Transactions of the Royal Society of Tropical Medicine and Hygiene (2007) **101**, 1208—1213

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevierhealth.com/journals/trst

Epidemiological monitoring of American tegumentary leishmaniasis: molecular characterization of a peridomestic transmission cycle in the Amazonian lowlands of Bolivia

Ana Lineth Garcia[a,b], Tatiana Tellez[a], Rudy Parrado[a], Ernesto Rojas[a], Hernan Bermudez[a], Jean-Claude Dujardin[b,*]

[a] *Centro Universitario de Medicina Tropical, Facultad de Medicina, Universidad San Simón, Av. Aniceto Arce 0371, P.O. Box 3023, Cochabamba, Bolivia*

[b] *Laboratory of Molecular Parasitology, Instituut voor Tropische Geneeskunde, Nationalestraat 155, B-2000 Antwerpen, Belgium*

Received 25 April 2006; received in revised form 4 September 2007; accepted 4 September 2007

KEYWORDS
Leishmania;
Lutzomyia;
Epidemiology;
Molecular diagnostic techniques;
PCR;
Bolivia

Summary Human-made and environmental changes constitute a major risk factor for the (re-)emergence and spread of leishmaniasis; surveillance of the transmission cycle is essential in this context. This study integrated entomological and molecular parasitological techniques to document the transmission pattern of a peridomestic focus of *Leishmania* in the Isiboro Secure area of Bolivia. First the spatial distribution and relative density of phlebotomine sand flies, genus *Lutzomyia*, were established. *Lutzomyia shawi* was the predominant species in domestic and peridomestic environments (90% from all collections). Second, direct application of the *hsp70* PCR to sand fly extracts detected *Leishmania* infections in *Lu. shawi* only, and gave an estimated infection rate of 0.21 to 0.38%. The cleavage of the *hsp70* amplicon with restriction enzymes (*hsp70* PCR—RFLP) allowed identification of *Le. (V.) braziliensis* and *Le. (V.) guyanensis* in *Lu. shawi* captured in the same village. These two parasite species were also found in humans from the study region, supporting the co-existence of two transmission cycles involving the same sand fly species. This study demonstrated the use of PCR—RFLP in the identification of *Leishmania* in sand fly pools which could lead to the development of methods for screening large sand fly populations in Latin America.

1. Introduction

Leishmaniasis is a widely distributed parasitic disease caused by several species of *Leishmania* and transmitted by

* Corresponding author. Tel. +32 3 2476358; fax: +32 3 2476359.
E-mail address: jcdujard@itg.be (J.-C. Dujardin).

0035-9203/$ — see front matter © 2007 Royal Society of Tropical Medicine and Hygiene. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.trstmh.2007.09.002

phlebotomine sand flies. The population at risk is estimated at 350 million with an overall prevalence of 12 million. The disease is re-emerging worldwide due to multiple risk factors such as drug resistance, immunosuppression, human migration and environmental changes (Desjeux, 2001). Monitoring these risk factors is essential for controlling the disease (Dujardin, 2006).

American tegumentary leishmaniasis (ATL) is associated with several species of *Leishmania* and with more than 30 species of *Lutzomyia* as potential vectors (Killick-Kendrick, 1999). The transmission cycle of ATL usually occurs in forest and sylvatic environments and the disease is a zoonosis (Lainson et al., 1994). Typically, young adult males are the most likely to be infected as a consequence of professional activities (e.g. settlers, farmers, soldiers). Over the last 10 years, there has been a great increase in the incidence of ATL (Davies et al., 2000; de Castro et al., 2005), which in many places affects people of all ages and both genders (Yadon et al., 2003). This may indicate a change in the ATL transmission cycle to a peri- or intradomestic situation.

