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African trypanosomiasis: Sensitive and rapid detection of the sub-genus Trypanozoon by loop-mediated isothermal amplification (LAMP) of parasite DNA

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Abstract

Control of human African trypanosomiasis (HAT) is dependent on accurate diagnosis and treatment of infected patients. However, sensitivities of tests in routine use are unsatisfactory, due to the characteristically low parasitaemias in naturally infected individuals. We have identified a conserved sequence in the repetitive insertion mobile element (RIME) of the sub-genus Trypanozoon and used it to design primers for a highly specific loop-mediated isothermal amplification (LAMP) test. The test was used to analyse Trypanozoon isolates and clinical samples from HAT patients. The RIME LAMP assay was performed at 62 °C using real-time PCR and a water bath. DNA amplification was detectable within 25 min. All positive samples detected by gel electrophoresis or in real-time using SYTO-9 fluorescence dye could also be detected visually by addition of SYBR Green I to the product. The amplicon was unequivocally confirmed through restriction enzyme NdeI digestion, analysis of melt curves and sequencing. The analytical sensitivity of the RIME LAMP assay was equivalent to 0.001 trypanosomes/ml while that of classical PCR tests ranged from 0.1 to 1000 trypanosomes/ml. LAMP detected all 75 Trypanozoon isolates while TBR1 and two primers (specific for sub-genus Trypanozoon) showed a sensitivity of 86.9%. The SRA gene PCR detected 21 out of 40 Trypanosoma brucei rhodesiense isolates while Trypanosoma gambiense-specific glycoprotein primers (TgsGP) detected 11 out of 13 T. b. gambiense isolates. Using clinical samples, the LAMP test detected parasite DNA in 18 out of 20 samples which included using supernatant prepared from boiled blood, CSF and direct native serum. The sensitivity and reproducibility of the LAMP assay coupled with the ability to detect the results visually without the need for sophisticated equipment indicate that the technique has strong potential for detection of HAT in clinical settings. Since the LAMP test shows a high tolerance to different biological substances, determination of the appropriate protocols for processing the template to make it a user-friendly technique, prior to large scale evaluation, is needed.

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1. Introduction

Human African trypanosomiasis (HAT) or sleeping sickness is endemic in sub-Saharan Africa where it is transmitted by tsetse flies of the Genus Glossina. The
disease is caused by trypanosomes belonging to the subgenus Trypanozoon namely Trypanosoma brucei rhodesiense and Trypanosoma brucei gambiense. T. b. rhodesiense causes disease in eastern, central and southern parts of Africa, while T. b. gambiense occurs in west and central Africa, extending from Angola to southern Sudan and Senegal. Other parasites of the sub-genus Trypanozoon are Trypanosoma brucei brucei and Trypanosoma evansi which cause diseases in livestock. However, a human case of T. evansi infection was recently reported in India (WHO, 2005; Truc et al., 2006), highlighting the need to develop definitive diagnostic tests for trypanosomes in humans.

Control of sleeping sickness relies on detection of the parasite and effective treatment of the patient. Routine diagnosis of the disease is based on direct visualisation of the parasite in blood, lymph node aspirates and CSF using a microscope (Van Meirvenne, 1999). This method has limited sensitivity due to fluctuating parasitaemia. In efforts to improve detection of trypanosomes, a number of diagnostic methods have been developed, including the mini-anion exchange centrifugation technique (mAECT; Lumsden et al., 1979), PCR (Welburn et al., 2001; Gibson et al., 2002; Radwanska et al., 2002; Jamonneau et al., 2003) and recently, a dipstick test (Deborggraeve et al., 2006) has been evaluated. Despite these advances, diagnosis of HAT remains unsatisfactory. The PCR-based tests have good sensitivity. However, the need for precision instruments and elaborate visualisation methods are obstacles to their wide application in clinical settings in Africa. Consequently, diagnosis of HAT involves a combination of parameters, such as origin of the patient, symptoms, demonstration of parasites by microscopy, or detection of specific antibodies using the Card Agglutination Test for Trypanosomiasis (CATT; Magnus et al., 1978). The paucity of definitive tests means that some patients go undetected and therefore become potential sources of infection to other people.

