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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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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.

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—besides 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), miniexon 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 with PRIMER PREMIER software: *Hsp70*sen (5′ GACGGTGCCTGCCTACTTCAA 3′) and *Hsp70*ant (5′ CCGCCCATGCTCTGGTACATC 3′). *hsp70* PCR amplification was carried out in a 50-μl solution containing 1× *Taq* polymerase buffer (Eurogentec, Seraing, Belgium), 1.5 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate, 5% dimethyl sulfoxide, 20 pmol of primers, and 2.5 U of *Taq* DNA polymerase (Eurogentec). The reaction mixtures were amplified in a MJ Research PTC-100 cycler with a heated lid at 94°C for 5 min followed by 33 cycles, each consisting of 30 s at 94°C, 1 min at 61°C, and 3 min at 72°C, and a final extension step of 10 min at 72°C.

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***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* (*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-RLFP 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-

TABLE 1. Strains of *Leishmania* analyzed in this study[a]

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/CUM68	Bolivia	M
	MHOM/BO/00/CUM152	Bolivia	M
	MHOM/PE/93/LC2143	Peru	C
	MHOM/PE/93/LC2176	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	Peru	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	*Choloepus*
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/LC2190	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

[a] C, M, and V, cutaneous, mucocutaneous, and visceral leishmaniasis, respectively. Species identification was determined by isoenzyme electrophoresis (2).

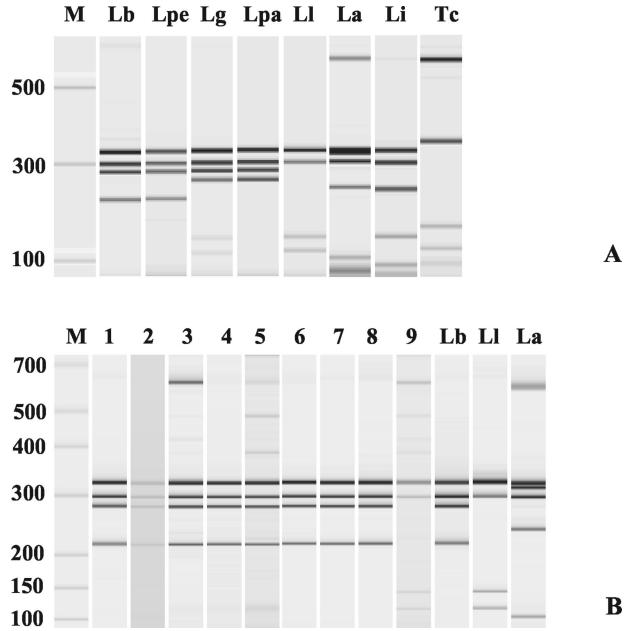

FIG. 1. *hsp70* PCR-RFLP patterns (HaeIII) after capillary electrophoresis (Bioanalyzer). M, size markers. (A) Reference strains of *L.* (*V.*) *braziliensis* (Lb), *L.* (*V.*) *peruviana* (Lpe), *L.* (*V.*) *guyanensis* (Lg), *L.* (*V.*) *panamensis* (Lpa), *L.* (*V.*) *lainsoni* (Ll), *L.* (*L.*) *amazonensis* (La), *L.* (*L.*) *infantum* (Li), and *T. cruzi* (Tc). (B) Biopsy specimens from Bolivian patients with leishmaniasis. Lanes 1 to 8, biopsies with *L.* (*V.*) *braziliensis*; lane 9, biopsy specimen with *L.* (*V.*) *lainsoni*. Reference strains of *L.* (*V.*) *braziliensis* (Lb), *L.* (*V.*) *lainsoni* (Ll), and *L.* (*L.*) *amazonensis* (La) are shown. M, size markers.

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).

