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**Distribution of *Lutzomyia ayacuchensis*, the vector of Andean-type cutaneous leishmaniasis, at different altitudes on the Andean slope of Ecuador**

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## **Abstract**

Distribution of the vector species is a major risk factor for the endemicity of leishmaniasis. In the present study, the vertical distribution of *Lutzomyia (Lu.) ayacuchensis*, the vector of *Leishmania (Leishmania) mexicana* in the Ecuadorian Andes was surveyed at different altitudes (300-2,500 m above sea level) of the Andean slope. The vector species *Lu. ayacuchensis* was identified at an altitude of 650 m and a higher areas, and higher distribution ratio of the species was observed at higher altitudes. In addition, high ratios of *L. (L.) mexicana* infection were detected in higher areas, but none in lower populations of sand flies. Since an association between sand fly populations and vector competence is suggested in *Lu. ayacuchensis*, haplotype analysis was performed on the species from different altitudes of the study areas; however, no apparent difference was observed among populations. These results suggested that *Lu. ayacuchensis* in Andean slope areas of Ecuador has the potential to transmit *L. (L.) mexicana* and spread leishmaniasis in these areas.

Keywords: Phlebotomine sand fly; Andean slope; *Lutzomyia ayacuchensis*; *Leishmania (Leishmania) mexicana*; Ecuador

## 1. Introduction

Leishmaniasis is a vector-borne parasitic disease caused by an obligate intracellular protozoan of the genus *Leishmania*. The disease is one of the most neglected diseases worldwide, having strong and complex associations with poverty, and it affects at least 12 million people (Alvar et al., 2012). Approximately 20 species of *Leishmania* are reported to be pathogenic to humans, and the parasites produce a wide range of clinical infections in both humans and vertebrate animals as zoonoses. In humans, the disease occurs in three distinct manifestations, cutaneous, mucocutaneous and visceral forms, and these clinical forms are largely associated with the *Leishmania* species responsible. The parasites are transmitted by female sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. At present, approximately 800 sand fly species have been recorded; however, less than 10% of them transmit each particular *Leishmania* species (Munstermann, 2004; Bates, 2007; Kato et al., 2010). Therefore, investigations on the prevalent parasite and vector species at given endemic areas is important for risk assessment and appropriate treatment of the disease.

Since 1982, we have been conducting epidemiological studies in the New World, especially in Ecuador. In this country, transmission of cutaneous and mucocutaneous leishmaniases occurs in rural populations living in bilateral regions of the Andes Mountains from the lowlands to the highlands up to an elevation of 2,500 m. The disease is widely spread in most provinces and is a considerable public health problem in Ecuador (Hashiguchi and Gomez, 1991). In the endemic areas, however, adequate epidemiological studies have not been done on a community basis, and no control measures have been applied to reduce or interrupt the transmission of the disease.

During our research activities, we have discovered a form of the disease in people

living on the Andean plateau/valleys; the disease form is very similar to 'Peruvian uta' but the causative agent and vector are completely different (Takaoka et al., 1990; Hashiguchi et al., 1991; Gomez and Hashiguchi, 1991; Kato et al., 2005, 2008a). These findings have increased the known distribution of Andean leishmaniasis in the Andes regions. Until very recently, the only form of leishmaniasis in the Andes was thought to be 'Peruvian uta' caused by *Leishmania (Viannia) peruviana*. In summary, in Peru the causative agent of the disease is *L. (V.) peruviana* and the suspected vectors are *Lutzomyia (Lu.) verrucarum*, *Lu. peruensis*, and *Lu. ayacuchensis* (Davies et al., 1993; Perez et al., 1994, 2007; Caceres et al., 2004; Kato et al., 2008a, 2011). In Ecuador, however, two species of the genus *Leishmania*, *Leishmania (Leishmania) mexicana* and *L. (L.) major*-like, seemed to be involved in Andean highland leishmaniasis (Hashiguchi et al., 1991). The only incriminated vector species is *Lu. ayacuchensis*, and the ratio infected by *L. (L.) mexicana* is high (1-8%) in highland areas, which is uncommon in most endemic areas of Ecuador (Takaoka et al., 1990; Gomez and Hashiguchi, 1991; Hashiguchi et al., 1991; Kato et al., 2005, 2008a). Therefore, distribution of the vector species is a major risk factor for the endemicity of leishmaniasis. In the present study, the vertical distribution of *Lu. ayacuchensis* and other man-biting sand fly species were surveyed at different altitudes (300-2,500 m above sea level) of the Andean slope, and the genetic divergence of *Lu. ayacuchensis* was analyzed by targeting the cytochrome oxidase I gene .

