

EPIDEMIOLOGY OF ANDEAN CUTANEOUS LEISHMANIASIS: INCRIMINATION OF *LUTZOMYIA AYACUCHENSIS* (DIPTERA: PSYCHODIDAE) AS A VECTOR OF *LEISHMANIA* IN GEOGRAPHICALLY ISOLATED, UPLAND VALLEYS OF PERU

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Abstract. The southernmost limit of the distribution of endemic Andean cutaneous leishmaniasis (CL), commonly known as Uta, is localized in the western Andean valleys of Ayacucho, Peru. This area is completely isolated from other regions endemic for this disease. Identification of the insect vector for Andean CL was carried out by combining entomologic and parasitologic approaches. Two *Lutzomyia* species were captured: *Lutzomyia ayacuchensis* and *L. noguchii*. The former species was considered responsible for transmission of *Leishmania* because 1) there was a coincidence in space and time between the presence of this insect and the distribution of Andean CL, 2) it was shown to be highly anthropophilic, 3) *Leishmania* parasites of the subgenus *Viannia* were detected by a specific polymerase chain reaction assay, 4) promastigotes isolated from this insect were shown by multilocus enzyme electrophoresis and molecular karyotyping to belong to the same deme of *Leishmania* (*Viannia*) *peruviana* as the one circulating in humans living in the study area, and 5) the complete cycle of *L. (V.) peruviana* was observed in experimental infections of *Lu. ayacuchensis*. Parasite and vector homogeneity found in Ayacucho contrasted with the heterogeneity reported for other areas endemic for Andean CL. The potential influence of ecologic determinants on this geographically isolated area is discussed.

INTRODUCTION

Andean cutaneous leishmaniasis (CL), commonly known as Uta, is a particular mild clinical form of tegumentary leishmaniasis. It is endemic in Peru and constitutes an important primary health problem in people living in the highlands of this country.^{1,2} This disease, which affects mainly children, has been reported between latitudes 5°S and 13°S.^{3–5} These coordinates include 1) western Andean valleys (from 800 to 3,000 meters above sea level, 2) inter-Andean valleys (from 1,900 to 3,200 meters above sea level), and 3) some eastern Andean valleys (between 300 and 1,900 meters above sea level) in Peru.

Peru is an very heterogeneous country in terms of ecology, and on the basis of the endemicity of several animal species (including butterflies), 48 different geographic and ecologic scenarios known as biogeographic units (BGUs), have been described.⁶ Andean CL is encountered in several of these BGUs; accordingly, one might expect an important heterogeneity of both parasites and vectors involved in the transmission of this disease.

Preliminary entomologic surveys in different Andean valleys of Peru have demonstrated extensive sand fly diversity. The following sand flies have been described in areas endemic for Andean CL: *Lutzomyia noguchii*, *Warileya phlebotomica*, *Lu. verrucarum*, *Lu. peruensis*,^{3,7–10} *Lu. tejadai*,¹¹ *Lu. ayacuchensis*,^{9,12} and *Lu. pescei*.^{7,13} Of them, the first two species are not anthropophilic.

Lutzomyia peruensis, *Lu. verrucarum*, and *Lu. tejadai* have been incriminated as vectors of Andean CL because 1) *Lu. peruensis* and *Lu. verrucarum* show the same spatial and temporal distribution as this disease,^{8,9} 2) sentinel hamsters had amastigotes in skin biopsy specimens when used as baits in areas with high densities of *Lu. peruensis*,¹⁴ 3) *Leishmania* sp. were obtained in culture from captured *Lu. peruensis*¹⁵ or from hamsters inoculated with their extracts,¹⁶ and 4) *Leishmania* of the subgenus *Viannia* was detected by a polymerase

chain reaction (PCR) in captured *Lu. peruensis*, *Lu. tejadai*, and *Lu. verrucarum*.^{11,17}

Lutzomyia ayacuchensis has been suspected to be an vector of Andean CL since its first description.^{9,12,18} This species was originally described in the geographically isolated valleys in Ayacucho, the southernmost limit of areas endemic for Andean CL in Peru.^{5,12} However, *Lu. ayacuchensis* was subsequently found in northern areas: 1) the North of Peruvian Andes (Piura in Huancabamba Province),⁹ 2) the neotropical rainforest (Cajamarca in San Ignacio Province), a Peruvian region located near the boarder with Ecuador,¹⁹ 3) areas endemic for Andean CL in the Llaucano Valley (Cajamarca in Chota Province),²⁰ and 4) the Andean valleys of Azuay and Chimborazo (Ecuador).^{21,22} Despite extensive entomologic surveys, no specimens of *Lu. ayacuchensis* have been captured in areas endemic for Andean CL between Ayacucho (in southern Peru) and Chimborazo or Cajamarca (in northern Peru).

