Isolation and molecular identification of *Leishmania* (*Viannia*) *peruviana* from naturally infected *Lutzomyia peruensis* (Diptera: Psychodidae) in the Peruvian Andes

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Leishmania (Viannia) peruviana *was isolated from 1/75* Lutzomyia peruensis *captured during May 2006 in an endemic cutaneous leishmaniasis region of the Peruvian Andes (Chaute, Huarochiri, Lima, Peru). Sand fly gut with promastigotes was inoculated into a hamster and the remaining body was fixed in ethanol.* L. (Viannia) *sp. was determined by polymerase chain reaction (PCR), and* Leishmania *species through molecular genotyping by PCR-restriction fragment length polymorphism analyses targeting the genes* cpb *and* hsp70, *resulting* L. (V.) peruviana. *The infected sand fly appeared 15 days after the rains finished, time expected and useful real time data for interventions when transmission is occurring.*

Key words: *Leishmania peruviana* - *Lutzomyia peruensis* - polymerase chain reaction-restriction fragment length polymorphism - Peru

An important step for the incrimmination of *Leishmania* vectors is the report of naturally infected sand fly species (Killick-Kendrick & Ward 1981). The proportion of sand flies infected with *Leishmania* in places frequented by humans is taken into account. Several methods have been applied for this requirement, being the sand fly gut dissection first used, although it is the less productive one because of the low *Leishmania* infection rate in sand flies (0.001-2.6) (Perez et al. 1994), the requirement of living sand flies and well trained personnel for sand fly gut dissection. To date this technique is still being used with the purpose of isolating the parasite from an individual sand fly. Sand flies can also be cryopreserved in liquid nitrogen to be dissected months later. Young et al. (1987) dissected 18,463 sand flies, obtaining 11 (0.059%) with *Leishmania* promastigotes in Colombia. In another technique, the whole sand fly body is homogenized in pools of up to 24 individuals and then inoculated into hamsters, where *Leishmania* infection produces lesions in at least six weeks. Finally, the parasites are isolated by aspiration of the lesions and transferred to culture medium. Using this technique the number of infected sand fly individuals is unknown and it is possible to have a mixture of *Leishmania* strains in a single pool. Sentinel hamsters have also been used to obtain *Leishmania* strains from sand flies (Herrer 1982a). The animals are exposed to sand fly bites in endemic areas and then aspirates of different parts of the hamster body are taken and inoculated into culture medium to isolate the parasites. Here indeed, the infected sand fly species are unknown. Detection of *Leishmania* DNA is now a commonly used technique. Sand flies can be preserved dried or frozen and large number of sand flies can be processed individually or in pools. By means of polymerase chain reaction (PCR) it is possible to amplify and detect DNA of less than one *Leishmania* parasite (Lopez et al. 1993). *Leishmania* species identification is now done by using specific primers designed for different *Leishmania* genes currently used as targets for molecular genotyping (Garcia et al. 2004, 2005).

The search for *Leishmania* vectors in Peru started in the Andes, where the diversity of sand fly species is low (1-6 species in a single valley). Cruzado (1987) reported four specimens of *Lutzomyia peruensis* infected with *Leishmania* in La Libertad. Herrer (1982b) obtained two (2.06%) isolates of *Leishmania* sp. from 97 specimens of *Lu. peruensis* homogenized and inoculated into hamsters, in the Rimac Valley (Lima). Perez et al. (1991) reported two isolations of *Leishmania* from sand flies, the first (strain IPRN/PE/87/Lp52) by inoculation of pools of homogenized *Lu. peruensis* (0.34%) and the second in a sentinel hamster, in the Huayllacallan Valley (Ancash). The strain Lp52 has been recently identified as *L. (Viannia) guyanensis* by means of PCR-restriction fragment length polymorphism (RFLP) analyses of kDNA minicircles (kDNAPCR-RFLP) (Callapiña 2001). Perez et al. (1994) worked with pools of 4-10 sand fly individuals for PCR detection of DNA of the *L. (Viannia)* subgenus, and found two pools of *Lu. peruensis* (0.2%) and six of *Lu. verrucarum* (0.13%) positive for *L. (Viannia)* sp. Caceres et al. (2002) in Ayacucho dissected 1849 *Lu. ayacuchensis*, obtaining five (0.27%) positives for *Leishmania* sp. by PCR, and four of which were cultured and determined as *L. peruviana* by multilocus enzyme electrophoresis and molecular karyotyping.

