Introduction

Human African trypanosomiasis (HAT) is a protozoan disease caused by the Trypanosoma brucei species, which are cyclically transmitted by tsetse flies. Two subspecies are pathogenic to man: Trypanosoma brucei (T.b.) gambiense in central and western Africa, and Trypanosoma brucei rhodesiense in eastern and southern Africa [1]. Currently, less than 10,000 cases per year are reported by the World Health Organization, of which over 70% occur in the Democratic Republic of Congo (DRC) [2].

Diagnostic algorithms for T.b. gambiense HAT generally start using the Card Agglutination Test for Trypanosomiasis (CATT) as initial screening for the presence of antibodies. Those testing positive in CATT are then subjected to parasitological tests for confirmation of the infection [3]. Parasitological confirmation relies on the microscopic search for parasites either in lymph, blood or cerebrospinal fluid (CSF). The most sensitive method is based on the mini-anion exchange centrifugation technique (mAECT), yielding an analytical sensitivity of <50 parasites per mL of blood [4,5]. However, given the low parasitemia associated with T.b. gambiense infection, some truly infected individuals remain negative in the mAECT.

Because of the limited sensitivity of parasitological confirmation tests, molecular methods have been developed [6,7] and they generally show high sensitivity and specificity [7]. They can be performed on various specimen types such as whole blood, blood stored on filter paper and CSF. However, the need for laboratory instruments for nucleic acid extraction, amplification and
Characteristics of study samples

For this retrospective evaluation we used DNA extracts from blood of study participants recruited consecutively in 2010 in Bandundu, the most HAT endemic province in DRC [11]. From all participants testing positive on CATT whole blood, the CATT was repeated with sequential plasma dilutions and the end titer was recorded [3]. For diagnostic purposes all were subjected to parasitological confirmation, irrespective of CATT results. Trypanosomes were detected by examination of lymph node aspirate (in subjects with swollen cervical nodes) or by blood examination (all study subjects) with the capillary centrifugation technique (CTC) [12], mAECT on whole blood [8], and mAECT on buffy coat [5]. For patients with parasites detected in the lymph or blood, or with a plasma CATT end titer ≥1:16 a lumbar puncture was done. Parasite detection in CSF was performed with the single modified centrifugation technique [4]. DNA was extracted from blood with the Maxwell® 16 Blood DNA Purification robot (Promega Corporation, Madison, WI, USA) from 200 μL blood stabilised in an equal volume of DNA stabilising GE buffer (6 M guanidium, 0.2 M EDTA, pH 7.5). Final DNA extraction volumes were 300 μL and extracts were stored at −20°C. Time between DNA extraction and LAMP testing was 1.5 to 2 years. All blood samples were also analysed with a Trypanozoon-specific 18S rDNA PCR in duplicate [13]. This PCR amplifies a 120 bp DNA sequence of the Trypanozoon 18S rRNA gene and the amplified product is visualized using conventional electrophoresis in agarose gels and ethidium bromide staining. All PCR testing was done in duplicate at the Institute of Tropical Medicine in Antwerp.

Participants were considered as HAT patients if parasites were detected by any parasitological method in any blood or lymph or CSF sample. Healthy endemic controls were recruited during active screening in the villages [14]. Healthy endemic controls are individuals presenting themselves for CATT screening but with no clinical symptoms of HAT, no previous history of HAT and negative results in CATT whole blood, trypanolysis and mAECT. Individuals with suggestive clinical findings, a positive CATT titer ≥1:4 and positive trypanolytic test that were not confirmed as cases on parasitological testing and who had no previous history of HAT, were classified as HAT suspects. Altogether, frozen DNA from blood of 350 study participants were tested by LAMP: 142 from confirmed HAT patients, 97 from HAT suspects and 111 from healthy endemic controls. In the confirmed HAT patient group, standard tests showed parasites in the blood in 131 cases while in 5 and 6 cases parasites were only detected as cases on parasitological testing and who had no previous history of HAT, were classified as HAT suspects. Trypanosomes were detected by examination of lymph node aspirate (in subjects with swollen cervical nodes) or by blood examination (all study subjects) with the capillary centrifugation technique (CTC) [12], mAECT on whole blood [8], and mAECT on buffy coat [5]. For patients with parasites detected in the lymph or blood, or with a plasma CATT end titer ≥1:16 a lumbar puncture was done. Parasite detection in CSF was performed with the single modified centrifugation technique [4]. DNA was extracted from blood with the Maxwell® 16 Blood DNA Purification robot (Promega Corporation, Madison, WI, USA) from 200 μL blood stabilised in an equal volume of DNA stabilising GE buffer (6 M guanidium, 0.2 M EDTA, pH 7.5). Final DNA extraction volumes were 300 μL and extracts were stored at −20°C. Time between DNA extraction and LAMP testing was 1.5 to 2 years. All blood samples were also analysed with a Trypanozoon-specific 18S rDNA PCR in duplicate [13]. This PCR amplifies a 120 bp DNA sequence of the Trypanozoon 18S rRNA gene and the amplified product is visualized using conventional electrophoresis in agarose gels and ethidium bromide staining. All PCR testing was done in duplicate at the Institute of Tropical Medicine in Antwerp.
incubator (LF-160 incubator, Eiken Chemical co,Taito-ku, Tokyo, Japan). LAMP amplified *Trypanosoma brucei* DNA was visualised using the provided UV-LED device. Amplified DNA emits green fluorescence while there is no fluorescence in negative samples. The provided positive and negative controls were taken in each run (14 tests) to validate the test results.

