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Leishmania (*Viannia*) Species Identification on Clinical Samples from Cutaneous Leishmaniasis Patients in Peru: Assessment of a Molecular Stepwise Approach

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We present an algorithm based on three PCR assays for *Leishmania* (*Viannia*) species identification and assessed its performance using 70 specimens from Peruvian patients. The succession of the assayed targets can be ordered according to species prevalence. Sequential progression through the algorithm reduced the number of samples here studied by approximately 30% after each step.

Cutaneous leishmaniasis (CL) is a vector-borne disease, which affects up to 1.5 million persons annually in tropical and subtropical regions worldwide (5). In several areas of endemicity like Peru, *Leishmania* species identification is important because different species with various clinical phenotypes coexist (11). There, higher incidence of mucocutaneous leishmaniasis (MCL) is attributed to *Leishmania* (*Viannia*) *braziliensis* (4), and low-pentavalent-antimonial treatment failures are associated with *Leishmania* (*Viannia*) *guyanensis* infections (1). Recognized associations between species and disease outcomes (1, 10, 12) have led the WHO expert committee on the control of leishmaniasis to recommend species identification for optimal treatment and control (16).

PCR identification of *Leishmania* (*Viannia*) from clinical specimens (8, 14, 15) has used different targets: mannose phosphate isomerase (*mpi*) (17) and cysteine proteinase B (*cpb*) and the 70-kDa heat shock protein (*hsp70*) (6, 7, 9). These targets have been used successfully on CL specimens collected by filter paper lesion impressions (FPLIs) (3).

Here, we report the development of a three-step strategy of PCR-based assays for *Leishmania* (*Viannia*) species identification and assess its performance on CL FPLIs. Reductions of time and cost to obtain results in areas where leishmaniasis is endemic were the criteria on which the algorithm was evaluated.

DNA isolation from cultured parasites and filter paper lesion impressions. International reference strains and cultured parasites obtained from 80 patients attending our reference center were harvested, and their DNA was extracted as previously described (13). DNAs from anonymized, FPLI specimens from 70 CL lesions collected from 57 Peruvian patients were obtained as reported previously (2, 3). Codes and geographical origins are presented in Table S1 in the supplemental material.

PCR assays for species identification. *Leishmania* (*Viannia*) species were identified using three PCR targets. The *mpi* gene was amplified in two separate reactions (*mpi* Lp and *mpi* Lb for *Leishmania* [*Viannia*] *peruviana* and *L.* [*V.*] *braziliensis*, respectively) according to the work of Zhang et al. (17). *cpb* and *hsp70* genes were amplified as previously reported (7, 9). Primer sequences and reaction conditions for each target are described in Table S2 in the supplemental material.

Analysis of cultured isolates and algorithm development. Eighty cultured isolates were analyzed using the three targets, of which 41 were identified as *L.* (*V.*) *braziliensis*, 23 as *L.* (*V.*) *peruviana*, 14 as *L.* (*V.*) *guyanensis*, and 1 as having an *L.* (*V.*) *braziliensis*/*L.* (*V.*) *peruviana* mixed pattern (see Table S1 in the supplemental material). Only 1 out of 80 remained unidentifiable after the three targets were assayed. Each one of the targets identified the three most prevalent species: *mpi* for *L.* (*V.*) *peruviana*, *cpb* for *L.* (*V.*) *braziliensis*, and *hsp70* for *L.* (*V.*) *guyanensis*. To differentiate genotypes in the lowest number of steps, the three targets were merged into one algorithm that decreases the number of samples to analyze in each step, reducing time and costs. According to the prevalence of species in our study site (Instituto de Medicina Tropical Alexander von Humboldt [IMTAvH], Lima, Peru), the *mpi*/*cpb*/*hsp70* order provided the lowest number of steps for species identification (Table 1).

PCR detection limit. The PCR detection limit was determined for each of the three targets using dilutions from 5 ng down to 0.1 pg of DNA. Amplification products were observed down to 1 pg of *Leishmania* DNA (approximately 10 parasites) after 30 and 45 cycles with *mpi* (both reactions) and *hsp70*, respectively. In contrast, 45 cycles of *cpb* PCR yielded amplification from 10 pg or more. Neither species cross-reaction nor nonspecific amplifications were observed with the targets.