## 2. Materials and Methods

### 2.1. Study sites

The main study sites are located in Chimborazo Province on the south east of Ecuador, and located on the Andean slope, ranging from 300 m to 2,500 m above sea level (a.s.l.) along a railway, which was recently reconstructed and restarted after an approximately 20-year break (Fig.1). The study area Canton Alausi has a population of ca. 4,000 and lies at 2,300-2,500 m a.s.l. (Fig. 1). A small village, Chanchan, has only 37 villagers in total, and Canton Huigra has a population of ca. 2,000 at an altitude of 1,200-1,300 m a.s.l. Vegetation in these three areas is sparse, and consists of typical Alpine flora. Another four sites on the Andean slope, Olympo (820 m a.s.l.), Ochoa (650 m a.s.l.), Naranjapata (500 m a.s.l.) and La Ventura (300 m a.s.l.), were also included. In these lower areas, there are scattered human dwellings and cultivated fields along with the railway. In the Andean highlands (Alausi, Chanchan, and Huigra), *L. (L.) mexicana* is endemic and *Lu. ayacuchensis* is incriminated as the vector species (Takaoka et al., 1990; Gomez and Hashiguchi, 1991; Hashiguchi et al., 1991; Kato et al., 2005, 2008a). No Andean type of cutaneous leishmaniasis (CL) cases caused by *L. (L.) mexicana* was reported in the lower areas, including Olympo, Ochoa, Naranjapata, and Ventura. In the neighboring areas, however, cases caused by *L. (V.) guyanensis* were reported from Cumanda, very close to the present study site at La Ventura (Kato et al., unpublished). In addition, *L. (V.) guyanensis* and *L. (V.) panamensis* are prevalent in other areas, such as Troncal, Zhucay, Manta Real, Ocaña (Cañar Province) and Naranjal (Guayas Province), also close to La Ventura.

### 2.2. Collection and identification of sand flies

Sand flies were collected mainly by human landing (protected human bait) collections from July to August, 2012 at seven sites on the Andean slope of Ecuador; Alausi, Chanchan, Huigra, Olympo, Ochoa, Naranjapata and La Ventura, Province of Chimborazo. Similar surveillance was performed in 1994 in our previous study. In addition, the man-biting activity of *Lu. ayacuchensis*, the vector of *L. (L.) mexicana* in Ecuadorian Andes, was also examined at Alausi on three days, 3rd, 17th and 28th, of August 2013.

The sand flies were dissected and identified based on the morphology of their spermathecae, measurements of wing veins, the ratio of palpus length to length of antenna and the color of the thorax (Young and Duncan, 1994). The infection of sand flies by *Leishmania* promastigotes in the gut was examined under a microscope. Dissected *Lu. ayacuchensis* were individually fixed in absolute ethanol and stored at room temperature for further molecular analyses.

### 2.3. DNA extraction

Individual ethanol-fixed sand flies were lysed in 50 µl of DNA extraction buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA and 0.1 % sodium dodecyl sulfate (SDS)] with 100 µg/ml of proteinase K. The samples were incubated at 37°C overnight, heated at 95°C for 5 min, and then, 0.5 µl portions were directly used as the templates for PCR amplification.

