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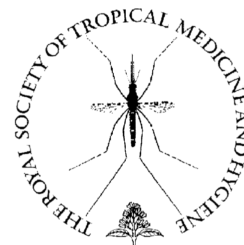

American tegumentary leishmaniasis: direct species identification of *Leishmania* in non-invasive clinical samples

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Summary Species identification is highly relevant for improved prognosis and adequate treatment of American tegumentary leishmaniasis (ATL). PCR-based methods are available for this purpose but should be simplified to improve accessibility. As a first step in this process, this paper describes a simplified protocol for collection of clinical samples. Using samples from 44 Bolivian patients with confirmed ATL, we demonstrated that *hsp70* PCR—RFLP on skin scrapings collected with a tooth pick allowed identification of the parasite species with a sensitivity of 95% and specificity of 100%. Our method should greatly facilitate individual patient management and epidemiological surveillance of ATL.
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1. Introduction

American tegumentary leishmaniasis (ATL) is a widely distributed infectious disease, caused by several species of *Leishmania*. The clinical features can vary from a single cutaneous ulcer to severe mucocutaneous involvement. Early diagnosis of ATL is important in order to avoid severe tissue damage, which in some cases can lead to extensive destruction of mucosal membranes known as mucosal leishmaniasis, or even death of the patient if the disease is not treated (Marsden, 1994). Mucosal leishmaniasis is essentially caused by *Leishmania (Viannia) braziliensis*. Since different *Leishmania* species can have different pathogenicity (Cupolillo et al., 2003) and also varying susceptibility to chemotherapeutic drugs (Croft et al., 2006), it is of great clinical and epidemiological importance that ATL diagnosis is not restricted to the detection of *Leishmania* but also comprises species identification.

In endemic areas, *Leishmania* diagnosis is usually based on clinical presentation and epidemiological data (Davies et al., 1997). However, in many cases, this does not allow adequate diagnosis of ATL due to the coexistence of ATL with other similar skin lesions such as sporotrichosis,

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bacterial ulcers, carcinomas and leprosy, which are common in endemic areas. In order to avoid misdiagnosis or underestimation of *Leishmania* incidence it is therefore crucial to demonstrate the presence of the *Leishmania* parasite (Escobar et al., 1992). This mainly relies on two methods; microscopical examination and parasite isolation (Escobar et al., 1992). However, both these techniques have several drawbacks including limited sensitivity (microscopy) and long delays (culture). Furthermore, neither of these techniques allows species identification. In this context, PCR represents an excellent alternative, and a series of assays based on RFLP analysis have already been developed for detection and species identification (Cupolillo et al., 1995; Garcia et al., 2004; Victoir et al., 1998). One of them, *hsp70* PCR—RFLP, was previously shown to be useful to detect and discriminate most neotropical *Leishmania* species directly with high sensitivity using skin biopsies (Garcia et al., 2004).

PCR—RFLP performance not only depends on the assay itself but also on the sampling and DNA extraction methods. Simple sampling methods providing a good quantity of good quality parasite DNA should be used (Lachaud et al., 2001). In addition, the traumatic impact of the sampling procedures should be minimized as much as possible. In order to simplify clinical sample collection in the field, we compared the sensitivity and reproducibility of *hsp70* PCR—RFLP on different skin specimens: dermal scrapings, syringe aspirates and biopsies in field conditions.

2. Materials and methods

2.1. Sample collection

During epidemiological studies in the Isiboro Secure area of Bolivia, we recruited 53 patients: 44 with suspected cutaneous leishmaniasis and nine with a suspected mucocutaneous form. From each patient we took a collection of different clinically well-documented skin specimens from the same ulcer in parallel: dermal scrapings, syringe aspirates and biopsies. All the clinical samples were obtained with informed consent of the patients and ethical clearance was given by the review board of the institution providing the samples, CUMETROP. Biopsies were taken with a 2 mm disposable punch from the border of the lesion and placed in a sterile Eppendorf tube. Syringe aspirates were collected from the indurated border of the lesion and used for cultivation in vitro. An aliquot of 200 μl from the same aspirate was stored in an Eppendorf tube for PCR analysis. Duplicate samples of dermal scrapings were collected from the bottom of the ulcers using a sterile wooden tooth pick; one sample was used to prepare a slide smear for microscopical examination and the other was stored in an Eppendorf tube containing 200 μl of PBS buffer for PCR analysis. All clinical samples were transported in standard coolers at 4 °C to the reference laboratory.