The potential dangers of the adaptation of sand flies to peridomestic and domestic habitats were identified in the New World by Forattini (1960). Since then, sand flies have been reported invading the domestic environment in response to human disturbance (Brandao-Filho et al., 1999; Meneses et al., 2005; Tolezano, 1994; Ximenes et al., 2000). It is essential therefore to identify the sand fly vectors involved in emerging foci. The vectorial competence of a sand fly is defined by several characteristics: (i) the overlap between its distribution and that of the disease, (ii) its anthropophily and (iii) the prevalence of natural *Leishmania* infections in the fly. The latter is usually determined by microscopy, isolation and culture. This is both inaccurate and laborious, as the flagellated forms of *Leishmania* are morphologically indistinguishable. PCR-based assays provide a powerful alternative capable of screening large numbers of flies, but most applications to sand flies concern the detection of the genus of *Leishmania* or its subgenera, rather than species (Caceres et al., 2004; Miranda et al., 2002). In regions where several *Leishmania* species are sympatric, it is important to use species-specific PCR assays to compare the infections encountered in humans and sand flies. We recently developed a series of PCR—RFLP assays allowing *Leishmania* species identification with a high sensitivity (Garcia et al., 2004) but they were not tested on sand flies. In the current study, we combined entomology and molecular parasitology to document the transmission pattern of a peridomestic focus of leishmaniasis in the Isiboro Secure area of Bolivia in the Amazon lowlands.

2. Materials and methods

2.1. Study area

The study was performed in the Isiboro Secure National Park in the Department of Cochabamba, central Bolivia. The area is characterized by Amazonian rainforest which has been cleared by intensive deforestation over the past two decades. The altitude ranges from 500 to 1600 m asl. The climate is tropical humid with average temperatures of 24 °C and rainfall of 2500 mm.

2.2. Sand fly collection

Selection of the three collection sites in the Isiboro Secure National Park, Isinuta, Samuzabet and Bolivar, was based on the presence of patients suspected to have been infected inside a residence. For each locality three collection stations were defined: station 1 (inside human dwellings), station 2 (domestic environment, 35 m from human dwellings) and station 3 (peridomestic environment, 100 m from human dwellings). Two collections of sand flies were carried out: (i) three consecutive nights per month in 2000 (February, April, May, June, August, September, December), in order to characterize sand fly species diversity and (ii) in 2004 (August to October), for evaluation of *Leishmania* infection. Captures were mostly done with an illuminated Shannon trap (Perez et al., 1991) operated by a team of three persons, during the peak activity period between 18:00 and 23:00 h. The Shannon traps were placed in stations 2 and 3 around dwellings where more than one family member (children and women) was ATL positive. Traps could not be placed inside the house as residents refused on the grounds of disturbance; in these cases automatic CDC light traps (Sudia and Chamberlain, 1988) were placed inside overnight. Collected sand flies were placed in liquid nitrogen using 1.5 ml cryotubes containing 5% dimethylsulfoxide in PBS.

2.3. Taxonomic identification

All the collected sand flies were dissected in a drop of sterile PBS and examined under a microscope. The head, wings, legs and abdominal segments containing the genitalia were removed. Taxonomic identification, using the keys of Young and Duncan (1994), was based on morphological characteristics of the genitalia. All females of the same species were stored in pools containing 30 to 50 insects (depending upon collection size) for further parasite detection and identification by *hsp70* PCR—RFLP. The infection rate was calculated by the method of pooled prevalence for variable pool size and perfect tests (assuming 100% sensitivity and specificity), with the pooled prevalence calculator (http://www.ausvet.com.au/pprev/content.php?page=PPVariablePoolSize). This method uses generalized linear modelling to calculate the maximum-likelihood estimates of prevalence and confidence limits, when multiple, different pool sizes are used (Williams and Moffitt, 2001).

2.4. Human sample collection

Clinical samples (dermal scrapings, syringe aspirates or biopsies) from 72 patients with confirmed ATL were collected from the three study localities as described elsewhere (Garcia et al., 2007). All clinical samples were transported to the reference laboratory in standard coolers at 4 °C. All the clinical samples were obtained with informed consent of the patients.