Early diagnosis of HAT is important in interrupting the transmission cycle of the parasite and progress of the disease to the late stage. Treatment of patients with late-stage disease, when parasites have invaded the CNS, is difficult due to the cost and long treatment schedules, which normally require hospital admission. Melarsoprol, the only drug that is effective for the late-stage T. b. rhodesiense form of disease, causes a post-treatment reactive encephalopathy in an estimated 10% of patients, half of which are fatal (Pépin and Milord, 1994; Kennedy, 2004). Modification of treatment regimes has not reduced mortality (Schmid et al., 2004) and treatment failure has been reported in the field (Legros et al., 1999; Matovu et al., 2001). Treatment of early stage HAT is much easier and safer, although some side effects have been reported when pentamidine or suramin are used (Lejon et al., 2003). Definitive diagnostic tests are therefore crucial for the early detection of cases. This would minimise false positives, and exposure of patients to drugs that are potentially dangerous (Inojosa et al., 2006), and whose efficacy may not be guaranteed.

Recently a rapid, simple and sensitive technique called loop-mediated isothermal amplification (LAMP) of DNA was developed by Notomi et al. (2000). LAMP is a novel strategy for gene amplification which relies on the autocycling strand displacement synthesis of DNA by Bst DNA polymerase under isothermal conditions (60–65 °C). The technique uses a set of six primers recognising eight sections of the target DNA (Nagamine et al., 2002). This increases amplification specificity, efficiency and rapidity. Moreover, the technique amplifies target DNA three-fold every half cycle, producing large amounts of product within 30–60 min (Notomi et al., 2000). The large amount of DNA formed allows visual detection of amplification through the addition of fluorescent dyes such as SYBR Green I (Poon et al., 2006) or Calcein (Boehme et al., 2007) and measurement of turbidity (Mori et al., 2001). LAMP has been used successfully in detection of human infectious agents for severe respiratory syndrome coronavirus (Hong et al., 2004), periodontitis (Yoshida et al., 2005), malaria (Poon et al., 2006), peptic ulcers (Minami et al., 2006) and tuberculosis (Boehme et al., 2007). Further, Kuboki et al. (2003) demonstrated the potential of LAMP based on a single copy target (PFRA gene) for the detection of several T. brucei sp. More recently Thékiso et al. (2007) have reported LAMP tests for T. evansi, Trypanosoma vivax, Trypanosoma congolense and T. b. gambiense. The LAMP test is attractive for diagnosis of HAT in sub-Saharan Africa where facilities are minimal, due to its speed, independence of specialised heating systems and results that can be visually inspected.

The availability of the sequenced genomes of several species of trypanosomes has provided information about genes that could be targeted as diagnostic markers. One of the targets is a non-autonomous retro-element, the repetitive insertion mobile element (RIME; Hasan et al., 1984). The RIME gene is ubiquitous, specific to the subgenus Trypanozoon (Wuyts et al., 1994; Tilley et al., 2003) and constitutes the most common mobile element in the T. brucei genome with approximately 500 copies per haploid genome (Bhattacharya et al., 2002). In the present study, we have used the conserved region in the RIME gene to develop a sensitive and specific LAMP test for the subgenus Trypanozoon. The test was evaluated and compared with PCR using Trypanozoon isolates and clinical samples.

2. Materials and methods

Institutional Ethical Clearance for the collection of human samples was obtained from the Livestock Health Research Institute (LIRI) Tororo, Uganda, and the Uganda National Council of Science and Technology (UNCST), Kampala. The use of mice was approved by Murdoch University.
2.1. Parasites and preparation of templates

A total of 59 T. b. rhodesiense and T. b. gambiense isolates were used in this study (Table 1). They were isolated from humans, hyenas, tsetse flies, pigs and a dog between 1968 and 2005 in west, east, central and southern parts of Africa. Ten T. b. brucei and five T. evansi isolates were included in the analysis. Trypanosomes were amplified in laboratory mice and DNA prepared using the published method (Sambrook and Russell, 2001) or the standard extraction commercial kits (Table 1). Stored infected mouse blood containing T. b. rhodesiense isolate ATCC 30027 was adjusted to achieve 1.0 × 10^6 trypanosomes/ml, then 10-fold dilutions were prepared. One portion was used for DNA extraction and the other boiled for supernatant.

2.2. Clinical samples

Twenty archived human blood, CSF and serum samples collected between 1991 and 2007 from HAT patients in Uganda were used. Upon isolation, each sample was divided into two portions. The first portion was inoculated into mice and the second for DNA either using a Sigma Genomic DNA extraction kit (St. Louis, MO USA) (JE samples) or using Gentra DNA purification Kit (Minneapolis, MN USA) (OM samples) (Table 2). In Tanzania, the blood was divided into two portions. The first portion was centrifuged anduffy coat collected. In the second portion, 15 μl of blood was mixed with 40 μl of ultra pure water (PCR grade) (Fisher Biotec), boiled for 3 min, centrifuged at 20,800g for 10 min and 10–15 μl of supernatant collected. CSF samples were boiled and centrifuged prior to addition into the reaction mixture while serum was added directly. Ten blood, CSF and serum samples from non-infected humans were used to check LAMP specificity. Two to 4 μl of supernatant was used in each LAMP reaction.