In this report, which combines classic entomologic studies with modern *Leishmania* characterization procedures, we present strong evidence that *Lu. ayacuchensis* is the single vector of Andean CL in the geographic isolated highlands of Ayacucho. Interestingly, in this endemic area, there is also only one reported genetic variant of *Leishmania peruviana*, in contrast with other endemic areas.¹⁸

MATERIALS AND METHODS

Study area. The study was performed in Saquihuacca (2,250 meters above sea level, 15°13'S, 73°59'W) in the district of Pullo, province of Parinacochas, department of Ayacucho (Figure 1). This locality is situated on the western side of the Andes, in the secondary valley of the Lampalla River, 15 km from the village of Miraflores (2,000 meters above sea level). This is a xerophytic area that is part of the Surco-South BGU and ecologically isolated and different from the occidental

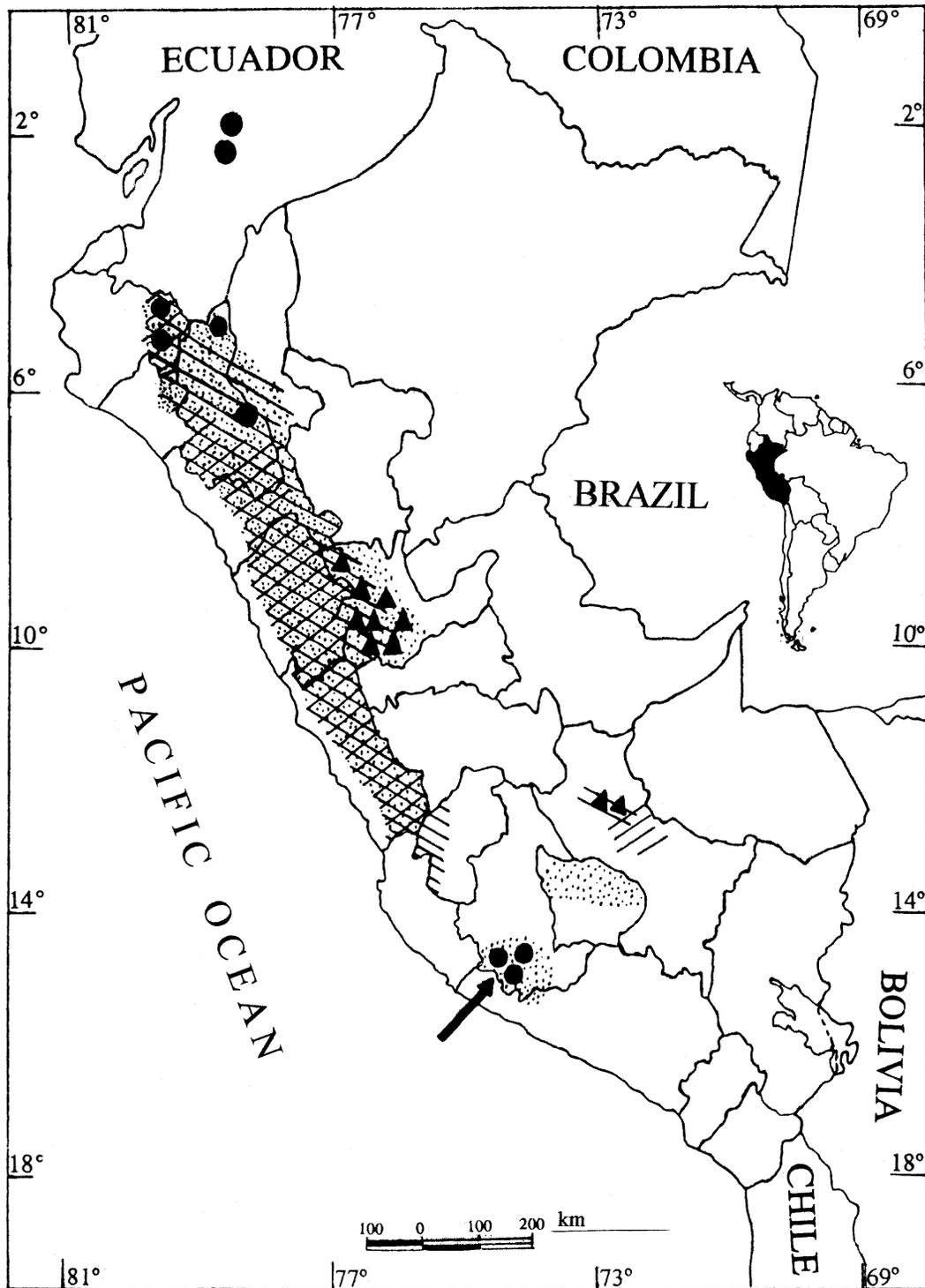


FIGURE 1. Map of Peru showing the distribution of areas endemic for Andean cutaneous leishmaniasis (dotted areas) and sand fly vectors: *Lutzomyia ayacuchensis* (●), *Lu. peruensis* (///////), *Lu. verrucarum* (~~~~~), and *Lu. tejadai* (▲). The arrow shows the study area, Parinacochas Province, Department of Ayacucho.

Andean valleys of the northern and central Peru.⁶ The predominant shrubs are *Caesalpineia tara* (tara), *Acacia* sp. (huarango), *Schinus* sp. (molle), *Carica candicans* (jerco), and Cactaceae.

From April to October, grassy areas (family Gramineae) and wild pasturage predominate after the rainy season. Cattle and herd of goats are put out to pasture in that area during

different periods of the year. Peasant families accompany their flocks for 2–3 months at that time, and live in *pircas* (temporary built stone dwellings). Leishmaniasis transmission occurs during this period of time in these locations.

Capture of sand flies. *Lutzomyia* sand flies were captured in February, April, July, September, and November for five consecutive days each month by 1) Shannon trap from 5:00 PM