2.5. Parasite detection and identification

The *hsp70* PCR—RFLP technique was used for parasite detection. *Lutzomyia* pools were ground and homogenized with a

disposable pestle (Kontes®, Fisher Scientific International, Suwanee, GA, USA) in a 1.5 ml microfuge tube containing 150 μl of TNE buffer (25 mM Tris, 100 mM NaCl, 5 mM EDTA, pH 8). Clinical samples were processed as described elsewhere (Garcia et al., 2007). DNA was extracted using DNAzol reagents according to the manufacturer's instructions (Invitrogen, SA, Merelbeke, Belgium). The *hsp70* PCR amplification was carried out as described elsewhere (Garcia et al., 2004). The *hsp70* PCR products were ethanol precipitated and resuspended in 30 μl pure water. Digestion with the restriction endonuclease *Hae*III was performed under the conditions suggested by the suppliers in a final volume of 10 μl. One microlitre of the digested product was then analysed in microchips (2100 capillary electrophoresis system; Agilent Technologies, Karlsruhe, Germany) (Labchip 7500; Caliper Technologies, Mountain View, CA, USA).

3. Results

3.1. Sand fly identification, spatial distribution and abundance

A total of 4433 female sand flies was collected during the entomological survey in 2000. Six species of sand fly were encountered, including one new species, with *Lu. shawi* (90%) predominating (Table 1). *Lutzomyia shawi* and *Lu. llanosmartinsi* were the only species found in collection stations 1 and 2 (indoor and domestic) but they constituted only 3.6% of all the sand flies captured. *Lutzomyia shawi* was present throughout the year with two density peaks towards the end and at the beginning of the rainy season (April and August, respectively) with numbers decreasing as conditions become extreme, either too dry or too rainy. The nightly peak of activity was observed between 19:00 and 20:00 h. The highly anthropophilic behaviour of *Lu. shawi* was demonstrated by the many sand flies caught trying to bite the Shannon trap operators (data not shown).

3.2. PCR analysis of natural infections

Overall, 2308 female sand flies were collected during August to October 2004. The 2168 *Lu. shawi* specimens were grouped into 67 pools (each containing 30 to 39 insects) and subjected to *hsp70* PCR analysis for the presence of a natural infection. Five of the pools were found to be *Leishmania*-positive by the presence of 1300 bp amplicons: 3 of 46 pools (total 1456 insects) in Bolivar, 2 of 17 (total 557 insects) in Isinuta and 0 of 4 (total 155 insects) in Samuzabeth. Using the method of pooled prevalence for variable pool size, we estimated that this corresponded to a minimal infection rate of 0.0021 (95% CI 0.0005—0.0055) and 0.0038 (95% CI 0.0004—0.0118) in Bolivar and Isinuta, respectively.

3.3. Identification of *Leishmania* species

The *hsp70* PCR—RFLP was then applied to the five positive pools for species identification. In all five cases, it was possible to observe patterns, albeit weak, with the capillary electrophoresis system used. Of the five pools, four were shown to be infected with *Le. (V). braziliensis* and one with *Le. (V). guyanensis* (Table 2). As can be seen from Table 2 the *Leishmania* species present in *Lu. shawi* were also found in humans; *Le. (V.) lainsoni* was also found in humans but not in sand flies in Bolivar and Isinuta.

4. Discussion

This study analysed the transmission pattern of a focus of ATL in the Amazonian lowlands of Bolivia and *Lu. shawi* was found to be the predominant sand fly species in domestic and peridomestic environments (90% from all collections). This species was also found inside houses, but in low numbers and this may have been due to the different capture methods used inside the residences (CDC trap). *Lutzomyia shawi* was the only insect species in which *Leishmania* infections were found and identified, with a minimal infection rate estimated at 0.21% (Bolivar) and 0.38% (Isinuta). The sensitivity of the method used was not experimentally verified (e.g. by mixing one artificially infected sand fly in a clean pool) and more than one infected sand fly might be necessary to identify a positive pool, hence the infection rate may be higher than the reported values. By direct application of PCR—RFLP to sand fly extracts, *Le. (V.) braziliensis* and *Le. (V.) guyanensis* could be identified.