2.2. Standard diagnostic procedures

Tissue scrapings smeared on glass slides were fixed with methanol, stained with Giemsa and examined microscopically. Culture in vitro was performed by inoculation of syringe aspirates into tubes containing NNN culture medium (4% Bacto blood agar base, 10% rabbit blood). The tubes were incubated at 26 °C and microscopically evaluated after 6 days. The Montenegro skin test (MST) was performed as follows: 0.1 ml of Montenegro antigen (leishmanin) was injected intradermally into the right forearm of the patient and, 48 h later, the diameter of induration was read by the ballpoint pen method. A value ≥5 mm was considered positive.

2.3. DNA preparation and PCR analysis

Genomic DNA was extracted from skin biopsies using the phenol—chloroform technique as described elsewhere (Garcia et al., 2004). Two microlitres of these DNA extracts were used for PCR amplification. For dermal scrapings, the tooth pick was removed and the 200 μl of transport buffer was used for DNA extraction using a DNAzol BD isolation kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. The syringe aspirates were extracted similarly. The resulting DNA pellets were resuspended in 30 μl of 8 mM NaOH. The *hsp70* PCR was carried out as described elsewhere (Garcia et al., 2004). The amplification reaction products (5 μl) were resolved by electrophoresis in a 1.5% agarose gel and visualized under UV light after ethidium bromide staining. The *hsp70* PCR products were precipitated, digested with *Hae*III and analysed by capillary electrophoresis as described elsewhere (Garcia et al., 2004).

3. Results

3.1. Detection of *Leishmania*

Of the 53 suspected ATL patients, 44 were confirmed by at least one of the three conventional diagnostic methods used

Table 1 Comparison of *hsp70* PCR and conventional diagnostic methods in identifying parasites present in clinical samples from 53 Bolivian patients with suspected American tegumentary leishmaniasis (ATL)

	PCR[a]	Culture	Microscopy	MST
Non-confirmed ATL patients	0/9	0/9	0/9	0/9
Confirmed ATL patients (total)	42/44 (95%)	32/44 (73%)	13/40 (33%)	37/40 (93%)
Lesion age ≤6 months	21/22 (95%)	18/22 (82%)	8/21 (38%)	16/18 (89%)
Lesion age >6 months	21/22 (95%)	14/22 (64%)	5/19 (26%)	21/22 (95%)

Numbers in parentheses indicate the sensitivity.
[a] Reference method for confirmation was positivity by culture, microscopy and/or Montenegro skin test (MST).

here; the nine non-confirmed ATL cases were all patients with cutaneous lesions. Using positivity with at least one of these three methods as a reference, *hsp70* PCR (all sampling methods confounded) had an overall sensitivity of 95% and the other methods had overall sensitivities of 73% (axenic culture), 33% (microscopy) and 93% (MST) (Table 1). The sensitivity of *hsp70* PCR was not affected by the age of the lesion or its stage of development, while for both culture and microscopy, the sensitivity dropped as the age of the lesion increased (from 82% to 64% and from 38% to 26%, respectively). The sensitivity of *hsp70* PCR was slightly higher with scrapings (39/41, 95%) and aspirates (40/42, 95%) than with biopsies (39/42, 93%). When we used DNA from biopsies, we observed an inhibitory effect leading to inconclusive results. Upon repetition of the experiment using diluted DNA extracts (i.e. two-fold dilutions with six samples and three-fold dilutions with four samples), we obtained clear DNA amplification. This problem was probably due to a higher proportion of human DNA vs. parasite DNA in biopsies, which may inhibit the PCR reaction. The specificity of *hsp70* PCR was found to be 100% since no amplification product was detected in samples from the nine non-confirmed ATL cases. However, routinely, any positive sample should be digested to confirm a *Leishmania* pattern to differentiate it from *Trypanosoma cruzi* (Garcia et al., 2004).