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The main goal of the present study was to find infected sand flies when they are most numerous, which is after the rainy period in the Andes, from the end of April to May (Perez et al. 1994), and also to carry out the *Leishmania* species determination through PCR amplification, followed by PCR-RFLP.

Sand flies for this study were captured in Chaute (Huarochirí, Lima), SL11º56.455' and WL76º30.392', at 2400 m of altitud, in May 2006, using a Shannon trap with protected human bait from 18:00 to 19:00 (Perez et al. 1988). All sand flies captured were placed in collecting flasks and provided with sugar solution (30%) in a piece of cotton, and transported to our laboratories in Lima where living sand flies are maintained in a sand fly colony room at constant temperature of 20ºC.

San dfly guts were dissected in saline solution with antibiotics and observed under compound microscope at 400x magnification. A total of 317 female sand flies were dissected in May 2006 (75 of *Lu. peruensis* and 242 of *Lu. verrucarum*).

A typical peripylarian infection was detected in a 1/75 *Lu. peruensis* captured by JE Perez on May 6, 2006. The stomodeal valve and midgut were full of promastigotes and traces of digested blood, the peritrophic membrane was not observed, the pylorus showed pear shaped promastigotes attached to its interior walls and no free promastigotes were observed in the hind gut. The infection was apparently old (6 to 9 days), the infected sand fly was attracted to the human bait, probably to take a bloodmeal. The gut was placed in another drop of saline solution with antibiotics, broken to release the parasites and then inoculated onto 5% blood agar base biphasic culture medium, and also inoculated onto the hind feet of a golden hamster (*Mesocricetus auratus*). The rest of the sand fly body (head, thorax, and abdomen) was fixed in absolute ethanol and processed for *Leishmania* DNA extraction, PCR using the primers MP1-L and MP3-H, which are specific for *L. (Viannia)* spp. (Arevalo et al. 1993), and molecular genotyping by PCR-RFLP using two different genes as targets, which encode major *Leishmania* antigens cysteine proteinase B (*cpb*), and heat shock protein 70 (*hsp70*) (Garcia et al. 2004, 2005).

Although the cultures were heavily contaminated, they were processed for amplification of *Leishmania* DNA. After seven weeks, the hamster did not show lesion, just a weak swelling and reddish area at the inoculation sites. The abnormal sites were aspirated and this material inoculated into culture medium, and maintained at 23ºC. *Leishmania* promastigotes were observed on the 3rd day of culture and the strain was coded IPRN/PE/2006/Chaute1.

Leishmania DNA amplification was successful from all materials which include fragments of sand fly body, contaminated culture and the positive culture from hamster lesions. The PCR employed, using the primers MP1-L and MP3-H, showed that the DNA isolated from the strain IPRN/PE/2006/Chaute1 belonged to any of the species belonging to the subgenus *L. (Viannia)*, where the characteristic amplification products of 72 base pair was shown by all materials (Arevalo et al. 1993). This fact was confirmed through a PCR technique using *cpb* gene (Fig. 1), the species of the subgenus *L. (Viannia)* including the strain IPRN/PE/2006/Chaute1 shown a similar pattern, a band of 1170 bp. The PCR-RFLP technique applied afterwards showed, using two different markers (*cpb* and *hsp70*), that the parasite was in fact *L.* (*V.*) *peruviana* (Figs 2, 3). Using the *cpb* gene alone, which shows a characteristic pattern for *L. (V.) braziliensis* after the restriction enzyme analysis with Taq1, we obtained a similar pattern to the reference strains *L. (V.) peruviana* (MHOM/PE/90/HB22) and *L. (V.) guyanensis* (IPRN/PE/87/Lp52) (Garcia et al. 2005). Using the *hsp70* gene as marker, the result is a specific pattern for *L. (V.) guyanensis*, the pattern obtained after the restriction enzyme analysis with BsuRI showed similarity with the reference strains *L. (V.) peruviana* (MHOM/PE/90/HB22) and *L. (V.) braziliensis* (MHOM/PE/93/LC2177); the identity of these strains was confirmed by Garcia et al. (2004, 2005).