Table 1 Phlebotominae species by subgenus or species group captured in the Isiboro Secure National Park, Bolivia

Taxonomic identification	Male	Female	Relative abundance *n* (%)	Collection environment[a]
Subgenus *Nyssomyia* Barreto				
Lu. shawi Fraiha, Ward & Ready	26	3965	3991 (90)	Stations 1, 2, 3
Lu. yuilli yuilli Young & Porter	0	151	151 (3.4)	Station 3
Subgenus *Psychodopygus* Mangabeira				
Lu. hirsuta Mangabeira	2	166	168 (3.8)	Station 3
Lu. llanosmartinsi Fraiha & Ready	0	111	111 (2.5)	Stations 1, 2, 3
Subgenus *Trychophoromyia* Barreto				
Lu. nuneztovari sp. # 5	2	8	10 (0.2)	Station 3
Subgenus *Lutzomyia*				
Lu. walkeri Newstead	0	2	2 (0.05)	Station 3

[a] Station 1: inside human dwellings (indoors); station 2: 35 m from human dwellings (domestic environment); station 3: 100 m from human dwellings (peridomestic environment).

Table 2 *Leishmania* species circulating among human hosts and sand flies in the Isiboro Secure National Park, Bolivia

Locality	Confirmed cases of ATL	Confirmed cases of ATL in children	*Leishmania* species in human patients[a]	*Leishmania* species in *Lutzomyia shawi*
Bolivar	35	8	*Le. (V.) braziliensis* (31) *Le. (V.) lainsoni* (4)	*Le. (V.) braziliensis* (3 pools)
Isinuta	30	4	*Le. (V.) braziliensis* (24) *Le. (V.) guyanensis* (2) *Le. (V.) lainsoni* (4)	*Le. (V.) braziliensis* (1 pool) *Le. (V.) guyanensis* (1 pool)
Samuzabeth	7	1	*Le. (V.) braziliensis* (7)	Not found

ATL: American tegumentary leishmaniasis.
[a] Number of samples characterized is in parentheses.

Lutzomyia shawi has been suggested as a potential vector of *Leishmania* in Latin America due to its high density and anthropophilic behaviour in endemic areas, together with the microscopical observation of *Leishmania*-like parasites in the insect (Bermudez et al., 1993; Young and Duncan, 1994). This study confirmed the earlier entomological data and, through the application of molecular tools, further elucidated these observations. Even though this study did not demonstrate the ability of *Lu. shawi* to complete the *Leishmania* life cycle (i.e. through experimental infection of animals by sand fly bites), these observations strongly support its vectorial competence in this region of Bolivia. *Lutzomyia shawi* belongs to the *Nyssomyia* subgenus that contains highly anthropophilic species that have been shown to be involved in the transmission of ATL, including *Lu. flaviscutellata*, the vector of *Le. (Le.) amazonensis* (Arias et al., 1985), *Lu. olmeca*, the vector of *Le. (Le.) amazonensis* and *Le. (Le.) mexicana* (Williams, 1970), *Lu. umbratilis*, the vector of *Le. (V.) guyanensis* (Lainson et al., 1994), and *Lu. withmani* and *Lu. intermedia*, the vectors of *Le. (V.) braziliensis* (de Castro et al., 2005).

To date, *Lu. shawi* has never been reported in or near houses. Previously, it was associated only with forest areas in Bolivia (Bermudez et al., 1993), Brazil (Ryan et al., 1987), Peru (Villaseca et al., 1993) and Colombia (CIPA, 1999). This report confirms the current changes in the epidemiological transmission profile of ATL, towards domestication (Campbell-Lendrum et al., 2001; Oliveira et al., 2004; Travi et al., 2002; Ximenes et al., 2000). This sand fly behaviour has been well characterized in deforested areas of Brazil (Brandao-Filho et al., 1999), Colombia (Montoya-Lerma et al., 1999), and Venezuela (Feliciangeli et al., 1999). In Bolivia, Martinez et al. (1999) have described the domesticity of *Lu. nuneztovari anglesi* vector of *Le. (Le.) amazonensis* in the sub-Andean foci of Cajuta, while Torrez et al. (1998) incriminated the same species as responsible for the domestic transmission of *Le. (V.) braziliensis* in the Yungas foci.