3.2. *Leishmania* species identification

All the samples that gave positive results with *hsp70* PCR were subjected to RFLP analysis of the amplification product. *Leishmania (V.) braziliensis* was identified in 39 of 42 positive samples (93%) while *L. (V.) lainsoni* was identified in three samples (7%). This confirms that the most common species circulating in the area is *L. (V.) braziliensis* (Figure 1). Although in many cases the visualized bands tended to have a lower intensity, there were no significant differences or non-specific patterns in the PCR—RFLP products of the different skin samples. The *hsp70* PCR—RFLP typing of *Leishmania* parasites in skin samples yielded the same result as typing of cultured parasites obtained from the same ulcer.

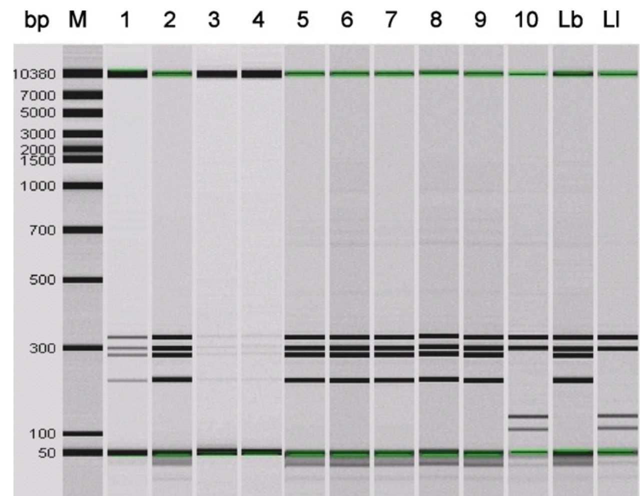

Figure 1 *hsp70* PCR—RFLP patterns (*Hae*III) after capillary electrophoresis (Bioanalyzer). M: marker ladder; lanes 1—9: skin scrapings from patients infected with *Leishmania (Viannia) braziliensis*; lane 10: skin scraping from patient infected with *L. (V). lainsoni*; Lb: *L. (V.) braziliensis* reference strain; Ll: *L. (V). lainsoni* reference strain. The bands in lanes 3 and 4 are very weak and this was probably due to the samples containing a very small amount of DNA.

4. Discussion

Species identification is becoming a major issue in diagnosis of tegumentary leishmaniasis for improved prognosis and adequate treatment. We have demonstrated that skin scraping with a tooth pick in combination with *hsp70* PCR—RFLP (Garcia et al., 2004) allows highly sensitive characterization of parasites present in clinical samples; this was also the case with old lesions, known to contain fewer parasites (Rodriguez et al., 1994). Collection of dermal scrapings with sterile sticks (Matsumoto et al., 1999) and exudate material with cotton swabs (Mimori et al., 2002) were previously suggested as alternatives to biopsy samples for detection of *Leishmania*, and we have confirmed here, using a single PCR assay (vs. different species-specific assays in the study of Matsumoto et al., 1999) their application for species identification. Using dermal scrapings is highly relevant in cases where a minimum of manipulation is required, for instance when lesions are situated on the face or when the patients are children. In addition, the simplified collection technique of dermal scraping allows sampling at ambulatory level by personnel with minimal training, and analysis at regional reference laboratories for definitive diagnosis. Dermal scraping may also facilitate the collection of a large number of samples in a short time in field conditions; this is difficult when biopsies are collected as the use of local anaesthesia and expensive disposable punches are required. When scraping is not possible, for instance with deep mucosal lesions, syringe aspiration might be suitable.

Our approach simplifies the molecular diagnosis of leishmaniasis, which is highly relevant particularly in endemic countries. It could also be useful in other endemic areas where different species of *Leishmania* may cause cutaneous disease, as well as in travel medicine clinics. Further efforts should be made to simplify the PCR itself (e.g. by loop-mediated isothermal amplification; Notomi et al., 2000) and the detection of PCR products (e.g. by oligochromatography, a method in which amplicons are detected by reverse hybridization on a dipstick; Mertens et al., 2004).

