Am. J. Trop. Med. Hyg., 61(5), 1999, pp. 846–849

Polez

LUTZOMYIA NUNEZTOVARI ANGLESI (LE PONT & DESJEUX, 1984) AS A VECTOR OF *LEISHMANIA AMAZONENSIS* IN A SUB-ANDEAN LEISHMANIASIS FOCUS OF BOLIVIA

EDDY MARTINEZ, FRANÇOIS LE PONT, MIGUEL TORREZ, JENNY TELLERIA, FERNANDO VARGAS, JEAN CLAUDE DUJARDIN, AND JEAN PIERRE DUJARDIN

Instituto Boliviano de Biología de Altura, Departamento de Enfermedades Tropicales, La Paz, Bolivia; Institut de Recherche pour le Développement La Paz, La Paz, Bolivia; Prince Léopold Institute of Tropical Medicine, Antwerp, Belgium; Génétique Moléculaire des Parasites et des Vecteurs, Institut de Recherche pour le Développement, Montpellier, France

Abstract. Recently, a new *Leishmania amazonensis* focus was described in a sub-Andean region (1,450–2,100 meters above sea level) of Bolivia. In this area, three anthropophilic sandfly species were identified: *Lutzomyia nuneztovari anglesi* Le Pont & Desjeux, 1984, which represented 86–99% of the captures, *Lu. galatiae* Le Pont et al., 1998, and *Lu. shannoni* Dyar 1929. Only *Lu. nuneztovari anglesi* was found naturally infected by flagellates (16 of 1,715 females). Three *Leishmania* stocks were isolated and analyzed by isoenzyme electrophoresis at 11 loci. No significant isoenzymatic differences were demonstrated between them and 7 stocks isolated from patients from the same area, and previously characterized as *L. amazonensis*. Moreover, in a simplified protocol, the experimental infection of *Lu. nuneztovari anglesi* by *L. amazonensis* was successful in 92% of the surviving specimens. These data are discussed in relation to the Killick-Kendrick criteria. These results strongly suggest that *Lu. nuneztovari anglesi* is the vector of *L. amazonensis* at Cajuata, Inquisivi, La Paz, Bolivia.

In Bolivia, only two *Leishmania* species have been identified as agents of human cutaneous leishmaniasis: *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis*. The first one is a widespread parasite in Bolivia,[1–4] while the second one has been reported only rarely.[5–7] *Leishmania amazonensis* is better known from sylvatic lowlands, especially in Amazonia,[8,9] where all proven vectors belong to the *Lutzomyia flaviscutellata* complex,[9,10] but the cycle is able to survive in plantation woodland and deforested areas as in Brazil.[11] In the Venezuelan and Ecuadorian Andes, parasites related to *L. amazonensis* have been described (*L. garnhami*[12] and *L. mexicana*,[13] respectively). *Leishmania amazonensis* is potentially very dangerous and occasionally induces a chronic and eventually fatal disease known as cutaneous diffuse leishmaniasis (CDL). The first Bolivian case reported by Prado Barrientos from the neighboring region of the Yungas was a typical case of CDL probably due to *L. amazonensis*.[14] Recently, we described an outbreak of cutaneous leishmaniasis in the province of Inquisivi, La Paz,[15] where the parasite was identified as *L. amazonensis* on the basis of biologic and molecular data. The present study provides evidence indicating that the vector of *L. amazonensis* is *Lu. nuneztovari anglesi* Le Pont and Desjeux, 1984.

MATERIALS AND METHODS

Study area. The *L. amazonensis* focus is located at Cajuata and surrounding communities in the province of Inquisivi in southeastern region (67º15′W, 16º42′S) of the Department of La Paz, Bolivia. The study area is at an altitude ranging from 1,450 to 2,100 meters above sea level.[15] It is a deforested valley with very steep slopes such that the bottom of the valley is shaded early in the afternoon. The human population lives in scattered adobe brick houses with corrugated iron roofs. Around the settlements, land is cultivated with coca plantations, root vegetables, and papaya crops, while residual deciduous forests with xerophytes and epiphytes cover the steepest places. The cumulative prevalence of cutaneous leishmaniasis was determined in the population by house-to-house studies. Two hundred inhabitants were examined in December 1995 and 215 inhabitants were examined in March 1996.

