Short Report: Molecular Mass Screening to Incriminate Sand Fly Vectors of Andean-type Cutaneous Leishmaniasis in Ecuador and Peru

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Abstract. Sand flies from the Andean areas of Ecuador and Peru were examined for Leishmania infections by using our recently established molecular mass screening method. Leishmanial minicircle DNA-positive sand flies were detected in 3 of 192 and 1 of 462 samples from Ecuador and Peru, respectively. Sand fly species were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the 18S ribosomal RNA (rRNA) gene, and the positive flies were Lutzomyia (Lu.) ayacuchensis and Lu. peruvensis, respectively. Furthermore, cytochrome b and mannose-phosphate isomerase gene sequence analyses identified the parasites from Ecuador and Peru as Leishmania (Leishmania) mexicana and L. (Viannia) peruviana, respectively. Thus, the mass screening method was confirmed to be a powerful tool for sand fly research.

Leishmaniasis is a protozoan disease caused by the genus Leishmania transmitted by female phlebotomine sand flies of the genus Phlebotomus in the Old World and Lutzomyia in the New World.1,2 It is well known that the prevalent sand fly species and infection rate of sand flies with Leishmania species are important risk factors in each endemic area, because only part of sand fly species transmit each particular species.3-6 The Andean highlands of Ecuador and Peru are unique endemic areas for leishmaniasis even though their ecologic features are quite different from those of tropical and subtropical areas where most endemic areas are located. In Ecuador, Andean-type cutaneous leishmaniasis, which occurs usually as a single small papular lesion, is caused by Leishmania (Leishmania) mexicana and very occasionally L. (L.) major-like.3,4 Lutzomyia (Lu.) ayacuchensis is the only proven vector for L. (L.) mexicana, although other sand fly species existed as minor populations in these areas.5-6 On the other hand, the main etiologic agent of Andean cutaneous leishmaniasis in Peru, commonly known as Uta, is L. (Viannia) peruviana.7 In these areas, Lu. peruvensis,8-10 Lu. verrucarum,9 and Lu. ayacuchensis11,12 are proven to transmit L. (V.) peruviana. Although information on the distributing sand fly species, endemic protozoan species, and vectors is accumulating for each endemic area, further details on the seasonality of prevalent sand fly species and their rates of infection with Leishmania species as a risk factor are required using larger populations. In the present study, using our recently established mass screening method, sand flies from areas where Andean-type cutaneous leishmaniasis is endemic in Ecuador and Peru were examined for Leishmania infections, and the infected parasite species and prevalent sand fly species were identified.

