MAJOR ARTICLE

Detection and Species Identification of *Leishmania* DNA from Filter Paper Lesion Impressions for Patients with American Cutaneous Leishmaniasis

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Background. Traditional detection of *Leishmania* from ulcers involves collection of invasive specimens that cause discomfort, require technical expertise, and carry risks of invasive procedures. We compared traditional diagnostic methods with a molecular noninvasive filter paper–based method for the diagnosis of cutaneous leishmaniasis.

Methods. Consecutive patients presenting to the *Leishmania* Clinic at Hospital Nacional Cayetano Heredia were enrolled. Polymerase chain reaction (PCR) was performed on lesion scrapings, aspirates, and filter paper impressions. The reference standard was any 2 of 5 tests positive: smear, aspirate culture, invasive-specimen PCR (scrapings and aspirates), filter paper PCR, and leishmanin skin test. Outcome measures were sensitivity and specificity. *Leishmania* speciation was performed by PCR–restriction fragment length polymorphism (RFLP) of positive specimens.

Results. Forty-five patients with 66 lesions were enrolled. Of 52 lesions diagnosed as cutaneous leishmaniasis, 50 were positive by PCR of invasive specimens versus 48 by PCR of filter papers ($P = .930$). Sensitivity and specificity of PCR on invasively obtained specimens were 94.2% (95% confidence interval [CI], 87.9%–100%) and 92.9% (95% CI, 79.4%–100%). Sensitivity and specificity of filter paper PCR were 92.3% (95% CI, 85.1%–99.5%) and 100%. Culture, smear, and leishmanin skin test all had inferior sensitivities, compared with PCR of invasive or noninvasive specimens ($P < .001$). Of 50 specimens positive by PCR, 19 had sufficient DNA for PCR-RFLP analysis.

Conclusions. Filter paper PCR constitutes a sensitive and specific alternative to traditional diagnostic assays. This novel, rapid, well-tolerated method has the potential for widespread use in the field and in pediatric populations where traditional specimen collection is most difficult to perform, and can potentially be used for rapid species identification.

The definitive diagnosis of cutaneous leishmaniasis (CL) can be challenging, particularly in resource-limited settings, where these diseases are predominantly endemic. Accepted gold standard diagnosis involves isolation of parasites either microscopically, or by culture, both of which involve obtaining specimens by invasive means [1–3]. Scrapings and aspirates of ulcer bases and borders are 2 of the most commonly obtained clinical specimens for the diagnosis of CL, the sensitivity of which ranges from 40%–75% on the low end for culture [4–7] to >90% for polymerase chain reaction (PCR) [1, 8–10]. These techniques can cause considerable discomfort, require technical expertise, carry all of the risks of invasive procedures including bleeding and infection, and are especially difficult to perform in the pediatric population, in remote field settings, and in those with intercurrent bacterial or fungal superinfection [1]. Thus, there is a dire need for less-invasive, more simple and sensitive diagnostic procedures.

PCR is a highly sensitive tool for the detection of

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Leishmania DNA [1, 8–11]. However, to date, this platform has mostly been performed using the aforementioned clinical specimens. PCR of filter paper lesion impressions is a potentially sensitive, noninvasive diagnostic approach to CL.

PCR from filter paper spot-inoculated with blood, bone marrow aspirate, or skin biopsies is a sensitive tool for detecting *Leishmania* DNA [12–15]. For CL, PCR of biopsy-inoculated filter paper alone demonstrated the same positivity rate as parasitological and immunological tests together, and was positive in 90.2% of confirmed cases [12–14]. Marques et al [14] recommend that clinical samples from patients with CL should be collected and preserved on filter paper for PCR testing at a reference laboratory. This method is simple, comparatively inexpensive, allows for easy specimen transport, and is adequate for field conditions in developing countries [14]. Thus, PCR of filter paper impressions of moist ulcer bases in American CL may offer a noninvasive alternative to scrapings, aspirates, and biopsies in clinically suggestive cases.