to 7:00 AM of the next day, 2) human bait from 5:00 PM to 8:00 PM, and 3) Centers for Disease Control (Centers for Disease Control and Prevention [CDC], Atlanta, GA) light traps from 5:00 PM to 6:00 AM of the next day. All traps were located in open fields, far from towns, where abandoned "pircas" still remained.

Lutzomyia were pooled in different batches according to the month and cite of capture. Pooled sand flies were stored under liquid nitrogen in 1.5-mL plastic centrifuge tubes containing 5% dimethylsulfoxide in phosphate-buffered saline. All material were suitable for use in analysis by a PCR. Equipment for manipulating insect material was free of DNA, either because it was disposable and new or because it was washed in a 1.5% bleach solution.^{23,24}

Dissection of sand flies and isolation of parasites. Sand flies kept under liquid nitrogen were thawed and processed separately. Each *Lutzomyia* female was placed on a microscope slide containing 0.5 mL of sterile saline solution. The wings and legs were then removed and further dissection was done on another microscope slide containing 0.3 mL of saline solution. The gut was extracted with fine sterile needles and microscopically observed to determine in which part of the gut trypanosomatids were present. *Lutzomyia* were identified according to the morphology of their spermathecae.^{12,25}

Guts that contained trypanosomatid-like forms were cut transversally to free them. The suspension was aspirated with a 1.0-mL disposable syringe containing 0.4 mL of saline solution with antibiotics (200 units/ml of penicillin and 200 mg/ml of streptomycin); 0.2 mL was inoculated into two tubes containing blood agar biphasic medium (USMARU),^{26,27} 0.1 mL was inoculated into the left hind paw of a golden hamster, and the remaining 0.1 mL was placed in a 1.5-mL plastic centrifuge tubes for subsequent PCR analysis. Culture media were maintained at room temperature for 30 days and observed weekly under an inverted microscope. Hamsters were observed during for months. Aspirates were performed twice during that period of time.