Sandfly collections, dissection, and parasite isolation. From October 1995 to September 1996, except for November 1995 and March 1996, monthly protected human bait captures (capturing the sand flies attracted to exposed legs; only the authors of this study were subjected to this procedure) in coffee crops or residual forest were organized on two consecutive nights each month between 6:00 PM and 10:00 PM. Female specimens were caught in individual glass tubes, and immediately dissected in saline solution (0.9%) on glass slides for microscopic examination. When positive for flagellates, the gut and head content was aspirated into a syringe with saline solution and subsequently inoculated into a hamster. Material from hamster lesions developing a granuloma at the inoculation site was aspirated into a syringe containing sterile saline solution and then cultured in tubes of diphasic medium (NNN and Schneider's). These were stored at 24ºC after changing from diphasic to monophasic medium (Schneider's). The study was approved by the Scientific Committee of the Instituto Boliviano de Biología de Altura.

Experimental infection of sand flies. Using local facilities but without temperature and humidity control, 140 wild female *Lu. nuneztovari anglesi* were blood fed on anesthetized hamsters experimentally infected with a patient strain from the same region and previously characterized as *L. amazonensis* by isoenzyme analysis. Of these 140 specimens, 6 were randomly selected 36 hr after the blood meal and checked for the presence of promastigotes in the digestive gut. After 60 hr, only 13 sand flies had survived, which were also examined. The other anthropophilic species (*Lu. galatiae* and *Lu. shannoni*) were not tested because their rarity did not permit us to gather a representative live sample after 24 or more hours. On the other hand, no member of these two species was found to be positive for flagellates after field dissection.

Isoenzyme electrophoresis. Three stocks of parasites iso-

TABLE 1

Anthropophilic *Lutzomyia* sand flies captured by human bite and detection of infection*

Month	*Lu. n. anglesi*		Infected (%)	*Lu. galatiae*		*Lu. shannoni*	
	F/hr/h	Dissected		F/hr/h	Dissected	F/hr/h	Dissected
October	28.5	199	2 (1.0)	4.2	34	0.25	2
December	34.5	250	4 (1.6)	1.2	10	0.62	5
January	36.0	138	2 (1.4)	2.0	16	0.62	5
February	6.0	43	0 (0.0)	0.4	3	0.25	2
April	81.0	112	2 (1.8)	0.4	3	0.37	3
May	47.9	243	1 (0.4)	3.5	28	0.00	0
June	102.1	303	2 (0.7)	6.4	51	0.25	2
July	66.1	225	1 (0.4)	1.9	15	0.12	1
August	75.7	87	1 (1.1)	2.8	22	0.75	6
September	82.0	115	1 (0.9)	1.7	14	0.37	3
Total		1,715	16 (0.93)		196		29

* F/hr/h = females captured/hour/human; % = percent of infected females.

lated from wild *Lu. nuneztovari anglesi* were compared with 7 stocks isolated from human lesions previously identified as *L. amazonensis,*[15] as well as with reference strains of *L.* (*L.*) *amazonensis* (IFLA/BR/67/PH8), *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2903), *L.* (*L.*) *chagasi* (MHOM/BR/74/PP75), *L.* (*L.*) *mexicana* (MNYC/BZ/62/M379), and *L.* (*L.*) *pifanoi* (MHOM/VE/57/LV135).