The infection rate in a peridomestic environment estimated in this study (*Lu. shawi*, 0.21—0.38%) is comparable to those reported in central Brazil, with infection rates of *Lu. withmani* fluctuating between 0.16 and 0.52% when captured near houses with recent cases of leishmaniasis (Galati et al., 1996; Ryan et al., 1990). Further monitoring is required to document this process of adaptation. In particular, more work should be done on the reservoir species: are they still rodents or Edentates as in the sylvatic cycle (Lainson and Shaw, 1987) or could other animals like dogs (Reithinger and Davies, 2002) play a major role as a *Leishmania* reservoir?

The observation of *Le. (V.) braziliensis* and *Le. (V.) guyanensis* in the same sand fly species deserves particular attention as this indicates a low species-specific vector competence, a feature already reported in Latin America. As mentioned earlier, *Lu. nuneztovari anglesi* can transmit two different species belonging to the subgenera *Viannia* and *Leishmania*, respectively (Martinez et al., 1999; Torrez et al., 1998). Alternatively, the single species *Le. (V.) peruviana* was shown to be transmitted by three different sand fly species, *Lu. peruensis* (Perez et al., 1991), *Lu. verrucarum* (Villaseca et al., 1993) and *Lu. ayacuchensis* (Caceres et al., 2004). This contrasts with some parasite—vector interactions which appear to be much more specific in the Old World, *Le. (Le.) tropica—Phlebotomus sergenti* (Kamhawi et al., 2000) or *Le. (Le.) major—P. papatasi* (Pimenta et al., 1994) for example. Recent studies have shown that species-specific vector competence may be linked to the structure of the lipophosphoglycan (LPG) of the parasite: the more side-chain sugar substitutions, the higher the specificity (Sacks, 2001). The report of little or no substitution in the LPG of *Le. (V.) braziliensis* and *Le. (V.) guyanensis* (Muskus et al., 1997; Soares et al., 2005) agrees with the results obtained in this study. Further work should be performed to ascertain if the third parasite species present in the region (*Le. (V.) lainsoni*) is also transmitted in (peri-)domestic conditions by *Lu. shawi*.

Finally, this report confirms the performance of *hsp70* PCR—RFLP as a tool for molecular epidemiological evaluation of leishmaniasis. This simple method was taxonomically validated on reference strains of *Leishmania* (Garcia et al., 2004), and in combination with a capillary electrophoresis system with a high detection threshold, it demonstrated the capacity for direct species identification in human tissues, with 100% sensitivity (Garcia et al., 2004). Simple agarose electrophoresis may also be used, with a slightly lower sensitivity (estimated at 93% in human tissues; Garcia et al., 2004). Applicability of this method to sand fly pools has been demonstrated by this work and could lead to further mass entomological studies in Latin America. These studies are essential to monitor the domestication of the *Leishmania* life cycle (one of the major risk factors for re-emergence and spread of leishmaniasis) and to

help health authorities to adapt their leishmaniasis control strategies.

Authors' contributions: ALG, HB and JCD designed the study protocol; ER and HB carried out the clinical assessment; ALG, TT and RP performed the field and laboratory work; ALG and JCD analysed and interpreted the data; ALG and JCD drafted the manuscript. All authors read and approved the final manuscript. ALG and JCD are guarantors of the paper.

Acknowledgements: We thank the Bolivian staff of CUMETROP and the patients for their collaboration, Prof. Marc Coosemans and Dr Vanessa Yardley for revising this manuscript and Prof. Dirk Berkvens for the calculation of pooled prevalence.

Funding: TDR (Grant 00476), BTC (Belgian Technical Cooperation) and FWO (Flemish Fund for Scientific research 1.5.047.02).

Conflicts of interest: None declared.

Ethical approval: The review board of the institution providing the samples, Centro Universitario de Medicina Tropical (CUMETROP), Cochabamba, Bolivia.