Experimental infection of sand flies. Twenty-five *Lu. ayacuchensis* female sand flies were captured in SaquiHuacca. Eggs obtained from them were transported to our laboratory in Lima and added to our sand fly colony. Seventy-nine fourth generation females were fed on the swollen footpads of hamsters experimentally infected with *L. peruviana* LCA04 (MHOM/PE/90/LCA04) or LCA06 (MHOM/PE/90/LCA06). These *Leishmania* isolates were obtained from skin lesions of patients infected in the study area.¹⁸ To check for *Leishmania* infection, a variable number of sand flies were dissected every day within a 10-day post-feeding period. The location of promastigotes in infected individuals were classified according to the criteria of Lainson and Shaw.²⁸ The maintenance and care of experimental animals complied with our University guidelines for the humane use of laboratory animals.

Molecular characterization of parasites. *Leishmania* parasites were characterized by three techniques: PCR, multilocus enzyme electrophoresis (MLEE), and molecular karyotyping.

The PCR for *Leishmania* detection, which is specific for the subgenus *Viannia*, was carried out using the MP1L and MP3H primers, as described elsewhere.²⁴ A 70-basepair amplification product indicated an infected sand fly.

For MLEE, 15 enzyme systems (revealing 17 loci) were analyzed by cellulose acetate electrophoresis: aconitase (ACON: EC 4.2.1.3), glucose-6-phosphate dehydrogenase

(G6PD: EC 1.1.1.49), glucose-6-phosphate isomerase (GPI: EC 5.3.1.9), aspartate aminotransferase (ASAT: EC 2.6.1.1), alanine aminotransferase (ALAT: EC 2.6.1.2), isocitrate dehydrogenase (NADP+) (IDH: EC 1.1.1.42), malate dehydrogenase (NADP+) (MDH: EC 1.1.1.37), malate dehydrogenase (NADP+) or malic enzyme (ME: EC 1.1.1.40), mannose-6-phosphate isomerase (MPI: EC 5.3.1.8), nucleoside phosphorylase (NP1, substrate inosine and NP2, substrate deoxyinosine: EC 2.4.2.1), peptidases S or leucyl aminopeptidases (PEP1, substrate L-leucyl-leucine-leucine and PEP2, L-leucyl-L-alanine: EC 3.4.11.1), 6-phosphogluconate dehydrogenase (6PGD: EC 1.1.1.44), and phosphoglucomutase (PGM: EC 5.4.2.2.). Electrophoresis and staining procedures were carried out according to the methods of Ben Abderrazak and others.²⁹

Molecular karyotyping was performed as described elsewhere.¹⁸ Analysis was made on the whole karyotype after staining with ethidium bromide or on three size-variable chromosomes recognized by specific DNA probes: pLb-134Sp (recognizing a portion of gp63 encoding genes)³⁰ and pLb-168 and pLb-22 (two random probes derived from a genomic library of *L. braziliensis* M2904.¹⁸ Random primer labeling with ³²P-dCTP and hybridization were performed according to the manufacturer's (Amersham, Rainham, United Kingdom) instructions.

RESULTS

Insect captures. A total of 4,886 sand flies were captured in the study area. Only two species were detected: *Lu. ayacuchensis* and *Lu. noguchii*. Among flies captured in CDC light traps, the former species was only slightly more abundant than the latter (400 versus 323 of 723 trapped individuals) (Table 1). Nevertheless, when anthropophilic traps were used (Shannon or human bait traps), there was a strong bias toward *Lu. ayacuchensis*. More than 90% of the females captured with Shannon traps were *Lu. ayacuchensis*, while only 6.2% were *Lu. noguchii*. A similar difference was obtained when human bait traps were used (Table 1). When anthropophily was evaluated, the proportion of male and female *Lu. noguchii* attracted by Shannon traps was quite similar (271 males and 227 females) (Table 1).

Sand fly dissection. Of the 3,856 *Lu. ayacuchensis* females captured, 1,849 were dissected. Nine (0.49%) were found to be infected with trypanosomatids. Of the 461 *Lu. noguchii* females captured, 352 were dissected. Five (1.42%) were found to be infected with trypanosomatids (Table 2).

