyperendemic focus where 41.78% of the population has been identified as skin-test-positive for *Leishmania* and where there has been a significant increase in incidence in the last few years (O'Daly et al. 1995). The village is situated at the foot of the coastal mountain chain (10°31′N, 66°34′W) at a distance of 3.5 km from the city of Guatire and 30 km from Caracas and at an altitude of ≈400 m above sea level. This zone is classified as premontane dry forest, with an average annual temperature of 18–24°C and an average annual rainfall of 830–1,300 mm (Ewel and Madriz 1968). The dry season lasts 5 months (December to April), and the rainy season lasts 7 months (May to November). A census carried out in June 1994 showed that the village consisted of 51 houses, with a total population of 221 inhabitants. Their principal economic activity is the production of fruit and flowers and temporary

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Table 1. Results of the dot-ELISA test for identification of blood meals in indoor phlebotomine sand flies according to condition and amount.

	Fresh blood		Digested blood		
Blood meals	Not identified (n [%])	Identified (n [%])	Not identified (n [%])	Identified (n [%])	Total
Partial	28 (54.9)	23 (45.1)	13 (81.3)	3 (18.7)	67 (32.7)
Full	32 (30.2)	74 (69.8)	16 (50)	16 (50)	138 (67.3)
Total	60 (29.3)	97 (47.3)	29 (14.1)	19 (9.3)	205 (100)

work in surrounding urban areas. A census of the domestic animals per house was also carried out to quantify the available hosts and their number in relation to humans.

Capture and treatment of sand flies: During 5 yr of epidemiological studies in El Ingenio, we observed that some inhabitants at risk of infection had contracted the disease more than once. For this reason, CDC light traps were placed inside the houses, since previous observations indicated that *L. ovallesi* and *L. gomezi* are highly phototropic.

Sand fly captures were carried out between June 1994 and March 1995, twice weekly from 1900 to 0700 h. During this period, a total of 974 CDC light traps were set inside the bedrooms of 10 (June–October) to 29 (November–March) houses. Captured sand flies were placed inside fine mesh cages (11 cm^3) that were stored inside plastic bags containing a damp paper towel to maintain humidity for transport to the laboratory. In the laboratory, males and unfed females were conserved in glass vials containing 70% ethanol for later identification, while bloodfed females were retained inside the cages for immediate dissection. Dissection was in phosphate-buffered saline (PBS) (pH 7.2) using fine needles and sterile glass slides. Sand fly species were identified by examination of spermathecae and cibaria, using phase contrast microscopy (400×). The digestive tracts were observed for blood condition (fresh or digested) and blood amount (partial or full) in 205 of 233 (88%) sand flies tested (Table 1) as well as for evidence of natural *Leishmania* infection. The intestinal contents were then absorbed onto small pieces of Whatman no. 1 filter papers. These were dried, preserved in gelatine capsules containing silica gel, and placed inside a plastic jar containing more silica. The vials were stored at room temperature for subsequent analysis of the blood meal sources.

Blood meal identification: The microplate method of the ELISA for identification of the blood meals of mosquitoes was adapted from Voller et al. (1974) by Edrissian and Hafizi (1982) and followed by Beier et al. (1988). Herein, this test was used for the antigen–antibody reaction on a small piece of nitrocellulose membrane (dot-ELISA). This technique was used for its sensitivity, specificity, simplicity, low cost, and rapid results.

The available commercial anti-sera IgG peroxidase conjugates were used (Sigma Inmunochemical Co.): anti-human (A-6029), anti-dog (A-6792), anti-chicken (A-9046), anti-pig (A-7042), anti-bovine (A-7414), and anti-mouse (A-5278). Sera from blood of selected hosts were obtained and stored at −40°C before use.

Preliminary tests were performed to determine optimal dilutions of the anti-sera, assay specificity, and assay sensitivity. Optimal dilution of the anti-sera using homologous serum gave a ratio of 1: 2,000 for anti-chicken; 1:250 for anti-mouse, and 1:500 for other hosts. Assay specificity was determined by testing cross-reactions of the sera when each anti-serum was challenged vs. all heterologous sera, diluted 1:20. Assay sensitivity was determined using laboratory-reared *Lutzomyia longipalpis* fed on human, dog, and chicken, maintained at room temperature (25 ± 2°C). Groups of 10 were killed 12, 24, 36, 48, 72, and 96 h after feeding. These laboratory-reared sand flies were processed in the same way as the sand flies collected in the field. In each trial, positive and negative controls were included, since plate-to-plate variation may occur. Negative controls were represented by the intestinal contents of laboratory-reared *L. longipalpis* males. Males as controls have previously been used in blood identification tests in mosquitoes (Rubio-Palis et al. 1994). For positive controls, host-sera PBS dilutions of 1:20 were used with species-specific anti-sera.