Conflicts of interest statement
The authors have no conflicts of interest concerning the work reported in this paper.

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References

Croft, S.L., Sundar, S., Fairlamb, A.H., 2006. Drug resistance in leishmaniasis. Clin. Microbiol. Rev. 19, 111—126.

Cupolillo, E., Grimaldi Jr, G., Momen, H., Beverley, S.M., 1995. Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of *Leishmania*. Mol. Biochem. Parasitol. 73, 145—155.

Cupolillo, E., Brahim, L.R., Toaldo, C.B., de Oliveira-Neto, M.P., de Brito, M.E., Falqueto, A., de Farias Naiff, M., Grimaldi Jr, G., 2003. Genetic polymorphism and molecular epidemiology of *Leishmania (Viannia) braziliensis* from different hosts and geographic areas in Brazil. J. Clin. Microbiol. 41, 3126—3132.

Davies, C.R., Llanos-Cuentas, E.A., Sharp, S.J., Canales, J., Leon, E., Alvarez, E., Roncal, N., Dye, C., 1997. Cutaneous leishmaniasis in the Peruvian Andes: factors associated with variability in clinical symptoms, response to treatment, and parasite isolation rate. Clin. Infect. Dis. 25, 302—310.

Escobar, M.A., Martinez, F., Scott Smith, D., Palma, G.I., 1992. American cutaneous and mucocutaneous leishmaniasis (tegumentary): a diagnostic challenge. Trop. Doct. 22 (Suppl. 1), 69—78.

Garcia, L., Kindt, A., Bermudez, H., Llanos-Cuentas, A., De Doncker, S., Arevalo, J., Wilber Quispe Tintaya, K., Dujardin, J.C., 2004. Culture-independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. J. Clin. Microbiol. 42, 2294—2297.

Lachaud, L., Chabbert, E., Dubessay, P., Reynes, J., Lamothe, J., Bastien, P., 2001. Comparison of various sample preparation methods for PCR diagnosis of visceral leishmaniasis using peripheral blood. J. Clin. Microbiol. 39, 613—617.

Marsden, P.D., 1994. Mucosal leishmaniasis due to *Leishmania (Viannia) braziliensis L(V)b* in Tres Bracos, Bahia-Brazil. Rev. Soc. Bras. Med. Trop. 27, 93—101.

Matsumoto, T., Hashiguchi, Y., Gomez, E.A., Calvopina, M.H., Nonaka, S., Saya, H., Mimori, T., 1999. Comparison of PCR results using scrape/exudate, syringe-sucked fluid and biopsy samples for diagnosis of cutaneous leishmaniasis in Ecuador. Trans. R. Soc. Trop. Med. Hyg. 93, 606—607.

Mertens, P., Vijgen, L., Olungu, C., Degallaix, S., Renuart, I., Van Ranst, M., Leclipteux, T., 2004. Detection of SARS-CoV by oligochromatography of RT-PCR amplicons. Clin. Microbiol. Infect. 10 (Suppl. 3), 526—527.

Mimori, T., Matsumoto, T., Calvopina, M.H., Gomez, E.A., Saya, H., Katakura, K., Nonaka, S., Shamsuzzaman, S.M., Hashiguchi, Y., 2002. Usefulness of sampling with cotton swab for PCR-diagnosis of cutaneous leishmaniasis in the New World. Acta Trop. 81, 197—202.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Rodriguez, N., Guzman, B., Rodas, A., Takiff, H., Bloom, B.R., Convit, J., 1994. Diagnosis of cutaneous leishmaniasis and species discrimination of parasites by PCR and hybridization. J. Clin. Microbiol. 32, 2246—2252.

Victoir, K., Bañuls, A.L., Arevalo, J., Llanos-Cuentas, A., Hamers, R., Noel, S., De Doncker, S., Le Ray, D., Tibayrenc, M., Dujardin, J.C., 1998. The gp63 gene locus, a target for genetic characterization of *Leishmania* belonging to subgenus *Viannia*. Parasitology 117, 1—13.