**Data analysis**

Sensitivity and specificity values and their 95% confidence intervals were calculated for the LAMP in the confirmed HAT patients and in healthy endemic controls, respectively. The sensitivity was defined as the proportion of confirmed HAT patients who are positive by the index test and specificity as the proportion of healthy endemic controls who are negative by the index test. Each DNA extract was tested in duplicate by LAMP. Agreement between LAMP and PCR and reproducibility of LAMP were assessed on all specimens (patients, suspects, controls) with Cohen’s Kappa and interpreted following the grading system described by Landis and Koch (1977) [15]. Data were analysed in Stata, version 11.1 (StataCorp, College Station, Lakeway, Texas, USA).

**Results**

**Diagnostic accuracy**

Of the 142 HAT patients, 132 and 124 were LAMP positive in the first and second run, respectively, with sensitivities of 93.0% (95% CI: 87.5%–96.1%) and 87.3% (95% CI: 80.9–91.8%), respectively (table 1). Of the 11 patients with trypanosomes detected only in lymph or in CSF, 7 were positive in both LAMP runs on blood. Of the 97 HAT suspects, 6 were positive in both replicates of LAMP, and 20 were positive in the first and second replicate, respectively. Of 111 healthy endemic controls, 4 tested positive twice with LAMP and 4 tested positive only once. Specificity estimates range from 92.8% (95% CI 86.4–96.3%) to 96.4% (95% CI 91.1%–98.6%). Sensitivities and specificities of PCR were in the same range as LAMP with overlapping confidence intervals (table 1).

**Agreement between molecular methods**

Assessed on all participants (patients, suspects and healthy controls), agreement between the two LAMP replicates was excellent with a kappa value of 0.82 (95% CI: 0.71–0.92) (table 2), which is in the same range as the PCR replicates (kappa value = 0.82, 95% CI: 0.72–0.92). Agreement between the first replicate of LAMP and the 18S PCR was also excellent with a kappa value of 0.82 (95% CI: 0.72–0.93). Kappa values of LAMP replicates were lower in the subgroups but in the same range as for PCR and with overlapping confidence intervals (table 2).

**Discussion**

In this diagnostic accuracy study, the LAMP showed a sensitivity of 87.3% and 93.0% in the two testing runs. Specificity was 92.8% and 96.4%, with a lowest lower limit of the 95% confidence interval of 86.4%. Agreement between LAMP replicates as well as between LAMP and PCR was excellent with kappa values above 0.8.

The sensitivity of the commercial LAMP kit tested here was equivalent to that of the 18S PCR test, which showed a sensitivity between 87.3% (95% CI: 80.9–91.8) and 90.1% (95% CI: 84.1–94.0) on the same DNA extracts. This is in line with the observation that both tests showed identical analytical sensitivities of 100 parasites per mL of blood in a head-to-head comparison using experimentally prepared blood samples (data not shown). While the LAMP detects the RIME DNA elements (500 copies per haploid genome) [16], the PCR targets the 18S rRNA gene (10–100 copies) [17]. In the 11 confirmed HAT patients with parasites only detected in the lymph or CSF, 7 were positive in both LAMP runs. In contrast, we also observed 5 false negative LAMP results in mAECT positive patients. In the HAT suspects, who could not be confirmed by the parasitological methods, we observed particularly poor agreement between the two LAMP repetitions (kappa = 0.35). These discordances are probably due to the fact that the target DNA concentration in such samples is at the detection limit of the test. If LAMP is to be used to confirm non-confirmed HAT suspects, testing multiple samples from the same patient may increase its sensitivity.

The specificity of the LAMP kit was in the same range as the 18S PCR, which showed a specificity between 96.4% (95% CI 91.1–98.6%) and 97.3% (95% CI 92.3–99.1%) on the same samples. The LAMP was twice positive in 4 of the 111 healthy endemic controls. Three of these LAMP positive controls were at least one time also positive by PCR. Some positive healthy endemic controls may actually be infected with *T. b. gambiense* because the parasitological confirmation algorithm using mAECT is not 100% sensitive, and this may lead to an underestimation of the specificity of the index tests. Another possible reason may be the absence of the LiTat 1.3 variable surface glycoprotein (VSG), which is the antigen used in the CATT and the trypanolysis test, in some strains of *T. b. gambiense* [18,19]. In addition, low antibody titers may be present in early or latent infections [20]. However, false positive LAMP results due to non-specific amplification reactions cannot be excluded. Since the LAMP detects the RIME DNA of all *Trypanozoon*, a transient human infection with *T. b. brucei* could also have led to a positive test result [21]. The recently developed LAMP assay that targets the *T. b. gambiense* specific glycoprotein (TgsGP) gene [16] can exclude an infection with other *Trypanozoon* and thus may be more specific. However, in the

