A Simplified and Standardized Polymerase Chain Reaction Format for the Diagnosis of Leishmaniasis

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Background. Definite diagnosis of Leishmania infections is based on demonstration of the parasite by microscopic analysis of tissue biopsy specimens or aspirate samples. However, microscopy generally shows low sensitivity and requires invasive sampling.

Methods. We describe here the development of a simple and rapid test for the detection of polymerase chain reaction--amplified Leishmania DNA. A phase 1 evaluation of the text was conducted in clinical samples from 60 nonendemic and 45 endemic control subjects and from 44 patients with confirmed cutaneous leishmaniasis (CL), 12 with mucocutaneous leishmaniasis (MCL), and 43 with visceral leishmaniasis (VL) from Peru, Kenya, and Sudan.

Results. The lower detection limits of the assay are 10 fg of Leishmania DNA and 1 parasite in 180 μL of blood. The specificity was 98.3% (95% confidence interval [CI], 91.1%–99.7%) and 95.6% (95% CI, 85.2%–98.8%) for nonendemic and endemic control samples, respectively, and the sensitivity was 93.2% (95% CI, 81.8%–97.7%), 91.7% (95% CI, 64.6%–98.5%), and 86% (95% CI, 72.7%–93.4%) for lesions from patients with CL or MCL and blood from patients with VL, respectively.

Conclusions. The Leishmania OligoC-TesT showed high specificity and sensitivity in clinical samples and was able to detect the parasite in samples obtained by less invasive means, such as blood, lymph, and lesion scrapings. The assay is a promising new tool for simplified and standardized molecular detection of Leishmania parasites.

Leishmaniasis is a vectorborne disease caused by protozoa of the genus Leishmania and is endemic in many areas of the tropics, subtropics, and Mediterranean basin [1]. Clinical presentations, depending on the infecting species and the immune status of the patient, range from self-healing skin sores to devastating cutaneous and mucocutaneous ulcers and the lethal visceral form [2]. The disease is responsible for important public health and economic problems in affected regions [3].

Definite diagnosis of Leishmania infections is currently based on microscopic demonstration of the parasites in skin biopsy specimens or mucosal aspirate samples for cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) and in spleen or bone marrow aspirate samples for visceral leishmaniasis (VL) [1]. However, this conventional method is hampered by its low and variable sensitivity and the need for invasive sampling techniques. Sensitivity may be increased by prior in vitro cultivation of the parasite, but this technique is cumbersome and time consuming. Serologic tests, such as the direct agglutination test (DAT) [4], support the clinical diagnosis of VL, but antibodies remain detectable for years after successful treatment [5, 6]. Furthermore, serologic tests are less useful in patients with VL coinfected with HIV [7] and in those with CL or MCL [8].

 Amplification of the parasite DNA by the polymerase chain reaction (PCR) has evolved into one of the most
specific and sensitive methods for *Leishmania* detection [9, 10].

Despite its promising features, PCR is restricted to well-equipped laboratory settings, partly due to technical complexity, from DNA extraction to PCR and product detection. There is a need for simplification, and a first step concerns the visualization of amplified DNA, which is usually identified using electrophoresis followed by ultraviolet transillumination in the presence of the carcinogenic ethidium bromide. Safer nucleic acid stains based on fluorescent dyes are much more expensive and not routinely used in most laboratories. Alternative methods for PCR product detection, such as real-time PCR [11], PCR-ELISA [12, 13], and mass spectrometry [14], have been developed but remain complex, expensive, and highly dependent on specific equipment. Along with the need for simplification of the PCR assay, there is a demand for standardization and optimization [10]. At present, standardization of PCR for *Leishmania* detection is largely neglected, and the abundance of in-house PCR assays may lead to diagnostic inconsistencies.

Oligochromatography provides a simple and rapid dipstick format for detection of PCR products (Coris BioConcept; patent WO 2004/099438A1) [15]. PCR products are visualized on the dipstick by hybridization with a gold-conjugated probe, allowing sequence-specific PCR product detection. This detection format takes only 5–10 min and requires no equipment other than a water bath and a pipette. Internal controls for amplification and chromatographic migration are incorporated in the assay. The technique has already been successfully applied for the diagnosis of human African trypanosomiasis [16], toxoplasmosis [17], and severe acute respiratory syndrome [18], and high sensitivity and specificity were reported.

