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Accurate and rapid species typing from cutaneous and mucocutaneous leishmaniasis lesions of the New World☆,☆☆,★

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ABSTRACT

The heat-shock protein 70 gene (*hsp*70) has been exploited for *Leishmania* species identification in the Old and New World, using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis. Three new *Leishmania*-specific *hsp*70 PCRs were recently described, and we applied 2 of these on 89 clinical samples from a total of 73 Peruvian patients with either cutaneous or mucocutaneous leishmaniasis. The new PCRs on average showed a 2- to 3-fold improved sensitivity in the tested sample types (lesion biopsies, aspirates, and scrapings), for both genus detection and species typing, and were most successful in biopsies. *Leishmania braziliensis*, *L. peruviana,* and *L. guyanensis* were encountered. About one third of the *L. braziliensis* parasites contained 2 *hsp*70 alleles. This study is a paradigm for the implementation of a globally applicable upgraded tool for the identification of *Leishmania* directly on human specimens from cutaneous and mucocutaneous lesions in the New World.

1. Introduction

Leishmaniasis is the collective term for several disease manifestations caused by the trypanosomatid genus *Leishmania* and is endemic in 88 countries on 4 continents. It has been categorized as one of the "most neglected diseases" (Hotez et al., 2004). In the Americas, it occurs in 3 clinical variations: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and diffuse CL (DCL) (Marzochi, 1992). American tegumentary leishmaniasis (ATL), as it is collectively called, is a zoonosis encountered from the south of the United States to the north of Argentina (Marzochi, 1992). As the severity of the disease varies according to the infecting *Leishmania* species, the presence of multiple *Leishmania* species with overlapping clinical features and geographical distribution requires not only the specific detection of *Leishmania* parasites, but also accurate species identification for evaluating prognosis and choosing appropriate therapy (Arévalo et al., 2007). The traditional diagnosis of ATL is performed using clinical and epidemiologic features, immunologic methods, and parasitologic tests such as direct examination of smears after Giemsa staining, culture, and histopathology (Grimaldi and Tesh, 1993), but none of these identifies the infecting parasite species.

Molecular methods are increasingly employed for diagnosis, clinical, and epidemiologic studies on leishmaniasis in an effort to detect infection and categorize *Leishmania* at the genus, species, or strain level (Schönian et al., 2008). Ideal assays should be easy to perform and interpret, rapid, sensitive, specific, and able to detect and identify parasites accurately in reservoirs and vectors to allow studying transmission dynamics (García et al., 2007b; Talmi-Frank et al., 2010). Many different polymerase chain reaction (PCR)–based methods have been described for the detection of *Leishmania* spp. in different skin lesion specimens (Boggild et al., 2010; Deborggraeve et al., 2008; López et al., 1993; Marfurt et al., 2003; Rotureau et al., 2006; Schönian et al., 2001; Simon et al., 2010; Victoir et al.,

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2003).With multilocus enzyme electrophoresis (MLEE) (Rioux et al., 1990) being increasingly replaced by genetic characterization for species identification (Bañuls et al., 2007; Rotureau et al., 2006), several techniques and targets have been developed (Nasereddin et al., 2008; Reithinger and Dujardin, 2007; Schönian et al., 2001). Unfortunately, many assays are homemade and therefore difficult to compare. For that reason, standardized tests that are validated and applicable globally on biological, clinical, and environmental samples are of crucial importance.

The cytoplasmic heat-shock protein 70 gene (*hsp*70) has been exploited for *Leishmania* species identification in the Old and New World using PCR, followed by sequencing or restriction fragment length polymorphism (RFLP) analysis (García et al., 2004, 2007a,b; Alves da Silva et al., 2010; Fraga et al., 2010; Montalvo et al., 2010a,b; 2012; Veland et al., 2012). Recently, 3 improved PCRs were developed for this target, with increased sensitivity and specificity, which facilitates their use for both *Leishmania* detection and identification (Montalvo et al., 2012). The aim of this work was to evaluate the performance of the original PCR of García et al. (2004) and the 2 improved *hsp*70 PCRs most useful in the New World on ATL samples from Peru, where ATL is a major health problem with more than 10,000 cases per year (PAHO, 2010). In Peru, the disease is caused predominantly by 3 *Leishmania* (*Viannia*) species: *L. (V.) peruviana, L. (V.) guyanensis*, and *L. (V.) braziliensis*. Other species occasionally found are *L. (Leishmania) amazonensis* and *L. (V.) lainsoni* (Arévalo et al., 2007; Lucas et al., 1998). CL is endemic in 70% of the country, resulting in high morbidity, lifelong scarring, and cumbersome treatment in many resource-deprived communities (Llanos-Cuentas et al., 2008).

2. Materials and methods

2.1. Reference strains and clinical isolates

Table 1 lists all 5 *Leishmania* reference strains and 20 clinical isolates used in this study. The reference strains were previously classified according to MLEE, and DNA was isolated from cultures at the Institute of Tropical Medicine Antwerp. DNA from 20 clinical isolates was obtained from cultured parasites from Peruvian patients consulting the Institute of Tropical Medicine Alexander von Humboldt (IMTAvH, Lima, Peru), following international ethical guidelines after review and approval by the Institutional Review Board of Peruvian University Cayetano Heredia (UPCH, Lima, Peru). Parasites were grown and typed according to the algorithm of Veland et al. (2012), which is based upon 3 genes: mannose phosphate isomerase, cysteine proteinase B, and heat-shock protein 70. This algorithm classified the clinical isolates as either *L. braziliensis*, *L. peruviana*, or *L. guyanensis* (Table 1). Some of the *L. braziliensis* isolates were found to contain an additional cysteine proteinase b (*cpb*) allele, identical to the *cpb* allele of *L. peruviana*.