Inclusion criteria for species identification in clinical specimens. Kinetoplast DNA (kDNA) PCR was performed on FPLIs to diagnose leishmaniasis (2, 3). PCR-positive FPLIs ($n = 70$) were classified into three groups according to resulting kDNA band intensities: (i) strong positive (≥10 pg of *Leishmania* DNA), $n =$

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TABLE 1 Algorithm step results according to amplification products and restriction patterns

Step order	Assay[c]	Gel result (bp)	Species identity	Next step
1st	*mpi* Lp PCR	312	*L.* (*V.*) *peruviana*	Stop
	mpi Lb PCR	Negative		
	mpi Lp PCR	312	*L.* (*V.*) *braziliensis*/*L.* (*V.*) *peruviana*	Stop
	mpi Lb PCR	312	Hybrid or mixed infection	
	mpi Lp PCR	Negative		
	mpi Lb PCR	312	*L.* (*V.*) *braziliensis* or *L.* (*V.*) *guyanensis*	2nd (*cpb* PCR-RFLP)
	mpi Lp PCR	Negative	Not identified	3rd (*hsp70* PCR-RFLP)
	mpi Lb PCR	Negative		
2nd	*cpb* PCR-RFLP	543, 343, 257	*L.* (*V.*) *braziliensis*[a]	Stop
		543, 343, 257, 220, 123	*L.* (*V.*) *braziliensis*[b]	Stop
		543, 257, 220, 123	Probable *L.* (*V.*) *guyanensis*	3rd (*hsp70* PCR-RFLP)
		Negative	Not identified	3rd (*hsp70* PCR-RFLP)
3rd	*hsp70* PCR-RFLP	338, 307, 286, 265	*L.* (*V.*) *guyanensis*	Stop
		(338 + 333), 307, 145, 120	*L.* (*V.*) *lainsoni*	Stop
		338, 307, 286, 225	*L.* (*V.*) *braziliensis*	Stop
		Negative	Not identified	Stop

[a] Typical *L.* (*V.*) *braziliensis cpb* PCR restriction fragment length polymorphism (PCR-RFLP) pattern.
[b] Unusual *L.* (*V.*) *braziliensis cpb* PCR-RFLP pattern that might be confounded with *L.* (*V.*) *braziliensis*/*L.* (*V.*) *peruviana* hybrids if *mpi* PCR were not available.
[c] *mpi* Lp and *mpi* Lb correspond to PCRs using reverse allele-specific primers for *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis*, respectively.

41; (ii) medium positive (between 10 and 0.1 pg), $n = 20$; and (iii) weak positive (<100 fg), $n = 9$. Different intensities of positive kDNA bands are clearly illustrated in Fig. 1. Only those specimens that were classified after kDNA PCR as strong positive or medium positive (Fig. 1, lanes 1 to 6) were adequate for species identification through the three-step algorithm. We established that a sample of lower intensity than the lane 6 band would not be amenable for species identification.

Algorithm performance on clinical specimens. Algorithm performance on clinical specimens was assessed using 70 FPLIs, where nine were excluded because of weak positive kDNA band after diagnostic PCR. After the three steps were performed on the remaining 61 specimens, species identification was successful in 53 (76% of all samples). From these, 17 were identified as *L.* (*V.*) *peruviana*, 19 as *L.* (*V.*) *braziliensis*, 15 as *L.* (*V.*) *guyanensis*, one as *Leishmania* (*Viannia*) *lainsoni*, and one as having an *L.* (*V.*) *bra-*