Cellulose acetate plates (Helena Laboratories, Beaumont, TX) were used. Running conditions and identification techniques were as described by Dujardin and others.[16] Each sample was mixed with a hypotonic enzyme stabilizer, held for 30 min on ice, centrifuged for 2 min at 3,500 × *g*, and immediately subjected to electrophoresis. All aliquots allowed the survey of as many as 12 different enzyme systems, including additional analyses for controls or verifications. The following 12 enzyme systems were assayed: aconitase (EC 4.2.1.3, ACON), glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PD), glucose phosphate isomerase (EC 5.3.1.9, GPI), α-glycerophosphate dehydrogenase (EC 1.1.1.8, αGPD), isocitrate dehydrogenase (EC 1.1.1.42, IDH), malate dehydrogenase (EC 1.1.1.37, MDH), peptidase 1, substrate L-leucyl-leucine (EC 3.4.11, PEP 1), 6-phosphogluconate dehydrogenase (EC 1.1.1.44, 6PGD), phosphoglucomutase (EC 2.7.5.1, PGM), malic enzyme (EC 1.1.1.40, ME), mannose phosphate isomerase (EC 5.3.1.8, MPI), and fructose-1,6 diphosphate (EC 3.1.3.11, FDP).

Numerical analysis. The proportion of loci with fixed differences, i.e., loci showing no allele in common, was estimated as the convenient genetic distance between stocks.[17,18] From these distances, an unweighted pair group method of analysis (UPGMA) tree was constructed.

RESULTS

House-to-house studies. Until 1995, leishmaniasis in the study area was known only from sporadic cutaneous cases with no history of mucocutaneous lesions (espundia) or fatal visceral leishmaniasis. In December 1995, 39 cases (19%) were clinically identified in 200 inhabitants examined, 12 (6%) had active lesions and the remaining patients had scars. In March 1996, 22 new cases were identified with active lesions (215 inhabitants examined). In the locality of Cajuata, where 45–55% of the houses had clinical cases, only *L. amazonensis* could be identified. The study area is clearly a circumscribed new focus of leishmaniasis with high endemicity.

Sandfly collections. From 86% to 99 % of the female sand flies captured on human bait during a one-year period were *Lu. nuneztovari anglesi*, the remaining ones were *Lu. galatiae* and *Lu. shannoni,* in decreasing order of abundance (Table 1).

Natural infection of *Lu. nuneztovari anglesi.* Only *Lu. nuneztovari anglesi* was found infected with flagellates in the midgut, the pharynx, cibarium, and proboscis. This natural infection was detected each month, except in February. Of the 1,715 female *Lu. nuneztovari anglesi* dissected, 16 (0.93%) were infected with promastigotes (Table 1).

Parasite isolation. Four to six weeks after inoculation of promastigotes from the gut and head of infected sand flies into the hind legs of hamsters, three hamsters developed nodular lesions, without ulceration, which progressively increased and developed into metastatic peripheral lesions (forelegs, nose, ears, tail, and mucocutaneous zones) after 6–8 months. Samples obtained from these lesions showed abundant free parasites as well as many vacuolated histiocytes containing parasites. Three stocks, each from a different hamster, were isolated. Development of parasites in the culture media was observed after 48 hr.

Experimental infection of sand flies. From the 6 sand flies examined 36 hr after experimental infection, all had flagellates in the gut. Sixty hours after infection, 13 (9%) sand flies had survived, of which 12 (92%) showed abundant flagellates in the midgut and head (pharynx, cibarium, and proboscis).

Isoenzyme electrophoresis. Of the 12 enzyme systems used, 10 were retained due to reproducibility and good histochemical identification on the gels. Since one of these systems (MPI) systematically produced two bands, 11 loci were estimated. The UPGMA dendrogram (Figure 1), based on Richardson's distances[17] (Table 2), illustrates the similarity between stocks isolated from patients and insects, their close proximity with the *L. amazonensis* reference strain, as well as their clear-cut differences from the *L. pifanoi, L. mexicana, L. chagasi,* and *L. braziliensis* reference strains.