2.2. Patient samples

In total, 89 samples were collected from 73 patients with CL (n = 81) or MCL (n = 8) between 2006 and 2009 at IMTAvH (Table 2). Ethical approval for the study was obtained from UPCH, following international guidelines. A written informed consent was obtained either from the patients or from their closest relative. Different sample types were collected: 40 skin biopsies, 12 aspirates, and 37 lesion scrapings. After thorough cleaning of the lesions, biopsy specimens were obtained from the active edge of the lesion with a sterile 4-mm-diameter biopsy puncher, aspirates from the indurate border of the lesion using a capillary tube, and lesion scrapings with a simple sterile lancet. Samples were stored in absolute ethanol at −20 °C until DNA was extracted with either the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) or the High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN, USA). The DNA was eluted in 20, 50, or 100 μL of pure water for aspirates, scrapings, and biopsies, respectively, and stored at −20 °C. All samples showed a positive amplification with a *Leishmania*-specific kDNA (López et al., 1993) or 18S-rDNA (Deborggraeve et al., 2008) PCR, 2 molecular diagnostic methods with high sensitivity (see below). From 53 samples, an intense *Leishmania*-specific amplicon was obtained (kDNA or 18S-rDNA), while the remaining 36 samples were weakly positive in kDNA PCR.

Table 1
Reference strains (5) and clinical isolates (20) used in the study.

Strain identification[a]	Clinical profile[b]	Country of origin	Reference technique[c]	*hsp*70 typing[d]
L. braziliensis				
MHOM/BR/75/M2904[e]	CL	Brazil	MLEE	*L. braziliensis*
CU00176	CL	Peru	Veland	*L. braziliensis*
CU00185[f]	CL	Peru	Veland	*L. braziliensis*
LH3662	MCL	Peru	Veland	*L. braziliensis*
LH3597	CL	Peru	Veland	*L. braziliensis*[g]
LH3844	CL	Peru	Veland	*L. braziliensis*
CU00183[f]	CL	Peru	Veland[g]	*L. braziliensis*
CU00181	CL	Peru	Veland[g]	*L. braziliensis*
CU00135	CL	Peru	Veland[g]	*L. braziliensis*
CU00186	CL	Peru	Veland[g]	*L. braziliensis*
CU00188	CL	Peru	Veland[g]	*L. braziliensis*[g]
L. peruviana				
MHOM/PE/92/HB22[e]	CL	Peru	MLEE	*L. peruviana*
LH3823[f]	CL	Peru	Veland	*L. peruviana*
LH3824	CL	Peru	Veland	*L. peruviana*
LH3855	CL	Peru	Veland	*L. peruviana*
LH3811	CL	Peru	Veland	*L. peruviana*
LH3807	CL	Peru	Veland	*L. peruviana*
L. guyanensis				
MHOM/BR/78/M5378[e]	CL	Brazil	MLEE	*L. guyanensis*
LH3850	CL	Peru	Veland	*L. guyanensis*
LH3842[f]	CL	Peru	Veland	*L. guyanensis*
LH3635	CL	Peru	Veland	*L. guyanensis*
LH3640	CL	Peru	Veland	*L. guyanensis*
LH3670	CL	Peru	Veland	*L. guyanensis*
L. lainsoni				
MHOM/PE/91/LC1581[e]	MCL	Peru	MLEE	*L. lainsoni*
L. amazonensis				
MHOM/BR/73/M2269[e]	CL	Brazil	MLEE	*L. amazonensis*

[a] Species grouped according to the reference technique.
[b] CL = Cutaneous leishmaniasis; MCL = mucocutaneous leishmaniasis.
[c] MLEE = Multilocus enzyme electrophoresis (Rioux et al., 1990); Veland = PCR and restriction fragment length polymorphism analysis of genes encoding mannose phosphate isomerase, cysteine proteinase b (*cpb*), and heat-shock protein 70, applied as in Veland et al. (2012).
[d] Result from typing scheme depicted in Fig. 1 (Montalvo et al., 2012), for each of the 3 RFLPs. No discrepant results were observed between RFLP-G, RFLP-F, and RFLP-N.
[e] Reference strains, identified by World Health Organization code.
[f] Isolates used for testing analytical sensitivity (Fig. 2).
[g] These assays showed 2 *cpb* alleles (Veland et al., 2012) and/or 2 *hsp*70 alleles (this article, B2 in Fig. 1), respectively.

2.3. PCR Amplification

kDNA was amplified in a 25-μL reaction containing 5 μL of DNA, 0.5 U of HotStarTaq Plus DNA Polymerase (Qiagen), 1× PCR buffer including 1.5 mmol/L $MgCl_2$, 1× Q-buffer, 200 μmol/L of each deoxynucleoside triphosphate, and 0.4 μmol/L of each primer. A pair of primers, MP1L and MP3H, specifically amplifies a 70-bp region of *Leishmania* (*Viannia*) kDNA minicircles as the diagnostic target (López et al., 1993), while a second pair of primers, HBBL and HBBR, amplifies a 197-bp region of the human beta-globin gene as an internal PCR control (Boggild et al., 2010).

Table 2
Samples analyzed and results.

