JCM Accepted Manuscript Posted Online 25 April 2018
J. Clin. Microbiol. doi:10.1128/JCM.00386-18

Development and evaluation of a novel LAMP assay for the diagnosis of Cutaneous and Visceral Leishmaniasis.

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Abstract

Introduction: A novel *Pan-Leishmania* LAMP assay was developed for diagnosis of Cutaneous and Visceral Leishmaniasis (CL & VL) which can be used in near-patient settings.

Methods: Primers were designed on the 18S rDNA and the conserved region of minicircle kDNA selected on the basis of high copy number. LAMP assays were evaluated for CL in a prospective cohort trial of 105 patients in South-West Colombia. Lesion swab samples from CL suspects were collected and tested using LAMP and compared to a composite reference of microscopy AND/OR culture to calculate diagnostic accuracy. LAMP assays were tested on 50 VL suspected patients from Ethiopia, including whole blood, peripheral blood mononuclear cells, and buffy coat. Diagnostic accuracy was calculated against a reference standard of microscopy of splenic or bone marrow aspirates. To calculate analytical specificity 100 clinical samples and isolates with fever causing pathogens including malaria, arboviruses and bacterial infections were tested.

Results & Conclusions: The LAMP assay had a sensitivity of 95% (95% CI: 87.2% - 98.5 %) and a specificity of 86% (95% CI: 67.3% -95.9 %) for the diagnosis of CL. On VL suspects the sensitivity was 92% (95% CI: 74.9 – 99.1%) and specificity of 100% (95% CI: 85.8-100%) in whole blood. For CL, LAMP is a sensitive tool for diagnosis and requires less equipment, time and expertise than alternative CL diagnostics. For VL, LAMP is sensitive using a minimally invasive sample as compared to the gold standard. The analytical specificity was 100%.

Introduction:

Infection with *Leishmania* parasites causes a spectrum of diseases from self-healing skin ulcers, cutaneous leishmaniasis (CL), to the potentially fatal form, visceral leishmaniasis (VL) affecting internal organs, in particular spleen, liver, bone marrow and lymph nodes. Accurate and opportune laboratory diagnosis followed by appropriate treatment is crucial in patient management, and to decrease transmission (1). Parasitological confirmation by microscopy of biopsies, sometimes in combination with culture techniques, remains the reference standard laboratory diagnosis for both VL and CL. For VL serological diagnostics are available in the form of rapid diagnostics tests (RDTs) and ELISAs optimally based on rK39 (2, 3), the Direct Agglutination Test (DAT)(4), and immunofluorescent antibody tests (IFA/IFAT). However, due to poor specificity, these tests are only recommended for use after prolonged fever, most commonly taken as more than 14 days of fever (3). No alternative serological diagnostic test exists for CL due to low concentrations of circulating antibodies; as regards molecular tools, several diagnostic protocols have been validated for CL but no reference standards are currently available.

Reference standard diagnostic tests suffer from challenges; microscopy can be poorly sensitive, lack standardisation of tissue collection, requires quality control and invasive sample types. The rK39 RDTs lack specificity and also sensitivity in certain regions, and culture methods require time, expertise and considerable infrastructure.

A recent advance in nucleic acid-based diagnostics has been the development of loop-mediated isothermal amplification (LAMP) of DNA. LAMP diagnostic kits have been developed for a variety of infectious diseases, including tuberculosis (5, 6), human African trypanosomiasis (HAT) (7) and malaria (8, 9). This nucleic acid amplification technique (NAAT) uses only one enzyme (*Bst* DNA polymerase) and is able to amplify large amounts of DNA within 40 minutes by the intricate design of primers and auto-strand displacement DNA synthesis. A thermocycler is not required because the enzyme works under isothermal conditions, reagents are dried-down with no requirement of a cold-

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chain. Results can be read visually, and there is no post-amplification handling or processing. This makes LAMP a powerful diagnostic test for disease endemic settings, bringing molecular diagnostics closer to the patient.

Recent modelling data for VL diagnostics shows that early diagnosis and treatment of patients has the potential to greatly reduce transmission of disease in elimination areas (1). Diagnostics that can specifically identify and allow treatment of patients earlier than is currently possible have the potential to reduce transmission.