An average of 12% fixed differences was found between stocks isolated from patients and from *Lu. nuneztovari anglesi* (Table 2). No differences were found between 4 stocks isolated from patients (P5, P11, P13, and P21) and 2 from

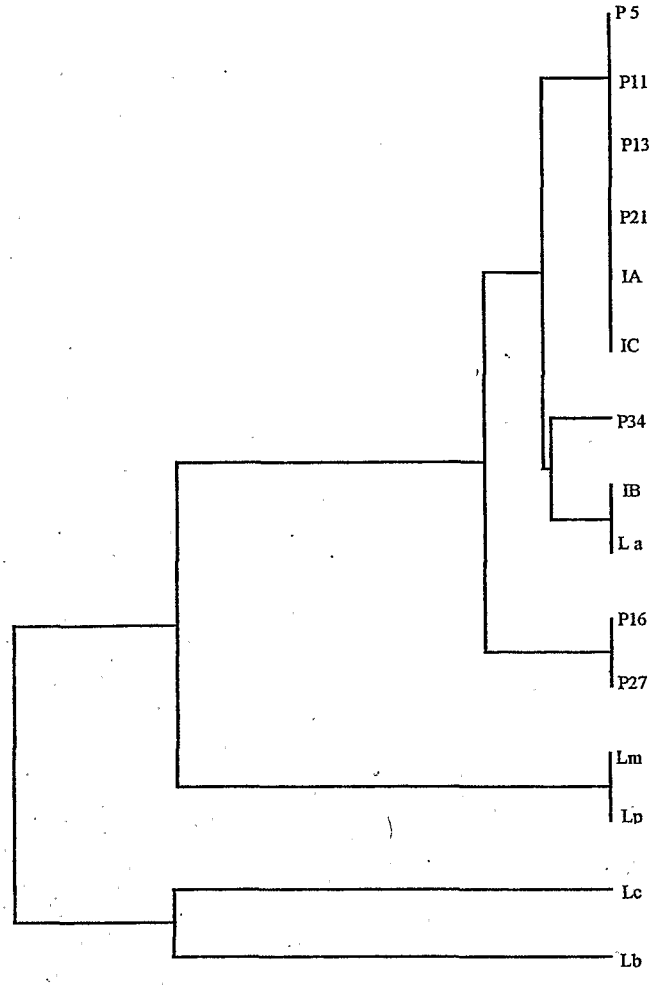

FIGURE 1. Unweighted pair group method of analysis tree derived from Richardson's distances, i.e., the proportion of loci showing no alleles in common between any two groups of an estimated total of 11 loci. Stocks isolated from patients = P5, P11, P13, P16, P21, P27, and P34; stocks isolated from *Lutzomyia nuneztovari anglesi* = IA, IB, and IC; reference strains = La (*Leishmania amazonensis*), Lm (*L. mexicana*), Lp (*L. pifanoi*), Lc (*L. chagasi*), and Lb (*L. braziliensis*).

Lu. nuneztovari anglesi (IA and IC) (Figure 1 and Table 2). The reference strain of *L. amazonensis* (IFLA/BR/67/PH8) showed 9% fixed differences, i.e., only one locus with no shared alleles[16] with two insect parasite stocks (IA and IC), and no difference compared with the third one (IB) (Table 2).

DISCUSSION

The present data provided the two essential criteria indicating the vectorial role of a sand fly:[10] 1) its anthropophily and 2) the repeated isolation and identification of the same species of *Leishmania* as that found in patients. The anthropophilic behavior of *Lu. nuneztovari anglesi* was already known in the nearby North Yungas province of Bolivia.[3] In our study, we also confirmed *Lu. nuneztovari anglesi* as an anthropophilic species in Inquisivi Province along with two other sand flies: *Lu. galatiae* and *Lu. shannoni.*