Patient no.	Sample type	Disease[a]	PCR Diagnosis[b]	Species[c]	PCR-G[d]	RFLP-G[e]	PCR-F[d]	RFLP-F[e]	PCR-N[d]	RFLP-N[e]
1	Aspirate	CL	K -S	*L. braziliensis*	P	U	P	B2	P	B2
2	Aspirate	CL	K -S	*L. braziliensis*	N	U	P	B1	P	B1
3	Aspirate	CL	K -S	*L. guyanensis*	P	G	P	G	P	G
4	Aspirate	CL	K -S	*L. peruviana*	P	P	P	P	P	P
5	Aspirate	CL	K -S	*L. braziliensis*	P	U	P	B1	P	B1
6	Biopsy	MCL	K -S	*L. braziliensis*	N	U	P	U	P	B2
7	Biopsy	MCL	K -S	*L. braziliensis*	N	U	P	B1	P	B1
8	Biopsy	CL	K -S	*L. peruviana*	N	U	P	U	P	P
9	Biopsy	CL	K -S	*L. guyanensis*	N	U	P	G	P	G
10	Biopsy	MCL	K -S	*L. braziliensis*	N	U	P	B2	P	B2
11	Biopsy	CL	K -S	*L. braziliensis*	P	U	P	B2	P	B2
12	Biopsy	CL	K -S	*L. guyanensis*	N	U	P	G	P	G
13	Biopsy	MCL	K -S	*L. braziliensis*	P	U	N	U	P	B2
13	Aspirate	MCL	K -S	*L. braziliensis*	N	U	P	B2	P	B2
14	Biopsy	CL	R-S	Undetermined	N	U	N	U	P	U
15	Biopsy	CL	R-S	*L. braziliensis*	N	U	N	U	P	B1
16	Biopsy	CL	R-S	*L. braziliensis*	N	U	N	U	P	B1
17	Biopsy	CL	R-S	*L. peruviana*	N	U	P	P	P	P
18	Biopsy	CL	R-S	*L. peruviana*	P	U	P	U	P	P
19	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	U	P	B1
20	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	U	P	B1
21	Biopsy	CL	R-S	*L. peruviana*	P	U	P	P	P	P
22	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	U	P	B1
23	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	B1	P	B1
24	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	B2	P	B2
25	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	B2	P	B2
26	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	B2	P	B2
27	Biopsy	CL	R-S	*L. peruviana*	N	U	P	P	P	P
28	Biopsy	CL	R-S	*L. peruviana*	N	U	P	P	P	P
29	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	B1	P	B1
30	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	B1	P	B1
31	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	B1	P	B1
32	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	B2	P	B2
33	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	U	P	B1
34	Biopsy	CL	R-S	*L. braziliensis*	N	U	P	U	P	B1
35	Biopsy	CL	K -S	*L. braziliensis*	P	B2	P	B2	P	B2
35	Scraping	CL	K -S	*L. braziliensis*	P	B2	P	B2	P	B2
36	Biopsy	CL	K -S	*L. braziliensis*	P	B1	P	B1	P	B1
36	Scraping	CL	K -S	*L. braziliensis*	P	B1	P	B1	P	B1
37	Biopsy	CL	K -S	*L. braziliensis*	P	B1	P	B1	P	B1
38	Biopsy	CL	K -S	*L. braziliensis*	P	B1	P	B1	P	B1
39	Scraping	CL	K -S	*L. braziliensis*	N	U	P	B1	P	B1
39	Biopsy	CL	K -S	*L. braziliensis*	P	B1	P	B1	P	B1
40	Biopsy	MCL	K -S	*L. braziliensis*	P	B1	P	B1	P	B1
40	Scraping	MCL	K -S	*L. braziliensis*	P	B1	P	B1	P	B1
41	Scraping	MCL	K -S	*L. braziliensis*	P	B1	P	B1	P	B1
42	Scraping	CL	K -S	*L. peruviana*	P	P	P	P	P	P
43	Scraping	CL	K -S	*L. peruviana*	P	P	P	P	P	P
44	Scraping	CL	K -S	*L. braziliensis*	P	B2	P	B2	P	B2
44	Aspirate	CL	K -S	*L. braziliensis*	N	U	P	B2	P	B2
45	Scraping	CL	K -S	*L. peruviana*	P	P	P	P	P	P
46	Scraping	CL	K -S	*L. braziliensis*	P	B1	P	B1	P	B1
47	Scraping	CL	K -S	*L. peruviana*	P	P	P	P	P	P
48	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
49	Scraping	CL	K -W	Undetermined	N	U	N	U	P	U
50	Scraping	CL	K -W	*L. guyanensis*	P	U	P	U	P	G
51	Aspirate	CL	K -W	Undetermined	N	U	N	U	P	U
52	Scraping	CL	K -W	Undetermined	N	U	N	U	P	U
53	Scraping	CL	K -W	Undetermined	N	U	N	U	P	U
54	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
55	Scraping	CL	K -W	*L. guyanensis*	P	U	P	U	P	G
56	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
57	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
58	Scraping	CL	K -W	*L. guyanensis*	P	G	P	G	P	G
59	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
60	Scraping	CL	K -W	*L. braziliensis*	P	B2	P	B2	P	B2
61	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
62	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
63	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
64	Scraping	CL	K -W	*L. braziliensis*	P	B1	P	B1	P	B1
65	Scraping	CL	K -W	*L. guyanensis*	N	U	P	G	P	G
66	Scraping	CL	K -W	*L. guyanensis*	P	G	P	G	P	G
67	Biopsy	CL	K -W	Undetermined	N	U	N	U	P	U
67	Scraping	CL	K -W	Undetermined	N	U	N	U	P	U
68	Biopsy	CL	K -W	Undetermined	N	U	P	U	P	U

Table 2 (*continued*)

Patient no.	Sample type	Disease[a]	PCR Diagnosis[b]	Species[c]	PCR-G[d]	RFLP-G[e]	PCR-F[d]	RFLP-F[e]	PCR-N[d]	RFLP-N[e]
68	Scraping	CL	K -W	Undetermined	N	U	N	U	P	U
68	Scraping	CL	K -W	Undetermined	N	U	N	U	P	U
69	Biopsy	CL	K -W	Undetermined	N	U	N	U	N	U
70	Biopsy	CL	K -W	Undetermined	P	U	P	U	P	U
70	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
70	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
70	Aspirate	CL	K -W	Undetermined	N	U	N	U	P	U
71	Aspirate	CL	K -W	Undetermined	N	U	N	U	P	U
71	Scraping	CL	K -W	Undetermined	N	U	N	U	P	U
72	Aspirate	CL	K -W	Undetermined	N	U	N	U	N	U
72	Scraping	CL	K -W	Undetermined	N	U	N	U	N	U
73	Biopsy	CL	K -W	*L. guyanensis*	P	U	P	G	N	U
73	Scraping	CL	K -W	*L. guyanensis*	P	U	P	G	P	G
73	Aspirate	CL	K -W	*L. guyanensis*	N	U	P	G	P	G

[a] CL = Cutaneous leishmaniasis; MCL = mucocutaneous leishmaniasis.
[b] K-S = Strong amplification using kDNA PCR; K-W = weak amplification using kDNA PCR; R-S = strong amplification using 18S-rDNA PCR.
[c] Species as determined from RFLP results (Fig. 1).
[d] Results from the 3 PCRs: P, positive; N, negative.
[e] Results from the 3 RFLPs, as in Fig. 1. U = Undetermined (weak or no PCR amplification).

