Culture-Independent Species Typing of Neotropical Leishmania for Clinical Validation of a PCR-Based Assay Targeting Heat Shock Protein 70 Genes

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Leishmaniasis is endemic in 88 countries, causing a burden estimated at 2.357,000 disability adjusted life years and 59,000 deaths (12). The disease is characterized by a considerable clinical and epidemiological pleomorphism, which is linked—tobesides host factors—with the important diversity of Leishmania species and their vectors. Clinical and epidemiological monitoring requires rapid and high throughput tools for species typing. This can be achieved with PCR assays combining a high detection level with the adequate discriminatory power. Currently, a few genetic targets are available: ribosomal DNA internal transcribed spacers (ITS) (5), gp63 genes (10), miniox genes (7), and kinetoplast DNA (3). However, there are still very few studies on their direct application to human tissues as well as their clinical and epidemiological validation in the New World, where several species can be sympatric (6, 11). We report here a complementary assay based on PCR amplification of the repeated heat shock protein 70 genes (1, 8), followed by restriction fragment length polymorphism analysis (hsp70 PCR-RFLP).

Development of hsp70 PCR assay. Reported sequences of Leishmania (Viannia) braziliensis and Leishmania (Leishmania) mexicana hsp70 genes (accession no. AF291716 and M87878) were aligned with the CLUSTAL W 1.8 program. The primers were designed from the conserved region between Leishmania species and their vectors.

PCR-restriction fragment length polymorphism analysis of heat shock protein 70 genes discriminates most neotropical Leishmania species, as well as Trypanosoma cruzi. The assay, combined with capillary electrophoresis in a microchip device, may be applied directly on clinical samples with a high sensitivity, hence supporting clinical and epidemiological monitoring of leishmaniasis.

hsp70 PCR on laboratory samples. We analyzed 59 reference strains (Table 1) of species reported in Latin America: L. (V.) braziliensis, Leishmania (Viannia) peruviana, Leishmania (Viannia) lainsoni, Leishmania (Viannia) guyanensis, Leishmania (Viannia) panamensis, Leishmania (Leishmania) amazonensis, and Leishmania (Leishmania) infantum (synonym Leishmania chagasi). Promastigotes were cultivated and harvested (10) and were DNA purified with DNAzol (Gibco, Merelbeke, Belgium). After hsp70 PCR, a single 1,300-bp product was observed in all Leishmania strains, corresponding to the expected size. The same product was encountered in Trypanosoma cruzi, but no amplification was detected with DNA from humans Mycobacterium tuberculosis, or Sporothrix schenckii.

hsp70 PCR-RFLP on laboratory samples. hsp70 PCR products were ethanol precipitated and resuspended in 20 μl of water. Digestion with restriction enzymes was performed according to the suppliers’ recommendations in a final volume of 10 μl. Electrophoretic resolution was first performed in 3% agarose, by using 9 μl of digestion products, and then in microchips (2100 Bioanalyzer capillary electrophoresis system; Agilent Technologies, Karlsruhe, Germany) (LabChip 1500 or 7500; Caliper Technologies, Mountain View, Calif.) with only 1 μl of digests because of their high sensitivity and discriminatory power. From the five restriction enzymes tested, AsuI, TaqI, AluI, and AvaI distinguished L. (L.) amazonensis from all Leishmania samples of subgenus Viannia, while HaeIII (BsuRI) distinguished all species but L. (V.) peruviana (Fig. 1A), a species found only in the Peruvian highlands (11). However, hsp70 sequencing data revealed a BsiI restriction site differentiating that species from L. (V.) braziliensis. There was no intraspecies polymorphism as with assays targeting rDNA ITS or gp63 (5, 10). T. cruzi, in which a 1,300-bp hsp70 amplicon was also encountered, showed a different cleavage pattern (Fig. 1A). This might be particularly useful for identifying mixed infections of Leishmania spp. and T. cruzi, which can be quite frequent in Latin America (4). Analytical sensitivity of the PCR-RFLP assay was higher with capillary (3 parasites/μl before PCR) than with agarose electrophoresis (30 parasites/μl).

