Am. J. Trop. Med. Hyg., 79(5), 2008, pp. 719–721

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. peruensis,* 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 of *Leishmania*.[1,2] 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.[3–6] *Lutzomyia* (*Lu.*) *ayacuchensis* is the only proven vector for *L.* (*L.*) *mexicana*, although other sand fly species existed as minor populations in these areas.[3–6] 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. peruensis*,[8–10] *Lu. verrucarum*,[9] and *Lu. ayacuchensis*[11,12] 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, Huigra (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. peruensis,* 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. peruensis,* and 28 *Lu. noguchii*) were subjected to PCR-RFLP analyses with the *Afa*I enzyme, and consequently the three species were clearly classified (data not shown). No genetic diversity affecting the RFLP pattern obtained with *Afa*I 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. peruensis,* 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. peruensis* 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

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TABLE 1
Mass screening of sand flies from areas endemic for leishmaniasis in the Ecuadorian and Peruvian Andes

		Identified*	Unidentified†	Total	Positive
Ecuador	*Lu. ayacuchensis*	0	191	191	3
	Lu. nevesi	0	1	1	0
Peru	*Lu. verrucarum*	79	105	184	0
	Lu. peruensis	88	70	158	1
	Lu. noguchii	28	45	73	0

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

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 *L.* (*L.*) *mexicana* (98.4–98.5%) when compared with other species (88.7–96.9%), whereas the positive one from Peru (Peru 3-5F) had greater homology (99.9%) with *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* than any of the others (88–98.8%). A phylogenetic tree showed that all 3 positive samples from Ecuador divided into the same clade as *L.* (*L.*) *mexicana,* whereas the Peruvian specimen had a closer relationship with *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* (Figure 1). These results indicated that the 3 positive sand flies from the Ecuadorian Andes were all infected with *L.* (*L.*) *mexicana,* and the one positive specimen from the Peruvian Andes was infected with *L.* (*V.*) *braziliensis* or *L.* (*V.*) *peruviana*. In a previous study, comparative enzymatic analyses of *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis* revealed that mannose phosphate isomerase (MPI) is the only reliable marker for distinguishing between the two species on the basis of isoenzyme profiles.[17] Furthermore, a recent study showed that the difference results from a single nucleotide change involving an amino acid substitution in the MPI genes.[18] We analyzed restriction enzyme sites of the MPI genes from the two species and found that the gene of *L.* (*V.*) *peruviana,* but not *L.* (*V.*) *braziliensis,* has an *Ava*II site at a different nucleotide position. Therefore, PCR-RFLP analysis of MPI gene was performed on the positive sample. As shown in Figure 2, *Ava*II cut the MPI fragments amplified from the Peru 3-5F sample and *L.* (*V.*) *peruviana,* but not from *L.* (*V.*) *braziliensis*, indicating that the positive *Lu. peruensis* was infected with *L.* (*V.*) *peruviana.* Thus, an additional simple PCR-RFLP analysis of the MPI gene allowed for differentiation between *L.* (*V.*) *peruviana* and *L.* (*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.

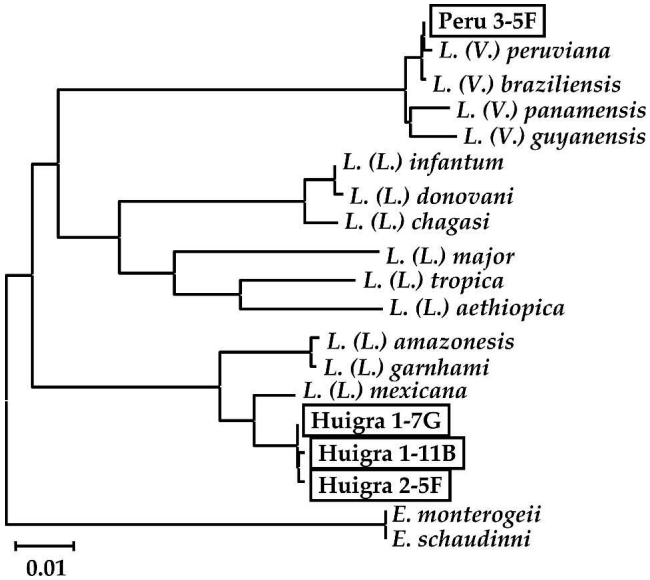

FIGURE 1. Phylogenetic tree of *Cyt* b gene sequences among species. The *Cyt* b genes of the parasites were amplified from the minicircle DNA-positive sand flies from Ecuador (Huigra 1-7G, Huigra 1-11B, and Huigra 2-5F) and Peru (Peru 3-5F), and the sequences were determined. Analyses were performed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

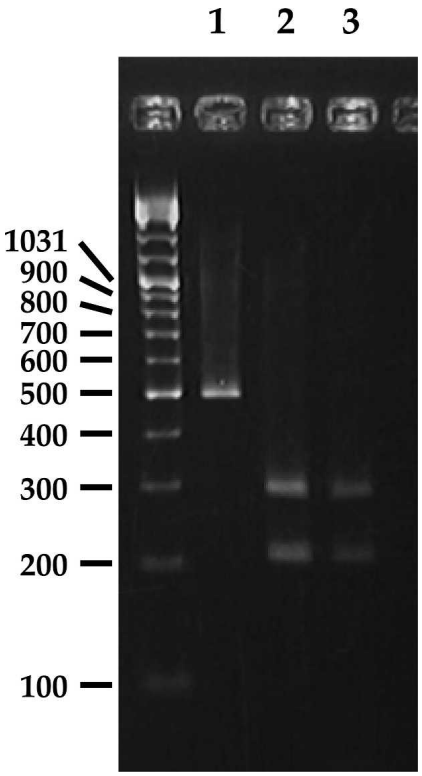

FIGURE 2. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of MPI genes from *L.* (*V.*) *braziliensis* (lane 1), *L.* (*V.*) *peruviana* (lane 2), and the positive sand fly from the Peruvian Andes, Peru 3-5F (lane 3). The PCR amplification was performed with MPI gene–specific primers, and the products were digested with *Ava*II.

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.

Received February 5, 2008. Accepted for publication July 12, 2008.

Acknowledgments: We are indebted to Roberto Sud A. (Ministerio de Salud Publica y Asistencia Social, Guayaquil, Ecuador) for his technical assistance throughout the study; to the director Dr. Luiggi Martini R. and the coordinator Dr. Ernesto Gutierrez V. (Instituto Nacional de Higiene y Medicina Tropical, Guayaquil, Ecuador) for their arrangement of our field activities; and to Dr. Teresa Flor, Dr. Jenny Muzzio Rodriguez, and Dr. Yim-Yan Wong Chum (Instituto Nacional de Higiene y Medicina Tropical, Guayaquil, Ecuador) for their support at the laboratory and field phases of the study. We would like to acknowledge the technical expertise of The DNA Core facility of the Center for Gene Research, Yamaguchi University, supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan.

Financial support: This study was supported by the Ministry of Education, Culture and Sports, Science and Technology (MEXT) of Japan (Grant Nos. 14256004, 18256004, and 18780230).

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