18S-rDNA was amplified as in Deborggraeve et al. (2008). The reaction mix (25 μL) contained 1× PCR buffer including 1.5 mmol/L $MgCl_2$, an additional 1 mmol/L of $MgCl_2$, 1× Q-buffer, 200 μmol/L of each deoxynucleoside triphosphate, 0.5 U of HotStarTaq Plus DNA polymerase (Qiagen), 0.1 mg/mL of acetylated bovine serum albumin (Promega, Madison, WS, USA), and 0.8 μmol/L of each of the primers 18S-L-F and 18S-L-R. As template, we included 5 μL of extracted DNA. As positive control, we used 1 ng DNA from isolate MHOM/PE/92/HB22. PCRs were considered positive if a specific amplicon of 115 bp was observed (Deborggraeve et al., 2008).

Three *hsp*70 PCRs were performed—PCR-G, PCR-F, and PCR-N—as in Montalvo et al. (2012). The reaction mix (50 μL) contained 1× PCR buffer including 1.5 mmol/L $MgCl_2$, an additional 1 mmol/L of $MgCl_2$ (only PCR-F and PCR-N), 1× Q-buffer, 200 μmol/L of each deoxynucleoside triphosphate, 1 U of HotStarTaq Plus DNA polymerase (Qiagen), and 0.8 μmol/L of each primer (0.4 μmol/L in PCR-G). As template, we included 10 ng of genomic DNA from reference strains and clinical isolates, or 5 μL of extracted DNA from clinical samples. A negative control tube containing all the components except for DNA was always included, as well as a positive control consisting of 1 ng DNA from isolate MHOM/PE/92/HB22. Thermal cycling parameters were as follows: initial denaturation at 95 °C for 5 min; 35 cycles consisting of 94 °C for 40 s, 61 °C for 1 min, 72 °C for 2 min (1 min for PCR-N, as this is a shorter fragment and hence requires less polymerization time); and a final extension step of 10 min at 72 °C. Finally, the reaction remained at 15 °C. After amplification, 10 μL of PCR amplicons was analyzed on a 2% agarose gel. PCRs were considered positive if a specific amplicon of the expected size (Montalvo et al., 2012) was observed (Fig. 1).

The limit of detection (analytical sensitivity) for each of the 3 PCRs was assessed on serial dilutions of genomic DNA from isolates representing the most prevalent *Leishmania* species in Peru (Table 1). Ten-fold dilutions were used starting from 1 ng down to 10 fg DNA added to the PCR reaction, as measured fluorometrically by Qubit (Invitrogen, Life Technologies, Paisley, UK).

2.4. RFLP Analysis

The PCR products were digested with different restriction endonucleases to allow identification of parasites following the stepwise scheme in Fig. 1 (Montalvo et al., 2012), whereby RFLP-G, RFLP-F, and RFLP-N are generated respectively from digesting PCR-G, PCR-F, and PCR-N amplicons. Digests of strong PCR amplicons were performed in 10 μL containing 1× optimal buffer recommended by the manufacturer, 7 μL of unpurified PCR product, and 5 U of enzyme. Weak amplicons were digested in 20 μL including 1× buffer, 17 μL of unpurified PCR product, and, again, 5 U of enzyme. Some amplicons were too faint for restriction digest analysis. The enzymes used are *Hae*III (MBI Fermentas, St. Leon-Rot, Germany), *Rsa*I (MBI Fermentas), *Bcc*I (New England Biolabs, Ipswich, MA, USA), *Bsa*JI (MBI Fermentas), and *Eco*RII (MBI Fermentas). Reactions were incubated between 3 h and overnight at 37 °C (55 °C for *Bsa*JI), and fragments were analyzed in a 3% small fragment agarose gel (Gentaur, Brussels, Belgium), running at 3.5 V/cm for 3 h. The species was inferred from the detected bands according to Fig. 1, using a stepwise analysis of 2 successive digests.

2.5. Statistical analysis

The diagnostic sensitivity of each PCR was calculated for the entire sample and various subsets, using a positive kDNA or 18S-rDNA PCR as gold standard. Univariate exact logistic regression analysis was used to detect any significant differences between proportions of PCR or RFLP success as a function of all samples and various subsets. Statistical significance was considered at the probability level of $P < 0.05$. Exact binomial confidence intervals (95%) were calculated for each proportion. Statistical analyses were conducted using the Stata/SE 11.2 software (Stata Corp., College Station, TX, USA, http://www.stata.com).

3. Results

3.1. Reference panel

The analytical sensitivity of PCR-G, PCR-F, and PCR-N was determined using 1 Peruvian reference isolate from each genotype (Fig. 2). The detection limit in each case was between 100 and 10 fg of DNA. PCR-N showed the highest product yield and was slightly (up to 10-fold) more sensitive than PCR-G, with PCR-F holding an intermediate position.

With the use of the algorithm in Fig. 1, the reference strains and isolates listed in Table 1 were characterized with the 3 *hsp*70 RFLPs. PCR amplicons were obtained for all 3 variants (RFLP-G, RFLP-F, RFLP-N) from all DNAs, and typing results are listed in Table 1. No disagreement was observed between the 3 RFLPs of the same sample. There is a perfect agreement with the reference methods used. The *L. braziliensis* isolates having 2 *cpb* alleles generally contained only 1