### 2.4. PCR amplification and sequence analysis of the *Lutzomyia ayacuchensis* cytochrome oxidase I gene

The *Lu. ayacuchensis* cytochrome oxidase I (COI) gene was amplified with

universal COI primers (LCO1490: GGTCAACAAATCATAAAGATATTGG and HCO2198: TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al., 1994). PCR amplification was carried out in a volume of 15  $\mu$ l with the primers (0.4  $\mu$ M each), Ampdirect Plus (Shimadzu Biotech, Tsukuba, Japan), and high fidelity DNA polymerase (KOD-Plus-ver.2; TOYOBO, Tokyo, Japan). After an initial denaturation at 95°C for 5 min, amplification was performed with 35 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min) and polymerization (72°C, 1 min), followed by a final extension at 72°C for 10 min. The PCR products were purified using a FastGene Gel/PCR Extraction kit (NIPPON Genetics, Tokyo, Japan) to remove excessive primers, and the sequences were directly determined with a forward primer by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

### *2.5. Data analysis*

The sequences were aligned with CLUSTAL W software (Thompson et al., 1994) and examined using the MEGA program (Molecular Evolutionary Genetics Analysis) version 5.2 (Tamura et al., 2011). The haplotype analysis was performed using DnaSP 5.0 (Rozas et al., 2003), and a haplotype network was constructed using the median-joining methods as implemented in the program NETWORK 4.6.1.2 (<http://www.fluxus-engineering.com/sharenet.htm>) (Bandelt et al., 1999).

### 3. Results

#### 3.1. Vertical distribution of sand flies along the Andean slope

Eight man-biting species of the genus *Lutzomyia*, *Lu. ayacuchensis*, *Lu. maranonensis*, *Lu. robusta*, *Lu. hartmanni*, *Lu. gomezi*, *Lu. trapidoi*, and *Lu. panamensis* were identified at different altitudes/sites of the present study areas (Table 1, Fig. 2). The vector species of Andean-type cutaneous leishmaniasis, *Lu. ayacuchensis*, was identified at altitude of 650 m and higher areas, and a higher distribution ratio of the species was observed at higher altitudes; from 23.4% in Ochoa (650 m a.s.l.) to 100% in Alausi (2,300 m a.s.l.) (Fig. 2). At lower sites, *Lu. gomezi*, *Lu. hartmanni*, and *Lu. trapidoi* were dominant, corresponding to our previous findings (Terayama et al., 2008). Therefore, the habitat of *Lu. ayacuchensis* is considered to be areas higher than 500 m a.s.l. in Ecuador. Similar findings were obtained in the surveillance of 1994, showing that *Lu. ayacuchensis* was identified at higher altitudes (>500 m) and the distribution ratio increased with increasing altitude (Fig. S1). In 1994, the ratio of species distribution fluctuated and a small number of *Lu. shannoni* (1.1%) were identified in Naranjapata in addition to the seven species mentioned above; however, no clear-cut difference was observed between the two surveys. Among them, only *Lu. ayacuchensis* was positive for natural infection with promastigotes in highland areas, Alausi (2.1%) and Chanchan (2.5%) in 2012, and Alausi (5.6%), Chanchan (1.8%) and Huigra (0.6%) in 1994, but no positive sand flies were detected in other study sites (Table 1). The parasites were all identified as *L. (L.) mexicana* by multilocus enzyme electrophoresis or cytochrome *b* gene analyses. The biting activity of *Lu. ayacuchensis* is shown in Fig. 3 in relation to the temperature and humidity at Alausi in 2013. Sand flies started to be captured around 17:00 before sunset, peaked between 19:00 and 19:30,

and decreased as the temperature dropped under 15 degrees centigrade (Fig. 3). Similar observations were made in other highland areas (Chanchan and Huigra). In lower areas, sand flies started to be captured after dark. Humidity was largely unaltered during the collection at Alausi, and it did not seem to be a major factor influencing the biting activity of sand flies (Fig. 3). In this trial, CDC and Shannon light traps were also tested. Among a total collection of 435 sand flies, 426 (97.9%) by human landing collection, 9 (2.1%) by CDC trap, and none (0.0%) by Shannon trap were captured. Thus, *Lu. ayacuchensis* showed an extremely high affinity for humans, but not to light traps.

