

Three new sensitive and specific heat-shock protein 70 PCRs for global *Leishmania* species identification

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Abstract The heat-shock protein 70 gene (*hsp70*) has been exploited for *Leishmania* species identification in the New and Old World, using PCR followed by restriction fragment length polymorphism (RFLP) analysis. Current PCR presents limitations in terms of sensitivity, which hampers its use for analyzing clinical and biological samples, and specificity, which makes it inappropriate to discriminate between *Leishmania* and other trypanosomatids. The aim of the study was to improve the sensitivity and specificity of a previously reported *hsp70* PCR using alternative PCR primers and RFLPs. Following in silico analysis of available sequences, three new PCR primer sets and restriction digest schemes were tested on a globally representative panel of 114 *Leishmania* strains, various other infectious agents, and clinical samples. The largest new PCR fragment retained the discriminatory power from RFLP, while two smaller fragments discriminated less species. The detection limit of the new PCRs was between

0.05 and 0.5 parasite genomes, they amplified clinical samples more efficiently, and were *Leishmania* specific. We succeeded in significantly improving the specificity and sensitivity of the PCRs for *hsp70* *Leishmania* species typing. The improved PCR-RFLP assays can impact diagnosis, treatment, and epidemiological studies of leishmaniasis in any setting worldwide.

Introduction

Leishmaniasis is one of the most neglected tropical diseases, with a major burden among the poorest segments of impoverished populations in Asia, Africa, South America and, in a lesser degree, Europe [1, 2]. It has been demonstrated that the clinical spectrum of the disease, prognosis, and response to treatment can vary according to the species [3–5]. Also epidemiological monitoring and vector and/or reservoir management rely on accurate parasite discrimination [6, 7]. Diagnosis of leishmaniasis currently focuses on parasite detection by microscopy, culture, and PCR. Molecular targets are either kinetoplast DNA (kDNA) [8] or genomic DNA (e.g. 18S rDNA [9]). Species discrimination requires species-specific PCRs, or PCR amplification followed by sequencing. Alternatively, digestion of the amplification product with a defined set of enzymes reveals restriction fragment length polymorphisms (RFLPs), which have been widely applied to discriminate *Leishmania* spp. from diverse geographical areas [10, 11].

Among the techniques used for both detection and typing, PCR amplification of the heat-shock protein 70 encoding gene (*hsp70*) has proven valuable [6]. However, it was initially used exclusively to analyze species of the *L. (Viannia)* subgenus, and the performance on other species, including those found in the Old World, had not been

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tested. Based on polymorphisms identified in a broad group of *hsp70* *Leishmania* sequences [12], an RFLP strategy was designed to allow identification of most medically important species, including those of the subgenus *L. (Leishmania)* [13]. For its application, efficient amplification of all *Leishmania* species is crucial in order to obtain a sufficient amount of gene product for RFLP analysis from various environmental, clinical, and biological samples, which is not the case for the *hsp70* protocol of García et al. [14], in particular for the *L. (Leishmania)* subgenus. In addition, this PCR also amplifies other organisms (e.g. trypanosomes) that might be present in these specimen, which can interfere with the RFLP pattern.

The goal of the present study was to improve the sensitivity and specificity of the *hsp70*-PCR by using alternative primer sets, without compromising the discriminatory power of RFLP for identifying all medically important species worldwide. We also aimed at testing the new assays on DNA from reference strains and clinical samples, to determine their sensitivity and species discrimination potential.

Materials and methods

Strains and DNA

Table 1 lists the species and geographical origin of the 121 reference strains and isolates included in our study, representative of 14 *Leishmania* and two *Trypanosoma* species. A detailed listing is available in the supplementary Table ESM-1. From these, 114 *Leishmania* strains were tested in PCR-RFLP, while five were included only in the in silico analysis. As DNA was obtained from

different sources, the reference species identification was based on various data such as zymodeme typing, RFLP analysis and gene sequencing. All DNAs were isolated from parasite cultures, and either parasites or DNA were obtained from the different institutes acknowledged at the end of this paper.

To test the analytical specificity of the PCRs, DNAs from clinical samples or cultures from various microorganisms and viruses were used: *Plasmodium falciparum*, *Schistosoma mansoni*, *Trypanosoma cruzi*, *Trypanosoma rangeli*, *Trypanosoma brucei*, *Candida albicans*, *Candida parasilopsis*, *Cryptococcus neoformans*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Enterococcus*, *Mycobacterium tuberculosis*, *Mycobacterium habana*, *Epstein-Bar virus*, *Herpes zoster virus*, *Herpes simplex virus*, *Citomegalovirus*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Neisseria meningitidis*. These were obtained from various sources acknowledged at the end of the manuscript.

DNA from confirmed positive clinical samples was obtained from the centers mentioned in the acknowledgements section. Different diseases and sample types were represented, including biopsies, blood, scrapings, and aspirates. DNA was extracted using a variety of methods. All samples were taken according to the ethical guidelines of the respective institutes.

Primer design and in silico analysis of *hsp70* coding sequences

Based on a previous alignment [12], three new PCR primer sets specific for the *Leishmania* genus were selected