2.3. RIME sequencing and LAMP primer design

Primers RIME 1, 5′GTTCACCACCCGTTGGCG and RIME 2, 5′CGTGGCCGCCAGCCTG were designed from the genetic databank sequence (Genbank Accession No. K01801) and used to amplify RIME monomer from members of the sub-genus Trypanozoon. After electrophoresis, a single band of ~500 bp was excised and purified with a DNA purification kit (MO BIO Lab, Solana, USA). The products were cloned into a TOPO vector (Invitrogen, Australia) and transformed in Escherichia coli cells. Plasmid purification was performed using a miniprep column kit (Qiagen, Australia) and the target product sequenced using both M13 forward and reverse primers in an ABI automatic DNA 3730 analyser (Applied Biosystems). The resulting sequences (GenBank Accession Nos. EF567424 for T. b. brucei; EF567425 for T. evansi and EF567226 for T. b. rhodesiense) were used to design eight conserved regions within the RIME sequence. The outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP) and backward inner primer (BIP) (Notomi et al., 2000) were designed using PrimerExplorer v3 software (http://primerexplorer.jp/lamp) while the loop forward (LF) and backward (LB) (Nagamine et al., 2002) primers were manually designed (Table 3). The forward and backward primers were designed such that the restriction enzyme NdeI cuts in between primer F1c and B1 (Yamada et al., 2006).

2.4. LAMP reaction

LAMP reactions of 25 μl were standardised for optimal reagent concentrations, temperature and time using T. b. rhodesiense isolate LVH 56 and T. b. gambiense isolate B014, following the Taguchi design (Cobb and Clarkson, 1994). Briefly, the FIP and BIP were varied from 0.8 to 2.4 μM, deoxynucleotides (dNTPs) from 100 to 400 μM, betaine from 0.2 to 0.8 μM and MgSO4 from 0 to 4 mM. The reactions were optimised at 2.0 μM each for FIP and BIP primer, 0.8 μM each for loop primer (LF and LB), 0.2 μM for each of the F3 and B3 outer primers, 200 μM each for deoxynucleoside triphosphate, 0.8 M betaine (Sigma, St. Louis, MO, USA), 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 1% Triton X-100 and 8 U of BstDNA polymerase large fragment (New England Biolabs, MA, USA). For real-time reactions 3.34 μM SYTO-9 fluorescence dye (Molecular Probes, OR, USA) was added. The template was 1 μl [~10 pg] for trypanosome DNA and 2–4 μl of processed supernatant, CSF and native serum. The LAMP test was carried out for 1 h at 58, 60, 62 and 64°C using the Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia) or in a water bath, and terminated by increasing the temperature to 80°C for 4 min.

2.5. Detection of LAMP product

Amplification of DNA in the LAMP reaction was monitored through electrophoresis in 1.0% agarose gels stained with ethidium bromide, direct visual inspection after addition of 1 μl of 1/10 dilution of SYBR Green I (Invitrogen, Australia), and by monitoring SYTO-9 dye fluorescence in the Rotor-Gene 3000. Real-time fluorescence data was obtained on the FAM channel (excitation at 470 nm and detection at 510 nm) as described by Monis et al. (2005). To confirm that the LAMP amplified the correct target: (i) the product was digested with specific restriction enzyme NdeI (New England Biolabs, MA, USA) which cuts between primers F1c and B1, (ii) melt curves were obtained and analysed as described by Monis et al. (2005), and (iii) a single LAMP band was cloned into a TOPO vector (Invitrogen, Australia), transformed into E. coli and sequenced. The resulting sequence was compared with target sequences using the DNAman software version 5.0 (Lynnon Biosoft, Canada).
Table 1
Trypanosome isolates used in the study