The combination of the presence of 343 bp for the *cpb* gene band of *L. (V.) braziliensis*, and the absence of a 224 bp for the *hsp70* gene band of *L. (V.) guyanensis*, distinguishes *L. (V.) peruviana* from these two species (cf. Garcia et al. 2005).

The isolation of the strain Chaute1 was carried out within the peak season of sand fly abundance, *Leishmania* infection both of sand flies and humans in Chaute. This situation was previously described by Perez et al. (1994), and it occurs seasonally just after the rains finish in the Andes (late April). The obtention of infected

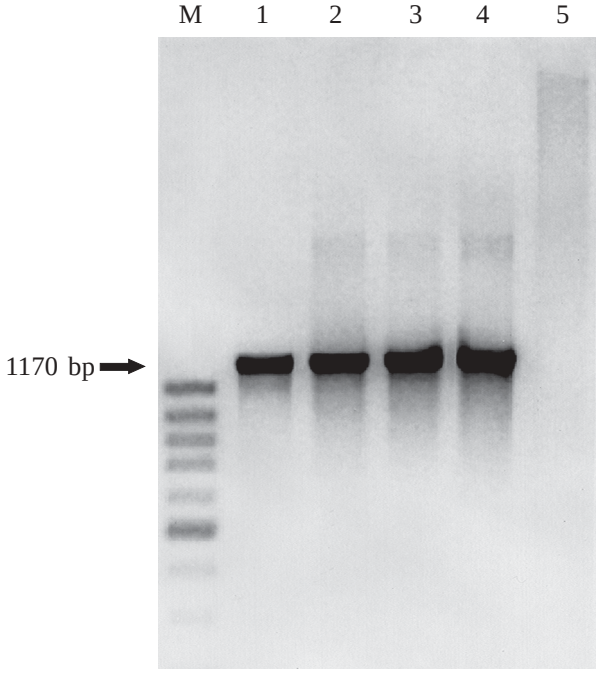

Fig. 1: agarose electrophoresis gel of *cpb* polymerase chain reaction products. M: 100 bp ladder; 1: IPRN/PE/2006/Chaute1; 2: *Leishmania (Viannia) braziliensis* (MHOM/PE/93/LC2177); 3: *L. (V.) peruviana* (MHOM/PE/90/HB22); 4: *L. (V.) guyanensis* (IPRN/PE/87/Lp52); 5: negative control.

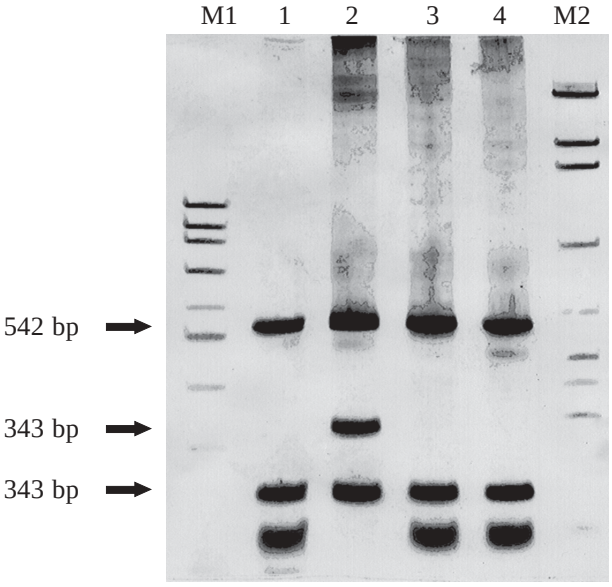

Fig. 2: *cpb* polymerase chain reaction-restriction fragment length polymorphism analyses patterns (Taq1) after polyacrylamide gel electrophoresis. M1: 100 bp ladder; 1: IPRN/PE/2006/Chaute1; 2: *Leishmania (Viannia) braziliensis* (MHOM/PE/93/LC2177); 3: *L. (V.) peruviana* (MHOM/PE/90/HB22); 4: *L. (V.) guyanensis* (IPRN/PE/87/Lp52); M2: pGEM ladder.