### 3.2. Haplotype analysis of the *Lutzomyia ayacuchensis* COI gene

High ratios of *Leishmania* infection in *Lu. ayacuchensis* were observed in highland areas (Alausi, Chanchan and Huigra), but not at lower sites (Table 1). Since an association between sand fly population and vector competence is suggested in *Lu. ayacuchensis* (Kato et al., unpublished), haplotype analysis was performed on the species from areas with different altitudes targeting a 621bp-fragment of the mitochondrial COI gene. Haplotype analysis showed that 12 flies from Alausi, 8 flies from Chanchan, 11 flies from Huigra, 12 flies from Olympo, and 5 flies from Ochoa belonged to 5, 5, 6, 3, and 4 haplotypes, respectively (Fig. 4A). In this analysis, a dominant haplotype, Hap2, in which sand flies from all 5 areas were included, was noted, but no marked genetic divergence was observed among populations (Fig. 4A and 4B). No relationship between haplotypes and vertical distribution was observed.

#### 4. Discussion

In the present study, sand fly species were surveyed at different altitudes of an Andean slope, and vertical distribution of *Lu. ayacuchensis*, the vector of *L. (L.) mexicana* in the Ecuadorian Andes, was elucidated. The distribution pattern was similar to that of 20 years ago. A haplotype analysis of *Lu. ayacuchensis* targeting the COI gene showed no apparent difference among populations at different localities where the infection ratio of the sand flies by *L. (L.) mexicana* is markedly different.

Cutaneous leishmaniasis (CL) caused by *L. (L.) mexicana* is endemic in Andean highland areas of southern Ecuador such as Huigra, Chanchan, Alausi, and Paute (Hashiguchi and Gomez, 1991). On the other hand, *L. (V.) guyanensis* and *L. (V.) panamensis* are widely prevalent in lowland subtropical regions, including areas close to La Ventura (Kato et al., unpublished). Surveillance of sand flies throughout the Andean slope identified the vertical distribution of the prevalent species; *Lu. ayacuchensis* at higher areas (>650 m a.s.l.), *Lu. maranonensis*, and *Lu. robusta* on the Andean slope (approximately 500-1,500 m a.s.l.), and *L. hartmanni*, *Lu. gomezi*, *Lu. trapidoi*, and *Lu. panamensis* at lower subtropical areas (<1,200 m a.s.l.). Of these, only *Lu. ayacuchensis* distributes at a higher area, Alausi (2,300 m a.s.l.), which is similar to the finding that only one species circulates in another highland area, Paute, Azuay Province, Ecuador (2,750 m a.s.l.) (Takaoka et al., 1990; Gomez and Hashiguchi, 1991; Hashiguchi et al., 1991).

In Ecuador, an aggressive man-biting species, *Lu. ayacuchensis* has been incriminated as the vector species of *L. (L.) mexicana* in the Andean highlands, including study areas (Takaoka et al., 1990; Gomez and Hashiguchi, 1991; Hashiguchi et al., 1991; Kato et al., 2005, 2008a), *Lu. gomezi* and *Lu. trapidoi* as possible vectors of

*L. (V.) guyanensis* and *L. (V.) panamensis*, respectively, in subtropical areas (Momori et al., unpublished), and *Lu. tortura* as a vector of *L. (V.) naiffi* in Amazonian areas (Kato et al., 2008b, 2013). To date, no sand flies infected by *Leishmania* species have been detected at subtropical study sites, and their vector competence has not been elucidated. Therefore, further vector research will be needed to understand the transmission mechanism of leishmaniasis. *Lutzomyia gomezi* and *Lu. trapdoi*, dominant species in Naranjapata and Ventura, may have the potential to spread leishmaniasis as reported in other areas.