| Species/sub-species | Identification code | Origin | Year of isolation | Original host |
|---------------------|---------------------|--------|-------------------|---------------|
| *Trypanosoma brucei rhodesiense* | LVH 56<sup>b</sup> | Lambwe valley, Kenya | 1978 | Human |
| *T. b. rhodesiense* | LVH 108<sup>b</sup> | Lambwe valley, Kenya | 1980 | Human |
| *T. b. rhodesiense* | KETRI 1883<sup>c</sup> | Lambwe valley, Kenya | 1970 | Reedbuck |
| *T. b. rhodesiense* | KETRI 1900<sup>c</sup> | Lambwe valley, Kenya | 1971 | Hyena |
| *T. b. rhodesiense* | KETRI 2544 | Lambwe valley, Kenya | 1981 | Human |
| *T. b. rhodesiense* | KETRI 2492<sup>c</sup> | Lambwe valley, Kenya | 1980 | Tsetse fly |
| *T. b. rhodesiense* | KETRI 2532<sup>c</sup> | Lambwe valley, Kenya | 1980 | Cow |
| *T. b. rhodesiense* | KETRI 3537<sup>c</sup> | Bugoma, Kenya | 1998 | Human |
| *T. b. rhodesiense* | KETRI 1883<sup>c</sup> | Lambwe valley, Kenya | 1970 | Reedbuck |
| *T. b. rhodesiense* | KETRI 1900<sup>c</sup> | Lambwe valley, Kenya | 1971 | Hyena |
| *T. b. rhodesiense* | KETRI 2544 | Lambwe valley, Kenya | 1981 | Human |
| *T. b. rhodesiense* | KETRI 2492<sup>c</sup> | Lambwe valley, Kenya | 1980 | Tsetse fly |
| *T. b. rhodesiense* | KETRI 2532<sup>c</sup> | Lambwe valley, Kenya | 1980 | Cow |
| *T. b. rhodesiense* | KETRI 3537<sup>c</sup> | Bugoma, Kenya | 1998 | Human |
| *T. b. rhodesiense* | KETRI 3624<sup>c</sup> | Busia, Kenya | 1999 | Human |
| *T. b. rhodesiense* | KETRI 3639<sup>c</sup> | Busia, Kenya | 2001 | Dog |
| *T. b. rhodesiense* | KETRI 3739<sup>c</sup> | Busia, Kenya | 1997 | Human |
| *T. b. rhodesiense* | KETRI 3007<sup>c</sup> | Busia, Kenya | 1987 | Pig |
| *T. b. rhodesiense* | KETRI 149<sup>c</sup> | Nyanza, Kenya | 1961 | Human |
| *T. b. rhodesiense* | KETRI 2473<sup>c</sup> | Nyanza, Kenya | 1970 | Human |
| *T. b. rhodesiense* | UTRO 2509<sup>b</sup> | Uganda | – | Human |
| *T. b. rhodesiense* | UTRO 2509<sup>b</sup> | Busoga, Uganda | 1979 | Dog |
| *T. b. rhodesiense* | UTRO 1911<sup>c</sup> | Busoga, Uganda | 1971 | Cow |
| *T. b. rhodesiense* | UTRO 2535<sup>c</sup> | Busoga, Uganda | 1990 | Human |
| *T. b. rhodesiense* | JE5 | Serere, Uganda | 2001 | Human |
| *T. b. rhodesiense* | JE6 | Serere, Uganda | 2001 | Human |
| *T. b. rhodesiense* | JE11 | Serere, Uganda | 1999 | Human |
| *T. b. rhodesiense* | JE12 | Serere, Uganda | 2003 | Human |
| *T. b. rhodesiense* | JE13 | Serere, Uganda | 2003 | Human |
| *T. b. rhodesiense* | JE14 | Serere, Uganda | 2001 | Human |
| *T. b. rhodesiense* | JE15 | Serere, Uganda | 2003 | Human |
| *T. b. rhodesiense* | TMRS 51a | Kibondo, Tanzania | 2004 | Human |
| *T. b. rhodesiense* | TMRS 51b | Kibondo, Tanzania | 2004 | Human |
| *T. b. rhodesiense* | TMRS 51c | Kibondo, Tanzania | 2005 | Human |
| *T. b. rhodesiense* | TMRS 52a | Urambo, Tanzania | 2005 | Human |
| *T. b. rhodesiense* | TMRS 52b | Urambo, Tanzania | 2004 | Human |
| *T. b. rhodesiense* | TMRS 52c | Urambo, Tanzania | 2006 | Human |
| *T. b. rhodesiense* | TMRS 53a | Mpanda, Tanzania | 2005 | Human |
| *T. b. rhodesiense* | TMRS 53b | Mpanda, Tanzania | 2005 | Human |
| *T. b. rhodesiense* | TMRS 53c | Mpanda, Tanzania | 2005 | Human |
| *T. b. rhodesiense* | TMRS 52a | Kasulu, Tanzania | 2001 | Human |
| *T. b. rhodesiense* | TMRS 52b | Kasulu, Tanzania | 2001 | Human |
| *T. b. rhodesiense* | TMRS 52c | Kasulu, Tanzania | 1991 | Human |
| *T. b. rhodesiense* | TMRS 52d | Mpanda, Tanzania | 1994 | Human |
| *T. b. rhodesiense* | ATCC 30027 | Tanganyika | 1934 | Human |
| *T. b. rhodesiense* | Gamella II<sup>b</sup> | Ethiopia | 1968 | Human |
| *T. b. rhodesiense* | 05<sup>b</sup> | Luangwa valley, Zambia | 1974 | Human |
| *T. b. rhodesiense* | TRPZ320<sup>b</sup> | Zambia | 1983 | Human |
| *T. b. rhodesiense* | EATRO 2636<sup>b</sup> | Mozambique | 1983 | Human |
| *Trypanosoma brucei gambiense* | MOS<sup>b</sup> | (Mbam) Cameroon | 1974 | Human |
| *T. b. gambiense* | Boula<sup>b</sup> | Bounenz, Congo | 1989 | Human |
| *T. b. gambiense* | NW2<sup>b</sup> | Uganda | 1992 | Human |
| *T. b. gambiense* | Dal 972<sup>b</sup> | Daloa, Ivory Coast | 1978 | Human |
| *T. b. gambiense* | Mba<sup>b</sup> | Daloa, Ivory Coast | 1978 | Human |
| *T. b. gambiense* | PT41<sup>b</sup> | Ivory Coast | 1992 | Human |
| *T. b. gambiense* | PT16<sup>b</sup> | Ivory Coast | 1992 | Human |
| *T. b. gambiense* | B014<sup>b</sup> | Fontem, Cameroon | 1988 | Human |
| *T. b. gambiense* | Fonti<sup>b</sup> | Fontem, Cameroon | 1993 | Human |
| *T. b. gambiense* | NW5<sup>b</sup> | Uganda | 1992 | Human |
| *T. b. gambiense* | JE16 | Adjuman, Uganda | 1992 | Human |
| *T. b. gambiense* | JE17 | Adjuman, Uganda | 1992 | Human |
| *T. b. gambiense* | KETRI 2565<sup>c</sup> | Sudan | 1982 | Human |
| *Trypanosoma brucei brucei* | LUMP 266<sup>b</sup> | Lambwe valley, Kenya | 1969 | Fly, Glossina pallidipes |
| *T. b. brucei* | KETRI 1814 | Kenya | 1970 | Rhino |
| *T. b. brucei* | KP14<sup>b</sup> | (Kouassi-Perita) Ivory coast | 1982 | Fly, G. palpalis |
| *T. b. brucei* | BS/18<sup>b</sup> | (Nsukka) Nigeria | 1962 | Pig |
Table 1 (continued)