We present here the development and evaluation of a *Leishmania*-specific PCR-oligochromatographic test (*Leishmania* OligoC-TestT), targeting a short sequence within the *Leishmania* 18S rRNA gene. After providing proof of concept in experimental samples, we evaluated (phase 1) the *Leishmania* OligoC-TestT in a larger series of clinical samples from nonendemic and endemic control subjects and patients with CL, MCL, or VL.

**METHODS**

**Parasite DNA**

DNA from *Leishmania* species (*L. donovani, L. amazonensis, L. infantum, L. braziliensis, L. peruviana, L. guyanensis, L. panamensis, L. mexicana, L. major, L. lainsoni, L. tropica, and L. aethiopica*) and from *Trypanosoma brucei gambiense* and *Trypanosoma cruzi* was obtained from the DNA reference bank at the Institute of Tropical Medicine Antwerp (ITMA). DNA from other pathogens (*Sporothrix schenckii, Plasmodium falciparum, Mycobacterium tuberculosis,* and *Schistosoma mansoni*) was obtained from other research groups.

**Samples**

Informed consent was obtained from patients or guardians and from persons without disease. The human and animal experimentation guidelines of the ITMA were followed. Ethical clearance for the study was obtained from the institutional review boards in Belgium, Kenya, Sudan, and Peru.

**Spiked blood.** *L. donovani* promastigotes were grown in glucose-lactalbumin-serum-hemoglobin medium [19] with 10% fetal calf serum at 26°C. A 10-fold dilution series of parasites, ranging from 10,000 parasites to 1 parasite per 180 μL of blood, was made in fresh blood obtained on EDTA from a healthy volunteer. Nonspiked blood was used as a negative control.

**Nonendemic controls.** DNA extracts from the blood of 20 persons with confirmed *T. brucei gambiense* infection from the Democratic Republic of the Congo (DRC), 20 with confirmed *P. falciparum* infection from Zambia, and 20 with confirmed *T. cruzi* infection from Chile were obtained from other research groups. *T. brucei gambiense* and *P. falciparum* infections were confirmed by direct parasite detection with microscopy, and *T. cruzi* infections were confirmed by kinetoplast DNA PCR followed by Southern blot hybridization [20].

**Patients with CL or MCL and healthy endemic controls (Peru).** Lesion biopsy specimens from 36 patients with CL, mucosal aspirate samples from 12 patients with MCL, and lesion scrapings from 8 patients with CL were collected in 2006 and 2007 at the Instituto de Medicina Tropical Alexander von Humboldt, Lima, Peru. CL and MCL were confirmed by means of microscopy and/or cultivation in 3 mL of Novy-MacNeal-Nicolle medium (Difco Laboratories) containing 15% defibrinated rabbit blood. Forty-one of the 56 CL and MCL lesion samples had a positive direct microscopy result, and the remaining 15 became positive after culture. All biopsy specimens were obtained from the active edge of the lesion with a sterile 4-mm-diameter biopsy puncher, and simple sterile lancets were used for the lesion scrapings. Dental biopsy specimens from 8 healthy endemic control subjects were collected in 2007 in a dental clinic in Lima, Peru. Biopsy specimens and lesion scrapings were stored in absolute ethanol at −20°C until DNA was extracted.

**Patients with VL and healthy endemic controls (Kenya and Sudan).** Sample collections in Kenya and Sudan were performed by the Kenya Medical Research Institute and Khartoum University, respectively. Blood samples from patients with confirmed VL and from healthy endemic control subjects were collected in 2007 in the Baringo district in Kenya (25 VL and 18 control samples) and Gedarif state in Sudan (18 VL and 19 control samples). A patient was classified as having confirmed VL if parasites were observed during microscopic analysis of lymph or bone marrow aspirate samples for Sudan and of spleen aspirate samples for Kenya. A person was classified as a healthy endemic control if there was no clinical suspicion for VL and if the DAT titer was below 1:3200 for Sudan and 1:12,000 for Kenya (threshold values used routinely at the 2 institutions). Along with the
blood samples, bone marrow from 12 and lymph from the remaining 6 of the 18 Sudanese patients with confirmed VL were collected for subsequent analysis with the *Leishmania* OligoC-TesT; 200 µL of blood, bone marrow, or lymph was used for DNA extraction.