Sand fly blood meal samples absorbed in filter paper were eluted individually in 100 μl of PBS (pH 7.4) in round-bottom wells of rigid inmunoassay plates and held overnight at 4°C covered with plastic film. Five microliters of each eluate was then transferred to 6 × 6-mm pieces of nitrocellulose membrane (Schleicher and Schuell, Keene, NH) in flexible plates of polyvinyl chloride (de Hubsch et al. 1988) and stored uncovered overnight at 37°C. On the next day, the plates were blocked with 150 μl of phosphate-buffered saline plus bovine serum albumin (PBS-BSA) and covered at room temperature for 30 min. Plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBS-Tw20). The anti-IgG host-specific peroxidase conjugate was mixed with 50 μl of each heterologous serum to reduce cross-reactions and enhance specificity. This mixture was diluted in

Table 2. Results of the dot-ELISA test: identification of natural blood meal sources in sand flies collected inside houses in El Ingenio, Venezuela.

Species	Total tested	Total identified	Human	Simple dog	Feeding chicken	Pig	Cattle	Mouse	Mixed feeding
Lutzomyia ovallesi	166	82	45	3	7	2	1	1	24
Lutzomyia migonei	57	31	0	12	9	0	1	2	7
Lutzomyia lichyi	5	4	3	0	0	0	0	0	1
Lutzomyia gomezi	6	2	0	0	0	0	0	0	2
Lutzomyia venezuelensis	1	1	0	0	0	0	0	1	0
Lutzomyia sp.	4	1	0	0	1	0	0	0	0
Total	233	121	48	15	17	2	2	4	34
%	100	51.9	20.6	6.4	7.3	0.9	0.9	1.3	14.6

0.05% PBS-Tw20 according to ratios previously determined for each host, and 150 μl was added to each well. To prevent evaporation, plates were covered and kept at 37°C. After washing wells 3 times with PBS-Tw20 at 37°C, 200 μl of peroxidase substrate (4 chloro 1-naphthol solution as described by Pappas et al. 1984) was added to each well and left in the dark. After 30 min, the reaction was stopped by washing with distilled H_2O. Samples were considered positive if well-defined blue-purple spots developed on antigen dots.

Data analysis: The Human Blood Index (HBI), the proportion of females of a vector species giving positive reaction for human blood, as proposed by Garrett-Jones (1964) for *Anopheles* spp., was calculated.

RESULTS AND DISCUSSION

During the study period, a total of 7,281 female sand flies were collected indoors, of which 237 contained blood meal (3.3%). The possibility that some of them may have fed outdoors and entered houses, attracted by the CDC light traps or seeking refuge (Morrison et al. 1993), should not be excluded. None of the 237 females were found to be naturally infected with *Leishmania* spp. or other trypanosomatids.

The sensitivity assay of the dot-ELISA technique used, based on the detection time after feeding, showed that clear positive reactions could be obtained 36 h after blood ingestion in laboratory-reared females fed on known hosts. This time exceeds the 24 h cited for sand flies by Ngumbi et al. (1992). However, this time may be influenced by different variables. Service (1986) stressed that temperature is an important factor in blood meal digestion. Similarly, Beier et al. (1988) reported that human blood meals were accurately detected by ELISA up to 32 h after feeding for dried mosquitoes, but only up to 23 h for frozen mosquitoes. Therefore, these results cannot be strictly compared, since test conditions are not fully known.

In our field samples, the percentages of blood meal identification were significantly higher in fresh-blooded (bright blood with red cells) sand flies than in digested-blooded (brown or black blood, no red cells) sand flies (Table 1: $n = 205$; $\chi^2 = 7.37$; df 1; $P < 0.01$). Similar results were obtained with fully engorged sand flies vs. partially engorged sand flies (Table 1: $n = 205$; $\chi^2 = 12.81$; df 1; $P < 0.01$).