References

Arias, J.R., Miles, M.A., Naiff, R.D., Povoa, M.M., de Freitas, R.A., Biancardi, C.B., Castellon, E.G., 1985. Flagellate infections of Brazilian sand flies (Diptera: Psychodidae): isolation in vitro and biochemical identification of *Endotrypanum* and *Leishmania*. Am. J. Trop. Med. Hyg. 34, 1098—1108.

Bermudez, H., Garcia, A.L., Troncoso, F., 1993. Leishmaniasis in the lowlands of Bolivia. Entomological studies on sandflies of the ''Valle del Sacta''. Tropical Carrasco of the Department of Cochabamba. Arch. Inst. Pasteur Tunis 70, 455—463.

Brandao-Filho, S.P., Campbell-Lendrum, D., Brito, M.E., Shaw, J.J., Davies, C.R., 1999. Epidemiological surveys confirm an increasing burden of cutaneous leishmaniasis in north-east Brazil. Trans. R. Soc. Trop. Med. Hyg. 93, 488—494.

Caceres, A.G., Villaseca, P., Dujardin, J.C., Banuls, A.L., Inga, R., Lopez, M., Arana, M., Le Ray, D., Arevalo, J., 2004. Epidemiology of Andean cutaneous leishmaniasis: incrimination of *Lutzomyia ayacuchensis* (Diptera: Psychodidae) as a vector of *Leishmania* in geographically isolated, upland valleys of Peru. Am. J. Trop. Med. Hyg. 70, 607—612.

Campbell-Lendrum, D., Dujardin, J.P., Martinez, E., Feliciangeli, M.D., Perez, J.E., Silans, L.N., Desjeux, P., 2001. Domestic and peridomestic transmission of American cutaneous leishmaniasis: changing epidemiological patterns present new control opportunities. Mem. Inst. Oswaldo Cruz 96, 159—162.

CIPA, 1999. Computed-aided Identification of Phlebotomine Sandflies of America. http://cipa.snv.jussieu.fr [accessed 17 August 2007].

Davies, C.R., Reithinger, R., Campbell-Lendrum, D., Feliciangeli, D., Borges, R., Rodriguez, N., 2000. The epidemiology and control of leishmaniasis in Andean countries. Cad. Saude Publica 16, 925—950.

de Castro, E.A., Luz, E., Telles, F.Q., Pandey, A., Biseto, A., Dinaiski, M., Sbalqueiro, I., Soccol, V.T., 2005. Eco-epidemiological survey of *Leishmania (Viannia) braziliensis* American cutaneous and mucocutaneous leishmaniasis in Ribeira Valley River, Parana State, Brazil. Acta Trop. 93, 141—149.

Desjeux, P., 2001. Worldwide increasing risk factors for leishmaniasis. Med. Microbiol. Immunol. 190, 77—79.

Dujardin, J.C., 2006. Risk factors in the spread of leishmaniases: towards integrated monitoring? Trends Parasitol. 22, 4—6.

Feliciangeli, M.D., Rodriguez, N., De Guglielmo, Z., Rodriguez, A., 1999. The re-emergence of American visceral leishmaniasis in an old focus in Venezuela. II. Vectors and parasites. Parasite 6, 113—120.

Forattini, O.P., 1960. On cutaneous leishmaniasis in the southern part of the state of Mato Grosso, Brazil. Rev. Bras. Malariol. Doencas Trop. 12, 69—73.

Galati, E.A., Nunes, V.L., Dorval, M.E., Oshiro, E.T., Cristaldo, G.M., Espindola, G., da Rocha, H.C., Garcia, W.B., 1996. Study of the phlebotomines (Diptera, Psychodidae), in area of cutaneous leishmaniasis in the State of Mato Grosso do Sul, Brazil. Rev. Saude Publica 30, 115—128.

Garcia, L., Kindt, A., Bermudez, H., Llanos-Cuentas, A., De Doncker, S., Arevalo, J., Wilber Quispe Tintaya, K., Dujardin, J.C., 2004. Culture-independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. J. Clin. Microbiol. 42, 2294—2297.