TABLE 1

Anthropophilic behavior of *Lutzomyia* sand flies from Ayacucho, Peru captured with anthropophilic (Shannon and HB) and non-anthropophilic (CDC) traps*

| Method of capture | <i>Lutzomyia</i> species | | | | | |
|-------------------|--------------------------|-------|-------|---------------------|-------|-------|
| | <i>Lu. ayacuchensis</i> | | | <i>Lu. noguchii</i> | | |
| | Females | Males | Total | Females | Males | Total |
| Shannon trap | 3,462 (93.8) | 94 | 3,556 | 227 (6.2) | 271 | 498 |
| HB | 102 (95.3) | 0 | 102 | 5 (4.7) | 2 | 7 |
| CDC light trap | 292 (56.0) | 108 | 400 | 229 (44.0) | 94 | 323 |
| Total | 3,856 | 202 | 4,058 | 461 | 367 | 828 |

* Values in parentheses are percentages. HB = human bait; CDC = Centers for Disease Control.

TABLE 2

Characterization of trypanosomatids found in female *Lutzomyia* sand flies in Ayacucho, Peru*

| Sand flies | No. captured | No. dissected | Trypanosomatids | <i>Viannia</i> | <i>Leishmania peruviana</i> |
|-------------------------|--------------|---------------|-----------------|----------------|-----------------------------|
| <i>Lu. ayacuchensis</i> | 3,856 | 1,849 | 9 | 5 | 4† |
| <i>Lu. noguchii</i> | 461 | 352 | 5 | 0 | 0 |
| Total | 4,317 | 2,201 | 14 | 5 | 4 |

* Female sand flies were naturally infected with either Trypanosomatids (detected by microscopy) or *Leishmania*. Further discrimination was attained by a polymerase chain reaction and multilocus enzyme electrophoresis for the subgenus *Viannia* and *L. peruviana*.

† Of the 5 PCR+ samples, only 4 could be isolated.

Molecular characterization of parasites. The 70-basepair PCR amplification product specific for the *Leishmania* subgenus *Viannia* was observed in five of the nine *Lu. ayacuchensis* that contained flagellate forms in their guts. In contrast, all five *Lu. noguchii* that contained trypanosomatids were PCR negative.

Of the five samples that were PCR positive, four led to successful parasite isolation by culture. These isolates were further characterized by more discriminative methods. Analysis by MLEE with the enzyme MPI identified all Ayacucho isolates as *L. peruviana* (Figure 2A). Moreover, exhaustive characterization of three of them (La36, La78, and La98) by MLEE for 17 loci showed that they all belonged to zymodeme 4, the same zymodeme circulating in human isolates in that region (Figure 2B).³¹ These three stocks were further characterized by molecular karyotyping, which showed a very similar chromosomal banding pattern (Figure 3A). Hybridization with the chromosome-specific probes pLb-134Sp, pLb-168, and pLb149 (Figure 3B–D) demonstrated that they belonged to the *L. (V.) peruviana* karyodeme PA2, the same karyodeme circulating in human isolates in that region.¹⁸

Experimental infection of sand flies. Twenty-six of forty-seven and twenty-seven of thirty-two *Lu. ayacuchensis* fed on footpad lesions in hamsters inoculated with the LCA04 and LCA06 isolates, respectively, were infected. Motile *Leishmania* were observed in the hindgut of infected sand flies after the third day post-feeding. This location (perypylaria) is a typical feature of the subgenus *Viannia*.

DISCUSSION

Leishmania peruviana, the causative agent of Andean CL, has been reported between latitudes 5°S and 13°S in the Peruvian Andes.^{2,3} The transmission cycle of *L. peruviana* has been reported in a variety of geographic and ecologic scenarios (BGUs).⁶ From Piura in northern Peru to Ayacucho in the southern part of this country, different isolates obtained from both patients and insect vectors have been extensively studied by molecular techniques. The genetic patterns have been shown to be polymorphic and have established a strong ecogeographic pattern of parasite genotypes along a north-south cline.^{22,30,31}

The Ayacucho region is particularly interesting because it constitutes the southernmost limit of the distribution of this disease (Figure 1). Furthermore, it is isolated from the neighbor endemic area by desert and seasonal small rivers (*quebradas*). This region has been reported as an area endemic for Andean CL since the end of 19th century.⁵