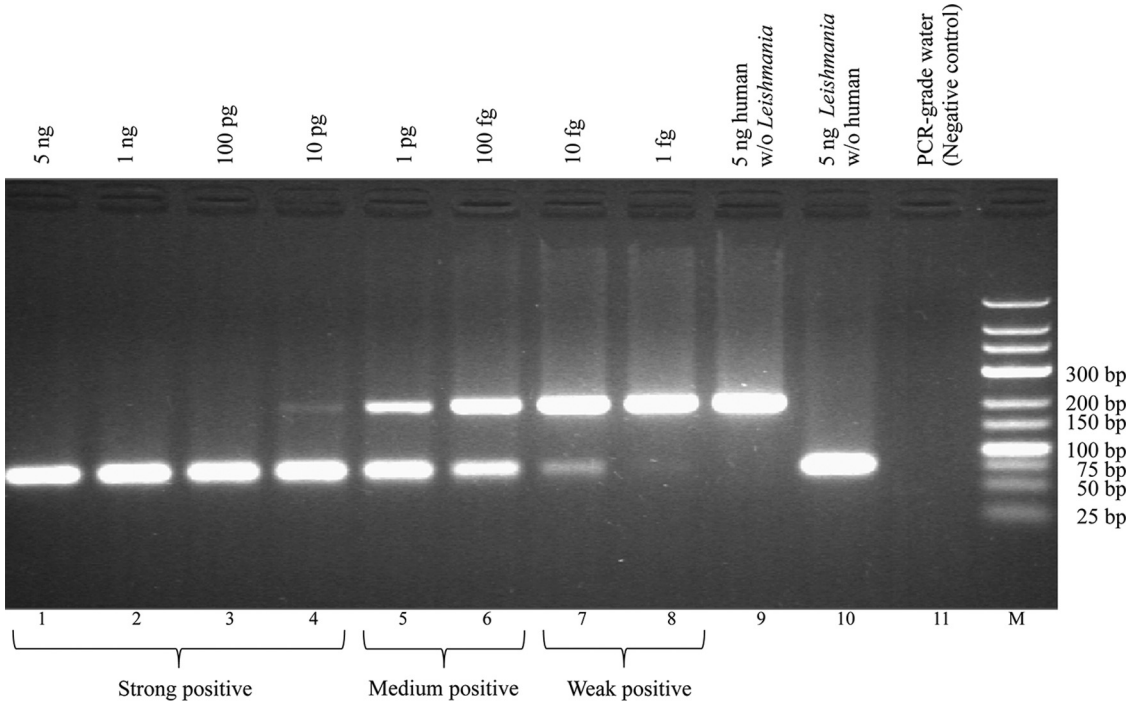

FIG 1 kDNA PCR band intensity categories used as references to allocate diagnosis of clinical specimens. PCR mixtures (lanes 1 to 8) contained 5 ng of human DNA plus different amounts (5 ng down to 1 fg) of *Leishmania* DNA. PCR product sizes correspond to 70 bp for *Leishmania* kDNA and 197 bp for human beta-globin gene (internal control). Abbreviations: w/o, without; M, molecular size ladder.

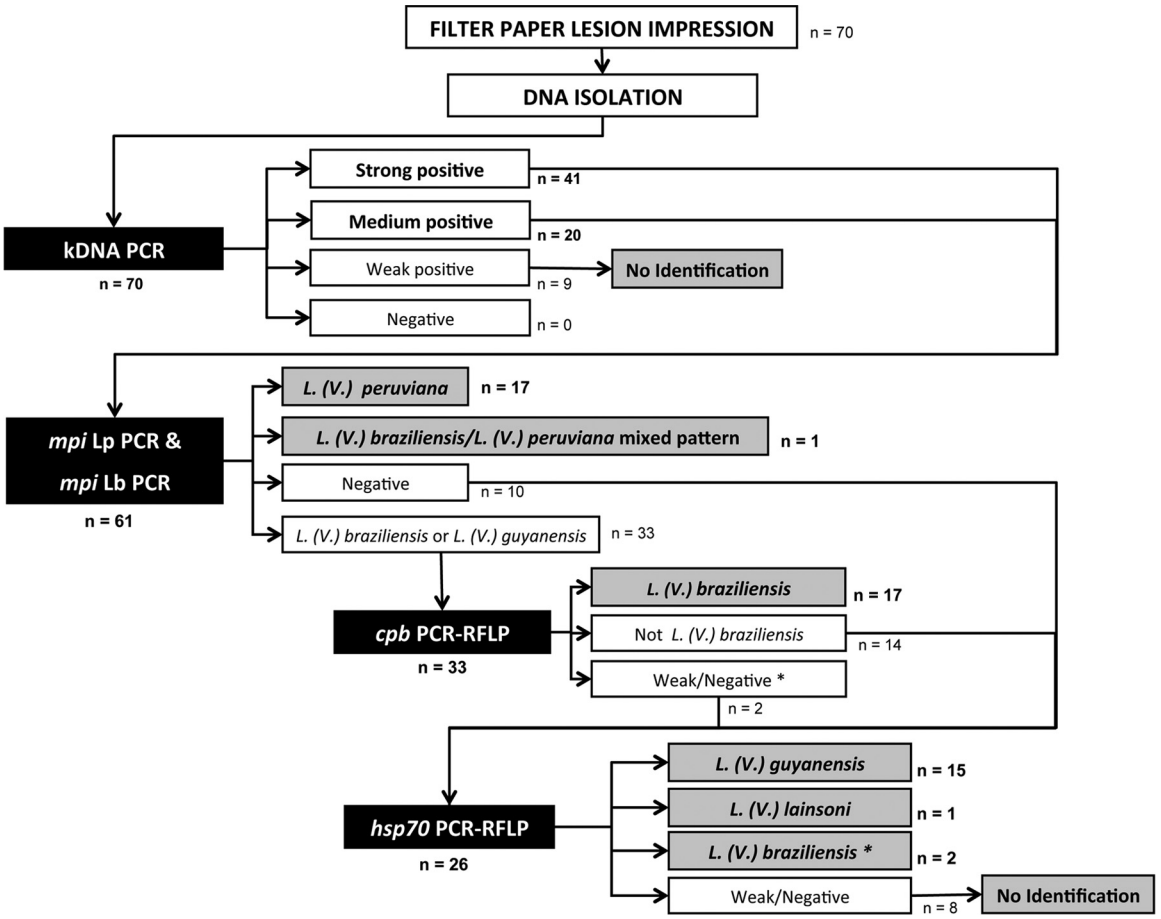

FIG 2 Reduction of numbers of clinical specimens through the algorithm steps. PCR steps are shown with black shading. Subsequent steps are indicated with arrows, while identification endpoints in the flow chart are indicated within gray-shaded boxes. The algorithm was evaluated using 70 specimens on filter paper lesion impressions, and the numbers processed in each step are indicated (*n*). *mpi* Lp and *mpi* Lb correspond to PCRs using reverse allele-specific primers for *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis*, respectively. ∗, two specimens that could not be typed with *cpb* PCR due to negative or weak *cpb* amplification but were identified with *hsp70* PCR-restriction fragment length polymorphism (PCR-RFLP).

ziliensis/L. (*V.*) *peruviana* mixed pattern (Fig. 2). Of the eight non-identified specimens, seven specimens did not amplify any target despite displaying a medium positive kDNA band, which may reflect low DNA levels (less than 1 pg) or highly inhibited specimens. The remaining specimen produced an incongruent pattern: an amplification product with the *mpi* Lb reaction allocated to either *L.* (*V.*) *braziliensis* or *L.* (*V.*) *guyanensis* and a *cpb* product indicative of *L.* (*V.*) *guyanensis*. Nevertheless, *hsp70* distinguished this specimen as *L.* (*V.*) *braziliensis*. In our experience, this result could be attributed to nucleotide polymorphisms that modified restriction enzyme recognition sites.

Progression through the algorithmic steps considerably reduces the number of specimens to be analyzed in the sequential step, thereby reducing time and resources. Thus, 30% of specimens analyzed were typed in the first 4 h (after kDNA PCR result) and 28% were identified over the next 24 h. The final 42% needed execution of the last step, which required 24 additional hours to obtain results. Consequently, one-third of patients had a definitive answer in the first 24 h after clinical specimen collection.

Algorithm flexibility allows switching steps according to species prevalence in a given region of endemicity. We recommend starting with the PCR target for the most predominant species. Our patient enrollment is principally from the Andean valleys where *L.* (*V.*) *peruviana* is predominant (1); therefore, *mpi* was the first target. Nevertheless, if this algorithm is to be applied in Cuzco, where *L.* (*V.*) *braziliensis* is mostly found (1, 11), the first target should be *cpb*. Therefore, it is expected that species identification will be obtained for most patients in the first step, leaving the minority for the subsequent *hsp70* step. In areas where *L.* (*V.*) *guyanensis* is the most prevalent, as occurs in the central high jungle (1, 11), the order of the targets should place *hsp70* in the first step followed by *cpb*.

The proposed algorithm strategy should be useful to guide patient management as well as for epidemiological studies and clinical trials. This concept can also be applied in Old World leishmaniasis and in other pathologies, especially in public health settings where resources are limited.

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