Garcia, A.L., Parrado, R., De Doncker, S., Bermudez, H., Dujardin, J.-C., 2007. American tegumentary leishmaniasis: direct species identification of *Leishmania* in non-invasive clinical samples. Trans. R. Soc. Trop. Med. Hyg. 101, 368—371.

Kamhawi, S., Modi, G.B., Pimenta, P.F., Rowton, E., Sacks, D.L., 2000. The vectorial competence of *Phlebotomus sergenti* is specific for *Leishmania tropica* and is controlled by species-specific, lipophosphoglycan-mediated midgut attachment. Parasitology 121, 25—33.

Killick-Kendrick, R., 1999. The biology and control of phlebotomine sand flies. Clin. Dermatol. 17, 279—289.

Lainson, R., Shaw, J.J., 1987. Evolution, classification and geographical distribution, in: Peters, W., Killick-Kendrick, R. (Eds), The Leishmaniases in Biology and Medicine. Academic Press, London, pp. 1—120.

Lainson, R., Shaw, J.J., Silveira, F.T., de Souza, A.A., Braga, R.R., Ishikawa, E.A., 1994. The dermal leishmaniases of Brazil, with special reference to the eco-epidemiology of the disease in Amazonia. Mem. Inst. Oswaldo Cruz 89, 435—443.

Martinez, E., Le Pont, F., Torrez, M., Telleria, J., Vargas, F., Dujardin, J.C., Dujardin, J.P., 1999. *Lutzomyia nuneztovari anglesi* (Le Pont & Desjeux, 1984) as a vector of *Leishmania amazonensis* in a sub-Andean leishmaniasis focus of Bolivia. Am. J. Trop. Med. Hyg. 61, 846—849.

Meneses, C.R., Cupolillo, E., Monteiro, F., Rangel, E.F., 2005. Microgeographical variation among male populations of the sandfly, *Lutzomyia (Nyssomyia) intermedia*, from an endemic area of American cutaneous leishmaniasis in the state of Rio de Janeiro, Brazil. Med. Vet. Entomol. 19, 38—47.

Miranda, J.C., Reis, E., Schriefer, A., Goncalves, M., Reis, M.G., Carvalho, L., Fernandes, O., Barral-Netto, M., Barral, A., 2002. Frequency of infection of *Lutzomyia* phlebotomines with *Leishmania braziliensis* in a Brazilian endemic area as assessed by pinpoint capture and polymerase chain reaction. Mem. Inst. Oswaldo Cruz 97, 185—188.

Montoya-Lerma, J., Cadena, H., Segura, I., Travi, B.L., 1999. Association of *Lutzomyia columbiana* (Diptera: Psychodidae) with a leishmaniasis focus in Colombia due to species of the *Leishmania mexicana* complex. Mem. Inst. Oswaldo Cruz 94, 277—283.

Muskus, C., Segura, I., Oddone, R., Turco, S.J., Leiby, D.A., Toro, L., Robledo, S., Saravia, N.G., 1997. Carbohydrate and LPG expression in *Leishmania viannia* subgenus. J. Parasitol. 83, 671—678.

Oliveira, C.C., Lacerda, H.G., Martins, D.R., Barbosa, J.D., Monteiro, G.R., Queiroz, J.W., Sousa, J.M., Ximenes, M.F., Jerônimo,

S.M., 2004. Changing epidemiology of American cutaneous leishmaniasis (ACL) in Brazil: a disease of the urban-rural interface. Acta Trop. 90, 155—162.

Perez, J.E., Villaseca, P., Caceres, A., Lopez, M., Zolessi, A., Campos, M., Guerra, H., Llanos-Cuentas, A., 1991. *Leishmania (Viannia) peruviana* isolated from the sandfly *Lutzomyia peruensis* (Diptera: Psychodidae) and a sentinel hamster in the Huayllacallan Valley, Ancash, Peru. Trans. R. Soc. Trop. Med. Hyg. 85, 60.