Characteristically, the infection rate of *Lu. ayacuchensis* by *L. (L.) mexicana* is constantly high (1-8%) in the Ecuadorian Andes although the ratio in sand flies is mostly less than 1% in subtropical areas (Takaoka et al., 1990; Gomez and Hashiguchi, 1991; Hashiguchi et al., 1991; Kato et al., 2005, 2008a). Although *Lu. ayacuchensis* is circulating in Andean “slope” areas (Olympo and Ochoa), infection of the species by *Leishmania* was not found in the present or past studies. In addition, no CL case caused by *L. (L.) mexicana* has been reported in these areas. These observations raise the possibility that vector competence may be different between the Andean “highland” and “slope” populations, since our recent study suggested an apparent association between population and vector competence in *Lu. ayacuchensis* (Kato et al., unpublished). A haplotype analysis based on COI genes showed no striking difference between the populations despite our expectations. Therefore, factors other than sand flies are considered to contribute such a distinct infection ratio, as well as the endemicity of leishmaniasis between Andean “highland” and “slope” areas. The presence or absence of appropriate reservoir animals due to particular flora and fauna may cause such differences. However, *Lu. ayacuchensis* of the Andean slope populations is considered

to have potential to transmit *L. (L.) mexicana*, and thus, the species in these areas can be a risk factor for the expansion of leishmaniasis.

In the present study, a vertical distribution of sand flies through an Andean slope (300-2,300 m a.s.l.) was identified, and the distribution range of *Lu. ayacuchensis*, the vector of *L. (L.) mexicana*, was determined. In addition, no marked genetic divergence was found in *Lu. ayacuchensis* populations between Andean “highland” and “slope” areas. These results suggest that *Lu. ayacuchensis* in Andean “slope” areas has potential to transmit *L. (L.) mexicana*, although CL caused by the parasite species, *L. (L.) mexicana*, is not endemic at the present time. Continuous further surveillance of sand flies regarding natural infection with *Leishmania* will be necessary in and around these areas as they are a risk factor of the endemicity of leishmaniasis.

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**Conflict of interest**

The authors have no conflicts of interest to declare.

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## Figure Legends

Fig.1. (A) Map of Ecuador and Chimborazo Province where the main study sites are located. Sand flies were collected at the sites on Andean slopes, ranging from 300 m to 2,500 m above sea level (a.s.l.), along a railway (broken line). (B) Elevation map of the study areas; Alausi (2,300 m a.s.l.), Chanchan (1,500 m a.s.l.), Huigra (1,200 m a.s.l.), Olympo (820 m a.s.l.), Ochoa (650 m a.s.l.), Naranjapata (500 m a.s.l.) and La Ventura (300 m a.s.l.).

Fig.2. The proportion of sand fly species captured at each study site from July to August in 2012. AL, Alausi; CH, Chanchan; HU, Huigra; OL, Olympo; OC, Ochoa; NA, Naranjapata; VE, La Ventura.

Fig.3. Man-biting activity of *Lutzomyia ayacuchensis* at Alausi on three days (3rd, 17th and 28th, of August 2013). Horizontal axis indicates collection time. Line plots show average temperature (●) and humidity (▲), and bar graphs and the numbers above the bars indicate the number of sand flies collected during each collection period.

Fig.4. (A) Variable nucleotides found in the alignment of the *Lutzomyia (Lu.) ayacuchensis* cytochrome oxidase I (COI) gene. The COI gene sequence of a 621bp-fragment was analyzed in 48 *Lu. ayacuchensis* collected from 5 sites (AL, Alausi; CH, Chanchan; HU, Huigra; OL, Olympo; OC, Ochoa). Dots denote identical sequences and numbers show their corresponding positions from a *Lu. ayacuchensis* COI gene fragment analyzed in this study. (B) Haplotype network of the COI sequences of *Lu. ayacuchensis* collected from 5 study sites. Each haplotype is represented by a

circle sized in proportion to the frequency of the haplotype. Each crossbar represents one nucleotide substitution.

Fig.S1. The proportion of sand fly species captured at each study sites in 1994. AL, Alausi; CH, Chanchan; HU, Huigra; OL, Olympo; OC, Ochoa; NA, Naranjapata; VE, La Ventura.