We herein compared several "traditional" methods for diagnosing American CL, including culture and PCR of lesion aspirates and scrapings, Giemsa-stained lesion smears, and the leishmanin skin test (LST) to the novel, noninvasive method of filter paper impression PCR. In addition, we performed species identification using PCR–restriction fragment length polymorphism (RFLP) of clinical specimens, which is important in countries like Peru where several members of the *Leishmania Viannia* complex can cause disease and portend different prognoses.

METHODS

Study site. The study was conducted at the *Leishmania* Clinic of the Instituto de Medicina Tropical "Alexander Von Humboldt" and Hospital Nacional Cayetano Heredia, in Lima, Peru, between January and April 2009, following institutional review board approval. The Institute houses a large outpatient clinic for the diagnosis and management of American tegumentary leishmaniasis, with an average of 30–40 new cases diagnosed per month [6, 7].

Study population. Consecutive patients presenting to the *Leishmania* Clinic for the evaluation of skin lesions were approached to participate in this study, and screened for eligibility criteria. We included patients who were referred to the *Leishmania* Clinic for suspected American CL; had a clinical indication for skin scraping and aspirate; and were able to give informed consent for the diagnostic procedures. We excluded patients with clinical evidence of intercurrent bacterial or fungal superinfection of the ulcer; and those undergoing active treatment for CL.

Sampling. After removing any overlying scab or crust with moistened gauze, single sheets of sterile, Fisher brand coarse-porosity 7-cm filter paper (Fisher Scientific) were gently pressed onto the moist ulcer base, which allowed for tissue fluid to be wicked onto the filter paper (Figure 1). Filter papers were then allowed to air-dry, and 6-mm punches were obtained and stored in 1.5-mL Eppendorf tubes containing 700 μL 100% ethanol for qualitative PCR testing. To avoid contamination between specimens, the single-hole punch was used on clean filter paper 10 times, then immersed and washed in soapy water for 10 min, allowed to air-dry, and then cleaned again with 2 separate isopropyl alcohol wipes.

After cleansing with topical antiseptic, lesion material was scraped from the ulcer base and border using a sterile lancet, and spread on a glass slide. Slides were prepared as described [6, 7]. Amastigotes were quantitated as follows: grade 0 = no amastigotes per high-power field; 1 = 1–10 amastigotes per 1000 high-power fields; 2 = 1–10 amastigotes per 100 high-power fields; 3 = 1–10 amastigotes per 10 high-power fields; 4 = 1–10 amastigotes per high-power field; 5 = 10–100 amastigotes per high-power field; 6 = >100 amastigotes per high-power field) [16]. Lancets were stored at −20°C in 1.5-mL Eppendorf tubes containing 700 μL 100% ethanol for qualitative PCR testing.

Skin lesions were aspirated in duplicate as previously described elsewhere [6, 7]. Aspirated fluid was inoculated in parallel and duplicate as follows: (A) 200 μL into 16×110 mm flat-sided tissue culture tubes (Nalge Nunc International) containing 3.0 mL modified Novy-MacNeal-Nicolle medium (blood agar base; DIFCO catalog number 245400) with 15% defibrinated rabbit blood, or (B) 60 μL of a 1:1 mixture of aspirate and 20% Roswell Park Memorial Institute medium 1640 (RPMI 1640; Invitrogen) supplemented with L-glutamine, 20% fetal bovine serum, 2 mM $NaHCO_3$, 0.25 mg/mL biopterin, and pH adjusted to 7.3 [20% RPMI microculture]) into sterile, nonheparinized 1×75 mm capillary tubes (Chase Scientific Glass). Capillary tubes were inoculated and sealed as described elsewhere [6, 7]. Cultures were labeled and incubated as previously described elsewhere [6, 7]. Remaining sample was stored at −20°C for qualitative PCR testing.

LST. LSTs were applied using 0.1 mL of in-house, sterile, heat-killed promastigote lysate in 0.005% thimerosal as described [6, 7], and read at 48 h after administration. A positive result was indicated by $\geqslant$5 mm of erythema and induration as previously described elsewhere [6, 7, 17].