Pimenta, P.F., Saraiva, E.M., Rowton, E., Modi, G.B., Garraway, L.A., Beverley, S.M., Turco, S.J., Sacks, D.L., 1994. Evidence that the vectorial competence of phlebotomine sand flies for different species o*f Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. Proc. Natl. Acad. Sci. USA 91, 9155—9159.

Reithinger, R., Davies, C.R., 2002. Canine leishmaniasis: novel strategies for control. Trends Parasitol. 18, 289—290.

Ryan, L., Lainson, R., Shaw, J.J., 1987. Leishmaniasis in Brazil. XXIV. Natural flagellate infections of sandflies (Diptera: Psychodidae) in Para State, with particular reference to the role of *Psychodopygus wellcomei* as the vector of *Leishmania braziliensis braziliensis* in the Serra dos Carajas. Trans. R. Soc. Trop. Med. Hyg. 81, 353—359.

Ryan, L., Vexenat, A., Marsden, P.D., Lainson, R., Shaw, J.J., 1990. The importance of rapid diagnosis of new cases of cutaneous leishmaniasis in pin-pointing the sandfly vector. Trans. R. Soc. Trop. Med. Hyg. 84, 786.

Sacks, D.L., 2001. *Leishmania*-sand fly interactions controlling species-specific vector competence. Cell Microbiol. 3, 189—196.

Soares, R.P., Cardoso, T.L., Barron, T., Araujo, M.S., Pimenta, P.F., Turco, S.J., 2005. *Leishmania braziliensis*: a novel mechanism in the lipophosphoglycan regulation during metacyclogenesis. Int. J. Parasitol. 35, 245—253.

Sudia, W.D., Chamberlain, R.W., 1988. Battery operated light trap, an improved model. J. Am. Mosq. Control Assoc. 4, 536—538.

Tolezano, J.E., 1994. Ecoepidemiological aspects of American cutaneous leishmaniasis in the state of Sao Paulo, Brazil. Mem. Inst. Oswaldo Cruz 89, 427—434.

Torrez, M., Lopez, M., Le Pont, F., Martinez, E., Munoz, M., Hervas, D., Yaksic, N., Arevalo, J., Sossa, D., Dedet, J.P., Dujardin, J.P., 1998. *Lutzomyia nuneztovari anglesi* (Diptera: Psychodidae) as a probable vector of *Leishmania braziliensis* in the Yungas, Bolivia. Acta Trop. 71, 311—316.

Travi, B.L., Adler, G.H., Lozano, M., Cadena, H., Montoya-Lerma, J., 2002. Impact of habitat degradation on phlebotominae (Diptera: Psychodidae) of tropical dry forests in Northern Colombia. J. Med. Entomol. 39, 451—456.

Villaseca, P., Llanos-Cuentas, A., Perez, E., Davies, C.R., 1993. A comparative field study of the relative importance of *Lutzomyia peruensis* and *Lutzomyia verrucarum* as vectors of cutaneous leishmaniasis in the Peruvian Andes. Am. J. Trop. Med. Hyg. 49, 260—269.

Williams, P., 1970. Phlebotomine sandflies and leishmaniasis in British Honduras (Belize). Trans. R. Soc. Trop. Med. Hyg. 64, 317—368.

Williams, C.J., Moffitt, C.M., 2001. A critique of methods of sampling and reporting pathogens in populations of fish. J. Aquat. Anim. Health 13, 300—309.

Ximenes, M.F., Castellon, E.G., de Souza, M.F., Freitas, R.A., Pearson, R.D., Wilson, M.E., Jeronimo, S.M., 2000. Distribution of phlebotomine sand flies (Diptera: Psychodidae) in the state of Rio Grande do Norte, Brazil. J. Med. Entomol. 37, 162—169.

Yadon, Z.E., Rodrigues, L.C., Davies, C.R., Quigley, M.A., 2003. Indoor and peridomestic transmission of American cutaneous leishmaniasis in northwestern Argentina: a retrospective case-control study. Am. J. Trop. Med. Hyg. 68, 519—526.

Young, D.G., Duncan, M.A., 1994. Guide to identification and geographic distribution of *Lutzomyia* sandflies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). Associated Publisher, Gainesville, FL.