There have been previous attempts to develop a LAMP test for Leishmaniasis (10, 11). However, these were in-house assays rather than diagnostic kits, and should not be considered for quality-controlled, standardised use. In addition, the design of some LAMP primer sets is geographically focused and therefore not suitable for all Leishmania endemic areas (11). Here we describe the development of a novel LAMP assay, with the advantages of quality control and standardisation that comes with product development. Data collected during development of the assay from cohorts of suspected CL patients in Colombia, and suspected VL patients from Ethiopia, are presented.

Material and Methods:

Target selection: A combination of literature searches and sequence alignment identified potential target genes conserved within the genus *Leishmania* that had low homology (<80%) to other targets, including the *Trypansoma* as the nearest taxonomic pathogen to *Leishmania*, and human DNA. The Leishmania were represented by the five reference genomes of *L. major* MHOM/IL/1981/Friedlin(12), *L. braziliensis* MHOM/BR/1975/M2904 (13), *L. mexicana* MHOM/GT/2001/U1103cl25 (14), *L. donovani* MHOM/NP/2003/BPK282/0cl4 (15) and *L. infantum* (JPCM5) MCAN/ES/1998/LLM-87 (13). To exclude targets that could be amplified due to trypanosomiasis, the *Trypansoma* were represented by *T. cruzi* CL Brenner (TcVI) (16), *T. cruzi* Sylvio ×10/1 (TcI) (17), *T. vivax* Y486 (18), *T. brucei* TREU927 (19), and *T. congolense* IL3000 (20). Sequence conversation and suitability as a LAMP target were assessed using genome-wide sequence alignment

with MAVID (21) following construction of an orthology map with Mercator (22), which identified CDS regions with Genscan, and measured orthology with BLAT and MUMmer. Sequences totalling 21 Mb in length were present in all five *Leishmania* (average ~32 Mb) and absent in all five *Trypansoma*. 14,030 candidate LAMP targets of >200 bp were identified - these spanned 7,942 Kb of sequence. Gene length, GC content and in silico copy number were evaluated using read information with Samtools v0.1.11 (23). Gene length, GC content and in silico copy number were evaluated using read information with Samtools v0.1.11 (23) and a priority list of targets for primer design was made.

Further sequence alignment showed only 18 substitutions at the 2,190 bp 18S rDNA gene using GenBank PopSet 254847845 (24) across nine *Leishmania* species (the five above plus *L. amazonensis*, *L. guyanensis*, *L. panamensis* and *L. tropica*). The 3' end (1954-2190) of the 18S rDNA gene showed some homology to *Trypanosoma*.

Copy-number calculation: In order to rank genes in priority for LAMP primer design, the copy number of each potential target was experimentally calculated as follows. Promastigotes were cultivated in RPMI-1640 culture medium containing 10% Fetal calf serum, at 27°C. DNA from 8 *Leishmania* species were extracted using the DNeasy extraction kit (Qiagen) at the log phase. For the purposes of this study we refer to VL causing species (*L. donovani* and *L. infantum*) and CL causing species (*L. tropica, L. major, L. braziliensis, L. mexicana, L. panamensis, L. guyanensis*). Alignments were made using T coffee software (www.tcoffee.crg.cat/) for all target genes except the kDNA where existing primer sets from Cavalcanti *et al.* (25) were used. Each target gene was amplified with HotStar Taq polymerase, using the following protocol: 94°C 10min., followed by 40 cycles of 94°C for 30s., 52°C, 55°C or 58°C for 30s and 72°C for 30S, and final hold of 72°C for 10 min. Resultant fragments were cloned as single copy vectors using TOPO TA cloning kit (Invitrogen). Colony PCR was used to select colonies containing the insert, and transformants were cultured in LB medium containing ampicillin (50µg/mL). DNA was extracted using the Qiagen plasmid midi kit, and DNA digestion was performed for 2 hours at 37°C with EcoRI and HINDIII/XBA restriction enzymes. A sybr green qPCR was performed for each target gene: 95°C 5 min. followed by 40 cycles of 95°C for

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116 10s and 60°C for 40s. A 10 fold dilution series of vector DNA was used as a standard curve, where the
117 vector was known to contain a single copy of the target gene, and the weight of the vector was
118 known. Additionally, a 5 fold dilution series of promastigote DNA was included, whereby the weight
119 of the whole genome was known (14). The DNA concentrations of the vector and genomic DNA were
120 determined using the ThermoScientific nanodrop 1000 spectrophotometer. The Cycle threshold (Ct)
121 of genomic DNA was compared with the Ct of vector DNA and copy number was calculated by the
122 standard formula:

123 $$\text{Copy number} = \frac{\text{Known Concentration of Vector DNA (ng/µl)/Vector weight (ng)}}{\text{Concentration genomic DNA (ng/ul) calculated by qPCR/Genome weight (ng)}}$$

125 The gene 7SL, was used as a control as a known single-copy gene (26).

126 *Primer design:* Genes 18S rDNA, Histone H3 and kDNA (Table 1) were chosen for primer design
127 based on their high copy-number. PrimerExplorer version 4.0 software
128 http://primerexplorer.jp/elamp4.0.0 was used to design LAMP primers on all 3 targets. Targets with
129 highest copy number were multiplexed to optimise sensitivity.

130 *Limit of Detection:* In order to determine the limit of detection (LOD) of each of the prototype LAMP
131 primer sets, serial dilutions of DNA from 8 species (see above) from different geographical areas,
132 including Asia and Africa were tested; this included 2 strains for CL causing species and 4 strains for
133 VL causing species, *L. infantum* and *L. donovani*. Cultured promastigotes of *Leishmania species* were
134 prepared, and DNA extracted using phenol/chloroform extraction method. A 10-fold dilution series
135 was tested from 1,000 parasites per µl to 0.001 p/µl. To ensure there was no cross reactivity of the
136 LAMP primer sets they were tested with serial dilutions of DNA from *Trypanosoma brucei, T. cruzi,*
137 *Plasmodium falciparum,* human cell lines THP1 and U937, salmon sperm, human whole blood and
138 buffy coat.

139 *Bank of pathogen samples:* In order to ensure the specificity of the LAMP assay, 50 clinical samples
140 or cultured isolates were tested. These included high, medium and low concentration samples of

Plasmodium falciparum, P. vivax, Trypanosome brucei brucei, Trypanosoma cruzi, Giardia lamblia, Cryptosporidium parvum; and 50 isolates of high, medium and low concentration dengue, chikungunya, Zika and bacterial species including *Escherichia coli* and *Klebsiella pneumoniae.*

Cutaneous Leishmaniasis clinical samples: A prospective collection of samples from suspected CL patients from CIDEIM, Colombia (27) was used to estimate the diagnostic sensitivity of the developed prototype multiplex LAMP kit (kDNA plus 18S rDNA primers). One lesion swab sample (Isohelix DNA buccal swabs, SK-1S) was taken per suspected patient by gently rubbing a swab over the ulcer ~10 times, and then stored at -20°C. Qiagen DNAeasy blood and tissue kit (Qiagen, USA) was used to extract DNA according to manufacturer's instructions and eluted in 50µl distilled water. Diagnostic performance of LAMP was compared with a composite reference standard of microscopy AND/OR culture positivity. Briefly, two slides with three lesion smears on each slide were microscopically examined for amastigotes; parasite isolation in semisolid culture medium was attempted from 4 independent lesion aspirates from each participant. Parasite isolation was traced for a maximum of one-month post-inoculation.

Ethics: This study was approved and monitored by the Centro Internacional de Entrenamiento e Investigaciones Médicas institutional review board for ethical conduct of research involving human subjects

Visceral Leishmaniasis clinical samples: Blood was collected from 50 VL suspected patients from University of Gondar Hospital, Amhara Regional State, Northern Ethiopia, in 2013. In order to determine the optimal extraction method with respect to parasite numbers, we compared the isolation of peripheral blood mononuclear cells (PBMC) with buffy coat, and with whole blood stored in heparin tubes from the same patient samples. PBMC were isolated by slowly layering two ml heparin blood on top of an equal volume of Histopaque (Sigma, Aldrich), the sample was centrifuged for 30 minutes at 2000 rpm. The PBMC fraction was removed and suspended in 600 µl PBS, and once again centrifuged for 1 minute at 8000 rpm. The cells were re-suspended in 180µl PBS. The buffy

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coat fraction was isolated by centrifuging two ml heparin blood for 5 minutes at 8,000 rpm. The buffy coat fraction was removed and suspended in PBS to a total volume of 180 µl. All PBMC, buffy coat and plain heparin blood samples (180 µl volumes) were mixed with an equal volume of AS1 buffer (Qiagen). DNA was extracted with Qiagen mini blood and tissue extraction kit according to protocol and resultant samples stored at -20°C. LAMP and qPCR were compared to microscopy of either bone-marrow or spleen-aspirate as the gold standard.

Ethics: This study was approved and monitored by the University of Gondar Institutional Review Board, Ethiopia, (Ref R/C/S/V/P/05/664/2013). Written informed consent was obtained from patients for the use of their specimens in the study.

Molecular methods:

LAMP: LAMP (Eiken Chemical, Japan) was performed as per manufacturer's instructions. 3µl extracted DNA was added to a LAMP tube plus 27µl water. Tubes were turned upside down for 2 minutes to release the dried-down reagents in the cap of the tube. Samples were briefly centrifuged and then placed in a real-time turbidimeter (Eiken) at 65°C for 40 minutes, and then 80°C for 2 minutes. A LAMP reaction was considered positive for *Leishmania* if fluorescence was observed visually and if a positive reaction was observed in the turbidimeter. Discrepancies between these two methods were recorded.

qPCR: qPCR was performed on DNA extracted from swab samples as described in Adams *et al.* 2014, based on amplification of the 18S rDNA gene for amplification of CL (27). kDNA qPCR was performed on all VL patient samples (N=50). 1.2 µL of DNA was added to 11.3 µL amplification mix containing 6.25 µL iQ Supermix (Bio-Rad, cat.no. 170-8862),0.25 µM forward primer kDNA(5'-TCCCAAACTTTTCTGGTCCT-3'), 0.25 µM reverse primer kDNA(5'-TTACACCAACCCCCAGTTTC-3'), 0.12 µM probe kDNA (5' FAM- TTCTGCGAAAACCGAAAAATGGGTGC-BHQ 3'). The qPCR protocol (Bio-Rad CFX-96) was as follows: 5 minutes at 95°C followed by 40 cycles of 10 s at 95°C and 40 s at 54°C.

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qPCR was used in this programme as a comparator molecular method to LAMP to understand results fully. Diagnostic accuracy of data, including sensitivity and specificity, was calculated for LAMP and qPCR. This study followed the STAARD guidelines, including blinding of index and reference diagnostic tests.

Results:

Target selection: The relative copy number of nine different target genes was calculated for the 8 *Leishmania* species tested, with an average across two strains. Results are shown for the highest copy number targets in Table 1, relative to the known single copy target of the 7SL gene.

Prototype primer sets: Primer design was attempted on 3 target genes, the 18S rDNA and the Histone 3 for *pan-Leishmania* assays and the kDNA for a VL specific assay. Each primer set was tested for the Limit of Detection (LOD) using a real-time LAMP turbidimeter. The kDNA had the lowest LOD at 0.0001 p/µl on *L. donovani* and *L. infantum*, and no amplification with other CL causing *Leishmania* species. The 18S rDNA had the next lowest LOD at 0.01-0.001 p/µl. The Histone LAMP primers had a similar LOD to the 18s rDNA at 0.01 p/µl, but could not amplify all *L. guyanensis* and *L. braziliensis*, indicating low sequence homology to some South American strains. The targets 18S rDNA and kDNA, were multiplexed to optimise sensitivity for detecting VL, and ability to detect all Leishmania species that cause CL; LOD was not affected by multiplexing. All testing of primer sets was performed using the dried-down LAMP assay developed by Eiken. No cross-reaction was observed with serial dilutions of DNA from *T. brucei, T. cruzi, P. falciparum,* salmon sperm, human cell lines THP1 and U937, human whole blood and buffy coat.

Bank of pathogen samples: Of 100 clinical samples and cultured isolates from different pathogens, none were positive with the Leish LAMP kit, showing an analytical specificity of 100%.

Cutaneous Leishmaniasis samples: 105 clinical suspects were enrolled. A complete description of demographic and clinical characteristics of study participants and *Leishmania* species is reported in

Adams *et al*., 2014. Parasites were isolated and identified in 64% of the participants; *L. panamensis* predominated overall, with representation from the *Viannia* and *Leishmania* subgenera. Compared to the reference standard of microscopy AND/OR culture, LAMP (kDNA + 18S rDNA) was 95% sensitive (95% CI: 87.22 % to 98.53 %) and 86% specific (95% CI: 67.32 % to 95.88 %). This compared well with qPCR data on the same samples, which showed a sensitivity of 97% (95% CI: 91%-100%) and specificity of 84% (95% CI: 64%-95%).

Visceral Leishmania samples: In this study, 50 VL suspects were enrolled, of which 26 were positive for VL by microscopy of the splenic (n=19 positive) or bone marrow (n=7 positive) aspirates. Of the 26 VL positive individuals 27% (n=7) were also positive for HIV.

Parasite load was quantified by qPCR in order to compare the extraction efficiency of the different sample types (whole blood, buffy coat and PBMCs). Of the 26 VL parasitologically positive individuals, the highest parasite load as determined by *ct* threshold on kDNA qPCR, was found in whole blood (n=19) followed by buffy-coat (n=5) and PBMC (n=1) of 25 qPCR positive individuals.

On VL suspects from Ethiopia, the sensitivity from whole blood was 92% (95% CI: 74.9 – 99.1%) and specificity of 100% (95% CI: 85.8-100%). The sensitivity of kDNA qPCR was 96% (95% CI: 80.1-99.9%) and specificity of 92% (95% CI: 73-99%). The sensitivity of both tests was the same on buffy coat samples but this decreased to 89% in PBMCs samples (see Table 2). The specificity was highest on LAMP from whole blood at 100% dropping to 96% on other sample types (see Table 2). qPCR had a specificity of between 92% and 95% depending on sample type. However, sample numbers are limited and confidence intervals overlap. Notably, all HIV positive patients (n=7) were positive for both qPCR and LAMP in all sample types.

No discrepancies were reported between the visual analysis of the LAMP tubes for fluorescence and the real-time turbidimeter data.

Discussion:

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This study presents data on the development of a LAMP diagnostic kit capable of detecting both VL and CL. Target genes were chosen based on high copy number and conservation across multiple strains and species of *Leishmania* across geographic areas. 18S rDNA and kDNA primer sets were multiplexed to ensure a sensitive reaction for VL (kDNA) and the capability to detect all species causing CL (18S rDNA). Since the differential diagnosis and sample types of VL and CL does not overlap, the combination of primers is considered appropriate. Testing was performed on geographically distinct strains and species of *Leishmania* to ensure production of a robust and reliable test. The multiplex LAMP was highly sensitive and specific, with a limit of detection between 0.01-0.001 parasites per µl for CL causing species, and 0.0001 parasites per µl for VL causing species on purified DNA. LAMP was 100% specific when tested on a range of fever causing organisms with overlapping epidemiology as *Leishmania;* this included, bacterial infections, malaria, arboviruses and other protozoan infections. LAMP was taken forward for testing on prospective clinical sample collections. In a cohort of suspected CL patients from South West Colombia LAMP was 95% sensitive (95% CI: 87.2%-98.5%) and 86% specific (95% CI: 67.3%-95.9 %). In a study on 50 suspected VL patients the LAMP showed a sensitivity of 92.3% (95% CI: 74.9%-99.1%) and a specificity of 100% (95% CI: 86.7%-100%) on whole blood. Due to the reduced number of sample handling steps and reduced *ct* values, the whole blood sample type is preferable for VL compared with PBMCs and buffy coat.

For CL samples, the LAMP test showed overlapping confidence intervals with qPCR, and was more sensitive than culture and microscopy alone. The lower specificity of both qPCR and LAMP may represent patients not detected by the composite reference standard, as no perfect gold standard test for CL exists. Multiple studies including (27, 28), have concluded that the molecular tools are more sensitive for diagnosing CL, and therefore, false positives in molecular tools may be considered truly positive patients. Follow-up studies of participants needs to be conducted to determine whether this is the case. Swab sampling on lesions was an appropriate collection method, although this would need adaptation to areas where non-ulcerated lesions of CL are prevalent.

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In VL samples, LAMP performed on DNA extracted from whole blood showed good sensitivity compared to microscopy of highly invasive biopsy samples. The kDNA qPCR was only slightly more sensitive (96%) as compared to LAMP (92%) in the Ethiopian collection. The extraction of DNA from whole blood resulted in a higher parasite load in qPCR than either processing PBMC or buffy-coat in spite of the fact that, for the latter two methods, ten times more blood was used for extraction (200 µl vs 2 ml). Using plain heparin blood for extraction is favourable since a smaller volume of blood is required, and the processing of this sample type requires fewer handling steps. Microscopic examination on bone-marrow or spleen aspirates will remain the gold standard because parasite numbers are high in these sample types. However, based on these results, LAMP could potentially be used to confirm infection in the majority of patients, and then aspiration performed in those that are LAMP negative but who remain VL suspects. This would circumvent aspiration in the majority of patients. Also in areas where it is not possible to take biopsy samples due to lack of appropriate medical facilities LAMP may be used as to confirm infection.

Modelling studies have suggested the use of highly specific diagnostic tools for the detection of VL in elimination zones (1). This would enable treatment of cases earlier than is currently possible in the diagnostic algorithm. Results for VL suspects in Ethiopia show a high specificity of 100% (95% CI: 85.8-100%), intimating that LAMP may be a suitable tool for this purpose.

LAMP has been developed as a platform diagnostic tool and is now available for malaria, tuberculosis and HAT, as well as a range of viral and bacterial infections. Here, this powerful diagnostic tool has now been designed for Leishmaniasis, and tested on *Leishmania* suspects from Colombia and Ethiopia. LAMP is simple and does not require expensive equipment and therefore can be used in basic laboratory facilities with minimal DNA extraction facilities. Further development work and evaluation data is required. For CL it would be useful to follow up patients with (false) positive LAMP reactions to see if they become positive with the gold standard. As alternative diagnostics exist for VL, the use of LAMP may be to confirm suspected patients in areas with poor infrastructure, and before 14 days of fever has passed.

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Acknowledgements:

We would like to thank all the local staff in Colombia and Gondar LRTC for enrolling patients, processing samples and performing reference standard diagnostic tests, especially Ruby Rios, Ricardo Marquez and Alexandra Cossio. We would also like to thank all the patients for their time and participating in this study.

References:

1. **Medley GF, Hollingsworth TD, Olliaro PL, Adams ER.** 2015. Health-seeking behaviour, diagnostics and transmission dynamics in the control of visceral leishmaniasis in the Indian subcontinent. *Nature* **528:**S102-108.
2. **Cunningham J, Hasker E, Das P, El Safi S, Goto H, Mondal D, Mbuchi M, Mukhtar M, Rabello A, Rijal S, Sundar S, Wasunna M, Adams E, Menten J, Peeling R, Boelaert M, Leishmaniasis WTV.** 2012. A Global Comparative Evaluation of Commercial Immunochromatographic Rapid Diagnostic Tests for Visceral Leishmaniasis. *Clinical Infectious Diseases* **55:**1312-1319.
3. **Boelaert M, Verdonck K, Menten J, Sunyoto T, van Griensven J, Chappuis F, Rijal S.** 2014. Rapid tests for the diagnosis of visceral leishmaniasis in patients with suspected disease. *Cochrane Database Syst Rev* **6:**CD009135.
4. **Chappuis F, Rijal S, Soto A, Menten J, Boelaert M.** 2006. A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *BMJ* **333:**723.
5. **Mitarai S, Okumura M, Toyota E, Yoshiyama T, Aono A, Sejimo A, Azuma Y, Sugahara K, Nagasawa T, Nagayama N, Yamane A, Yano R, Kokuto H, Morimoto K, Ueyama M, Kubota M, Yi R, Ogata H, Kudoh S, Mori T.** 2011. Evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis. *International Journal of Tuberculosis and Lung Disease* **15:**1211-1217.
6. **Boehme CC, Nabeta P, Henostroza G, Raqib R, Rahim Z, Gerhardt M, Sanga E, Hoelscher M, Notomi T, Hase T, Perkins MD.** 2007. Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *Journal of Clinical Microbiology* **45:**1936-1940.
7. **Njiru ZK, Mikosza AS, Armstrong T, Enyaru JC, Ndung'u JM, Thompson AR.** 2008. Loop-Mediated Isothermal Amplification (LAMP) Method for Rapid Detection of *Trypanosoma brucei rhodesiense*. *PLoSNeglTropDis* **2:**e147.
8. **Hopkins H, Gonzalez IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C, Agaba B, Kyabayinze DJ, Sutherland CJ, Perkins MD, Bell D.** 2013. Highly sensitive detection of malaria parasitemia in a malaria-endemic setting: performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda. *J Infect Dis* **208:**645-652.
9. **Polley SD, Gonzalez IJ, Mohamed D, Daly R, Bowers K, Watson J, Mewse E, Armstrong M, Gray C, Perkins MD, Bell D, Kanda H, Tomita N, Kubota Y, Mori Y, Chiodini PL, Sutherland**

CJ. 2013. Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. *J Infect Dis* **208:**637-644.

10. **Adams ER, Schoone GJ, Ageed AF, Safi SE, Schallig HD.** 2010. Development of a reverse transcriptase loop-mediated isothermal amplification (LAMP) assay for the sensitive detection of Leishmania parasites in clinical samples. *AmJTropMedHyg* **82:**591-596.
11. **Verma S, Avishek K, Sharma V, Negi NS, Ramesh V, Salotra P.** 2013. Application of loop-mediated isothermal amplification assay for the sensitive and rapid diagnosis of visceral leishmaniasis and post-kala-azar dermal leishmaniasis. *Diagn Microbiol Infect Dis* **75:**390-395.
12. **Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, Sisk E, Rajandream MA, Adlem E, Aert R, Anupama A, Apostolou Z, Attipoe P, Bason N, Bauser C, Beck A, Beverley SM, Bianchettin G, Borzym K, Bothe G, Bruschi CV, Collins M, Cadag E, Ciarloni L, Clayton C, Coulson RM, Cronin A, Cruz AK, Davies RM, De Gaudenzi J, Dobson DE, Duesterhoeft A, Fazelina G, Fosker N, Frasch AC, Fraser A, Fuchs M, Gabel C, Goble A, Goffeau A, Harris D, Hertz-Fowler C, Hilbert H, Horn D, Huang Y, Klages S, Knights A, Kube M, Larke N, Litvin L, et al.** 2005. The genome of the kinetoplastid parasite, Leishmania major. *Science* **309:**436-442.
13. **Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC, Quail MA, Peters N, Adlem E, Tivey A, Aslett M, Kerhornou A, Ivens A, Fraser A, Rajandream MA, Carver T, Norbertczak H, Chillingworth T, Hance Z, Jagels K, Moule S, Ormond D, Rutter S, Squares R, Whitehead S, Rabbinowitsch E, Arrowsmith C, White B, Thurston S, Bringaud F, Baldauf SL, Faulconbridge A, Jeffares D, Depledge DP, Oyola SO, Hilley JD, Brito LO, Tosi LR, Barrell B, Cruz AK, Mottram JC, Smith DF, Berriman M.** 2007. Comparative genomic analysis of three Leishmania species that cause diverse human disease. *NatGenet* **39:**839-847.
14. **Rogers MB, Hilley JD, Dickens NJ, Wilkes J, Bates PA, Depledge DP, Harris D, Her Y, Herzyk P, Imamura H, Otto TD, Sanders M, Seeger K, Dujardin JC, Berriman M, Smith DF, Hertz-Fowler C, Mottram JC.** 2011. Chromosome and gene copy number variation allow major structural change between species and strains of Leishmania. *Genome Res* **21:**2129-2142.
15. **Downing T, Imamura H, Decuypere S, Clark TG, Coombs GH, Cotton JA, Hilley JD, de Doncker S, Maes I, Mottram JC, Quail MA, Rijal S, Sanders M, Schonian G, Stark O, Sundar S, Vanaerschot M, Hertz-Fowler C, Dujardin JC, Berriman M.** 2011. Whole genome sequencing of multiple Leishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. *Genome Res* **21:**2143-2156.
16. **Weatherly DB, Boehlke C, Tarleton RL.** 2009. Chromosome level assembly of the hybrid Trypanosoma cruzi genome. *BMC Genomics* **10:**255.
17. **Franzen O, Ochaya S, Sherwood E, Lewis MD, Llewellyn MS, Miles MA, Andersson B.** 2011. Shotgun sequencing analysis of Trypanosoma cruzi I Sylvio X10/1 and comparison with T. cruzi VI CL Brener. *PLoS Negl Trop Dis* **5:**e984.
18. **Jackson AP, Allison HC, Barry JD, Field MC, Hertz-Fowler C, Berriman M.** 2013. A cell-surface phylome for African trypanosomes. *PLoS Negl Trop Dis* **7:**e2121.
19. **Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, Lennard NJ, Caler E, Hamlin NE, Haas B, Bohme U, Hannick L, Aslett MA, Shallom J, Marcello L, Hou L, Wickstead B, Alsmark UC, Arrowsmith C, Atkin RJ, Barron AJ, Bringaud F, Brooks K, Carrington M, Cherevach I, Chillingworth TJ, Churcher C, Clark LN, Corton CH, Cronin A, Davies RM, Doggett J, Djikeng A, Feldblyum T, Field MC, Fraser A, Goodhead I, Hance Z, Harper D, Harris BR, Hauser H, Hostetler J, Ivens A, Jagels K, Johnson D, Johnson J, Jones K, Kerhornou AX, Koo H, Larke N, et al.** 2005. The genome of the African trypanosome *Trypanosoma brucei*. *Science* **309:**416-422.
20. **Jackson AP, Berry A, Aslett M, Allison HC, Burton P, Vavrova-Anderson J, Brown R, Browne H, Corton N, Hauser H, Gamble J, Gilderthorp R, Marcello L, McQuillan J, Otto TD, Quail MA, Sanders MJ, van Tonder A, Ginger ML, Field MC, Barry JD, Hertz-Fowler C, Berriman**

M. 2012. Antigenic diversity is generated by distinct evolutionary mechanisms in African trypanosome species. *Proc Natl Acad Sci U S A* **109:**3416-3421.

21. **Bray N, Pachter L.** 2004. MAVID: constrained ancestral alignment of multiple sequences. *Genome Res* **14:**693-699.

22. **Dewey CN.** 2007. Aligning multiple whole genomes with Mercator and MAVID. *Methods Mol Biol* **395:**221-236.

23. **Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S.** 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25:**2078-2079.

24. **de Almeida ME, Steurer FJ, Koru O, Herwaldt BL, Pieniazek NJ, da Silva AJ.** 2011. Identification of Leishmania spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2. *J Clin Microbiol* **49:**3143-3149.

25. **de Paiva Cavalcanti M, Felinto de Brito ME, de Souza WV, de Miranda Gomes Y, Abath FG.** 2009. The development of a real-time PCR assay for the quantification of Leishmania infantum DNA in canine blood. *Vet J* **182:**356-358.

26. **Zelazny AM, Fedorko DP, Li L, Neva FA, Fischer SH.** 2005. Evaluation of 7SL RNA gene sequences for the identification of Leishmania spp. *Am J Trop Med Hyg* **72:**415-420.

27. **Adams ER, Gomez MA, Scheske L, Rios R, Marquez R, Cossio A, Albertini A, Schallig H, Saravia NG.** 2014. Sensitive diagnosis of cutaneous leishmaniasis by lesion swab sampling coupled to qPCR. *Parasitology* **141:**1891-1897.

28. **Faber WR, Oskam L, van Gool T, Kroon NC, Knegt-Junk KJ, Hofwegen H, van der Wal AC, Kager PA.** 2003. Value of diagnostic techniques for cutaneous leishmaniasis. *J Am Acad Dermatol* **49:**70-74.

Tables:

Table 1. Three highest copy number targets with low homology to other related pathogens. Copy number taken from literature and experimentally.

Target	Copy number Literature	Copy number experimental
Kinetoplast DNA (visceral leishmaniasis)	> 10,000	7,500-22,500
18S ribosomal DNA	20-200	300-2200
Histone H3	X	80-380
7SL RNA	1	1

Table 2. Correlation between Microscopy, PCR and LAMP on blood, PBMC and buffy-coat from 50 VL suspected patients from Ethiopia, Gondar teaching hospital. TP = True Positive, FN = False Negative, TN = True Negative, FP = False Positive. PBMC = Peripheral Blood Mononuclear Cells. 95% CI = 95% Confidence Intervals.

Sample	**Whole Blood**		**PBMCs**		**Buffy-coat**	
Test	**PCR (95% CI)**	**LAMP (95% CI)**	**PCR (95% CI)**	**LAMP (95% CI)**	**PCR (95% CI)**	**LAMP (95% CI)**
TP	25	24	25	23	25	24

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FN	1	2	1	3	1	2
TN	22	24	23	23	23	23
FP	2	0	1	1	1	1
Sensitivity	96.1% (80.1-99.9)	92.3% (74.9-99.1)	96.1% (80.1-99.9)	88.5% (69.9-97.6)	96.1% (80.1-99.9)	92.3% (74.9-99.1)
Specificity	91.7% (73 - 99)	100% (86.7-100)	95.8% (78.9-99.9)	95.8% (78.9-99.9)	95.8% (78.9-99.9)	95.8% (78.9-99.9)

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