Isolation of kinetoplastid DNA from filter papers, aspirates, and lancets. Prior to DNA extraction, frozen aspirates and 6-mm filter paper punches were incubated at room temperature with 500 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA) for 5 min to remove contaminants. Samples were then centrifuged at 8050 g for 10 min, and the supernatant was discarded. Tissue traces were removed from stored lancets using sterile micropipette tips and transferred to microcentrifuge tubes. Kinetoplastid DNA (kDNA) isolation was performed with the phenol-

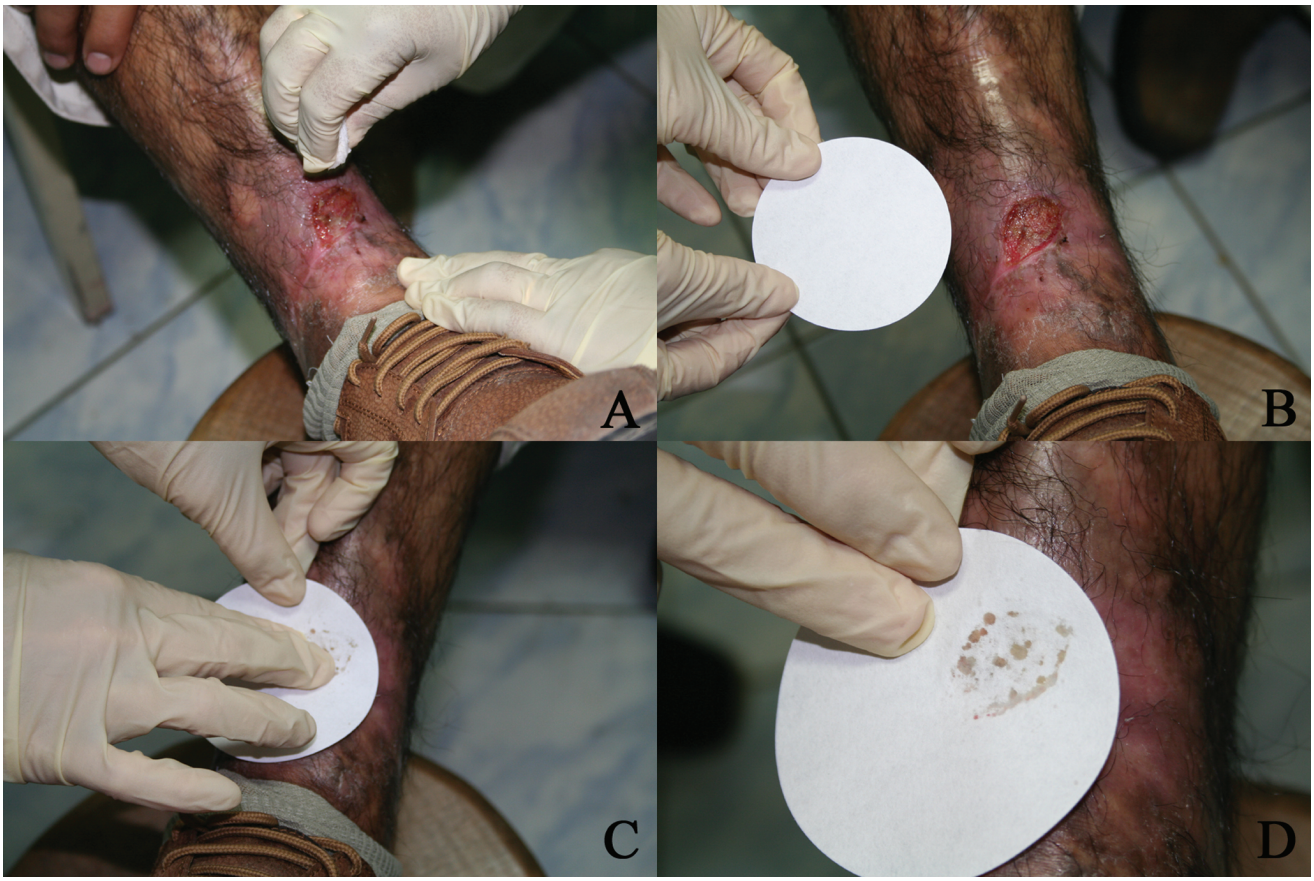

Figure 1. Specimen collection using the Fisher brand 7-cm filter paper lesion impression method. *A,* cleansing of an ulcer suspected to be cutaneous leishmaniasis with topical antiseptic; *B,* preparation of the filter paper for specimen collection; *C,* application of the filter paper to the ulcer base; *D,* evidence of tissue fluid and ulcer exudates wicked onto the filter paper.

chloroform-isoamyl alcohol method as previously described elsewhere [18].

kDNA PCR. kDNA PCR was performed using the HotStar Taq DNA Polymerase kit (Qiagen). Final volume of the reaction mixture was 25 μL. PCR conditions were as follows: 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s; primer annealing at 55°C for 30 s; extension at 72°C for 30 s, and a final extension step at 72°C for 10 min (iCycler iQ; Bio-Rad). The first primer, specific for *Leishmania* subgenus *Viannia,* had the following sequences: MP1-L (fwd) 5′-TACTC-CCCGACATGCCTCTG-3′ and MP3-H (rev) 5′-GAACGGGG-TTTCTGTATGC-3′, and generated a product 70 bp long [19]. Sequences of control primers, which amplify a region of the human β hemoglobin gene, were: HBBL (fwd) 5′-GGCAGACT-TCTCCTCAGGAGTC-3′ and HBBR (rev) 5′-CTTAGACCTC-ACCCTGTGGAGC-3′, and generated a product with a length of 197 bp. Amplicons were visualized on 4% agarose gels (Promega) and stained with ethidium bromide.

Species identification by PCR-RFLP. Three PCR assays targeting different sequences specific to *Leishmania* subgenus *Viannia* species including *L. (V.) braziliensis, L. (V.) peruviana,* and *L. (V.) guyanensis,* the principal causative species in Peru, were used for the species identification following initial kDNA PCR. Only samples producing strong bands on the kDNA PCR were selected for subsequent PCR and RFLP, based on the otherwise low likelihood of successful species identification directly from a clinical specimen, rather than from a culture. PCR assays were performed using the HotStar Taq DNA Polymerase kit (Qiagen). Final volume of the reaction mixture was 25 μL in each case.

The first assay, targeting the cysteine proteinase B (*Cpb*) gene, employed primers with the following sequences, which distinguish between *L. (V.) braziliensis* and non–*L. (V.) braziliensis* species, and generated a product 1170 bp long: *Cpb* (fwd) 5′-TGTGCTATT CGAGGAGTTCAA-3′ and *Cpb* (rev) 5′-TTACC-CTCAGGAATCACTTTGT-3′ [20, 21]. *Cpb* PCR conditions were as follows: 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 30 s; primer annealing at 60°C for 30 s; extension at 72°C for 60 s, and a final extension step at 72°C for 6 min (iCycler iQ; Bio-Rad) [20, 21].

The second assay, targeting heat shock protein 70 (*hsp70*), employed primers with the following sequences, which distin-

guish between *L. (V.) guyanensis* and non–*L. (V.) guyanensis* species, and generated a product 1422 bp long: *hsp70* (fwd) 5′-GACGGTGCCTGCCTACTTCAA-3′ and *hsp70* (rev) 5′-CCGCCCATGCTCTGGTACATC-3′ [21, 22]. *Hsp70* PCR conditions were as follows: 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 30 s; primer annealing at 60°C for 60 s; extension at 72°C for 60 s, and a final extension step at 72°C for 6 min (iCycler iQ; Bio-Rad) [21, 22].

The third and final assay, targeting the mannose phosphate isomerase gene (MPI), employed allele specific primers with the following sequences, which distinguish between *L. (V.) braziliensis* and *L. (V.) peruviana,* and generated a product 312 bp long: MPI (fwd) 5′-GCTCTTCCTGTCGGACAGCGAGC-3′ (common to both species) and MPI (rev) 5′-GTCGGCAGCGTCACGGAGGTCG-3′ [specific for *L. (V.) braziliensis*] and MPI (rev) 5′-GTCGGCAGCGTCACGGAGGTCC-3′ [specific for *L. (V.) peruviana*] [23]. MPI PCR conditions were as follows: 95°C for 15 min, followed by 33 cycles of denaturation at 94°C for 30 s; primer annealing at 69°C for 30 s; extension at 72°C for 30 s, and a final extension step at 72°C for 5 min (iCycler iQ; Bio-Rad) [23]. All PCR products were visualized on 1.5% agarose gels (Promega) and stained with ethidium bromide.

RFLP analysis of* Cpb *and* Hsp70 *PCR products (PCR-RFLP). Following *Cpb* and *Hsp70* PCR amplification as above, products were separately digested overnight at 65°C for the *cpb* assay, or 37°C for the *hsp70* assay, in a total volume of 20 μL, with 5 U of each restriction enzyme. The following enzymes were used in each reaction: *Cpb* (*Taq*I) and *hsp*70 (*Hae*III) (Fermentas). Reactions were stopped with Proteinase K (20 mg/mL). RFLP products were then analyzed separately using 12% polyacrylamide gel electrophoresis (MiniProtean III; Bio-Rad), and stained with silver stain (Promega). MPI PCR was used to confirm the identity of *L. (V.) peruviana* in cases where the *cpb* restriction fragment pattern was non–*L. (V.) braziliensis,* and the *hsp70* restriction fragment pattern was non–*L. (V.) guyanensis,* and also served as an internal control of the *cpb* assay.

Composite reference standard. We defined a lesion as CL when any 2 of 5 tests were positive, where tests refer to LST, lesion smear, culture, PCR of aspirates and scrapings, or PCR of filter paper. These 5 tests served as the composite reference standard against which each individual diagnostic test was compared.

Sample size calculation. Based on existing literature [6, 7,24, 25], we estimated the overall sensitivity of invasive molecular methods to be 96%, and the sensitivity of microculture (the most sensitive nonmolecular invasive method) to be 83%. In order to achieve a sensitivity of filter paper lesions impression PCR equal to or better than microculture, assuming an $\alpha = 0.05$ and a power of 80%, 66 lesions were required per group. For sensitivity analysis, the aforementioned composite reference standard was applied, and the unit of analysis was the lesion. Lesions were assumed to not be correlated within patients as previously described elsewhere [6, 7].

Statistical analysis. Descriptive statistics (mean, standard deviation, median, range) were calculated for continuous variables, and differences were compared using the 2-tailed *t* test. Categorical variables were quantitated by proportions, and differences between the groups were compared using Yates-corrected χ^2 analysis. Differences in sensitivities were compared using the *z* test. Statistical analyses were performed using SigmaStat, version 2.03 (SPSS). Level of significance was set at $P < .05$.

RESULTS

Forty-five patients with 66 skin lesions were enrolled in the study: 26 males and 19 females. Median age was 32 years (range, 5–70 years), and median duration of exposure in the risk area was 36 months (range, 1 day–70 years). Work in agriculture and residence or tourism in an endemic region were the principal risk occupations (29%, 20%, and 16%, respectively). Students accounted for only 9% of the cohort. Median duration of lesions was 3 months (range, 1 month–3 years). Twenty-two patients (49%) presented with multiple lesions, with a median number of lesions per patient of 1 (range, 1–10 lesions per patient). Two of the study participants (5%) had evidence of intercurrent mucosal and cutaneous involvement. The majority of skin lesions were purely ulcerative (94%), with a much smaller number of lesions having a primarily nodular or verrucous presentation (4.5% and 1.5%, respectively). Lesions were primarily located on the upper extremity (41%), face (27%), or lower extremity (26%).

Using the composite reference standard (at least 2/5 tests positive), 52 lesions (79%) fulfilled criteria for a diagnosis of CL. Fifty-eight lesions (88%) were positive by at least one test, 45 (68%) were positive by 3 or more tests, 36 (55%) were positive by 4 or more tests, and 9 (14%) were positive by all 5 tests. When the individual patient was used as the unit of analysis, sensitivities and specificities of individual assays did not change appreciably from the per-lesion analysis.

Culture. Of the 52 lesions that were positive by at least 2 of 5 diagnostic tests, 38 were culture positive. The overall sensitivity and specificity of culture was 73% (95% confidence interval [CI], 60.9%–85.1%] and 100%, respectively (Table 1). Culture had superior sensitivity only to LST ($P = .047$).

Smears. Thirty-one lesions were positive by Giemsa-stained smear, yielding a sensitivity of 59.6% (95% CI, 46.3%–72.9%) (Table 1). In those lesions that were smear positive, median smear amastigote density was grade 3 (1–10 amastigotes/10 high-power fields). In those lesions that fulfilled the composite reference standard diagnostic criteria, median smear density was higher in those that were also culture positive (den-

Table 1. Analysis of 5 Diagnostic Tests Used in the Evaluation of 66 Lesions Suspected to Be Cutaneous Leishmaniasis

Assay	No. of lesions that tested positive	No. of lesions that tested negative	Sensitivity, %	Specificity, %	PPV, %	NPV, %
LST[a]	33	32	54.9	64.3	84.8	28.1
Smear	31	35	59.6	100.0	100.0	40.0
kDNA PCR of invasive specimens[b]	50	16	94.2	92.9	98.0	81.3
kDNA PCR of noninvasive specimens[c]	48	18	92.3	100.0	100.0	77.8
Culture	38	28	73.0	100.0	100.0	50.0

NOTE. kDNA, Kinetoplastid DNA; LST, leishmanin skin test; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value.

[a] One individual did not undergo leishmanin skin testing.
[b] Includes lesion aspirates and scrapings.
[c] Includes filter paper lesion impressions.

sity grade 3 [1–10 amastigotes/10 high-power fields]) compared with those that were culture negative (density grade 0) ($P < .001$).

PCR of scrapings and aspirates. Fifty lesions were positive by PCR of invasively obtained specimens, yielding a sensitivity of 94.2% (95% CI, 87.9%–100%) (Table 1). However, only 49 of those positive by PCR fulfilled composite reference standard diagnostic criteria, so in 1 lesion, PCR of invasive specimens was the only positive test. Compared to the composite reference standard, specificity of PCR from invasive specimens was 92.9% (95% CI, 79.4%–100%) (Table 1). PCR of invasive specimens was more sensitive than LST ($P < .001$), smear ($P < .001$), and culture ($P = .002$).

PCR of filter paper impressions. Forty-eight lesions were positive by PCR of filter paper lesion impressions, yielding a sensitivity and specificity of 92.3% (95% CI, 85.1%–99.5%%) and 100.0%, respectively (Table 1). PCR of filter paper lesion impressions was more sensitive than LST ($P < .001$), smear ($P < .001$), and culture ($P = .007$). PCR of filter paper lesion impressions was equally sensitive as PCR of invasive specimens ($P = .930$).

RFLP. Of the 50 lesions with PCR-positive clinical specimens, sufficient amplifiable DNA for speciation by RFLP was present in 19 sets of aspirates, scrapings, and filter paper impressions. Of the 18 kDNA-positive filter papers, aspirates, and lancets with definitive RFLP results, species identification was as follows: *L. (V.) braziliensis,* 1 lesion; *L. (V.) peruviana,* 9 lesions; *L. (V.) guyanensis,* 7 lesions; and *L. (V.) braziliensis–peruviana* hybrid, 1 lesion (Table 2).

LST. Thirty-three lesions were from patients with positive LST results, yielding a sensitivity and specificity of 54.9% (95% CI, 41.4%–68.4%) and 64.3% (95% CI, 39.2%–89.4%), respectively (Table 1).

DISCUSSION

We have demonstrated in a clinical evaluation of ulcerative lesions suspicious for American CL in Peru that kDNA PCR of filter paper lesion impressions offers at least comparable diagnostic sensitivity and specificity versus PCR of invasively obtained specimens such as lesion aspirates and scrapings, and performs in a superior manner to LST, smear, and culture. While the performance characteristics of PCR on each specimen were comparable, that filter paper lesion impressions are completely noninvasive, easy to perform, and well tolerated by patients makes them an attractive alternative to traditional diagnostic methods. In addition, filter paper impressions can theoretically be obtained in the setting of bacterial or fungal superinfection, unlike scrapings or aspirates, where incising a superinfected ulcer would be contraindicated. Thus, the patient population in whom this diagnostic maneuver can be performed may be broader than that of scrapings and aspirates. However, it must be recognized that this is only a theoretical advantage to filter paper PCR and was not evaluated in this study. Future assessment of the performance characteristics of filter paper PCR in the setting of superinfection is warranted.

We have also demonstrated that species identification directly from a non invasive clinical specimen can be achieved. While

Table 2. Species Identification of 19 Sets of Specimens Out of 50 Polymerase Chain Reaction (PCR)–Positive Lesions Subsequently Tested with PCR–Restriction Fragment Length Polymorphism (RFLP) Targeting the Cysteine Proteinase B, Heat Shock Protein 70, and Mannose Phosphate Isomerase Genes

Leishmania species	No. (%) of those tested
L. (Viannia) braziliensis	1 (5.3)
L. (Viannia) guyanensis	7 (36.8)
L. (Viannia) peruviana	9 (47.4)
Hybrid *L. (Viannia) braziliensis–peruviana*	1 (5.3)
Not identifiable	1 (5.3)
Not tested[a]	31

[a] Only specimens with sufficient amplifiable DNA from the kinetoplastid DNA PCR of lancets, aspirates, and filter paper impressions were selected for direct clinical specimen PCR-RFLP.

only 19/50 kDNA-positive lancets, aspirates, and filter papers produced a sufficient band to proceed with direct specimen RFLP, that species identification occurred in 18/19 of these specimens constitutes a major advance in our approach to speciation, which has historically relied on cultured promastigotes and labor-intensive isozyme analysis [1]. Future studies which work to optimize the performance of PCR-RFLP of filter paper lesion impressions are warranted.

Noninvasive diagnostic testing in CL is a relatively novel approach, with a dearth of published studies supporting its utility [26]. On the other hand, PCR of filter papers spot-inoculated with invasively obtained clinical specimens such as blood, splenic aspirates, and skin biopsies, has been shown to be a sensitive means of diagnosing leishmaniasis [12–15]. By applying the noninvasive diagnostic approach to filter paper based specimen collection, we have been able to bypass the intermediary step altogether. In doing so, we have reduced the inherent risks to the patient such as bleeding and infection, diminished the cost of testing by obviating the need for anesthesia, needles, syringes, and sharps biohazard disposal, all of which exceed the cost of filter papers, and have provided clinicians with an expedient, low-tech alternative to the highly operator-dependent aspirate and scraping.

Because of its simplicity, portability, and tolerability, this method has the potential to be deployed widely in remote endemic areas, provided that a mechanism by which to transport the filter papers to a larger laboratory or reference center exists. At present, many remote areas offer only the very insensitive lesion smear, if any diagnostic workup is performed at all. Certainly, in better resourced centers, filter paper lesion impressions should be viewed as superior to traditional tests such as lesion smear, culture, and LST, and at least comparable to molecular testing of invasive specimens. Efforts to promote this sensitive noninvasive method in settings where diagnostic capabilities are limited, as well as in well-resourced reference centers should be pursued.

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