TABLE 2. Diagnostic performance of 34 *hsp70* PCRs on samples from Bolivian patients with clinical suspicion of tegumentary leishmaniasis compared to microscopy, culture, and IDRM[a]

Clinical status	No. of results									
	Microscopy		Culture		*hsp70* PCR		Total[b]	IDRM		Total
	+	−	+	−	+	−		+	−	
Cases	8	20	26	2	28	0	28	17	4	21
Noncases	0	6	0	6	0	6	6	0	5	5
Total samples	8	26	26	8	28	6	34	17	9	26

[a] IDRM available for only 26 patients. Case definition based on positivity by microscopy, culture, or *hsp70* PCR.
[b] Total results from microscopy, culture, and *hsp70* PCR.

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 *gp63* 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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REFERENCES

1. **Arora, S. K., G. S. Kapoor, and S. Sehgal.** 1998. Heterogeneity in heat shock protein genes in *Leishmania* isolates. Immunol. Cell Biol. **76:**186–189.
2. **Bañuls, A. L.** 1998. Apport de la génétique évolutive à la taxonomie et à l'épidémiologie du genre *Leishmania.* Ph.D. thesis. University of Montpellier, Montpellier, France.
3. **Bastrenta, B., R. Buitrago, F. Vargas, F. Le Pont, M. Torrez, M. Flores, N. Mita, and S. F. Breniere.** 2002. First evidence of transmission of *Leishmania* (*Viannia*) *lainsoni* in a Sub Andean region of Bolivia. Acta Trop. **83:**249–253.
4. **Bastrenta, B., N. Mita, R. Buitrago, F. Vargas, M. Flores, M. Machane, N. Yacsik, M. Torrez, F. Le Pont, and S. F. Brenière.** 2003. Human mixed infections of *Leishmania* spp. and *Trypanosoma cruzi* in a Sub Andean Bolivian area: identification by polymerase chain reaction/hybridization and isoenzyme. Mem. Inst. Oswaldo Cruz **98:**255–264.
5. **Cupolillo, E., G. Grimaldi, Jr., H. Momen, and S. M. Beverley.** 1995. Intergenic region typing (IRT), a rapid molecular approach to the characterization and evolution of *Leishmania.* Mol. Biochem. Parasitol. **73:**145–155.
6. **Marfurt, J., A. Nasereddin, I. Niederwieser, C. L. Jaffe, H. P. Beck, and I. Felger.** 2003. Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. J. Clin. Microbiol. **41:** 3147–3153.
7. **Marfurt, J., I. Niederwieser, N. D. Makia, H. P. Beck, and I. Felger.** 2003. Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP. Diagn. Microbiol. Infect. Dis. **46:**115–124.
8. **Quijada, L., M. Soto, C. Alonso, and J. M. Requena.** 1997. Analysis of post-transcriptional regulation operating on transcription products of the tandemly linked *Leishmania infantum* hsp70 genes. J. Biol. Chem. **272:**4493–4499.
9. **Rico, A. I., S. O. Angel, C. Alonso, and J. M. Requena.** 1999. Immunostimu-

latory properties of the *Leishmania infantum* heat shock proteins HSP70 and HSP83. Mol. Immunol. **36:**1131–1139.
10. **Victoir, K., A. L. Banuls, J. Arevalo, A. Llanos-Cuentas, R. Hamers, S. Noël, S. De Doncker, D. Le Ray, M. Tibayrenc, and J. C. Dujardin.** 1998. The gp63 gene locus, a target for genetic characterization of *Leishmania* belonging to subgenus *Viannia.* Parasitology **117:**1–13.
11. **Victoir, K., S. De Doncker, L. Cabrera, E. Alvarez, J. Arevalo, A. Llanos-Cuentas, D. Le Ray, and J. C. Dujardin.** 2003. Detection and direct identification of *Leishmania* species in biopsies from patients with American tegumentary leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. **97:**80–87.
12. **World Health Organization.** 2002. The world health report 2002. Reducing risks, promoting healthy life. World Health Organization, Geneva, Switzerland. [Online.] http://www.who.int/whr/2002/en.