The next crucial criterion i.e., insects and patients with the same parasite,[19] was supported by isoenzyme comparisons. The genetic distance between insects and patients stocks could be zero (IA and IC, Figure 1) or, on average, < 13%. These genetic distances between insect and patient strains, as well as the level of their genetic differences with *L. amazonensis* (zero in one case, see IB, Figure 1), were commensurate with intraspecific variation.[17] As a matter of comparison, we verified that the proportion of fixed differences between various reference strains of *L. amazonensis* analyzed elsewhere by isoenzymes could reach 20% (Guerrini F, 1993. *Génétique des Populations et Phylogenie des Leishmania du Nouveau Monde.* Ph.D. Thesis, Université Montpellier II, Université des Sciences et Techniques du Languedoc, France). We also provided most of the additional supporting observations consistent with the hypothesis of *Lu. nuneztovari anglesi* as the vector of *L. amazonensis* in the study area.

Among the anthropophilic sand flies, *Lu. nuneztovari anglesi* was by far the most abundant one (86–99%). It was the only anthropophilic species found naturally infected with flagellates. A notable observation was its monthly infection

TABLE 2
Richardson's distances between stocks isolated and reference strains*

	Patient stocks							Insect stocks			Reference strains				
	P5	P11	P13	P16	P21	P27	P34	IA	IB	IC	Lb	Lc	Lp	La	Lm
P5	0.00														
P11	0.00	0.00													
P13	0.00	0.00	0.00												
P16	0.00	0.00	0.00	0.00											
P21	0.00	0.00	0.00	0.22	0.00										
P27	0.00	0.00	0.00	0.00	0.25	0.00									
P34	0.17	0.14	0.14	0.40	0.00	0.40	0.00								
IA	0.00	0.00	0.00	0.27	0.00	0.30	0.10	0.00							
IB	0.14	0.13	0.13	0.36	0.11	0.30	0.10	0.09	0.00						
IC	0.00	0.00	0.00	0.27	0.00	0.30	0.10	0.00	0.09	0.00					
Lb	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.00				
Lc	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.73	0.00			
Lp	0.71	0.75	0.75	0.64	0.67	0.60	0.70	0.73	0.64	0.73	0.91	1.0	0.00		
La	0.14	0.13	0.13	0.36	0.11	0.30	0.10	0.09	0.00	0.09	1.0	1.0	0.64	0.00	
Lm	0.71	0.86	0.86	0.70	0.75	0.67	0.78	0.80	0.70	0.80	0.90	1.0	0.00	0.70	0.00

* Proportion of loci with fixed differences (unshared alleles) of a total of 11 loci. This distance is known as Richardson's distances.[16,17] Reference strains: Lb = *L. braziliensis*; Lc = *L. chagasi*; Lp = *L. pifanoi*; La = *L. amazonensis*; Lm = *L. mexicana.*

(except in February), which suggests that the transmission cycles runs permanently throughout the year.

The full development of parasites in its digestive tube could be assessed by experimental infection.[10,19] Among living specimens available, *Lu. nuneztovari anglesi* showed a high rate of infection (6 of 6 after 36 hr and 12 of 13 after 60 hr). High mortality of the specimens after 60 hr (121 of 134) was probably due to inadequate field conditions.

We could not demonstrate that *Lu. nuneztovari anglesi* was able to transmit the parasite by bite,[10,19] but the natural and experimental infection of its proboscis was strong evidence supporting this behavior.[10,19] The animal reservoir was not determined in this study, but the present data strongly support *Lu. nuneztovari anglesi* as the vector of *L. amazonensis* in the Inquisivi Province of Bolivia. The remaining anthropophilic species, *Lu. galatiae* and *Lu. shannoni,* could not be ruled out as possible vectors. However, their low biting rate and prevalence make their possible role a secondary one.