In Ayacucho, only two species of *Lutzomyia* belonging to

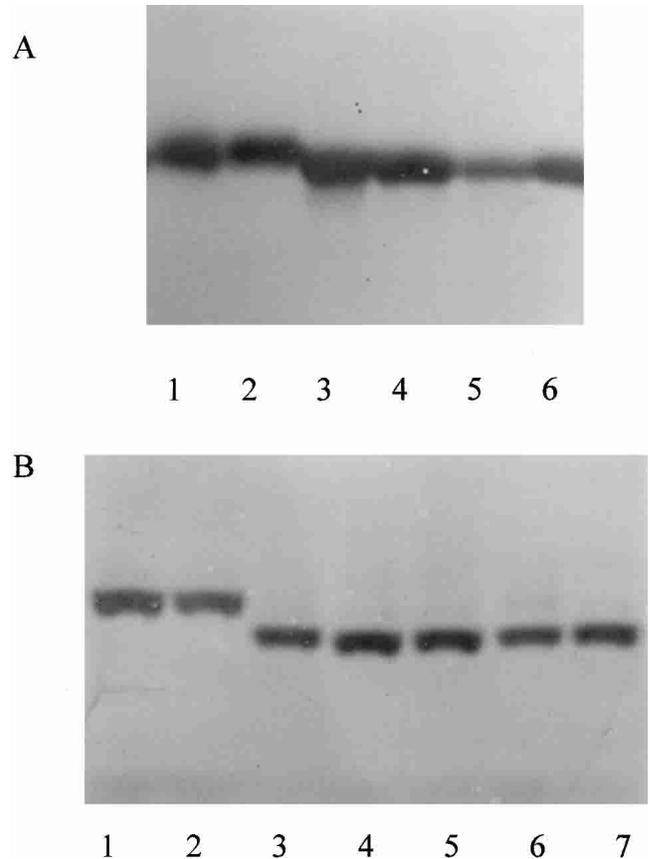


FIGURE 2. Multilocus enzyme electrophoresis profiles of parasites with **A**, mannose-6-phosphate isomerase and **B**, nucleoside hydrolase (substrate deoxyinosine) enzyme systems. All isolates were obtained from humans, except for La36 and La98, which were obtained from *Lutzomyia ayacuchensis*. **A**, Lanes 1 and 2, two *Leishmania braziliensis* reference stocks; lanes 3 and 4, La36 and La98; lanes 5 and 6, *L. peruviana* originating from Huancabamba biogeographic units and Surco biogeographic units, respectively. **B**, Lanes 1 and 2, two *L. peruviana* originating from Huancabamba biogeographic units; lanes 3 and 4, La36 and La98; lanes 5–7, three *L. peruviana* originating from Surco biogeographic units.

subgenus *Helcoctomyia* have been reported: *Lu. ayacuchensis* and *Lu. noguchii*. Our results support the hypothesis that *Lu. ayacuchensis* is the primary, if not the only, vector of Andean CL in the region of Parinacochas (Ayacucho). First, this study showed that *Lu. ayacuchensis* females were very anthropophilic. More than 90% of the females captured in Shannon traps and by human baits were *Lu. ayacuchensis*, whereas *Lu. noguchii* was poorly represented in captured sand flies. This difference cannot be explained because of a lower abundance of *Lu. noguchii* because flies captured in CDC light traps showed a similar proportion of females of both species (56% and 44% for *Lu. ayacuchensis* and *Lu. noguchii*, respectively). Second, *Leishmania* belonging to the subgenus *Viannia* were detected in five specimens of *Lu. ayacuchensis* by a specific PCR assay. Four of them grew in culture and were subsequently characterized by MLEE and molecular karyotyping as *L. (V.) peruviana*. These characterization methods clearly demonstrated that sand fly isolates belong to the same genetic cluster group as those isolated and characterized from human patients with Andean CL who lived in this endemic area.¹⁸