The census of the animals in the 29 houses surveyed generated the following results: there were 151 humans, 23 dogs, 12 cats, 4 pigs, 45 chickens, 37 pigeons, and 6 peacocks, showing that humans were the major source of blood meals in this environment. Blood meal identification was possible in 121 of 233 (51.9%) sand flies examined. Unidentified blood meals (48.1%) suggest that this high proportion may be explained in part by fresh but small quantities of blood or digested blood older than 36 h, as well as by the presence in the area of animals other than those tested for, and presumably wild, that are an important source of blood. Additional studies with field-collected sand flies and antisera of feral animals are required to more completely comprehend the sand fly feeding habits in this locality, as well as to investigate the role of natural reservoirs of leishmaniasis.

Lutzomyia ovallesi, a proven vector of cutaneous leishmaniasis in this area, was the predominant species entering the house (5,005 of 7,281; 68.7%) and bloodfed (166 of 233; 71.2%). In this habitat, where human blood meal sources predominated, 45 of 166 (27.1%) had fed only on human blood (Table 2), and 22 of 166 (13.3%) were found to have mixed blood meals containing human blood (Table 3). The HBI for *L. ovallesi* caught inside houses, calculated as number of flies fed on human blood in relation to the number of flies whose gut content was identified, was 0.82 (67 of 82). Additionally, *L. ovallesi* showed mixed meals containing up to 4 different host blood sources, indicating that this species is an opportunistic feeder with catholic feeding habits. Four of these females with mixed feeds (20.8%) reacted with anti-mouse serum (Table 2).

Lutzomyia gomezi comprised only 3.3% of the total female flies that entered the house ($n = 240$) and 2.6% of the total fed. The 2 specimens positive for blood meal source showed mixed feeding: one on human and bovine blood and the other on hu-

Table 3. Results of the dot-ELISA test: identification of mixed feeds in sand flies collected inside houses in El Ingenio, Venezuela.

	Lutzomyia ovallesi (n = 24)	*Lutzomyia migonei* (n = 7)	*Lutzomyia gomezi* (n = 2)	*Lutzomyia lichyi* (n = 1)
Human + cattle	4		1	
Human + dog	2			
Human + pig	8			1
Dog + cattle	2			
Pig + mouse		1		
Human + dog + mouse	1			
Human + dog + cattle	1		1	
Human + dog + pig	3			
Human + dog + pig + mouse	1			
Human + pig + mouse	2			
Cattle + dog + mouse		2		
Total	24	7	2	1

man, dog, and bovine blood. In the apparent absence of cattle in the village, a great range of dispersal of both *L. ovallesi* and *L. gomezi* (Alexander and Young 1992) or cross-reactions with sylvatic animal blood meals may explain the positive results obtained with bovine anti-serum. The small sample size of fed *L. gomezi* precludes any conclusion on the feeding behavior of this species in north-central Venezuela.

Reduced numbers of *L. ovallesi,* collected from tree buttresses and leaf litter and tested for blood meal sources in Panama, gave positive reactions to rodents and edentates, while *L. gomezi* reacted with rodents, marsupials, and carnivores (Tesh et al. 1971). A greater number was studied by Christensen and de Vasquez (1982), who found that *L. ovallesi* (n = 52) had fed on humans and hosts such as armadillos, sloths, and rodents, while *L. gomezi* (n = 30) had fed on armadillos, sloths, and opossums. In Guatemala, Rowton et al. (1992) pointed out that, since *L. ovallesi* has been collected at both canopy and ground levels (Williams 1970), its ability to move vertically may have permitted this species to increase its contact with humans, since many local residences in Petén are built beneath the forest canopy, and only underbrush is cleared. A similar situation may also occur in El Ingenio.

With respect to other species considered to be anthropophilic, it was noticed that no *L. migonei* blood meals reacted with human blood. This species, the second most abundant species collected by CDC traps inside the houses, fed primarily on dogs and chickens (Tables 1 and 2). *Lutzomyia migonei,* reported to be naturally infected with promastigotes in Yaracuy, Venezuela (Pifano 1940), is often caught in chicken houses and is a suspected vector of *L. braziliensis* in Brazil (Azevedo et al. 1990).

Of the 5 specimens of *L. lichyi* collected, 3 were found to have fed on human blood. This species has never been reported to be naturally infected with *Leishmania* species, but Warburg et al. (1991) infected it experimentally in the laboratory.

The people of El Ingenio are usually inside their houses from dusk until morning, during the hours of activity of the vector species (Feliciangeli 1997). This fact, as well as the frequency with which *L. ovallesi* enters houses and feeds on humans, may help to explain many of the cases of cutaneous leishmaniasis among young children in this area.

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