It is worth noting that *Lu. nuneztovari anglesi* has been suspected as a vector of *L. braziliensis* in a nearby focus of the Yungas.[20,21] This uncommon feature, i.e., the transmission of a peripyloric parasite in one focus and a suprapyloric one in another nearby focus, should be verified by exploring the species homogeneity of *Lu. nuneztovari anglesi.*

Acknowledgments: We thank M. Lehane for revising the manuscript, and the Institut de Recherche pour le Développement at La Paz for field assistance.

Financial support: This investigation received financial support from the UNDP/World/Bank/WHO Special Program for Research and Training in Tropical Diseases grant no. 940902, and from an Institut de Recherche pour le Développement Allocation de Recherches no. 98087.

Authors' addresses: Eddy Martínez, Miguel Torrez, Jenny Telleria, and Fernando Vargas, Departmento de Enfermedades Tropicales, Instituto Boliviano de Biología de Altura, C. C. Sanjinez s/n, Miraflores, CP 641, La Paz, Bolivia. François Le Pont, Institut de Recherche pour le Développement La Paz, CP 9214, La Paz, Bolivia. Jean Claude Dujardin, Prince Léopold Institute of Tropical Medicine, Antwerp, Belgium. Jean Pierre Dujardin, Unité Mixte de Recherche, Centre National de la Recherche Scientifique–Institut de Recherche pour le Développement 9926, Génétique Moléculaire des Parasites et des Vecteurs, Institut de Recherche pour le Développement, BP 5045, 911 Avenue Agropolis, 34032, Montpellier Cedex 01, France.

Reprint requests: Eddy Martínez, Departmento de Enfermedades Tropicales, Instituto Boliviano de Biología de Altura, C. C. Sanjinez s/n, Miraflores, CP 641, La Paz, Bolivia.