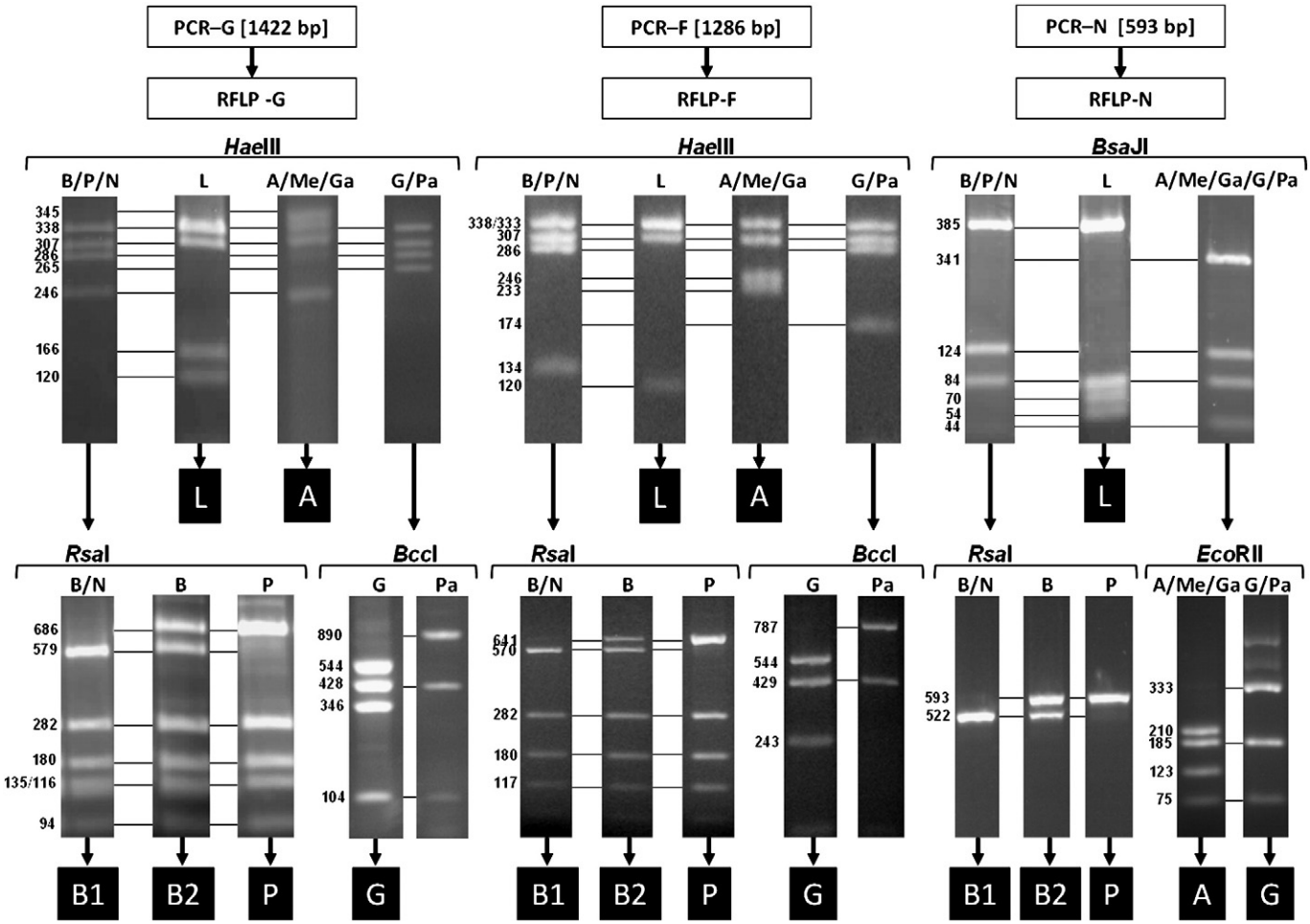

Fig. 1. Algorithm and restriction patterns used for the identification of New World *Leishmania* species in Peru with the *hsp*70 PCRs [adapted from Montalvo et al. (2012)]. The RFLP assays shown on top allow categorizing the species into several groups, whereby a further distinction is made by the digests indicated below. Size indication in base pairs is depicted on the left of each distinct RFLP enzyme panel, and between square brackets for each PCR product on top. *Leishmania* species abbreviations: A = *L. amazonensis*; B/B1/B2 = *L. braziliensis*, whereby B2 contains both the B1 and *L. peruviana* alleles; G = *L. guyanensis*; Ga = *L. garnhami*; L = *L. lainsoni*; Me = *L. mexicana*; N = *L. naiffi*; P = *L. peruviana*; Pa = *L. panamensis*. Patterns are often shared among species, as indicated on the top of each digest. The final conclusion for species identification in Peru is given in the black boxes. Both B1 and B2 are considered *L. braziliensis*. When identifying *L. guyanensis* (G) with RFLP-G and RFLP-F, the second digest is used only for confirmation, to avoid confusion with *L. panamensis* (Pa). Occasionally, some faint additional fragments are seen in the gels, which are the result of a partial digest. The size of these bands is not indicated, and they should not be considered when analyzing the patterns.

*hsp*70 allele, except for CU0188 which contained 2 alleles in both loci. Equally so, isolate LH3597 has 2 *hsp*70 alleles, but only 1 *cpb* allele.

3.2. Clinical samples: diagnostic sensitivity

We analyzed a panel of 89 clinical samples from 73 patients with confirmed molecular diagnosis of leishmaniasis (kDNA or 18S-rDNA PCR), using PCR-G, PCR-F, and PCR-N for detection of the genus *Leishmania* (Table 2). The sensitivity results are shown in Fig. 3, both for the total set of samples and for several subsets. It is striking that PCR-N in all but 1 (*L. guyanensis*) case was able to detect the highest number of positives, followed by PCR-F and, finally, by PCR-G, irrespective of the sample type, clinical presentation, diagnostic PCR signal strength ("load" in Fig. 3), or species investigated. When

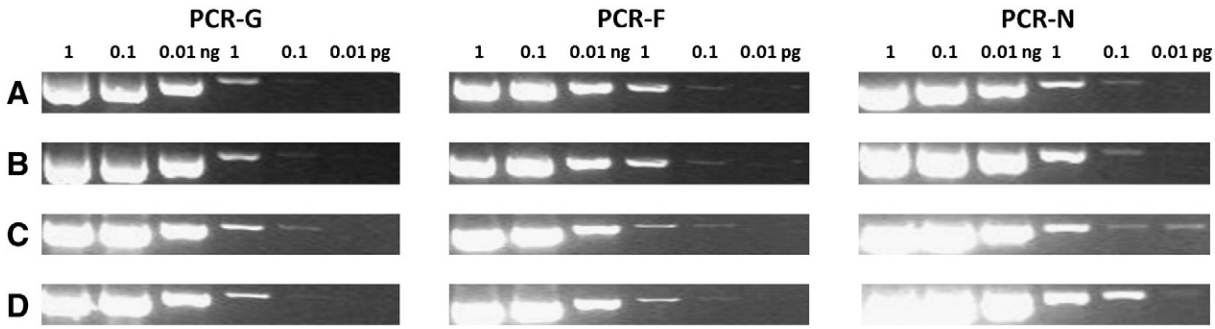

Fig. 2. Analytical sensitivity of *hsp*70 PCRs in Peruvian *Leishmania* isolates: *L. peruviana* isolate LH3823 (A), *L. braziliensis* isolate CU00185 (B), *L. braziliensis* isolate CU00183 (C), and *L. guyanensis* isolate LH3842 (D) (see also Table 1). The *hsp*70 PCR variant appears on top, along with the DNA amount used, ranging from 1 ng down to 10 fg in steps of 10× dilutions.