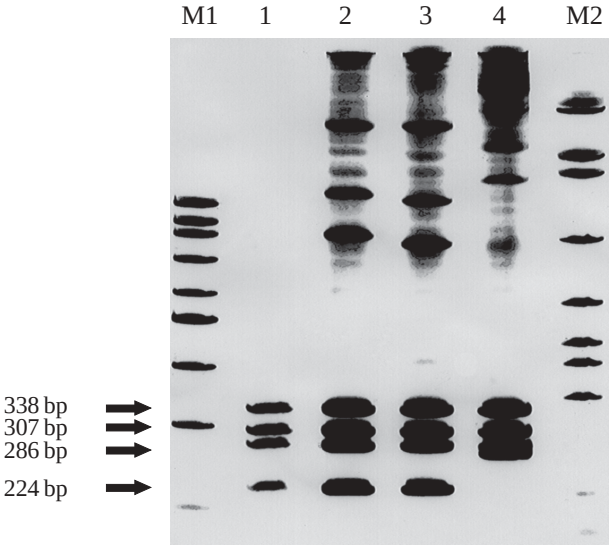

Fig. 3: *hsp70* polymerase chain reaction-restriction fragment length polymorphism analyses patterns (BsuRI) after polyacrylamide gel electrophoresis. M1: 100 bp ladder; 1: IPRN/PE/2006/Chaute1; 2: *Leishmania (Viannia) braziliensis* (MHOM/PE/93/LC2177); 3: *L. (V.) peruviana* (MHOM/PE/90/HB22); 4: *L. (V.) guyanensis* (Lp52); M2: pGEM ladder.

sand flies is more successful on this time of the year in the Andean *Leishmania* endemic areas. Sand flies are also a nuisance insect, an average of 472 sand fly bitings can be received by night in Chaute in the sand fly peak season.

It is understood that the chance to find naturally infected sand flies with *Leishmania* is normally very low, but is necessary to obtain living pathogens from sand flies for further studies. It is convenient to determine if the *Leishmania* strains proceeding from sand flies and animals are the same as those proceeding from humans. Studies like that should be performed with some periodicity as surveillance to detect changes in the genetical patterns (infectivity, virulence) of *Leishmania* species strains through time in a given endemic area. *Leishmania* infection rate of animals, which are the source of infection of sand flies, is variable, it is 0.2-2.8% in wild mice and 2.6-11.9% in dogs (Llanos-Cuentas et al. 1999). The isolation of the strain Chaute1 represents 1.33% of the *Lu. peruensis* captured in May 2006 in Chaute, the rate found is higher than the records in other Andean areas (Caceres et al. 2002).

L. verrucarum was not found infected with *Leishmania* in this study, which contrast with the findings of Perez et al. (1994), where six infected individuals were detected by means of PCR. This fact probably correspond to light infections probably missing in the observation under microscope and the fact that PCR technique is much more effective for the detection of early infections and those that may not succeed in the sand fly. This is the main reason why it is highly recommended.

The study of *Lu. peruensis* becomes relevant. This species coexist with *Lu. verrucarum* and both species are vectors of Andean cutaneous leishmaniasis which is caused mainly by *L. (V.) peruviana.* In the Department of Ancash (central Peruvian Andes), *L. (V.) guyanensis* also affects the human population in the same areas, being the second *Leishmania* species found in *Lu. peruensis* (Perez et al. 1994).

Information about infected sand flies can be available in few days combining sand fly gut dissection with PCR detection of *Leishmania* DNA and *Leishmania* species identification by PCR-RFLP. Sand fly dissectors should work routinarely closer to PCR procedures equipped with a set of specific primers. The knowledge of the *Leishmania* and its vector species in a real time can result in appropriate intervention when the transmission is taking place in a given area.

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