Clinical samples. Thirty-four biopsy samples (4 mm) from Bolivian patients with clinical suspicion of tegumentary leish-
| Species                  | International code | Origin   | Pathology |
|-------------------------|--------------------|----------|-----------|
| L. (V.) braziliensis    | MHOM/BO/00/CUM27   | Bolivia  | M         |
|                         | MHOM/BO/00/CUM29   | Bolivia  | M         |
|                         | MHOM/BO/94/CUM43   | Bolivia  | M         |
|                         | MHOM/BO/00/CUM45   | Bolivia  | M         |
|                         | MHOM/BO/94/CUM49   | Bolivia  | M         |
|                         | MHOM/BO/94/CUM52   | Bolivia  | M         |
|                         | MHOM/BO/00/CUM56   | Bolivia  | M         |
|                         | MHOM/BO/00/CUM152  | Bolivia  | M         |
|                         | MHOM/PE/93/LC2143  | Peru      | C         |
|                         | MHOM/PE/00/LC2177  | Peru      | C         |
|                         | MHOM/PE/00/LC2320  | Peru      | M         |
|                         | MHOM/PE/94/LC2368  | Peru      | M         |
|                         | MHOM/BO/94/CUM41   | Bolivia   | C         |
|                         | MHOM/BO/94/CUM153  | Bolivia   | C         |
|                         | MHOM/BO/94/CUM42   | Bolivia   | C         |
|                         | MHOM/BO/00/LC2123  | Peru      | C         |
|                         | MHOM/BO/00/CUM97   | Bolivia   | C         |
|                         | MHOM/PE/00/LC2355  | Peru      | C         |
|                         | MHOM/PE/00/LC2284  | Peru      | C         |
|                         | MHOM/PE/00/LC2367  | Peru      | C         |
| L. (V.) peruviana       | MHOM/PE/90/HB22    | Peru      | C         |
|                         | MHOM/PE/90/HB44    | Peru      | C         |
|                         | MHOM/PE/90/HB67    | Peru      | C         |
|                         | MHOM/PE/90/HB83    | Peru      | C         |
|                         | MHOM/PE/90/LCA09   | Peru      | C         |
|                         | MHOM/PE/90/LH249   | Peru      | C         |
|                         | MHOM/PE/90/LH827   | Peru      | C         |
|                         | MHOM/PE/90/LC1015  | Peru      | C         |
|                         | MHOM/PE/90/LCA04   | Peru      | C         |
| L. (V.) guyanensis      | MHOM/PE/91/LC1446  | Peru      | C         |
|                         | MHOM/PE/91/LC1447  | Peru      | C         |
|                         | MHOM/PE/91/LC1448  | Peru      | C         |
|                         | MHOM/PE/94/LC2309  | Peru      | C         |
|                         | MHOM/PE/00/LC2797  | Perú      | C         |
|                         | MHOM/BR/75/M5378   | Brazil    | C         |
|                         | MHOM/GF/85/LEM699  | French Guyana | C       |
|                         | IPRN/PE/00/Lp52    | Peru      | Lutzomyia peruensis |
|                         | MHOM/PE/00/LH941   | Peru      | C         |
|                         | MHOM/PE/00/LH705   | Peru      | C         |
| L. (V.) panamensis      | MHOM/PA/71/LS94    | Panama    | C         |
|                         | MCHO/PA/00/M4039   | Panama    | Choleopus |
| L. (V.) lainsoni        | MHOM/BO/95/CUM71   | Bolivia   | C         |
|                         | MHOM/BO/94/CUM78   | Bolivia   | C         |
|                         | MHOM/BO/94/CUM88   | Bolivia   | C         |
|                         | MHOM/BO/95/CUM129  | Bolivia   | C         |
|                         | MHOM/PE/92/LC1581  | Peru      | M         |
|                         | MHOM/PE/00/LH619   | Peru      | C         |
|                         | MHOM/PE/93/LC2029  | Peru      | C         |
|                         | MHOM/PE/00/LC1290  | Peru      | C         |
|                         | MHOM/PE/00/LH1154  | Peru      | C         |
|                         | MHOM/PE/00/LH762   | Peru      | C         |
| L. (L.) amazonensis    | MHOM/BO/00/CEN001  | Bolivia   | C         |
|                         | MHOM/BO/00/CEN018  | Bolivia   | C         |
|                         | MPRO/BR/77/LV78    | Brazil    | Proechimys |
|                         | IFLA/BR/67/PH8     | Brazil    | Lutzomyia flaviscutellata |
|                         | MHOM/BR/73/M2269   | Brazil    | C         |
| L. (L.) infantum       | MHOM/FR/1978/LEM75 | France    | V         |
| T. cruzi               | CANIII             | Brazil    | Chagas' disease |