viewed over all samples, these differences were statistically significant, but this was not always the case in the different subsets, possibly due to the limited sampling size. PCR detection in biopsies was significantly better as in scrapings, the difference with aspirates being nonsignificant. This was the case when considering compiled results over all 3 PCRs (i.e., successful amplification in either of the 3 PCRs), as well as in PCR-F and PCR-N. For PCR-G, no significant differences were found between the 3 sampling types. As an illustration of the difference in sensitivity in several samples, Fig. 4 presents the PCR results of all 89 samples.

3.3. Clinical samples: species typing

In a subsequent step, species were determined with RFLP (Fig. 1, Table 2), and the efficiency of species typing of the 3 RFLP assays was assessed, as shown in Fig. 5. Typing efficiency was defined as the percentage of samples in which the species could be determined with PCR-RFLP, which is different from PCR sensitivity (i.e., genus detection) because RFLP needs a sufficient amplicon amount for visualization of all digested fragments. Consequently, PCRs resulting in a weak amplification, even though they can be used for detection purposes, are not always useful for RFLP typing. Therefore, typing efficiencies are, by definition, lower than PCR sensitivity, even though they followed the same trends: RFLP-F and RFLP-N being significantly the most efficient, followed by RFLP-G. This trend is generally observed in all sample categories. Biopsies analyzed with RFLP-N are significantly the best combination for typing purposes. In general, when combined results from all 3 RFLPs are considered, typing has the most chance of success on biopsies. For RFLP-F, no significant differences were seen for the 3 sampling types, while RFLP-G worked better in scrapings as compared to biopsies.

It should be noted that, in all cases, where RFLP could be performed, species typing could be completed, and where more than 1 of the RFLPs could be analyzed from the same sample the results were perfectly congruent. From 12 patients, 2 or all 3 sample types were available, in which case all gave congruent results in terms of the identified species. Where species identification failed in one of the samples, it consistently failed in the other samples as well, possibly indicative of a low parasite load in the lesion. From the 89 samples, 40 were typed as *L. braziliensis* (15 of which with 2 *hsp*70 alleles; B2 in Fig. 1), 11 as *L. peruviana*, and 11 as *L. guyanensis*. Twenty-seven samples could not be typed. Of the 73 patients, 34, 11, and 9 were found infected with *L. braziliensis*, *L. peruviana*, and *L. guyanensis*, respectively, while in the remaining 19 the species could not be determined.

4. Discussion

Of the 3 PCRs evaluated in this study, PCR-N was diagnostically the most sensitive as assayed in various clinical samples. PCR-F was found less sensitive, and, finally, PCR-G showed the lowest sensitivity. In line with these findings, the typing efficiency, i.e., the number of samples that could be successfully typed with the corresponding RFLP analyses RFLP-N, RFLP-F, and RFLP-G, followed the same order. This seems logical as the PCRs generate fragments of 1422 (PCR-G), 1286 (PCR-F), and 593 (PCR-N) nucleotides, and smaller fragments are generally easier to amplify. Nevertheless, this could not be demonstrated by the analytical sensitivities determined on dilutions of cultured parasite DNA, which are in line with Montalvo et al. (2012). Opposed to the relatively pure DNA generally obtained from cultures, DNA from clinical samples often contains more PCR inhibitors. These impact the amplification of shorter fragments less severely, thereby explaining the more pronounced sensitivity improvement by PCR-N in clinical versus cultured samples. The sensitivity (37%) of PCR-G contrasts sharply with previous studies reporting 93% to 100% (García et al., 2007a,b), even though we used PCR conditions that, in our hands, performed better than those reported (data not shown). This difference can be accounted for by the lack of standardization of sampling and DNA extraction, and the fact that between 1 and 4 years had passed between our sample taking and analysis. Because the quality of samples and extracted DNA degrades with time, the obtained sensitivities of all PCRs described here will be higher when assayed on fresh material.