| Species/sub-species | Identification code | Origin                        | Year of isolation | Original host |
|---------------------|---------------------|-------------------------------|-------------------|--------------|
| T. b. brucei        | J10<sup>b</sup>     | Luangwa valley, Zambia        | 1973              | Hyena        |
| T. b. brucei        | STIB 215<sup>b</sup>| Serengeti, Tanzania           | 1971              | Lion         |
| T. b. brucei        | Katereka<sup>a</sup>| Uganda                        | 1990              | Cow          |
| T. b. brucei        | TSW187/78E<sup>b</sup>| Ivory coast                   | 1978              | Pig          |
| T. b. brucei        | LVBG 3N<sup>b</sup>  | Lambwe valley, Kenya          | 1980              | Cow          |
| T. b. brucei        | H3<sup>b</sup>       | Luangwa valley, Zambia        | 1974              | Lion         |
| Trypanosoma evansi  | SA17<sup>c</sup>    | Isiolo, Kenya                 | 2003              | Camel        |
| T. evansi           | KETRI 3426<sup>c</sup>| Ukunda, Kenya                | 1978              | Camel        |
| T. evansi           | KETRI 3093<sup>c</sup>| Colombia, South America       | 1979              | Horse        |
| T. evansi           | SA263<sup>c</sup>    | Samburu, Kenya                | 2003              | Camel        |
| T. evansi           | KETRI 2439<sup>c</sup>| Kulal, Kenya                 | 1979              | Camel        |
| T. evansi           | KETRI 3565<sup>c</sup>| Athi River, Kenya             | 1994              | Camel        |
| Trypanosoma congolense forest | Cam 22<sup>b</sup> | Mbetta, Cameroon           | 1984              | Goat         |
| T. congolense kilifi | WG5<sup>b</sup>     | Kenya                         | 1980              | Sheep        |
| T. congolense savannah | KETRI 1869<sup>c</sup> | Kenya                      | –                 | –            |
| Trypanosoma simiae  | Ken 4<sup>b</sup>    | Keneba, The Gambia            | 1988              | Fly          |
| T. simiae tsavo     | KETRI 1864<sup>c</sup>| Kenya                        | –                 | Fly          |
| Trypanosoma godfreyi| Ken 7<sup>b</sup>    | Kenya                         | 1988              | Fly, Glossina morsitans |
| Trypanosoma vivax   | Y58<sup>b</sup>      | Nigeria                       | –                 | –            |