Table 1 Strains and isolates^a

Species ^b	Country of origin
<i>L. aethiopica</i> (n=8)	6 Ethiopia, 2 Kenya
<i>L. tropica</i> (n=9)	1 India, 1 Iraq, 3 Kenya, 2 Palestinian Territory, 1 USSR, 1 unknown
<i>L. donovani</i> (n=12)	2 Ethiopia, 3 India, 3 Kenya, 4 Sudan
<i>L. infantum</i> (n=21)	3 Brazil, 1 China, 5 France, 2 Italy, 1 Malta, 1 Morocco, 1 Portugal, 7 Spain
<i>L. major</i> (n=7)	1 Burkina Faso, 1 Israel, 1 Kenya, 1 Saudi Arabia, 1 Spain, 1 Sudan, 1 Tunisia
<i>L. amazonensis</i> (n=4)	3 Brazil, 1 Bolivia
<i>L. garnhami</i> (n=1)	1 Venezuela
<i>L. mexicana</i> (n=2)	1 Belize, 1 Peru
<i>L. lainsoni</i> (n=10)	4 Bolivia, 6 Peru
<i>L. braziliensis</i> (n=19)	7 Bolivia (4 bra* ^c), 4 Brazil, 8 Peru (5 bra* ^c)
<i>L. peruviana</i> (n=12)	12 Peru
<i>L. naiffi</i> (n=2)	2 Brazil
<i>L. guyanensis</i> (n=10)	1 Bolivia, 3 Brazil, 1 French Guiana, 5 Peru
<i>L. panamensis</i> (n=2)	2 Panama
<i>Trypanosoma cruzi</i>	1 Brazil
<i>Trypanosoma rangeli</i>	1 Honduras

^aA detailed listing is provided in Table ESM-1

^bSpecies designations are according to current literature, and were determined as explained in Materials and Methods

^cNumber of *L. braziliensis* strains, from the total listed, having a mixed RFLP pattern (*L. braziliensis* and *L. peruviana*), indicated as bra* in Fig. 2. See also Table ESM-1

(Table 2). The fragments flanked by these primers contain polymorphisms for species differentiation already identified [13]. In silico RFLP analysis and prediction of the resulting fragments was performed with the software packages MEGA4 [15] and DNAMAN version 4.02 (Lynnon Biosoft, Quebec, Canada), as well as the online tools NEBcutter (tools.neb.com/NEBcutter2) and DistinctiEnz (www.bioinformatics.org/ydocreza/cgi-bin/restriction/DistinctiEnz.pl).

PCRs

Four *hsp70*-PCR protocols were performed (Table 2). PCR-G [14] was done according to previous reports [13]. PCR-F, PCR-N, and PCR-C were done in 50 μ l total volume containing 1x Q solution; 1x standard PCR buffer; a total of 2.5 mM MgCl₂; 0.8 μ M of each primer; 200 μ M of each deoxynucleoside triphosphate; and 1U HotStarTaq Plus DNA Polymerase (Qiagen, Hilden, Germany). Except when testing sensitivity or clinical samples, 10 ng of DNA was used. Concentrations were measured by spectrophotometry using the Nanodrop (ND1000, Thermo Scientific, Wilmington, DE, USA). Negative no-template controls were always included, along with a positive control consisting of 1 ng DNA from *L. braziliensis* strain MHOM/BR/75/M2903. The amplification conditions for the three new PCR variants were: denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 61°C for 1 min (except PCR-C: 65°C), extension at 72°C for 1 min (except PCR-F: 2 min); and a final extension step of 10 min at 72°C. Thermal cycling was performed in either a PTC-150 (MJ Research, Waltham, MA, USA), a MyCycler™ (Bio-Rad, Foster City, CA, USA), or an iCycler (Bio-Rad). Analysis on a 2% agarose gel was used to verify the amplified product size.

Analytical sensitivity and specificity

The analytical sensitivity was determined for all four PCRs (Table 2) using several strains representing each species or species complex (Table ESM-1). Ten-fold DNA

dilutions in water were tested, ranging from 10 fg to 10 ng/ μ l, whereby 1 μ l was used for PCR. The presence of *Leishmania* DNA in the four lowest dilutions was confirmed by a diagnostic 18S rDNA-PCR [9]. The specificity of the PCRs was analyzed using the DNAs described above as template.

RFLP analysis

RFLP analysis was performed on amplicons from PCR-F, PCR-N, and PCR-C from 114 strains (Tables 1 and ESM-1). These are further referred to as RFLP-F, RFLP-N, and RFLP-C. Digests were performed in a total of 10 μ l containing 5 μ l of amplicon solution in 1x optimal buffer provided by the manufacturers, using 2U *Hae*III (MBI Fermentas, St Leon-Rot, Germany); 2U *Bcl*I (New England Biolabs, Ipswich, MA, USA), 2U *Rsa*I (MBI Fermentas), 2U *Mlu*I (MBI Fermentas); 2U *Bsa*HI (MBI Fermentas); 2U *Bsa*JI (MBI Fermentas), 2U *Hind*II (MBI Fermentas), or 20U *Eco*RII (MBI Fermentas). For double digests with enzymes *Eco*RII and *Mlu*I, 10U and 20U were used respectively in buffer O, as the activity of *Mlu*I in this buffer is only 50%. Reactions were incubated overnight at 37°C, except for *Bsa*JI (55°C). Subsequently, restriction patterns were analyzed by electrophoresis in a 3% small fragment agarose gel (Gentaur, Brussels, Belgium), running at 3.5 V/cm for 3 h. The GeneRuler™ 100 bp DNA Ladder (MBI Fermentas) was used as a size reference marker.

Results

For all 14 *Leishmania* species, the amplicon size for each PCR (data not shown) agrees with the in silico analysis (Table 2) and previous reports [13, 14]. A selection of results from the sensitivity study, showing one representative strain of each species or species complex, is depicted in Fig. 1. The 18S rDNA-PCR confirms the presence of *Leishmania* DNA in each dilution, down to 10 fg/ μ l. The *hsp70* fragments amplified in all species. The detection

Table 2 PCR primers

	<i>hsp70</i> -PCR ^a	Primer	Annealing ^b	Primer sequence 5'-3'	Amplicon size
<i>bp</i> base pairs	PCR-G	HSP70-sen HSP70-ant	435-455 1856-1836	GACGGTGCCTGCCTACTTCAA CCGCCATGCTCTGGTACATC	1422 bp
^a PCR-G: García et al. [14]. PCR-F, PCR-N, PCR-C: designed in this study	PCR-F	F25 R1310	480-498 1765-1744	GGACGCCGGCACGATTKCT CCTGGTTGTTGTTTCAGCCACTC	1286 bp
^b The annealing position of the primers is given relative to the GenBank accession XM_001684512 (<i>L. major</i> Friedlin)	PCR-N	F25 R617	480-498 1072-1051	GGACGCCGGCACGATTKCT CGAAGAAGTCCGATACGAGGGA	593 bp
	PCR-C	F251 R991	706-726 1446-1425	GACAACCGCCTCGTACGTTTC GTCGAACGTCACCTCGATCTGC	741 bp

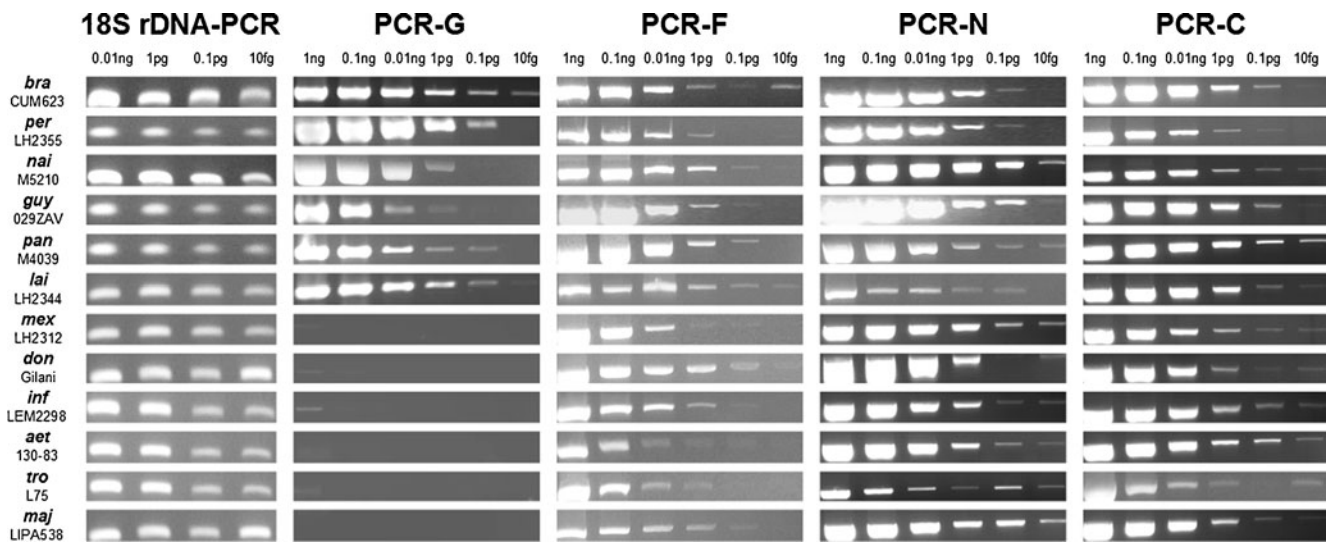


Fig. 1 Analytical sensitivity of the four PCRs (Table 2), compared to the 18S rDNA-PCR, as analyzed on agarose gel. Only the relevant part of the gels is shown. One representative isolate is depicted from each of the following *Leishmania* species: *bra*, *L. braziliensis*; *per*, *L. peruviana*; *nai*, *L. naiffi*; *guy*, *L. guyanensis*; *pan*, *L. panamensis*; *lai*,

L. lainsoni; *mex*, *L. mexicana* species complex including *L. garnhami* and *L. amazonensis*; *don*, *L. donovani*; *inf*, *L. infantum*; *aet*, *L. aethiopica*; *tro*, *L. tropica*; *maj*, *L. major*. The DNA amount used per assay, ranging from 1 ng to 10 fg, is indicated on top

limit of PCR-G differed according to the subgenus: whilst for *L. (Viannia)* parasites the procedure was able to detect between 10 (0.05 parasite genomes) and 100 (0.5 parasite genomes) fg of DNA, the detection limit for parasites belonging to subgenus *L. (Leishmania)* ranged from 0.1 to more than 1 ng. The overall sensitivity of PCR-F was better, detecting 10 fg of *L. (Viannia)* and 10–100 fg of *L. (Leishmania)*. The detection limit of PCR-N and PCR-C was comparable for both subgenera, between 10 and 100 fg. When analyzing five clinical sample sets from different countries and diseases (Table 3), it was clear that PCR-F performed much better in amplifying *Leishmania* DNA as

compared to PCR-G, as it amplified between two and ten times more samples. PCR-N and PCR-C performed only slightly better than PCR-F. With regard to the analytical specificity (figure available on request), no amplification was observed for PCR-F and PCR-N from species other than *Leishmania*. PCR-C showed an amplicon when DNA from *M. tuberculosis* was used, but its size differed from the *Leishmania* fragment. PCR-G amplified DNA from *Leishmania*, *Trypanosoma cruzi*, *T. rangeli*, and *T. brucei*.

Nucleotide polymorphisms affecting restriction endonuclease recognition sites for differentiation of *Leishmania* species

Table 3 PCR success rate in clinical samples

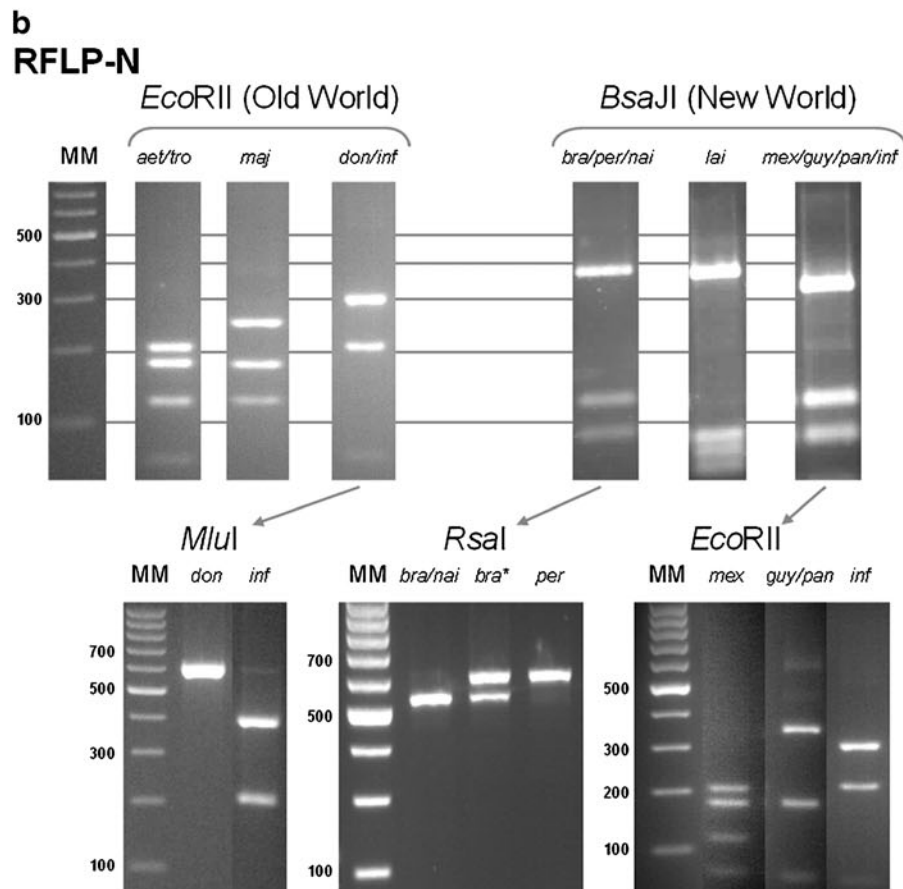
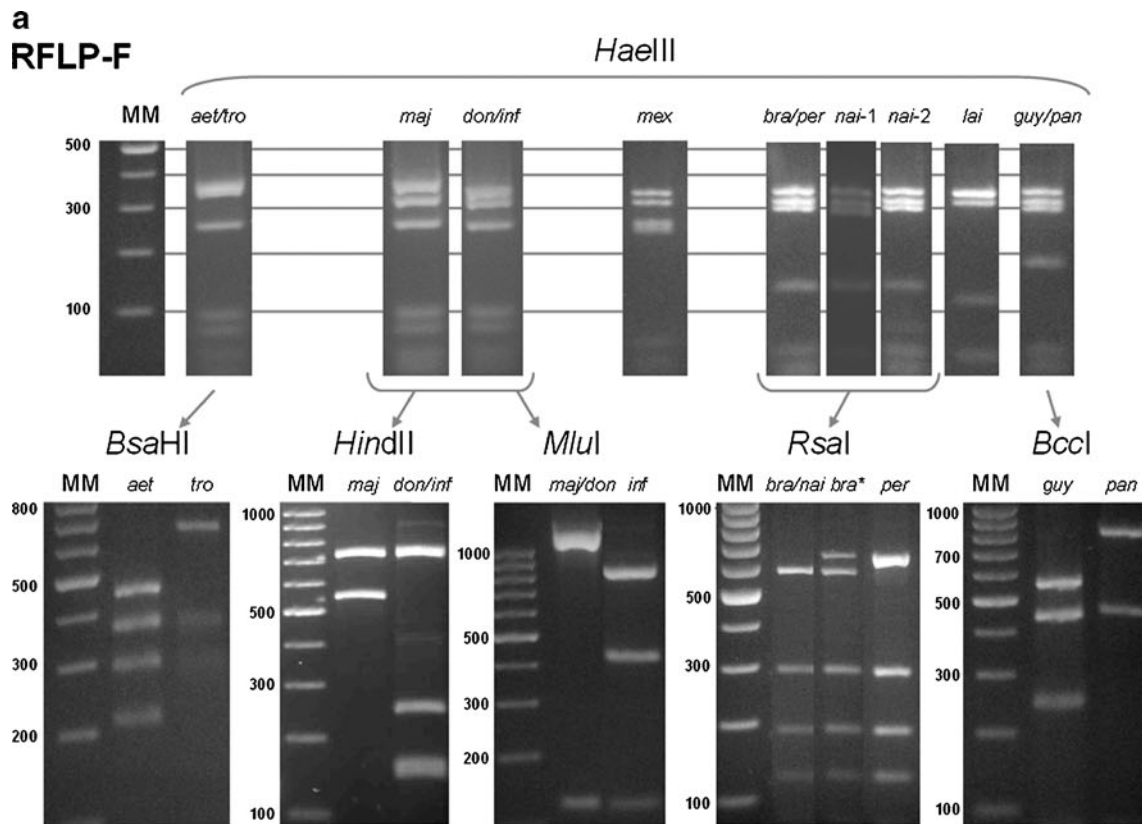
Country	Disease	Confirmed positives ^a	Positives ^b			
			PCR-G	PCR-F	PCR-N	PCR-C
Italy	VL	20	5	13	18	19
Peru	CL/ MCL	15	6	13	15	ND
Portugal	Can	6	3	6	6	6
Sudan	VL	30	3	20	23	22
Tunisia	CL	31	2	20	25	22

Can canine leishmaniasis, *CL* cutaneous leishmaniasis, *MCL* mucocutaneous leishmaniasis, *VL* visceral leishmaniasis, *ND* not done

^a Total number of confirmed positive samples tested

^b Number of samples positive in the PCRs listed in Table 2

Fig. 2 RFLP agarose gel patterns obtained from amplicons of the four PCRs in Table 2, and in different species and species complexes. Species abbreviation is as in Fig. 1, whereby *bra** indicates isolates with a composite *L. braziliensis* – *L. peruviana* pattern. Per obtained pattern, only one representative image is depicted, in relation to the size markers (MM) on their left. This size marker is the GeneRuler™ 100 bp DNA Ladder (MBI Fermentas, St. Leon-Rot, Germany), showing fragments in multiples of 100 base pairs (bp), whereby some band sizes are indicated on the left in bp, and a grid across the figure is provided in the upper panels. **a** RFLP-F: the upper panel represents the groups as defined by digestion with *Hae*III, the digests in the lower panel allow further separation within these groups. *nai*-1: MDAS/BR/79/M5533; *nai*-2: MDAS/BR/78/M5210. **b** RFLP-N: the upper panel digests allow a first separation of either Old World or New World species, which can be further distinguished using the digests below. **c** RFLP-C: *Hae*III allows a first separation (*upper panel*), from which groups can be further typed using the digests in the lower panel. No intra-species variability was seen in any of the species when analyzing the isolates in Table 1 and ESM-1, except for *L. braziliensis* and *L. naiffi*, for which different patterns are shown next to each other in the relevant RFLPs



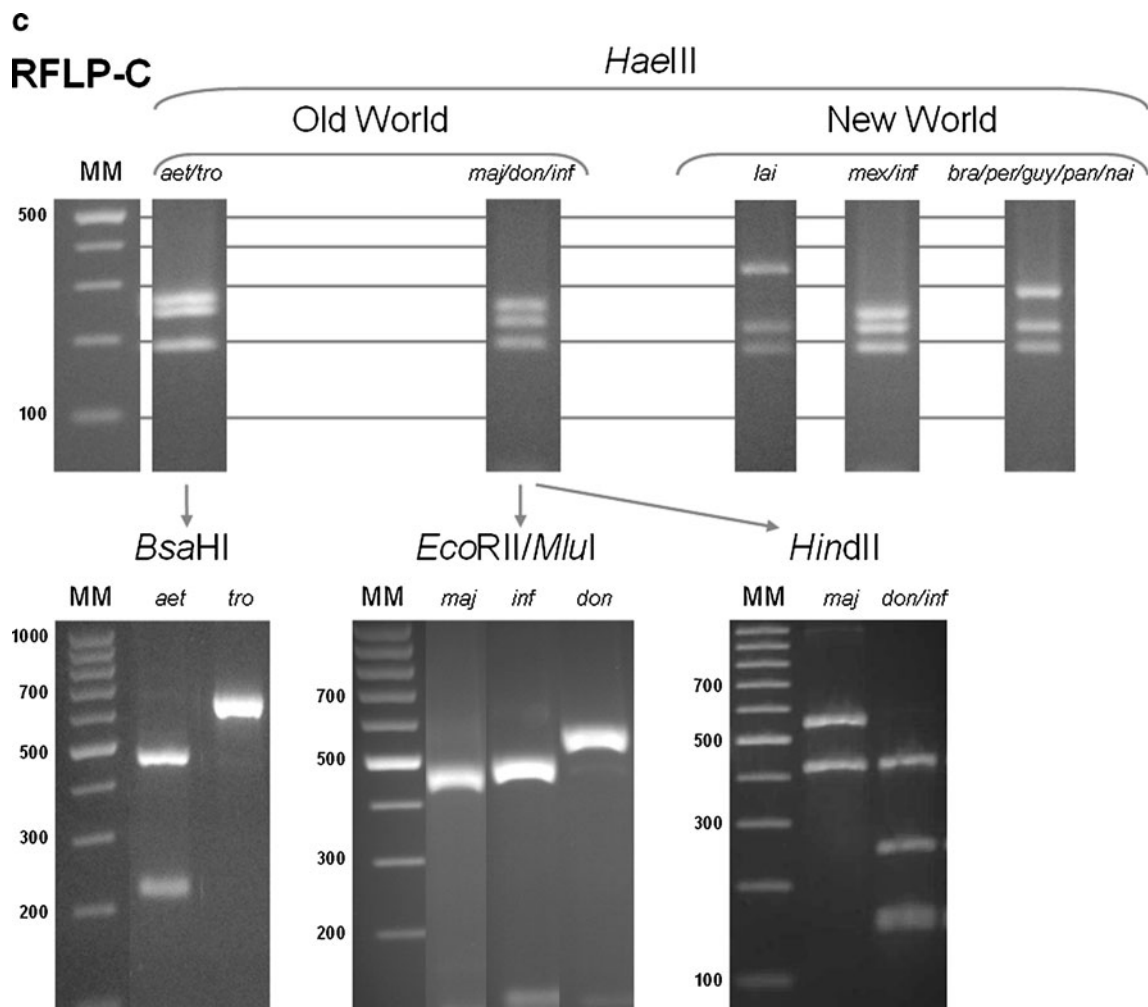


Fig. 2 (continued)

using PCR-F, PCR-N, and PCR-C, were identified in silico. Different schemes for discriminating medically important species from the New and Old World were designed, each relying on one to three digests for identification of all groups. They were applied on strains from all species from a diverse geographical origin (Table 1 and ESM-1, Fig. 2), which confirmed the in silico obtained results.

The discriminative power for each new RFLP analysis is as follows (Fig. 2). RFLP-F (Fig. 2a) typed five species from the Old World (*L. major*, *L. tropica*, *L. aethiopica*, *L. donovani*, *L. infantum*) and five from the New World (*L. infantum*, *L. lainsoni*, *L. peruviana*, *L. guyanensis*, *L. panamensis*). Of the 19 *L. braziliensis* isolates, ten shared the *HaeIII* pattern with *L. naiffi* MDAS/BR/79/M5533, while *L. naiffi* MDAS/BR/78/M5210 showed an extra faint band. In the *RsaI* digest, these 10 *L. braziliensis* isolates could not be distinguished from *L. naiffi*, but the remaining nine *L. braziliensis* showed a composite pattern with *L. peruviana* (indicated as bra* in Table 1 and ESM-1, and Fig. 2). Species from the *L. mexicana* complex (*L. mexicana*, *L.*

amazonensis, *L. garnhami*) could not be separated, but the complex as a whole can be identified using *HaeIII*. RFLP-N (Fig. 2b) allowed differentiating four groups from the Old World (*L. tropica*/*L. aethiopica*, *L. major*, *L. donovani*, *L. infantum*) and seven from the New World (*L. lainsoni*, *L. infantum*, *L. braziliensis*/*L. naiffi*, *L. peruviana*, *L. guyanensis*/*L. panamensis*, *L. mexicana* complex, composite *L. braziliensis* bra*). Finally, RFLP-C (Fig. 2c) identified all five tested species from the Old World, as well as *L. lainsoni* from the New World. No intra-species differences were observed in any of the RFLPs, apart from the aforementioned cases of composite *L. braziliensis* (bra* in Table 1 and ESM-1, and Fig. 2) and *L. naiffi*.

Discussion

PCR-RFLP analysis of a 1422 bp *hsp70* gene fragment (PCR-G) was already demonstrated to be valuable for discrimination of *Leishmania* species, both in the subgenera

L. (Viannia) and *L. (Leishmania)* [6, 11, 13, 14]. However, sensitivity in the latter was poor. The new PCR variants (PCR-F, PCR-N, PCR-C) presented here showed an increased sensitivity of a factor 10,000 in *L. (Leishmania)*, while sensitivity in the *L. (Viannia)* subgenus was comparable to PCR-G. In clinical samples however, we showed a significant improvement in amplification efficiency when comparing to PCR-G in both subgenera. This brings the typing potential of *hsp70* to the same level for all *Leishmania* species, in view of the discriminative potential of the corresponding RFLPs. This was theoretically expected, as the new PCR primer sets were designed on the basis of all available *Leishmania* sequences, opposed to PCR-G having been developed specifically for *L. (Viannia)*. An adequate sensitivity of the PCRs is of crucial importance for analyzing clinical and biological samples, which often contain a small number of parasites [16, 17]. It should be noted that in case the new PCRs show no or insufficient amplification from one PCR round, the new primer combinations can also be used in a (hemi-) nested PCR approach to further increase sensitivity, as was already applied in some clinical samples (data not shown). In such case either PCR-G or PCR-F can be used as the outer PCR.

The new PCR versions also showed an improved specificity for *Leishmania*. As already reported previously [14], our analysis confirmed the amplification of trypanosomatid species other than *Leishmania* with PCR-G, i.e. *Trypanosoma cruzi*, *T. rangeli*, and *T. brucei*. In the New World, *T. rangeli*, *T. cruzi* and *Leishmania* are sympatric in many areas [18, 19], emphasizing the importance of genus-specific PCRs. On the contrary, African trypanosome endemic areas are distinct from *Leishmania* foci, but in Uganda already both parasites occur [20], indicating a potential migration that could lead to both parasites being sympatric in certain regions in the future. None of the three newly designed PCRs amplified any of the trypanosomes. As for other organisms that potentially could cause mixed infections, such as bacteria, yeasts, and viruses, those included in the study did not amplify with any of the PCRs tested, except for an *M. tuberculosis* culture sample from which a product of different size as compared to *Leishmania* was amplified in PCR-C. These results highlight the potential of PCR-F, PCR-N, and PCR-C to be used as a first line molecular diagnostic tool to complement microscopy and simple serological tests where available, whereby a positive amplification is indicative of leishmaniasis. The amplified amplicon can then subsequently be digested in order to determine the species or species complex. It must be noted that for diagnostic purposes the PCRs should be evaluated in every specific setting, and negative PCRs must be confirmed with the most sensitive assays available, such as 18S rDNA [9] or kDNA PCR [21, 22].

The PCR-RFLP experiments on DNA from strains and isolates generally corroborated the results from in silico analysis. It should be emphasized that species discrimination for all RFLPs is based upon a step-wise approach, whereby a first digest (upper panels in Fig. 2) discriminates among broader groups, if needed followed by additional digests (lower panels in Fig. 2) to discriminate within these groups. Table ESM-2 gives an overview of the digests needed to discriminate all groups in the three RFLPs. Certainly upon implementation in a new setting, but also as a general practice, it is highly recommended to run all samples next to well-characterized references in order to compare fragment sizes on gel.

In the New World, identification is important for instance in the subgenus *L. (Viannia)*, as some species can cause mucocutaneous disease, and a differential response to antimonial treatment has been documented [5, 23]. *L. guyanensis*, *L. panamensis*, *L. lainsoni*, *L. peruviana*, and *L. infantum* (syn. *L. chagasi*) can be discriminated using RFLP-F and RFLP-N, with the exception of *L. guyanensis* and *L. panamensis* being identical in RFLP-N (Table ESM-2). Species from the *L. mexicana* complex (*L. mexicana*, *L. garnhami*, *L. amazonensis*) can be distinguished as a group, but cannot be classified into their respective species. This is not surprising as also sequence analysis failed to cluster them accordingly [12], questioning their monophyly and species status. The discrimination of *L. braziliensis* and *L. naiffi* is not straightforward, as they often share the same pattern in RFLP-F and RFLP-N. For *L. naiffi*, the only exception is RFLP-F when using *HaeIII*, showing an additional fragment for one of the two tested isolates (Fig. 2a). Separating both species is necessary when sympatric transmission occurs, as in Brazil, where discrimination was suggested on the basis of PCR-G and restriction endonuclease *BstUI* [24]. However, not all *L. braziliensis* isolates are identical to *L. naiffi*, as almost half of them (9/19 bra* in Table 1 and ESM-1, see also Table ESM-2) displayed a mixed pattern, combining the *L. braziliensis* fragments with those of *L. peruviana*, being indicative of the presence of at least two *hsp70* alleles. They originated from a wide geographical area (Bolivia and Peru), and may represent *L. peruviana* – *L. braziliensis* hybrids [25, 26], or genetic variation present within *L. braziliensis*. As yet, the clinical relevance of discriminating these parasites with both alleles is unclear. In conclusion, RFLP-F is recommended for species determination in the New World if *L. guyanensis* needs to be discriminated from *L. panamensis*. PCR-N has a slightly better analytical sensitivity as PCR-F (Fig. 1), and is expected to be less sensitive to PCR inhibitors because of its smaller size (Table 2), and can thus be used as an alternative. RFLP-C is of very limited use for separating New World species (Table ESM-2).

All medically important species from the Old World [27] can be identified by RFLP-F and RFLP-C, while in RFLP-N

no distinction can be made between *L. tropica* and *L. aethiops*. As RFLP-C is based upon a smaller PCR fragment, it may be less inhibited in clinical and biological specimen, and is therefore the method of choice. Taking into consideration the broad potential of RFLP-F across the entire globe, it might be a convenient option for travel clinics dealing with import *Leishmania* of unknown origin. In principle, *L. major* can be distinguished from *L. donovani* and *L. infantum* in RFLP-F after the first digest with *HaeIII* (Fig. 2a), but the size difference of the largest fragment is only 13 base pairs, often necessitating a second confirmation digest with *HindIII*, as is the case in RFLP-C (Fig. 2c). Discriminating *L. donovani* from *L. infantum* requires yet an additional digest both in RFLP-F and RFLP-C.

Which one of the four now available PCRs (Table 2) is the most adequate, depends upon the particular situation. Origin of infection, sympatry of species, imported versus endemic pathology, clinical presentation, and type of samples, are determinants that will govern the choice of the most appropriate typing assay. Other studies have proposed PCR-based methods for distinguishing among neotropical and/or Old World *Leishmania* [24, 28–33]. Even though some of these included additional species as compared to those analyzed here, they generally focused on a more confined geographical representation of the genus. In addition, restriction digest schemes were often more complicated than those proposed here.

In conclusion, we have designed an adequate and flexible toolbox for diagnosing and typing most medically relevant *Leishmania* species worldwide. Specificity and sensitivity were significantly increased in relation to previously described *hsp70* assays. The proposed methods can therefore be applied in an endemic context, as well as in travel clinics dealing with import leishmaniasis. Analysis of *hsp70* can be advocated as a standardized analysis tool for *Leishmania* typing, whereby RFLP could also be replaced by sequencing of the proposed amplicons wherever available. Currently, validation of the technology in endemic and travel clinic settings is ongoing, and we anticipate that our method can contribute significantly to case management, and epidemiological as well as clinical studies.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Yamey G, Torreele E (2002) The world's most neglected diseases. *British Med J* 325:176–177, Erratum *British Med J* 325:352
2. Alvar J, Yactayo S, Bern C (2006) Leishmaniasis and poverty. *Trends Parasitol* 22:552–557
3. Herwaldt BL (1999) Leishmaniasis. *Lancet* 354:1191–1199
4. Arévalo J, Ramírez L, Adauí V, Zimic M, Tulliano G, Miranda-Verástegui C, Lazo M, Loayza-Muro R, De Doncker S, Maurer A, Chappuis F, Dujardin JC, Llanos-Cuentas A (2007) Influence of *Leishmania* (*Viannia*) species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. *J Infect Dis* 195:1846–1851
5. Llanos-Cuentas A, Tulliano G, Araujo Castillo R, Miranda-Verástegui C, Santamaría-Castrellon G, Ramírez L, Lazo M, De Doncker S, Boelaert M, Robays J, Dujardin JC, Arévalo J, Chappuis F (2008) Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous leishmaniasis in Peru. *Clin Infect Dis* 46:223–231
6. García AL, Parrado R, De Doncker S, Bermúdez H, Dujardin JC (2007) American tegumentary leishmaniasis: direct species identification of *Leishmania* in non-invasive clinical samples. *Trans R Soc Trop Med Hyg* 101:368–371
7. Pérez JE, Veland N, Espinosa D, Torres K, Ogasuku E, Llanos-Cuentas A, Gamboa D, Arévalo J (2007) Isolation and molecular identification of *Leishmania* (*Viannia*) *peruviana* from naturally infected *Lutzomyia peruensis* (Diptera: Psychodidae) in the Peruvian Andes. *Mem Inst Oswaldo Cruz* 102:655–658
8. Ampuero J, Pereira Rios A, Carranza-Tamayo CO, Romero GAS (2009) Genus-specific kinetoplast-DNA PCR and parasite culture for the diagnosis of localized cutaneous leishmaniasis: applications for clinical trials under field conditions in Brazil. *Mem Inst Oswaldo Cruz* 104:992–997
9. Deborggraeve S, Laurent T, Espinosa D, Van der Auwera G, Mbuchi M, Wasunna M, El-Safi S, Al-Basheer AA, Arévalo J, Miranda-Verástegui C, Leclipteux T, Mertensa P, Dujardin JC, Herdewijn P, Büscher P (2008) A simplified and standardized polymerase chain reaction format for the diagnosis of leishmaniasis. *J Infect Dis* 198:1565–1572
10. Gadisa E, Kuru T, Genet A, Engers H, Aseffa A, Gedamu L (2010) *Leishmania donovani* complex (Kinetoplastida, Trypanosomatidae). Comparison of deoxyribonucleic acid based techniques for typing isolates from Ethiopia. *Exp Parasitol* 126:203–208
11. Montalvo AM, Fraga J, Montano I, Monzote L, Marín M, Van der Auwera G, Dujardin JC, Vélez ID, Muskus C (2010) Differentiation of *Leishmania* (*Viannia*) *panamensis* and *Leishmania* (*V.*) *guyanensis*

- sis using *BceI* for *hsp70* PCR-RFLP. *Trans R Soc Trop Med Hyg* 104:364–367
12. Fraga J, Montalvo AM, De Doncker S, Dujardin JC, Van der Auwera G (2010) Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. *Infect Genet Evol* 10:238–245
 13. Montalvo AM, Fraga J, Monzote I, Montano I, De Doncker S, Dujardin JC, Van der Auwera G (2010) Heat-shock protein PCR-RFLP: a universal simple tool for *Leishmania* species discrimination in the New and Old World. *Parasitology* 137:1159–1168
 14. García AL, Kindt A, Bermúdez H, Llanos-Cuentas A, De Doncker S, Arévalo J (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
 15. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599
 16. Rodríguez N, Guzman B, Rodas A, Takiff H, Bloom BR, Convit J (1994) Diagnosis of cutaneous leishmaniasis and species discrimination of parasites by PCR and hybridization. *J Clin Microbiol* 32:2246–2252
 17. Ramírez JR, Agudelo S, Muskus C, Alzate JF, Berberich C, Barker D, Vélez ID (2000) Diagnosis of cutaneous leishmaniasis in Colombia: the sampling site within lesions influences the sensitivity of parasitological diagnosis. *J Clin Microbiol* 38:3768–3773
 18. Bastrenta B, Mita N, Buitrago R, Vargas F, Flores M, Machane M, Yacsik N, Torrez N, Le Pont F, Brenière F (2003) Human mixed infections of *Leishmania* spp. and *Leishmania-Trypanosoma cruzi* in a sub Andean Bolivian area: identification by polymerase chain reaction/hybridization and isoenzyme. *Mem Inst Oswaldo Cruz* 98:255–264
 19. Mendes DG, Lauria-Pires L, Nitz N, Lozzi SP, Nascimento RJ, Monteiro PS, Rebelo MM, Rosa Ade C, Santana JM, Teixeira AR (2007) Exposure to mixed infections with *Trypanosoma cruzi*, *Leishmania braziliensis* and *Leishmania chagasi* in the human population of the greater Amazon. *Trop Med Int Health* 12:629–636
 20. Mugasa CM, Deborggraeve S, Schoone GJ, Laurent T, Leeftang MM, Ekangu RA, El-Safi S, Saad AA, Basiye FL, De Doncker S, Lubega GW, Kager PA, Büscher P, Schallig HDFH (2010) Accordance and concordance of PCR and NASBA followed by oligochromatography for the molecular diagnosis of *Trypanosoma brucei* and *Leishmania*. *Trop Med Intern Health* 15:800–805
 21. Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL (2006) Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. *J Clin Microbiol* 44:1435–1439
 22. Romero GAS, Ferreira-Noronha E, Pímez C, Silva-Pires FES, Fernandes O, Saad-Nehmec N, Cupolillo E, Firoozmand L, Cardoso da Graca G, Volpini A, Lopes-Santos S, Romanha AJ (2009) Sensitivity and reproducibility of a PCR assay for *Leishmania* detection using skin biopsy imprints on filter paper. *Acta Trop* 109:74–77
 23. Arévalo J, Ramírez L, Adai V, Zimic M, Tulliano G, Miranda-Verástegui C, Lazo M, Loayza-Muro R, De Doncker S, Maurer A, Chappuis F, Dujardin JC, Llanos-Cuentas A (2007) Influence of *Leishmania (Viannia)* species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. *J Inf Dis* 195:1846–1851
 24. da Silva LA, de Sousa CdS, da Graça GC, Porrozzini R, Cupolillo E (2010) Sequence analysis and PCR-RFLP profiling of the *hsp70* gene as a valuable tool for identifying *Leishmania* species associated with human leishmaniasis in Brazil. *Infect Genet Evol* 10:77–83
 25. Dujardin JC, Bañuls AL, Llanos-Cuentas A, Alvarez E, De Doncker S, Jacquet D, Le Ray D, Arévalo J, Tibayrenc M (1995) Putative *Leishmania* hybrids in the Eastern Andean valley of Huanuco, Peru. *Acta Trop* 59:293–307
 26. Nolder D, Roncal N, Davies CR, Llanos-Cuentas A, Miles MA (2007) Multiple hybrid genotypes of *Leishmania (Viannia)* in a focus of mucocutaneous leishmaniasis. *Am J Trop Med Hyg* 76:573–578
 27. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, Boelaert M (2007) Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* 5:873–882
 28. Cupolillo E, Grimaldi G, Momen H, Beverley SM (1995) Intergenic Region Typing (IRT) A rapid molecular approach to the characterization and evolution of *Leishmania*. *Mol Biochem Parasitol* 73:145–155
 29. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I (2003) Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the minixon sequence and subsequent restriction fragment length polymorphism analysis. *J Clin Microbiol* 41:3147–3153
 30. Marfurt J, Niederwiese I, Divine MN, Beck HP, Felger I (2003) Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP. *Diagn Microbiol Infect Dis* 46:115–124
 31. Rotureau B, Ravel C, Couppie P, Pratlong F, Nacher M, Dedet JP, Carme B (2006) Use of PCR-restriction fragment length polymorphism analysis to identify the main new world *Leishmania* species and analyze their taxonomic properties and polymorphism by application of the assay to clinical samples. *J Clin Microbiol* 44:459–467
 32. Oshaghi MA, Ravasan NM, Hide M, Javadian EA, Rassi Y, Sedaghat MM, Mohebbi M, Hajjarian H (2009) Development of species-specific PCR and PCR-restriction fragment length polymorphism assays for *L. infantum* / *L. donovani* discrimination. *Exp Parasitol* 122:61–65
 33. Simon S, Veron V, Carme B (2010) *Leishmania* spp. identification by polymerase chain reaction-restriction fragment length polymorphism analysis and its applications in French Guiana. *Diagn Microbiol Infect Dis* 66:175–180