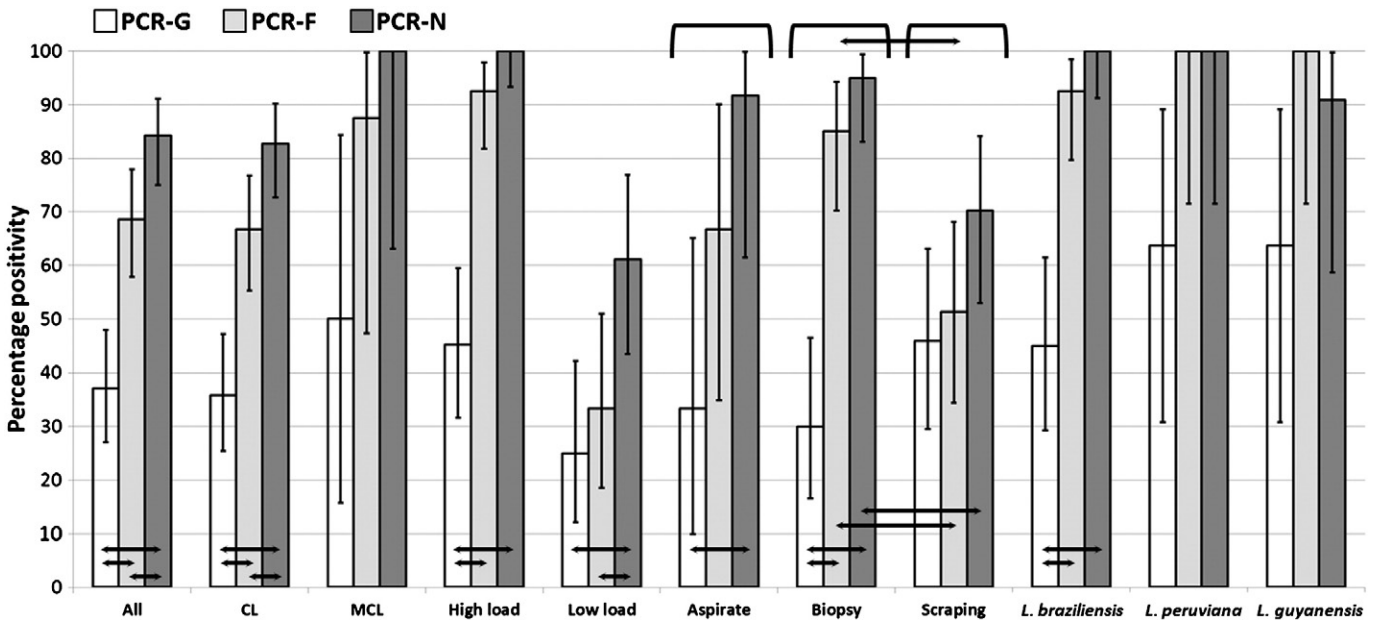

Fig. 3. Diagnostic sensitivity of each *hsp*70 PCR variant in different sample groups: type of disease (cutaneous leishmaniasis [CL]/mucocutaneous leishmaniasis [MCL]); parasite load as determined qualitatively from the kDNA or 18S-rDNA PCR signal (high/low); sampling (aspirate/biopsy/scraping); species as identified in this study (Table 2). Error bars indicate the 95% confidence interval. Statistically significant differences ($P < 0.05$) were determined for each sample group between the 3 PCRs. Across sample types (aspirate/biopsy/scraping), compiled results from the 3 PCRs were compared (horizontal top brackets), as well as results from each PCR separately. Significant differences are indicated by horizontal arrows. As the infecting species could not be determined in all samples, the data per species are biased because they only list the number of positive results in the subpopulation of samples that could be typed.

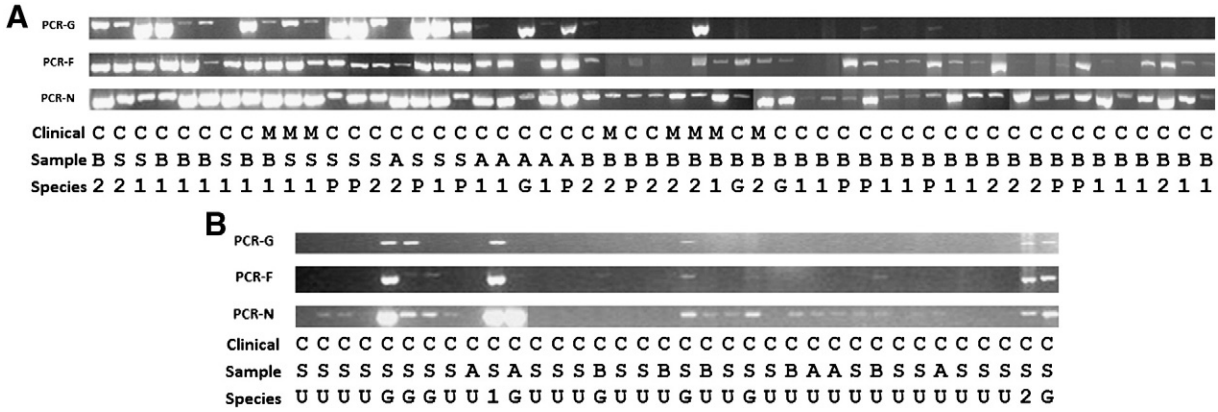

Fig. 4. Agarose gel analysis of PCR-G, PCR-F, and PCR-N from 53 clinical samples shown to be strongly positive for *Leishmania*-specific diagnostic PCRs based on kDNA or 18S-rDNA (Panel A) and from 36 weakly positive kDNA PCR samples (Panel B). Clinical presentation (C = CL, M = MCL); sample type (B = biopsy, A = aspirate, S = lesion scraping); and RFLP-identified species (as in Fig. 1 and Table 2, whereby 1 = B1 and 2 = B2) are indicated below for each sample. Only the relevant part of the gels is shown.

As judged from the characterization of previously characterized parasites, the species typing based on all RFLPs used here agrees perfectly with the reference methods, in line with Montalvo et al. (2012) describing validation on a global *Leishmania* panel. For practical purposes, there is no reason to separate *L. braziliensis* with 1 from those with 2 *hsp*70 alleles (B1 and B2 in Fig. 1), as both forms were found in patients suffering from MCL, a complication typically associated with an *L. braziliensis* infection (Guerra et al., 2011). Furthermore, Odiwuor et al. (2012) showed that the majority of *L. braziliensis* strains dispose of more than 1 *hsp*70 allele, only in some cases displaying polymorphism at the RFLP digestion site *Rsa*I used here. *L. guyanensis*, which was frequently found among the analyzed samples, can be detected with all RFLPs, but RFLP-N does not allow separating it from *L. panamensis*, so far not identified in Peru. Two other species sometimes encountered in Peru, *L. lainsoni* and *L. amazonensis* (Arévalo et al., 2007; Llanos-Cuentas et al., 2008; Lucas et al., 1998; Veland et al., 2012), were not found in the studied samples. They too can be identified with the RFLPs used here, but *L. amazonensis* cannot be discriminated from *L. garnhami* or *L. mexicana*, 2 other members of the *L. mexicana* species complex (Montalvo et al., 2010a,b; 2012). The same holds for *L. naiffi*, which, in most cases, cannot be distinguished from *L. braziliensis* (Montalvo et al., 2010a,b; 2012). In summary, we tailored the globally usable assay in Montalvo et al. (2012) for use in Peru, taking into account the known endemicity of 5 species (Arévalo et al., 2007; Boggild et al., 2010; Llanos-Cuentas et al., 2008; Lucas et al., 1998; Veland et al., 2012; Victoir et al., 2003). If as a consequence of changing climate conditions and/or human migration other species were to invade Peru, misclassifications could be avoided by sequencing rather than by RFLP analysis.