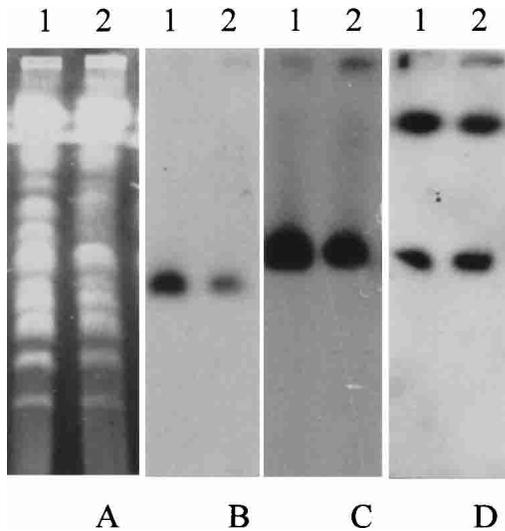


FIGURE 3. Molecular karyotyping of parasites after staining with ethidium bromide (A) and hybridization with chromosome-specific probes pLb-134Sp (B), pLb-168 (C), and pLb-22 (D). Lane 1, *Leishmania peruviana* LCA08 isolated from a human; lane 2, La36 isolated from *Lutzomyia ayacuchensis*.

Lutzomyia ayacuchensis kept in a colony become infected with *L. (V.) peruviana* when fed on animals experimentally infected with this parasite. This finding shows that these parasites can be successfully transmitted from a vertebrate host to a sand fly. Even when these results do not fully demonstrate vector competence, taken together with all our data, they strongly support the role of *Lu. ayacuchensis* as a vector of *L. peruviana*.

Our results also indicate that the transmission of leishmaniasis in Ayacucho involves only a single anthrophilic species of *Lutzomyia*. In contrast, other endemic areas show more than one insect vector. For example, *Lu. peruensis* and *Lu. verrucarum* have been found in the western Andean valleys in central Peru (Surco-North and Surco-Center BGUs),^{9,15-17,20} and *Lu. tejadai* has been found in the inter-Andean valleys of central Peru (Huanuco BGU).^{11,20} In contrast, *Lu. ayacuchensis* has never been observed in these other BGUs, and *Lu. peruensis*, *Lu. verrucarum*, and *Lu. tejadai* have never been found in Ayacucho (Surco-South BGU) (Figure 1).

A remarkable finding in this study was that *L. peruviana* isolated from naturally infected insect vectors in Ayacucho corresponded to a genetic homogeneous population based on their molecular karyotype and zymodeme. There was a single karyodeme of parasites (PA2) reported to circulate in Ayacucho among both human and invertebrate hosts.¹⁸ The homogeneity of both parasites and insect vectors could be explained by geographic isolation.

The Nasca Desert and the Andean highlands (more than 3,200 meters above sea level) constitute major natural barriers that keep both sand flies and rodents (the latter animal a suspected wild reservoir of leishmaniasis) isolated from other endemic areas. This isolation is reinforced by the restricted ecologic diversity of the Ayacucho region, a primary xerophytic environment that contrasts with other regions endemic for Andean CL that show secondary and more complex environments.

We do not exclude the possibility that other karyodemes or zymodemes do exist, either native or imported, but their prevalence would suggest a very low contribution to the genetic pool of *Leishmania* circulating in Ayacucho. Moreover, this particular area shows very low human immigration rates because of its very depressed economy.

Parasites homogeneity could also be explained by a strict parasite/vector association in natural conditions. Nevertheless, such an association is not exclusive because the PA2 karyodeme has been previously reported in the Surco-Center BGU (8 of 14 isolates studied) where *Lu. ayacuchensis* was never reported.¹⁸ In contrast, *Lu. ayacuchensis* captured in the southern Ecuador (Azuay and Chimborazo)^{21,22} have been found to be infected with *L. mexicana*.³²⁻³⁴

Finally, another interesting aspect of *Lu. ayacuchensis* is that this species was found in two different areas of Peru separated by a considerable distance. One area was in the extreme northern part of Peru (inter-Andean valleys of Huancabamba, Huancabamba BGU,^{9,20} inter-Andean valleys of Chinchipe¹⁹ and Llaucano²⁰) and the other was in Ayacucho. These two areas are 1,200 km apart, without any reported *Lu. ayacuchensis* despite intensive surveys. Further studies (morphologic, hybrid fertility, genotypic) of the insect are necessary to better characterize these allopatric populations and evaluate the need for reclassifying them as subspecies.

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