* C, M, and V, cutaneous, mucocutaneous, and visceral leishmaniasis, respectively. Species identification was determined by isoenzyme electrophoresis (2).
maniasis (cutaneous and mucosal) were obtained with informed consent from the Isiboro secure area, between 1994 and 2000. Frozen biopsy specimens were lysed at 65°C for 3 h in 50 µL of TNE buffer (25 mM Tris, 100 mM NaCl, 5 mM EDTA [pH 8]) containing 5% sodium dodecyl sulfate and 200 µg of proteinase K/µL. After ethanol precipitation, DNA pellets were resuspended in 15 µL of buffer TE (10 mM Tris, 1 mM EDTA [pH 7.4]), and 2 µl was used for hsp70 PCR.

First, the capacity to detect parasites was considered. Sensitivity was compared with other methods, by using a laboratory case definition (positivity with microscopy, culture, or hsp70 PCR itself) and scored as follows (Table 2): 100% (hsp70 PCR), 92.9% (axenic culture), 80.9% (intradermal reaction of Montenegro [IDRM]), and 28.6% (microscopy). hsp70 PCR is thus slightly more sensitive than similar assays designed for species identification (85 to 89.7%; 6, 11). The relatively high sensitivity observed here for axenic culture can be explained by the fact that in CUMETROP, several aspirates are taken from the same patient to increase the isolation rate. The specificity of hsp70 PCR was 100%, but routinely, any positive sample should be digested to confirm a Leishmania pattern (versus T. cruzi, for instance). Concordance was highest between PCR and culture (kappa = 0.82).

Second, species identification was performed by cutting the hsp70 amplicons of the 28 PCR-positive samples with HaeIII; 26 and 28 patterns were detected in agarose gels and microchips, respectively. Species identification was L. (V.) braziliensis (27 samples, including the two that were only PCR positive) and L. (V.) lainsoni (1 sample) (Fig. 1B).

**Conclusion.** Our study brings new and original aspects to the field of Leishmania genetic characterization. Microchip capillary electrophoresis increases the performance of PCR-RFLP assays. hsp70 genes represent an adequate target for sensitive typing of neotropical Leishmania species in host tissues. They bring complementary information to other markers: (i) encoding for a major antigen (9), they allow probing of the genetic variability of molecules possibly involved in immunopathology, and (ii) presenting a lower rate of genetic variation than g63 genes or rDNA ITS, for instance, they may be applied at other taxonomical levels (combining species and genus typing). This new marker also paves the way to future multigenic PCR-based approaches, essential for direct population studies in the host. Further work should be undertaken to compare on the same clinical samples the sensitivity, specificity, and discrimination power of the different PCR-RFLP assays currently available and to confirm the performance of hsp70 PCR-RFLP in other trypanosomatids.

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