**Table 1.** Sensitivities and specificities of replicate RIME LAMP and 18S PCR on the blood of HAT patients and healthy endemic controls.

| Test | HAT patients (n = 142) | Healthy endemic controls (n = 111) |
|------|-----------------------|-----------------------------------|
|      | Positive results | Sensitivity% (95% CI) | Positive results | Specificity% (95% CI) |
| LAMP Run 1 | 132 | 93.0 (87.5–96.1) | 4 | 96.4 (91.1–98.6) |
| LAMP Run 2 | 124 | 87.3 (80.9–91.8) | 8 | 92.8 (86.4–96.3) |
| PCR Run 1 | 124 | 87.3 (80.9–91.8) | 4 | 96.4 (91.1–98.6) |
| PCR Run 2 | 128 | 90.1 (84.1–94.0) | 3 | 97.3 (92.3–99.0) |

Note: n: number of specimens, CI: confidence interval.

doi:10.1371/journal.pntd.0002504.t001
same publication the authors showed that the diagnostic sensitivity of the TgoGP LAMP is lower than the sensitivity of the RIME LAMP.

Reproducibility of LAMP was excellent and as good as that of PCR, with kappa values of 0.81 and 0.82 respectively when all samples were considered. Within the sub groups lower kappa values were observed, which is due to the fact that in these more homogenous groups the expected agreements were much higher. Values observed within the groups were in the same ranges for LAMP and PCR. The LAMP-amplified DNA is visualised by a UV-LED device attached to the LF-160 incubator. This single-tube and easy read-out avoids the risk for sample contamination due to post-amplification manipulations. Another advantage is that Loopamp Trypanosoma brucei Detection Kit is thermostable at 30°C which greatly enhances the feasibility of use in peripheral health facilities in tropical countries. Although in the present study LAMP was performed on DNA extracted with the Maxwell® DNA Purification robot, simplified DNA extraction methods that are compatible with LAMP are currently under development. The requirement of electrical power supply to operate the incubator for the amplification step constitutes a potential drawback for use in remote health facilities, even if it can be circumvented by using an alternative power source such as a photovoltaic panel.

In recent years there has been a sharp decline in HAT prevalence in most of the endemic countries and the classical case finding approach by mobile screening units is becoming less cost-effective. There is thus an urgent need to consider alternative ways of surveillance and case detection, and the LAMP technology could play a role [22]. Though still more complicated than the parasitological methods, LAMP is feasible for use at the level of a district hospital laboratory and could be useful as part of a testing algorithm for samples collected at more peripheral levels. LAMP can be applied on samples collected elsewhere without the need to be processed the same day. Either CATT or one of the newly developed rapid tests [23] can be used to screen suspects for HAT at health center or at village level; LAMP can then be used in a more centrally located laboratory as a second step in the diagnostic algorithm. LAMP data for serologically positive individuals who remained negative in parasitological testing may be particularly informative. However, future research should determine if HAT suspects with positive LAMP testing need further diagnostic work-up before being put on treatment and if the detection of LAMP positive individuals from the same geographical origin should be a trigger for intensified surveillance efforts. Although we feel that LAMP is best suited for use in central laboratories, the feasibility and cost-effectiveness of including LAMP in the screening process by the mobile teams may be determined in specific evaluation studies but should also take into account the test specimen preparation prior to the LAMP itself.

In conclusion, the study shows that the LAMP has similar diagnostic accuracy as the 10S rDNA PCR and can replace PCR for accurate and simplified detection of Trypanosoma DNA in clinical specimens. LAMP may have an important role to play in disease surveillance. However, one should note that the specificity of LAMP is not 100%, that HAT treatment is complex and toxic, and that the positive predictive value of tests in low incidence settings is low. Based on this study we cannot yet recommend initiating treatment of patients based on LAMP results; further evidence from prospective clinical studies under field conditions is needed, as well as cost-effectiveness analysis of competing algorithms. Feasibility studies of LAMP are currently conducted in the D.R. Congo [http://www.finddiagnostics.org/programs/hat-ond/hat/molecular_diagnosis.html].

Supporting Information

Checklist S1 STARD checklist showing that all essential elements of a diagnostic evaluation study are included in the manuscript.
(PDF)

Figure S1 STARD flowchart describing the design of the study and the flow of the participants.
(PDF)

Table S1 Excel spreadsheet with the raw data of the reference tests and index test in the study population.
(XLS)

Acknowledgments

This Project was a collaborative effort between the Institute of Tropical Medicine of Antwerpen, Belgium and The Foundation for Innovative New Diagnostics (FIND) of Geneva, Switzerland. We gratefully acknowledge FIND for their useful suggestions on the protocol, and for providing the necessary LAMP test kits for this study.

Author Contributions

Conceived and designed the experiments: PM EH PL PB MB SD. Performed the experiments: PM SD. Analyzed the data: PM EH MB PB SD DMN PPP VL WVdV PL. Contributed reagents/materials/analysis tools: PM EH MB PB SD DMN PPP VL WVdV PL. Wrote the paper: PM EH PB VL MB SD.

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