The use of *hsp*70 PCR-F or PCR-N as a first-line molecular diagnostic tool for leishmaniasis (i.e., *Leishmania* genus detection) may be considered in order to save cost and time when species typing is required. Indeed, the amplicon obtained from molecular diagnosis in such case can be used directly in typing assays. PCR-N probably has a higher success rate, but cannot discriminate *L. guyanensis* from *L. panamensis*, even though this is a minor drawback as the latter species has so far not been documented in Peru. Contrary to PCR-F and PCR-N, PCR-G cannot be used as a first-line diagnostic tool because of its lower sensitivity for *Leishmania* in general and for the subgenus *L.* (*Leishmania*) (e.g. *L. amazonensis*) in particular, and because it also amplifies other endemic Trypanosomatidae such as

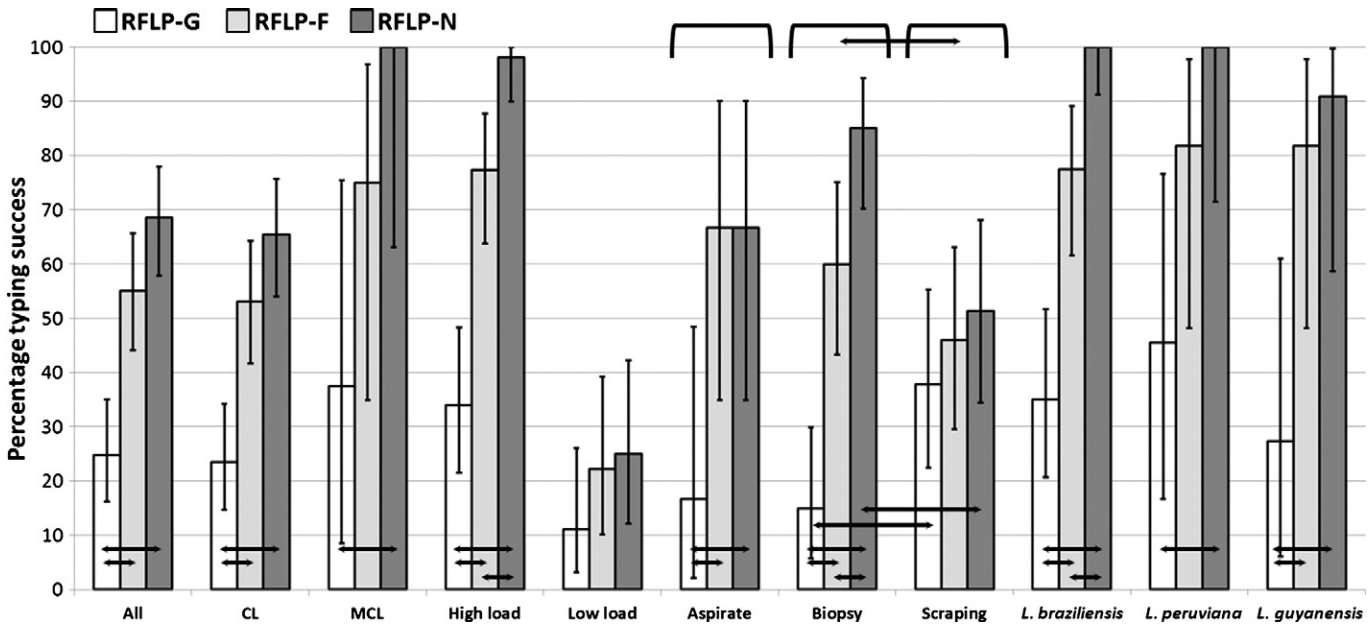

Fig. 5. RFLP typing success of each *hsp*70 RFLP variant in different sample groups. All features and abbreviations as in Fig. 3.

Trypanosoma rangeli and *T. cruzi* (Montalvo et al., 2012). If no species typing is required, or if PCR-F and PCR-N are negative, one must revert to more sensitive molecular methods such as those based on kDNA and 18S-rDNA (Boggild et al., 2010; Deborggraeve et al., 2008). These loci are present in higher copy numbers and are thus easier to detect (Floeter-Winter and Uliana, 1993; Folgueira and Requena, 2007; Simpson et al., 1987).

In terms of sampling, biopsies are better fit for typing as are scrapings. This may be due to differences in parasite load and PCR inhibitors in these sample types. This difference across sample types was, however, not generally observed in the few paired samples available. Probably, the sensitivity and typing success rate of our methods are, in reality, higher as reported here, because they are based upon samples that were up to 4 years old at the time of analysis.

Compared to the recently published algorithm for *Leishmania* species typing in Peru (Veland et al., 2012), the method proposed here has the advantage that only 1 PCR product is amplified instead of 3, which saves money and facilitates timely delivery of results to the clinicians. In addition, the algorithm could type none of the 36 samples that amplified weakly in kDNA PCR (data not shown), as opposed to 10 successful typings using our approach (Table 2). The sensitivity of our methods could be improved even further by using PCR-F as a nested PCR, with PCR-G as the first-round (outer) PCR. Equally so, PCR-N can be amplified from PCR-G or PCR-F. Even though we have used these methods already in a diagnostic setting (results not shown), they have not systematically been tested. The resolution of our assays can be enhanced by sequencing rather than by RFLP analysis, the latter being based on less polymorphisms.

In the total of 54 patients for whom typing was achieved by *hsp*70 RFLP, *L. braziliensis* was the predominant species with 63% (34 patients), followed by *L. peruviana* with 20% (11 patients) and *L. guyanensis* with 17% (9 patients), which agrees with reported species (Arévalo et al., 2007; Boggild et al., 2010; Llanos-Cuentas et al., 2008; Lucas et al., 1998; Veland et al., 2012; Victoir et al., 2003). It is worth mentioning that the only species encountered in MCL samples was *L. braziliensis* (6 patients). This is in line with previous reports showing that MCL is predominantly associated with *L. braziliensis* (Guerra et al., 2011; Lucas et al., 1998).

In conclusion, we present in this article an assay for *Leishmania* diagnosis and typing, both for epidemiologic monitoring and for use in clinical studies or patient follow-up. The method allows identification of all species previously reported in Peru, based on a single PCR product. It is an application of a broader and universal *Leishmania* typing strategy on the basis of the HSP70 gene, adapted to a specific setting, and, as such, illustrates the potential of this gene to impact strategic decisions towards the efficient management of leishmaniasis.

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