REFERENCES

1. Desjeux P, Le Pont F, Mollinedo S, Tibayrenc M, 1986. Les *Leishmania* de Bolivie. I. *Leishmania braziliensis Vianna,* 1911 dans lés Départements de La Paz et du Beni. Premiers isolements de souches d'origine humaine. Caractérisation enzymatique. Coll. Int. CNRS/INSERM, 1984. Roux JA, ed. *Leishmania. Taxonomie et Phylogenèse.* Montpellier: Institut Méditerranéen d'Etudes Epidemiologiques et Ecologiques (IMEEE), 401–410.
2. World Health Organization, 1990. Control of the leishmaniases. *World Health Organ Tech Rep Ser 793.*
3. Le Pont F, Desjeux P, Torres JM, Fournet A, Mouchet J, 1992. *Leishmanioses et Phlébotomes en Bolivie.* Paris: ORSTOM-INSERM.
4. Dedet JP, 1993. *Leishmania* et leishmanioses du continent Américain. *Ann Inst Pasteur/Actualités 4 (suppl 1):* 3–25.
5. La Fuente C, Recacoechea M, Tibayrenc M, Urjel R, Darras C, Cardozo L, 1986. Leishmaniasis en Bolivia: presencia de dos complejos de *Leishmania* en los llanos orientales del Departamento de Santa Cruz—Bolivia. *Bol Cient CENETROP 12:* 1–15.
6. Dujardin JC, Gajendran N, Hamers R, Matthijsen G, Urjel R, Recacoechea M, Villarroel G, Bermudez H, Desjeux P, De Doncker S, Le Ray D, 1987. Leishmaniasis in the lowlands of Bolivia. VII. Characterization and identification of Bolivian isolates by PFG karyotyping. D. Hart, ed. *Leishmaniasis: the First Centenary (1885–1985). New Strategies for Control.* NATO ASI Series A. Volume 163. New York: Plenum Press, 137–148.
7. Grimaldi Jr G, David JR, McMahon-Pratt D, 1987. Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. *Am J Trop Med Hyg 36:* 270–287.
8. Lainson R, Shaw J, 1987. Evolution, classification and geographical distribution. Peters W, Killick-Kendrick R, eds. *The Leishmaniasis in Biology and Epidemiology.* Volume 1. London: Academic Press, 1–120.
9. Grimaldi Jr G, Momen H, Naiff R, McMahon-Pratt D, Barrett T, 1991. Characterization and classification of leishmanial parasites from humans, wild mammals and sand flies in the Amazon region of Brazil. *Am J Trop Med Hyg 44:* 645–641.
10. Killick-Kendrick R, 1990. Phlebotomine vectors of the leishmaniasis: a review. *Med Vet Entomol 4:* 1–24.
11. Shaw J, Lainson R, 1987. Ecology and epidemiology: New World. Peters W, Killick-Kendrick R, eds. *The Leishmaniasis in Biology and Epidemiology.* Volume 1. London: Academic Press, 291–363.
12. Scorza JV, Valera M, De Scorza C, Carnevalli M, Moreno E, Lugo-Hernandez A, 1979. A new species of *Leishmania* parasite from the Venezuelan Andes region. *Trans R Soc Trop Med Hyg 73:* 293–298.
13. Hashiguchi Y, Gomez E, De Coronel V, Mimori T, Kawabata M, Furuya M, Nonaka S, Takaoka H, Alexander JB, Quizhpe A, Grimaldi G Jr, Kreutzer R, Tesh R, 1991. Andean Leishmaniasis in Ecuador caused by infection with *Leishmania mexicana* and *L. major*-like parasites. *Am J Trop Med Hyg 44:* 205–217.
14. Prado Barrientos L, 1948. Um caso atípico de leishmaniose cutáneo-mucosa (Espundia). *Mem Inst Oswaldo Cruz 46:* 415–418.
15. Martínez E, Le Pont F, Torrez M, Tellería J, Vargas F, Muñoz M, de Doncker S, Dujardin JC, Dujardin JP, 1998. A new focus of cutaneous leishmaniasis due to *Leishmania amazonensis* in a sub-Andean region of Bolivia. *Acta Trop 71:* 97–106.
16. Dujardin JP, Le Pont F, Cruz M, Leon R, Tarrieu F, Guderian R, Echeverria R, Tibayrenc M, 1996. Cryptic speciation in *Lutzomyia (Nyssomyia) trapidoi* (Fairchild & Hertig) (Diptera: Psychodidae) detected by multilocus enzyme electrophoresis. *Am J Trop Med Hyg 54:* 42–45.
17. Richardson BJ, Baverstock PR, Adams SM, 1986. *Allozyme electrophoresis: A Handbook for Animal Systematics and Population Studies.* Orlando, FL: Academic Press.
18. Andrews RH, Handman E, Adams M, Baverstock PR, Mitchell G, 1988. Genetic characterization of *Leishmania* isolates at 37 enzyme loci. *Int J Parasitol 18:* 445–452.
19. Killick-Kendrick R, Ward RD, 1981. Ecology of *Leishmania.* Workshop No. 11. *Parasitology 82:* 143–152.
20. Le Pont F, Mouchet J, Desjeux P, 1989. Leishmaniasis in Bolivia. VI. Observations on *Lutzomyia nuneztovari anglesi* Le Pont & Desjeux, 1984 the presumed vector of tegumentary leishmaniasis in the Yungas focus. *Mem Inst Oswaldo Cruz 84:* 277–278.
21. Torrez M, Lopez M, Le Pont F, Martínez E, Muñoz M, Hervas D, Yaksic N, Arevalo J, Sossa D, Dujardin JP, 1998. *Lutzomyia nuneztovari anglesi* (Diptera: Psychodidae) as a probable vector of *Leishmania braziliensis* in the Yungas (Bolivia). *Acta Trop 71:* 311–316.

ISSN=0002-9637

VOLUME 61 NOVEMBER 1999 NUMBER 5

The American Journal of TROPICAL MEDICINE & HYGIENE

OFFICIAL JOURNAL OF
AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE