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ARTICLE

Universal PCR assays for the differential detection of all Old World *Leishmania* species

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Abstract For the epidemiological monitoring and clinical case management of leishmaniasis, determination of the causative *Leishmania* species gains importance. Current assays for the Old World often suffer from drawbacks in terms of validation on a geographically representative sample set and the ability to recognize all species complexes. We want to contribute to standardized species typing for Old World leishmaniasis. We determined the ribosomal DNA internal transcribed spacer 1 sequence of 24 strains or isolates, and validated four species-specific polymerase chain reactions (PCRs) amplifying this target. They discriminate *L. aethiopica*, *L. tropica*, *L. major*, and the *L. donovani* complex, use the same cycling conditions, and include an internal amplification control. Our PCRs amplify 0.1 pg of *Leishmania* DNA, while being 100% specific for species identification on an extensive panel of geographically representative strains and isolates. Similar results were obtained in an endemic reference laboratory in Kenya. Species could also be identified in clinical specimens. The presented PCRs require only agarose gel detection, and have several other advantages over many existing assays. We outline potential problems, suggest concrete solutions for transferring the technique to other settings, and deliver the proof-of-principle for analyzing clinical samples.

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Introduction

Leishmaniasis describes a spectrum of tropical and subtropical diseases, which range from self-healing localized cutaneous lesions to aggravated visceral disease with multi-organ involvement, often fatal if left untreated. The diseases are endemic in 88 countries, where an estimated 12 million people are infected, while 350 million being at risk [1, 2]. The etiological agents are parasites of the kinetoplastid genus *Leishmania*, in which two subgenera and some 20 medically important species are currently recognized [3]. *Leishmania* distribution is dichotomized into the Old (East-Africa, the Mediterranean region, and the Indian subcontinent) and the New (Central and South-America) World groups. Five species are endemic in the Old World: *L. major*, *L. aethiopica*, *L. tropica*, *L. donovani*, and *L. infantum*, whereby the latter two comprise the so-called *L. donovani* complex. The distribution of these species is highly dynamic and is expected to be increasingly modulated by global climate changes and associated demographic developments, making species identification on the basis of geographical origin unreliable [4].

The characterization of *Leishmania* species is important for clinical case management and the epidemiological monitoring of the parasite spread [5–7]. Cutaneous leishmaniasis in the Old World can be caused by any of the four

following species, each with different disease progression characteristics: *L. infantum* can also visceralize and cause the lethal visceral leishmaniasis; *L. major* is often self-curing [8]; *L. aethiopica* and *L. tropica* are recalcitrant to treatment [9, 10]. Visceral leishmaniasis caused by *L. donovani* in East-Africa often does not respond to first-line therapy [11], and is frequently associated with a secondary pathology called post-kala-azar dermal leishmaniasis [12]. As species cannot be identified on the basis of morphology and clinical symptoms alone [13, 14], other techniques are needed.

The current gold standard for species discrimination is multi-locus enzyme electrophoresis [15], a cumbersome technique requiring mass parasite cultivation. Molecular methods based upon the polymerase chain reaction (PCR) amplification of a *Leishmania* DNA fragment have replaced this technique for practical purposes. Many such PCR assays have been reported in the literature (reviewed briefly in [16] for the Old World), but they mostly suffer from a lack of validation on all species, fail testing a geographically representative panel of isolates to account for intra-species variability, and are often hampered by limited sensitivity. In addition, several assays require post-PCR handlings not always available in endemic regions, such as sequencing, restriction digests, or melting curve analysis, which are required to reveal polymorphisms within the amplified fragment.

The internal transcribed spacer 1 of the ribosomal DNA repeat unit (rDNA-ITS1) has previously been exploited for Old World species discrimination [17] using restriction fragment length polymorphisms [18–20], reverse hybridization assays [21], and melting curve analysis [22]. There are an estimated 20 to 200 identical copies in the *Leishmania* genome, making it a good target for analyzing low parasite quantities. In this study, we describe four species-identification PCRs based on rDNA-ITS1 for the discrimination of *L. aethiopica*, *L. tropica*, *L. major*, and the *L. donovani* complex. We also determined the sequence of this region from 24 isolates or strains.

Materials and methods

Reference strains

DNAs from reference strains and isolates were obtained from cultured parasites, and were either grown and extracted at the Institute of Tropical Medicine Antwerp (ITMA, Antwerp, Belgium) or kindly donated by several institutes acknowledged at the end of this manuscript. Concentration of the parasite DNA was measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Isolates were characterized by rDNA-ITS1 sequencing or a discriminative PCR targeting cysteine proteinase B [16]. For sequencing, PCR amplicons (Fig. 1) were generated with primers rDNA-10 F (5′ CAATACAGGTGATCGGACAGG 3′) and rDNA-14R (5′ CACGGGGATGACACAATAGAG 3′), and this was followed by direct sequencing without cloning. Table 1 lists the origin of all samples used during the evaluation and validation steps, and also the accession numbers of the determined rDNA-ITS1 sequences. The definitions of *L. infantum* and *L. donovani* are as in [23].

Primers and internal controls

Species-specific PCR primers were designed on the basis of an alignment of rDNA-ITS1 sequences newly determined

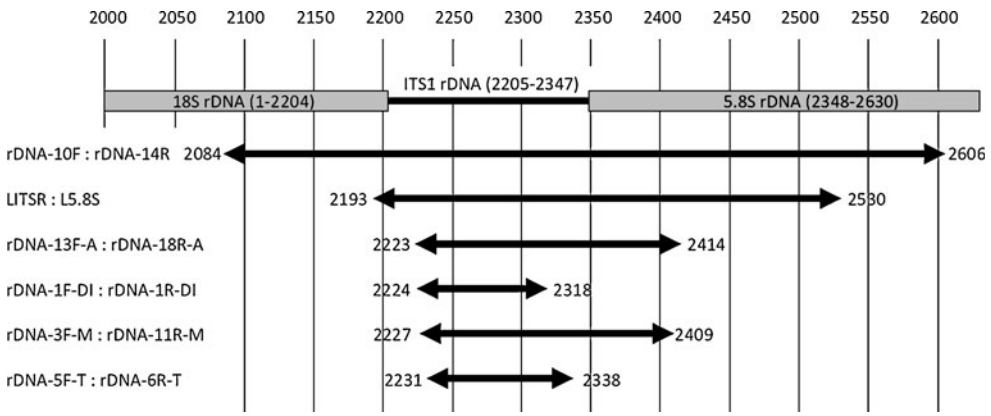

Fig. 1 Position of the sequenced and species-specific polymerase chain reaction (PCR) fragments relative to the first nucleotide of the 18 S rDNA gene in chromosome 27 of *Leishmania major* strain Friedlin (http://www.ebi.ac.uk, accession number CP000079, release 102), drawn to scale. The 18 S, ITS1, and 5.8 S rDNA fragments are indicated at the top, according to the current annotation. The primers are as in Table 2, and the annealing position of the 5′ base is indicated, also delineating the start and end of each amplicon. As the annealing position of all primers is given relative to *L. major*, the fragment sizes calculated from these do not correspond to those indicated in Table 2, except for *L. major* itself

Table 1 Origin of strains used for the evaluation and validation

Species	Country	Evaluation[a]	Validation[a]	Accession numbers[b]
L. aethiopica	Ethiopia	8	8	FN677344, FN677347, FN677348, FN677349, FN677350, FN677351, FN677352, FN677353, FN677354, FN677355, FN677356
	Kenya	2	2	FN677346
L. donovani[c]	Ethiopia	2	2	
	India	2		
	Kenya		1	FN677363, FN677364
	Sudan	2	3	FN677358, FN677359, FN677360, FN677361, FN677362
L. infantum[c]	France	1	1	
	Italy	1	2	
	Malta		1	
	Portugal	1		
	Spain	3	2	
L. major	Unknown	2	1	
	Burkina Faso		1	
	Israel	1	1	
	Kenya	1	1	
	Saudi Arabia	1	1	
	Spain	1	1	
	Sudan	1	1	
	Tunisia	1	1	FN677342
	USSR (former Soviet Union)	1	1	
	Uzbekistan	1	1	FN677357
L. tropica	Unknown		2	
	Iraq	1	1	
	Israel	2	2	FN677341
	Kenya	4	2	
	Palestinian territory	2	2	FN677343, FN677345
	USSR (former Soviet Union)	1	1	

[a] Where possible, different parasite strains were used for the evaluation and validation

[b] Accession numbers are listed according to species and origin

[c] Species of the *L. donovani* complex (*L. donovani* and *L. infantum*) are defined as in [23]

(Table 1) and downloaded from the EBI sequence database (http://www.ebi.ac.uk). Several primer combinations per species were tested, and a final set (Fig. 1, Table 2) was selected for extensive optimization, evaluation, and validation experiments. For each of the four species-specific PCRs, internal positive control templates were developed (Figs. 2 and 3). These internal controls are added to the respective PCRs, and upon successful amplification, they show a product of about 100 bp longer than the *Leishmania* species-specific amplicon. Such internal controls allow to check for correct PCR mix setup and sample inhibition. To obtain these internal controls, phage lambda DNA fragments were amplified with composite cloning primer pairs, each consisting of a 3′ lambda-specific segment with a *Leishmania* species-specific 5′ extension matching the PCR primers in Table 2. The amplified phage lambda sequences were selected to have a base composition comparable to the respective *Leishmania* targets. The phage lambda amplicons, flanked by the *Leishmania* primer annealing sites, were cloned in vector pCR4-TOPO using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), and verified by sequencing (Fig. 2).

Species-specific PCRs

Five PCRs were used in this study, which are listed in Table 2 and depicted schematically in Fig. 1. The total reaction volume was 25 μl of 1× CoralLoad PCR buffer (Qiagen, Venlo, The Netherlands), including 0.1 mg/ml acetylated BSA (Promega, Madison, WI, USA), 200 μM of

Table 2 Overview of species-specific polymerase chain reactions (PCRs)[a]

PCR	PCR specificity	Amplicon size *Leishmania*[b]	Internal control[c]	Amplicon size internal control	$MgCl_2$ (mM)[d]	Polymerase (units)	Primers (sequences 5′ to 3′)[e]		Concentration primers (μM)
LA	*L. aethiopica*	199	pICR1Aet21 (5.61×10^{-8} ng)	303	2.5	0.5	rDNA-13F-A	(AAAATATACAAAACTCGGGC)	0.5
							rDNA-18R-A	(CCGCCAACAAAAGACGG)	0.5
LDI	*L. donovani* complex	93	pICR1Don2 (6.67×10^{-9} ng)	220	2.5	0.5	rDNA-1F-DI	(AAACATATACAACTCGGGGAGA)	0.1
							rDNA-1R-DI	(TTACTGCAAATTTTGAGTACAAAAC)	0.8
LM	*L. major*	183	pICR1Maj10 (5.23×10^{-9} ng)	285	2	0.5	rDNA-3F-M	(CATATACAACTCGGGGAGGCT)	0.5
							rDNA-11R-M	(AAAAACCGAAACGCCGTC)	0.5
LT1	*L. tropica*	112	pICR1Tro45 (5.94×10^{-8} ng)	206	2.5	0.5	rDNA-5F-T	(CAAAACTCGGGGAGGCCTATA)	0.5
							rDNA-6R-T	(ATAASGTCGATCGGCCTTTTG)	0.5
LT2	*L. tropica*	112	pICR1Tro45 (5.94×10^{-8} ng)	206	3.0	1.0	rDNA-5F-T	(CAAAACTCGGGGAGGCCTATA)	0.5
							rDNA-6R-T	(ATAASGTCGATCGGCCTTTTG)	0.5
							LITSR	(CTGGATCATTTTCCGATG)	0.02
							L5.8S	(TGATACCACTTATCGCACTT)	0.02

[a] Only reagents differing between the PCRs are listed. Common reagents in all PCRs (buffer, BSA, dNTPs, dUTP) are described in the Materials and methods section

[b] These amplicon sizes can deviate with a few nucleotides, depending on the exact strain or isolate

[c] Internal control constructs are depicted in Fig. 2, and the amount used in each PCR is given in parentheses. The concentration of the internal control template was estimated spectrophotometrically using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA)

[d] This is the final concentration used in the PCR, inclusive of the 1.5 mM already present in the CoralLoad PCR buffer

[e] Primer annealing positions are depicted schematically in Fig. 1

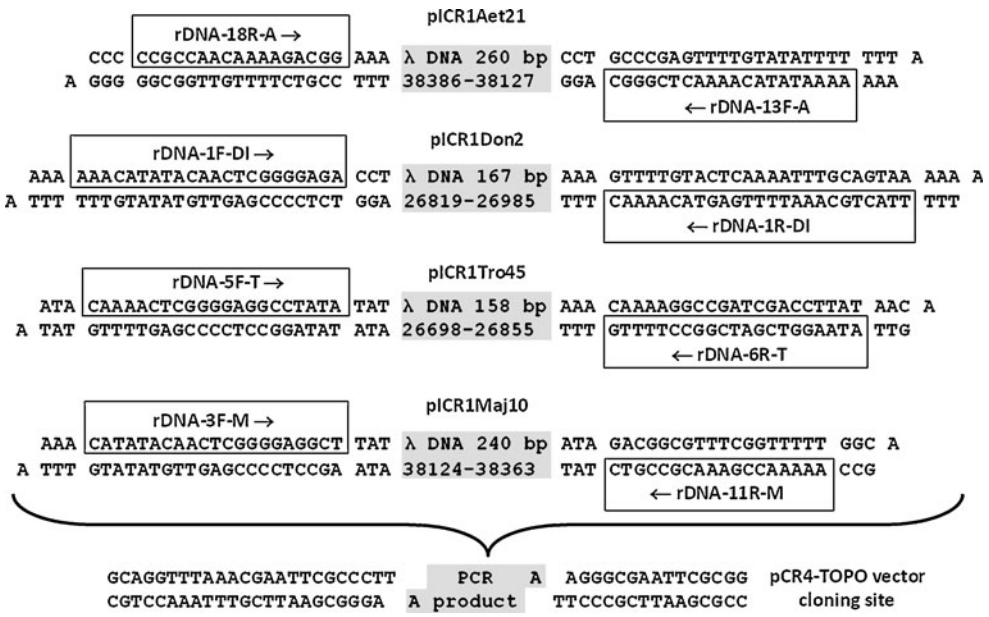

Fig. 2 Overview of the four cloned PCR products used as internal controls (Table 2). These PCR products were cloned in the pCR4-TOPO vector (http://tools.invitrogen.com/content/sfs/vectors/pcr4topo_map.pdf), from which the cloning site sequence is shown below. Amplicons were inserted using the T:A overhang strategy, and the orientation in the obtained plasmids is as shown in the figure. The internal phage lambda fragments are described in the *gray boxes*, with positions referring to accession J02459 (http://www.ebi.ac.uk, accession number J02459, release 102). The regions corresponding to the *Leishmania* species-specific primer sequences (Table 2) are indicated in the *outlined boxes*, with the *arrows* showing the 5′–3′ orientation

each dNTP supplemented with 400 μM of dUTP, in a 200-μl tube or plate. Other components are listed in their respective concentrations in Table 2, and HotStarTaq Plus DNA Polymerase (Qiagen) was used in each case. PCR LT2 was not used in the evaluation and validation experiments. Primers were ordered from Sigma-Aldrich (Bornem, Belgium), dNTPs from Eurogentec (Seraing, Belgium), and dUTPs from Roche (Vilvoorde, Belgium). Cycling conditions for all PCRs were identical: 5 min at 95°C; followed by 40 cycles of 30 s at 95°C, 40 s at 55°C, and 40 s at 72°C; and finally 5 min at 72°C. All PCRs were performed in a BioMetra T3000 thermocycler (BioMetra, Göttingen, Germany). The products were analyzed on a 2% or 2.5% agarose gel stained with ethidium bromide, and scored positive when an amplicon of the expected size (Table 2) was observed.

Optimization and evaluation

Following the development of the PCR protocols, the detection limit of the PCRs was assessed by using 10, 1, 0.1, and 0.01 pg DNA from five different parasite isolates or strains from each species. This was repeated with the addition of DNA extracted from 9 μl naïve human blood in each PCR, for which 2.5 μl of 180 μl blood extract eluted in 50 μl was added to the reaction. The discriminatory power for identifying the correct species was assessed using 100 pg of the DNAs listed in Table 1. The optimal

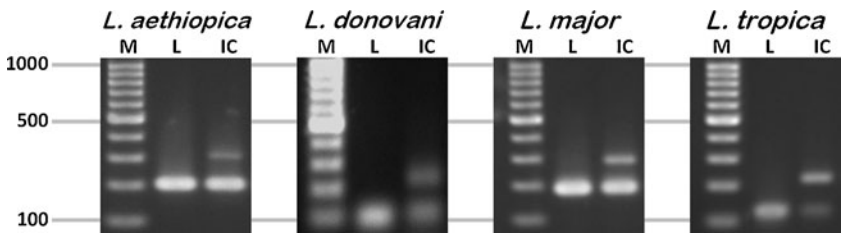

Fig. 3 Agarose gel image of the *Leishmania* species-specific PCR products (*L*), and the same products with internal control amplicon (*IC*). The GeneRuler 100 bp DNA size marker (Fermentas, St.Leon-Rot, Germany) is depicted on the left (*M*), with indicated fragments of 100 (smallest fragment), 500 (brightest fragment), and 1,000 (largest fragment) nucleotides. The expected sizes are shown in Table 2

concentration of the recombinant internal control plasmids was determined as the lowest concentration that consistently amplified in repeated experiments, as tested at least in triplicate in a 10-fold dilution series, followed by further refining by using four intermediate concentrations. The sensitivity of the internal control amplification to PCR inhibitors was tested by adding 0.01, 0.1, and 1% of sodium dodecyl sulphate (SDS) to the reaction.

Validation and implementation

For the purpose of validating the final PCR protocols, a blinded test panel was compiled, including the strains listed in Table 1. DNA from each strain was included twice in the panel: once at an amount of 100 pg per PCR, and once at an amount of 0.2 pg including a DNA equivalent of 1 μl naïve human blood per PCR. In addition, the panel contained three negative controls and three naïve blood extracted DNAs as a quality check for contamination and cross-reactivity. This makes a total of 90 samples, which were supplemented by six negative controls by the person performing the test. Positive results were considered to be valid if no contamination was observed in these six added negative controls, and an amplicon of the expected *Leishmania* size (Table 2) was obtained. Negative PCR results were valid if no product of the expected size was observed, and the internal control amplicon (Table 2, Fig. 2) was amplified (Fig. 3). In case one of these conditions was not met, the PCR was repeated. All PCR mixes were prepared in bulk, containing all reagents except polymerase and *Leishmania* template. They were stored at −20°C and, when needed, an aliquot was taken, to which polymerase and template were added. For the purpose of implementation in an endemic reference laboratory, the tests were transferred to the Kenya Medical Research Institute (KEMRI, Nairobi, Kenya), where PCR mixes were produced locally. The tests in KEMRI were performed by a different person to those at ITMA, but using the same blinded test panel DNA.

Clinical samples

We tested our assays on various clinical samples, containing different species of *Leishmania* and other pathogens. *L. donovani* samples were obtained from the blood, bone marrow, and lymph node of visceral leishmaniasis patients from Sudan. *L. infantum* samples were obtained from the bone marrow of Italian visceral leishmaniasis patients and spleen taken from Portuguese dogs suffering from canine leishmaniasis. *L. major* and *L. tropica* samples were obtained from the skin lesions of cutaneous leishmaniasis patients in Tunisia and Kenya. In addition, our assays were performed on the blood from patients suffering from *Plasmodium falciparum* malaria (Uganda) and human African trypanosomiasis (Democratic Republic of Congo), and on the sputum from tuberculosis patients (Democratic Republic of Congo). DNA from these samples was extracted using various methods, and the laboratories acknowledged at the end of this manuscript contributed to the test set.

Results

Sequences

The determined rDNA-ITS1 sequences were submitted to the EBI sequence database, and were assigned the accession numbers listed in Table 1.

Optimization and evaluation

Internal control templates and amplicons were successfully obtained and are depicted in Figs. 2 and 3. Their amplification was inhibited completely by 0.01% SDS, indicating their ability to detect PCR inhibitors. All PCRs showed the potential to amplify as little as 0.1 pg of DNA, which is about half a parasite's genome. This detection limit was unaffected by the presence of DNA isolated from human blood, nor by the addition of the internal control template in its proper limiting concentration. For some DNA samples, the detection limit was 1 pg; for others, it was possible to amplify 0.01 pg, variations probably caused by DNA degradation. No cross-reactivity of any specific PCR to other species was observed during the evaluation phase, except for one out of ten *L. aethiopica* DNAs that reacted in the LM PCR specific for *L. major*.

Validation

Table 3 lists the results of a blinded panel validation, containing at least ten isolated DNAs of each species or species complex. The results obtained at ITMA are given, as well as those obtained at KEMRI when discordant. In total, six assays were included in the validation, both using 100 or 0.2 pg parasite DNA, the latter spiked with naïve human blood DNA. The six assays comprise the four species-specific assays LA, LDI, LM, and LT1 (Table 2) evaluated separately, but also as a combined tool (fifth assay). In the separate evaluation, only the results of one PCR at a time are taken into account (positive or negative), while in the combined assay, results from the four separate PCRs are considered simultaneously. In the latter case, a species will be assigned only if one out of four PCRs scores positive, and is listed as undetermined if none or more than

Table 3 Overview of the blinded panel validation results

		Sample type or *Leishmania* species				
		L. aethiopica	*L. donovani* complex	*L. major*	*L. tropica*	Negative[a]
	Total number of samples[b]	10	12	10	10	3
100 pg	LA PCR[c]	10	0	0	0	0
	LDI PCR[c]	0	12	0	0	0
	LM PCR[c]	0	0	10	0	0
	LT1 PCR[c]	1[g]	0	0	10	0
	Combined assay correct[e]	9	12	10	10	
	Combined assay incorrect[e]	0	0	0	0	
	LT2 PCR[c, f]	2	0	0	10	0
0.2 pg	LA PCR[c]	8 (9)[d]	0	0	0	0
	LDI PCR[c]	0	11	0	0	0
	LM PCR[c]	0[g]	0	8 (10)[d]	0	0
	LT1 PCR[c]	1	0	0	4	0
	Combined assay correct[e]	8 (9)[d]	11	8 (10)[d]	4	
	Combined assay incorrect[e]	1 (*L. tropica*)[g]	0	0	0	
	LT2 PCR[c, f]	2	0	0	7	0

[a] Negatives are TE in the 100-pg panel and naïve human blood DNA in the 0.2-pg panel

[b] The geographic origin of the isolates and strains is listed in Table 1

[c] See Table 2. For each PCR assay, the number of positive PCRs per species or negative sample type is given

[d] When there was no agreement between the numbers for the ITMA and KEMRI validation, the KEMRI result is given in parentheses

[e] For the combined assay, the number of correctly and incorrectly identified isolates from each species is listed, as determined from a parallel evaluation of LA, LDI, LM, and LT1 PCRs

[f] The LT2 PCR was not tested in KEMRI, and was not taken into account in the combined assay

[g] The *L. aethiopica* isolate MHOM/ET/67/L86 was identified as *L. tropica*

one PCR shows a positive result. The reason for evaluating the PCRs separately is that, in reality, it is unlikely that all four PCRs are performed on all samples, and, more often than not, a PCR will be used to confirm only the expected species. Finally, PCR LT2 makes up the sixth assay, which was performed following the validation of the other assays because of a poor sensitivity of LT1.

Implementation

As a proof-of-principle to assess the performance when using the developed PCRs in another laboratory, the species-specific PCRs were implemented and validated at KEMRI, a reference laboratory in Kenya, where leishmaniasis is endemic. Even though the same products and type of thermocycler were used, there was a problem in the amplification of the internal control amplicons, as well as with a slight contamination in the *L. donovani* complex PCR. This contamination showed a very faint PCR product and was visible in one out of the six user-added negative controls, but in none of the negative controls of the blinded test panel. When ignoring these faint amplicons, and the fact that, in many negative PCRs, no internal control amplicon was amplified, the results were almost identical to those obtained at ITMA (Table 3). PCR LT2 (Table 2) was not tested in KEMRI.

Clinical sample analysis

Figure 4 depicts the results of four species-specific PCRs in clinical samples. The species could be successfully identified in the various *Leishmania* samples, as only one of the four PCRs showed a *Leishmania* product of the correct size (Table 2). In all other cases, only the internal control amplicon was amplified. In LT2, occasionally, a weak PCR product is seen in samples not containing *L. tropica*, but this product is slightly smaller than 100 bp, and clearly below the molecular weight of the product seen in the actual *L. tropica* samples. Equally so, a very weak amplicon is observed from the LA PCR in the left skin lesion scrapping *L. tropica* sample, but this is much fainter than the LT2 PCR amplicon. No products were amplified from patients suffering from tuberculosis, sleeping sickness, or malaria.

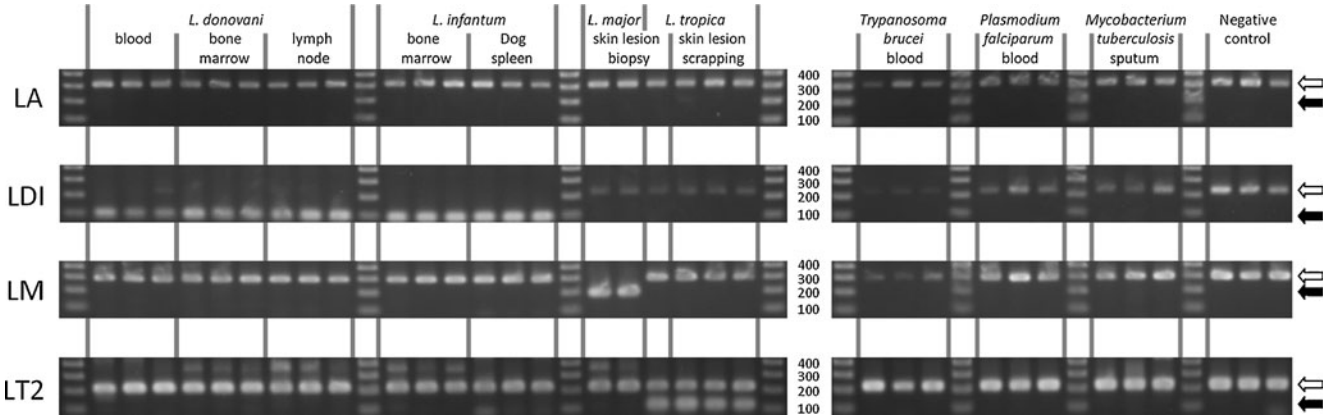

Fig. 4 Results from four species-specific PCRs on clinical samples. The PCRs are indicated on the left, as in Tables 2 and 3. Species in the clinical samples are depicted on the top, as well as the type of sample analyzed. Only the agarose gel region between 100 and 400 bp is shown, as indicated by the size marker fragments identified between the left and right gel. The size of the amplified internal control and *Leishmania* fragments is as in Table 2 and Fig. 3, and is respectively indicated by white and black arrows on the right

Discussion

In this study, we present four ITS1-rDNA PCRs that can faithfully distinguish the Old World *Leishmania* species *L. aethiopica*, *L. major*, *L. tropica*, and the *L. donovani* species complex comprised of *L. donovani* and *L. infantum*. Because the number of sequences available for this region was limited for certain species, we added additional ones to the public repositories (Table 1).

Compared to other assays devoid of post-amplification steps other than agarose gel analysis (reviewed in [16]), our approach has several advantages. First, our assay is universally applicable in the Old World. Intra- and inter-species variability was validated by testing all species from diverse geographical origins (Table 1). It does not allow to differentiate *L. infantum* from other members of the *L. donovani* complex, for which alternatives are available [16, 24–26]. Second, the detection limit is sufficient to analyze clinical samples, as between 5 and 1/20 parasite genomes are amplifiable, and we showed amplification in actual clinical samples. Third, our assays include dUTP, which allows to counter the effect of amplicon contamination using the dUTP/UNG system [27]. This can be particularly important in low-resource settings, where basic precautions to avoid carry-over contamination are often not easily adhered to, leading to false-positive results. Fourth, all four assays use the exact same thermal amplification conditions, which allows parallel analysis. As a plus, PCR mixes containing all components except *Leishmania* template and enzyme can be stored at −20°C, facilitating batch quality control and saving time. Fifth, internal controls allow checking for consistent amplification across different runs and PCR sample inhibition [28]. Failure to amplify this control in a negative *Leishmania* sample points to the presence of inhibitors or an erroneously prepared PCR mix, in which case, no conclusions must be drawn from the PCR. In a positive sample, it might simply indicate that the *Leishmania* template outcompetes the control for primer annealing. Finally, as illustrated in Fig. 1, our assays can be used as a nested PCR in case of insufficient sensitivity, whereby the *Leishmania*-specific primers LITSR and L5.8S [29] generate the outer PCR amplicons. In such a case, all four specific PCRs must be run in parallel in the second PCR round to ensure specificity.

As can be seen in Table 3, when taking results from the validation PCRs LA, LDI, LM, LT1, and the combined assay, at the 100-pg level, the species discrimination and identification performance is 100%, except for *L. aethiopica* isolate MHOM/ET/67/L86, which cross-reacted in the LT1 PCR. Species identification of this isolate was, however, based on its unique zymodeme LON33 [30], and the rDNA-ITS1 region (accession FN677356) clearly showed an *L. tropica* sequence, explaining the positive amplification in the *L. tropica* PCR. Nevertheless, a limiting amount of *L. aethiopica* rDNA-ITS1 template is present in the DNA sample, as evidenced by the additional positive *L. aethiopica* PCR only at the 100-pg level, pointing to a hybrid strain or a mixed culture. The other PCRs showed no cross-reaction at the 0.2-pg level, but some isolates could not be identified. This was the case especially for *L. tropica*, where only four out of ten DNAs could be typed. Given the fact that amplification failure may be caused by DNA degradation or mismatched primers, it is not surprising that some are not detected at the 0.2-pg level, corresponding to one parasite only. As the *L. tropica* PCR, however, identified merely four out of ten *L. tropica* DNAs when supplied with 0.2 pg, we used an alternative PCR (LT2 in Table 2) including an outer *Leishmania*-specific DNA primer pair (Fig. 1, [29]) to boost the reaction. This allowed an additional three *L. tropica* to be identified from 0.2 pg, but resulted in an extra *L. aethiopica* DNA being detected (Table 3). LT2 must,

therefore, be used only in parallel with PCRs LA, LDI, and LM.

Upon evaluating the blinded test panel in an endemic reference center in Kenya, two problems were identified: the internal control amplicon did not amplify consistently, and a slight *L. donovani* contamination was observed in one out of six negative controls. Despite using the same products in the PCR mix, this illustrates the necessity to re-optimize the internal control template concentration during implementation. This becomes even more important when changing PCR reagents, such as the type of Taq DNA polymerase. Equally so, depending on the geographical region and type of clinical samples analyzed, one should be aware of potential cross-reactivity against species other than *Leishmania*. And even though our analysis did not identify cross-reactions against three other pathogens, such problems are region- and sample-dependent, and a validation should be part of any implementation process. In this respect, we also highlight that our assays are to be used in second line for *Leishmania* species identification, only after performing diagnostic—generally more sensitive—assays for parasite detection. During implementation, it is highly recommended to run all four PCRs in parallel to assess the performance of each individual PCR by comparing to the combined assay, as was done in Table 3.

Once validated, PCR mixes without DNA polymerase can be prepared in bulk and aliquoted, which allows quality control and saves time. When functioning properly, there is no need to use all four PCRs for analyzing each sample (combined assay in Table 3), and one could suffice by using an appropriate selection depending on the exact purpose of the study and the species expected to occur: in endemic settings, this would typically be the sympatric species; in travel clinics, all species can be found. At most, 100 pg *Leishmania* DNA should be used in the assays, as cross-reactivity for higher concentrations has not been checked for. In clinical samples, it is unlikely that a larger amount of *Leishmania* DNA is present, but if uncertain, it is advisable to run all four PCRs in parallel to increase the chance of finding unexpected cross-reactions.

Our assays were able to identify the infecting *Leishmania* species in various clinical samples (Fig. 4), and did not show cross-reaction against *Mycobacterium tuberculosis*, *Plasmodium falciparum*, or *Trypanosoma brucei*. These tests were performed as a proof-of-principle only, as in particular real-life settings, the diagnostic specificity and sensitivity must be determined using standardized sampling methods, and accounting for the *Leishmania* species and other pathogens circulating in the region.

Our study presents the first step towards a standardized Old World *Leishmania* species typing assay. It focuses on validation in cultured samples, with emphasis on the implementation of the PCRs in other settings, as a starting point for their use in clinical and epidemiological studies for which the proof-of-principle was delivered. Validation of the PCRs and potential unexpected reactions are essential topics to cover before starting any analysis. We are currently in the process of using the here reported PCRs in endemic reference laboratories on an extended set of clinical samples, and anticipate that the here described versatile PCRs can contribute to the adequate management and follow-up of Old World leishmaniasis.

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