Sand flies were collected in February and March 2007 in the Andean areas of Ecuador and Peru, where Andean-type cutaneous leishmaniasis is endemic. The sites were as follows: in Ecuador, Huıgra (2°20’S, 78°58’W, Department of Chimborazo), at an altitude of 1,200–1,500 m: collections were made using protected human bait on a fruit farm for two nights. In Peru, Huayllacayán Valley, Yumpe and the surrounding 5 areas (10°15’S, 77°29’W, Department of Ancash), at an altitude of 2,000–2,400 m: collections were made using CDC light traps and Shannon traps at four points mainly on fruit farms for three nights. The sand flies were fixed in 70% ethanol and stored at room temperature. Parts of the specimens from Peru were morphologically identified prior to fixation. The sand fly species was identified by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)-based method as described previously.13,14 To this end, 18S ribosomal RNA (rRNA) gene sequences of the three species, Lu. verrucarum, Lu. peruvensis, and Lu. noguchii, which are prevalent in research areas, were determined, and their restriction enzyme sites were analyzed. On the basis of the analytical results, morphologically identified sand flies (79 Lu. verrucarum, 88 Lu. peruvensis, and 28 Lu. noguchii) were subjected to PCR-RFLP analyses with the AfiI enzyme, and consequently the three species were clearly classified (data not shown). No genetic diversity affecting the RFLP pattern obtained with AfiI was observed (data not shown). Unidentified specimens captured in Ecuador and Peru were subjected to a PCR-RFLP analysis of the 18S rRNA gene for molecular typing of the species. As the result, the sand flies were identified as Lu. ayacuchensis and Lu. nevesi in the Ecuadorian Andes, and Lu. verrucarum, Lu. peruvensis, and Lu. noguchii in the Peruvian Andes (Table 1). Infection of Leishmania within individual sand flies was examined by our recently established mass screening method.13 The Leishmania minicircle DNA-positive samples in a single PCR assay were regarded as positive for the infection because the reproducibility of the method has been confirmed.13 As the result, 3 and 1 positive flies were detected in the Ecuadorian and Peruvian Andes, respectively (Table 1). The positive ones were identified as Lu. ayacuchensis in Ecuador and Lu. peruvensis in Peru (Table 1). The infection rate appears to be lower in Peru (0.2%) than Ecuador (1.6%), corresponding to the previous findings.3-5,8-10,12,15 The factors causing such a
difference remain to be elucidated. However, it is conceivable that the distribution of reservoir animals is greater, and the cycle of transmission between reservoirs and vectors is better maintained in the Ecuadorian Andes, because climate, ecology, flora, and fauna differ considerably between the two countries. To identify the parasite species within the minicircle DNA-positive sand flies, parasite Cyt b gene sequences were determined from the specimens.13,15,16 The Cyt b gene sequences of all three positive samples from Ecuador (Huigra 1-7G, 1-11B, and 2-5F) had the highest degree of homology with the sequence of \( \text{Cyt}_b \text{Endotrypanum} \) homology (99.9%) with \( \text{Cyt}_b \) genes from the two species and found that the gene of \( L. \) \( \text{V. peruviana} \), but not \( L. \) \( \text{V. braziliensis} \), has an \( \text{AvaiI} \) site at a different nucleotide position. Therefore, PCR-RFLP analysis of \( \text{MPI} \) gene was performed on the positive sample. As shown in Figure 2, \( \text{AvaiII} \) cut the \( \text{MPI} \) fragments amplified from the Peru 3-5F sample and \( L. \) \( \text{V. peruviana} \), but not from \( L. \) \( \text{V. braziliensis} \), indicating that the positive \( L. \) \( \text{peruisuis} \) was infected with \( L. \) \( \text{V. braziliensis} \). Thus, an additional simple PCR-RFLP analysis of the \( \text{MPI} \) gene allowed for differentiation between \( L. \) \( \text{V. peruviana} \) and \( L. \) \( \text{V. braziliensis} \).

In conclusion, the utility of a molecular mass screening method was confirmed by comparative examination of sand flies from areas endemic for Andean type cutaneous leishmaniasis in Ecuador and Peru. Further continuous efforts using the method in various endemic areas in different seasons will accumulate detailed information on the risk factors for leishmaniasis, such as the prevalent sand fly species and the seasonal variation in the infection rate and transmission risk.

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**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Identified</th>
<th>Unidentified</th>
<th>Total</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecuador</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( L. ) ( \text{ayacuchensis} )</td>
<td>0</td>
<td>191</td>
<td>191</td>
<td>3</td>
</tr>
<tr>
<td>( L. ) ( \text{nevesi} )</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Peru</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( L. ) ( \text{verrucarum} )</td>
<td>79</td>
<td>105</td>
<td>184</td>
<td>0</td>
</tr>
<tr>
<td>( L. ) ( \text{peruensis} )</td>
<td>88</td>
<td>70</td>
<td>158</td>
<td>1</td>
</tr>
<tr>
<td>( L. ) ( \text{noguchii} )</td>
<td>28</td>
<td>45</td>
<td>73</td>
<td>0</td>
</tr>
</tbody>
</table>

* Morphologically identified sand fly specimens were subjected to polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis for molecular typing of the species.

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**FIGURE 1.** Phylogenetic tree of Cyt b gene sequences among species. The Cyt b genes of the parasites were amplified from the minicircle DNA-positive sand flies from Ecuador (Huigra 1-7G, Huigra 1-11B, and Huigra 2-5F) and Peru (Peru 3-5F), and the sequences were determined. Analyses were performed based on the sequences together with those from 13 \( \text{Leishmania} \) species and 2 \( \text{Endotrypanum} \) species. The scale bar represents 0.01% divergence.

**FIGURE 2.** Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of MPI genes from \( L. \) \( \text{V. braziliensis} \) (lane 1), \( L. \) \( \text{V. peruviana} \) (lane 2), and the positive sand fly from the Peruvian Andes, Peru 3-5F (lane 3). The PCR amplification was performed with MPI gene-specific primers, and the products were digested with \( \text{AvaiII} \).
In addition, elucidation of the relationships between Leishmania and vector species, which requires enormous effort with current/traditional methods, by use of the present method, will contribute to not only epidemiologic research on leishmaniasis, but also basic studies on parasite-vector interactions.

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