**DNA Extraction**

DNA from spiked blood samples and from biopsy specimens and lesion scrapings was extracted using the QIAamp DNA Blood Mini Kit and the QIAamp DNA Mini Kit (Qiagen), respectively. DNA from blood, bone marrow, and lymph node samples from patients with VL and healthy endemic controls was extracted according to the method of Boom et al. [21]. DNA was eluted in 50 µL of pure water or Tris-EDTA (TE) buffer and stored at −20°C, except for the Sudanese clinical samples, which were eluted in 100 µL of TE buffer.

**Primers, Probes, and Internal Control (IC) DNA**

Primers, probes, and IC DNA were synthesized by Biomers.net (figure 1). Sequences of the 18S rRNA gene of the Trypanosomatidae parasites *Leishmania donovani* (L.d.; GenBank accession no. X07773), *Trypanosoma brucei gambiense* (T.b.; GenBank accession no. AJ009141), and *Trypanosoma cruzi* (T.c.; GenBank accession no. AF303660) were aligned and biotinylated at the 5' end. The 5'-acg and 5'-a linkers were added to avoid steric hindrance by the biotin-avidin binding during hybridization.

The IC DNA has the same length as the *Leishmania* target sequence. Both sequences are identical except for a 17-bp central part.

The detection probe was designed, synthesized, and conjugated with gold particles using the procedure described in patent WO 2004/099438A1 [15]. The *Leishmania* capture probe and the IC capture probe, which hybridize to the central part of the *Leishmania* amplicon and the IC DNA amplicon, respectively, were synthesized and biotinylated at the 5' end. The 5'-acg and 5'-a linkers were added to avoid steric hindrance by the biotin-avidin binding during hybridization. The migration control sequence is the reverse complement of the detection probe sequence.

**PCR Amplification**

The 50-µL PCR mixture was prepared by adding 5 µL of sample DNA and 1 U of HotStar Taq polymerase (Qiagen) to 44.8 µL of *Leishmania* Ampli-Mix (Coris BioConcept). This premade PCR mix contains all components to allow PCR amplification, the primers 18S-L-F and 18S-L-R and the IC DNA at a concentration of 3.2 × 10⁻¹⁸ mol/L. The commonly used dTTP was replaced by dUTP to allow elimination of carryover contamination with uracil-DNA N-glycosylase (UNG). An initial denaturation step at 94°C for 15 min to activate the HotStar Taq polymerase was followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a single final extension at 72°C for 5 min. Amplification was done in 200-µL thin-wall PCR tubes (Abgene) in a T3 thermocycler 48 (Biometra).

**Oligochromatography**

**Preparation.** The *Leishmania* Oligo-Strip was constructed as described by Deborggraewe et al. [16] but with the following modifications. The lower absorbent pad on the test side was impregnated with the detection probe and the *Leishmania* capture probe. The lower absorbent pad on the control side was impregnated with the detection probe and the IC capture probe. On both sides of the nitrocellulose membrane, 2 lines were coated, a line with Neutralite avidin (Belovo SA) and a line with the migration control probe.

**Principle.** A schematic overview of the *Leishmania* OligoC-TesT principle is presented in figure 2. The assay was repeated when the control lines indicated migration or PCR failure.

**Assay procedure.** After PCR amplification, the PCR product was denatured at 94°C for 30 s and transferred immediately to ice. Then, 40 µL was mixed with an equal volume of migration buffer preheated at 55°C, and the *Leishmania* Oligo-Strip was dipped into the solution. Test results were read after 10 min.
RESULTS

Analytical sensitivity. The detection limit of the assay was evaluated with a 10-fold serial dilution of L. donovani DNA in water containing 0.1 mg/mL acetylated bovine serum albumin. The lower detection limit was 10 fg per assay (figure 3A), which is approximately 1/20th of the DNA content of 1 parasite. The analytical sensitivity was also evaluated on DNA extracted from blood samples spiked with decreasing numbers of living L. donovani promastigotes. The assay could detect 1 parasite in a 180-μL blood sample (figure 3B), while nonspiked control blood samples always remained negative.

Analytical specificity. The analytical specificity of the assay was assessed with purified DNA from target Leishmania species (1 ng of DNA per assay) and from relevant nontarget pathogens (50 ng of DNA per assay) (figure 3C). The Leishmania OligoC-TesT showed a positive result for all Leishmania species tested: L. amazonensis, L. donovani, L. infantum, L. braziliensis, L. peruviana, L. guyanensis, L. panamensis, L. mexicana, L. major, L. lainsoni, L. tropica, and L. aethiopica. A negative test result was observed with

**Specificity in nonendemic control blood samples.** The Leishmania OligoC-TesT was performed in DNA extracts from 20 persons with T. brucei gambiense, 20 with P. falciparum, and 20 with T. cruzi infection. The test result was positive in 1 sample from a T. brucei gambiense–infected person and negative in all of the other samples (table 1). This indicates a diagnostic specificity of 98.3%, with a 95% confidence interval (CI) (scored by Wilson’s method [22]) of 91.1%–99.7%.

**Diagnostic accuracy in patients with CL, MCL, or VL and endemic controls.** The Leishmania OligoC-TesT had positive results in 33 of the 36 biopsy samples from patients with CL, all 8 lesion scrapings from patients with CL, and 10 of the 12 aspirate samples from patients with MCL (table 1). Hence, the sensitivity of the Leishmania OligoC-TesT in the 56 clinical samples from patients with CL or MCL was 92.9% (95% CI, 83.0%–97.2%), higher than the sensitivity of microscopy for the same samples. Results of microscopy were available for 52 samples and were positive in 41; thus, the sensitivity of microscopy was 78.8% (95% CI, 66%–87.8%). Test results were negative in all 8 dental biopsy specimens from healthy endemic control subjects.

A positive Leishmania OligoC-TesT result was observed in 23 of the 25 (92% [95% CI, 75.0%–97.8%]) and 14 of the 18 (77.8% [95% CI, 54.8%–91.0%]) blood samples from patients with confirmed VL from Kenya and Sudan, respectively. Eleven of the 12 bone marrow samples and all 6 lymph node samples from Sudanese patients with confirmed VL were positive in the assay. Leishmania DNA was detected in 1 of the 18 and 1 of the 19 blood samples from healthy control subjects in Kenya and Sudan, respectively. Thus, the specificity of the test for acute VL disease was 94.6% (95% CI, 82.3%–98.5%).

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**Figure 3.** Analytical sensitivity and specificity of the Leishmania OligoC-TesT. The upper line is the migration control, and the lower line is the Leishmania test line. A, Test results for a serial dilution of *Leishmania donovani* DNA in water containing 0.1 mg/mL acetylated bovine serum albumin. Dipsticks 1–9 are for 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 0 fg per assay, respectively. B, Test results for a serial dilution of *L. donovani* promastigotes in naive human blood. Dipsticks 1–6 are for 10,000, 1000, 100, 10, 1, and 0 parasites in 180 µL of blood. C, Test results for different *Leishmania* species (1 ng of DNA per assay) and nontarget pathogens (50 ng of DNA per assay). Dipsticks 1–20 are for *L. amazonensis, L. donovani, L. infantum, L. braziliensis, L. peruviana, L. guyanensis, L. panamensis, L. mexicana, L. major, L. lainsoni, L. tropica, L. aethiopica, Sporothrix schenckii, Plasmodium falciparum, Trypanosoma brucei, Trypanosoma cruzi, Mycobacterium tuberculosis, Schistosoma mansoni, a negative control for polymerase chain reaction, and a negative control for DNA extraction.
DISCUSSION

Accurate detection of the parasite is of utmost importance in the diagnosis of Leishmania infections and in disease control. We here report on the development, proof-of-concept analysis, and phase 1 evaluation of a simplified molecular test for detecting Leishmania organisms in clinical samples. The Leishmania OligoC-TesT is based on PCR amplification of Leishmania DNA followed by simple and rapid detection of the PCR product in dipstick format. The lower detection limits of the assay were evaluated in L. donovani promastigotes, and 10 fg of purified DNA and 1 parasite in 180 μL of human blood could be detected.

Fluctuations in the analytical sensitivity between different isolates might be expected. In 1998, Inga et al. [23] reported a lower copy number of the rRNA genes in L. (Viannia) peruviana from southern Peru compared with L. (V.) peruviana from northern Peru. In our study, however, we did not observe a significantly lower sensitivity of the Leishmania OligoC-TesT in 13 patients originating from L. (V.) peruviana–endemic regions in southern Peru, because only one of these patients had a negative test result. No cross-reaction with nontarget human pathogens was observed, and all Leishmania species tested had a positive test result. Considering the high similarity of the 18S rRNA gene sequence in Leishmania and Endotrypanum, Crithidia, Wallaceina, and Leptomonas organisms [24], our assay might have positive results with these nonpathogenic lower trypanosomatids. Although the chances of finding such protozoa in immunocompetent patients are negligible, they may show up as opportunistic infections in immunocompromised patients [25]. If needed, confirmation could be provided by direct sequencing of the PCR product, because Leishmania-specific point mutations are present (authors’ unpublished data).

The Leishmania OligoC-TesT showed a high diagnostic specificity when tested in 60 nonendemic control blood samples from persons infected with T. brucei gambiense, P. falciparum, or T. cruzi. One T. brucei gambiense–infected person from the DRC had a positive Leishmania OligoC-TesT result. We cannot exclude the possibility that this could be due to a Leishmania infection, because VL has been occasionally reported in the DRC [26, 27].

When evaluated in 56 clinical samples from patients with confirmed CL or MCL (definition based on microscopy and/or culture) collected in Peru, the Leishmania OligoC-TesT showed an overall diagnostic sensitivity of 92.9%, whereas all 8 dental biopsy specimens from healthy control subjects were negative. This finding is similar to the observed sensitivities of other PCR assays reviewed by Vega-López [8]. Direct microscopy of the lesion showed a lower sensitivity (78.8%). The lack of a positive PCR result in some of the confirmed CL and MCL lesion samples might be explained by (1) heterogeneity in parasite distribution

| Table 1. Diagnostic accuracy of the Leishmania OligoC-TesT on clinical samples from nonendemic control subjects, endemic control subjects, and patients with cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), or visceral leishmaniasis (VL). |
|------------------------------|-----------------|-----------------|-----------------|
| Sample                      | Total no.       | Positive OligoC-TesT result, no. | Specificity, % (95% CI) | Sensitivity, % (95% CI) |
| Nonendemic control subjects |                 |                              |                              |                         |
| Patients with HAT (DRC)     | 20              | 1                            | 95 (76.4–99.1)               | ...                      |
| Patients with malaria (Zambia) | 20              | 0                            | 100 (83.9–100)               | ...                      |
| Patients with Chagas disease (Chile) | 20              | 0                            | 100 (83.9–100)               | ...                      |
| Endemic control subjects    |                 |                              |                              |                         |
| Biopsy specimens (Peru)     | 8               | 0                            | 100 (67.6–100)               | ...                      |
| Blood (Kenya)               | 18              | 1                            | 94.4 (74.2–99)               | ...                      |
| Blood (Sudan)               | 19              | 1                            | 94.7 (75.4–99.1)             | ...                      |
| Patients with CL            |                 |                              |                              |                         |
| Biopsy specimens (Peru)     | 36              | 33                           | ...                          | 91.7 (78.2–97.1)         |
| Lesion scrapings (Peru)     | 8               | 8                            | ...                          | 100 (67.6–100)           |
| Patients with MCL           |                 |                              |                              |                         |
| Biopsy specimens (Peru)     | 12              | 11                           | ...                          | 91.7 (64.6–98.5)         |
| Patients with VL            |                 |                              |                              |                         |
| Blood (Kenya)               | 25              | 23                           | ...                          | 92 (75–97.8)             |
| Blood (Sudan)               | 18              | 14                           | ...                          | 77.8 (54.8–91)           |
| Bone marrow (Sudan)         | 12              | 11                           | ...                          | 91.7 (64.6–98.5)         |
| Lymph node (Sudan)          | 6               | 6                            | ...                          | 100 (61–100)             |

NOTE. CI, confidence interval (by Wilson’s method); DRC, Democratic Republic of the Congo; HAT, human African trypanosomiasis.
in the lesion, (2) delay between sample collection and testing, or (3) false-negative microscopic results caused by staining artifacts. This is supported by the fact that 3 of the 4 *Leishmania* OligoC-TesT negative samples were also negative in a second PCR analysis performed in Peru targeting the *Leishmania* kinetoplast DNA [28] (authors' unpublished data). *Leishmania* DNA could be detected in all lesion scrapeings, which is encouraging, because this offers a less invasive sampling procedure compared with biopsy punctures. The higher sensitivity for lesion scrapeings can be explained by the lower amount of PCR-inhibiting factors. This is confirmed by the observation of Garcia et al. [29], who reported a higher sensitivity of PCR for lesion scrapeings than for biopsy specimens in 44 Bolivian patients with CL or MCL. The similar sensitivities of our method for CL and MCL samples is encouraging, considering the generally low parasite load in the latter.

The sensitivity of 86% in blood from patients with confirmed VL is promising and suggests that the OligoC-TesT can contribute significantly to less invasive VL diagnosis. Patients with clinically suspected infection who have positive serologic results and positive *Leishmania* OligoC-TesT results in blood might not need to undergo the invasive bone marrow or spleen sampling. Previous PCR studies found the same range of sensitivity in blood [9, 30]. The lower sensitivity of the test in blood samples from Sudan (77.8%) versus those from Kenya (92%) could be due to bias in parasite load or experimental differences during DNA extraction. Further evaluations with different types of extraction methods might be recommended. Despite the low numbers of available lymph node samples, the observation that all of them showed positive *Leishmania* OligoC-TesT results is encouraging. Indeed, if molecular diagnosis in lymph nodes proves to have high sensitivity, this would be a major step forward in the search for a noninvasive method for diagnosing VL. Previous studies showed similar findings in a larger number of lymph node samples [31, 32]. Further studies of molecular diagnosis in the lymph would be interesting, especially for East African VL, for which the majority of patients have enlarged lymph nodes [33].

Two of 37 DAT-negative blood samples from Kenyan and Sudanese endemic control subjects were positive with the *Leishmania* OligoC-TesT. Although PCR contamination can never be 100% excluded and low sensitivity of the DAT in Sudan has been reported [34], these positive results in the healthy control group are probably due to asymptomatic infections. In VL-endemic regions, there is a high prevalence of asymptomatic infections, which are generally not treated because the available drugs are very toxic. Therefore, the interpretation of PCR results should always take into account possible asymptomatic infections [35].

Multiple attempts to simplify and speed up the detection of PCR products have been reported, such as PCR-ELISA [12, 13] and fluorescent self-probing amplicons [36], but none of them merges simplicity and speed with “low-tech” approaches. The *Leishmania* OligoC-TesT combines the sensitivity and specificity of PCR with the simplicity and speed of membrane oligochromatography. Detection of PCR products is performed in 10 min and does not require any equipment except for a water bath and a pipette. The cost of the *Leishmania* Ampli-Mix components is similar to that of conventional PCR mix, and the cost of the Oligo-Strip is much lower than that of commercial agarose for the same number of samples. The global cost of diagnosis is reduced with the OligoC-TesT, because the internal amplification control eliminates the need for additional PCRs to check inhibition.

A common drawback of PCR in the diagnostic process is carryover contamination leading to false-positive results. The implementation of the dUTP/UNG decontamination system and the single-step detection format of the *Leishmania* OligoC-TesT facilitate contamination control. Presentation of the *Leishmania* OligoC-TesT as a self-containing kit with a ready-to-use PCR mix and dipsticks will allow quality control and standardization. However, sampling and DNA extraction are also important steps for accurate molecular diagnosis and need to be standardized. The described tool may help in the rapid and simple molecular diagnosis of leishmaniasis and in epidemiologic studies for which a sensitive marker of infection is required, although the current format is not directly designed for high-throughput applications.

There is a need for further phase 2 and 3 studies to estimate the diagnostic accuracy of the *Leishmania* OligoC-TesT in patients with suspected infection and with positive serologic test results, healthy endemic controls, and patients with other pathologic conditions. Furthermore, a multicentric study in which different PCR methods are evaluated on the same samples would be highly valuable. In addition, a standardized oligochromatographic format for identification of *Leishmania* species would be welcome, given the high importance of accurate species identification in patient management, disease control, and epidemiologic studies. Recently, a first prototype to discriminate between *L. infantum* and *L. donovani* by means of the oligochromatographic approach has been developed (T. Laurent, G.V.d.A., M. Hide, et al., unpublished data). The combination of the dipstick with novel isothermal nucleic acid amplification techniques, such as nucleic acid sequence-based amplification [37, 38] and loop-mediated isothermal amplification [39], may circumvent the need for thermocycling reactions and therefore further simplify molecular diagnosis.

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