The JE samples were processed using a Sigma Genomic DNA extraction kit, USA.

TMR5 samples were processed using a Qiangen DNA extraction kit, Australia.

<sup>a</sup> Ten picograms were used for each sample and the reactions were performed in triplicate and repeated after 2 weeks.

<sup>b</sup> Wendy Gibson, University of Bristol, UK. DNA processed through the method of Sambrook and Russell (2001).

<sup>c</sup> Trypanosomiasis Research Centre, Kenya. The DNA was prepared using Qiangen DNA extraction kit, Australia.

2.6. Sensitivity and specificity of LAMP

To determine the analytical sensitivity of the LAMP test, 10-fold dilutions were made from ~100 ng of DNA purified from T. b. rhodesiense isolate LVH 56 and T. b. gambiense isolate B014. The assay was carried out using both cold and pre-heated templates (Table 4). The LAMP test was compared with PCR tests specific for the Trypanozoon sub-genus (Masiga et al., 1992; Wuyts et al., 1994; Tilley et al., 2003). Specificity of the test was determined with approximately 1 ng of DNA from human, tsetse fly, bovine, camel, Plasmodium falciparum and trypanosomes belonging to subgenus Nannomonas (T. congolense savannah, T. congolense kilifi, T. congolense forest, Trypanosoma simiae, T. simiae tsavo, Trypanosoma godfreyi), T. vivax and Trypanosoma lewisi.

3. Results

3.1. Detection and confirmation of LAMP product

Optimum results were obtained when the reaction temperature was maintained at 62 °C. All positive LAMP reactions produced a characteristic ladder of multiple bands on an agarose gel, indicating that stem-loop DNA with inverted repeats was formed (Notomi et al., 2000). The developed LAMP test was reproducible in laboratories in Kenya, Uganda and Tanzania and no false positives were observed. Upon addition of SYBR Green I to the reaction products, all positive reactions turned green while the negative ones remained orange (Fig. 1). No colour change was recorded with non-infected human samples (blood, CSF or serum). Digestion of LAMP product with NdeI restriction enzyme gave the predicted sizes of 89 and 134 bp. The melting curves for RIME LAMP amplification of T. b. rhodesiense human samples JEB, TMRS10S and T. b. gambiense samples OM64, OM51 (Table 2) produced a single peak at 84.5 °C, suggesting amplicons of the same sequence (Fig. 2). The analysis of five clones obtained showed 100% identity with the target sequence, and revealed that the length varied with sequence repeats of primers F1c, F2, LFc, F1, B1c, LB, B2c, B1 and the sequence between F1c and B1 (Yamada et al., 2006).

3.2. Sensitivity and specificity of LAMP

Ten-fold serial dilutions of genomic DNA from T. b. rhodesiense, T. b. gambiense and supernatant prepared from infected mouse blood containing 1.0 × 10⁶ trypanosomes/ml of isolate ATCC 30027 were used to determine the lower detection limit for the LAMP assay at 62 °C. The RIME primer set without loop primers had a detection limit of 10⁻⁴ dilution (~100 trypanosomes/ml). In contrast, the RIME reaction with loop primers had a detection limit of 10⁻⁵ dilution (~0.001 trypanosomes/ml) for T. b. rhodesiense, T. b. gambiense and ATCC 30027 (Table 4). Amplification was detectable within 20–30 min for the test with loop primers, however the optimal time was set at 35 min to allow for very low DNA concentrations. A 10-fold increase in sensitivity was obtained when pre-heated templates (DNA and supernatant) were used. We observed inhibition of the LAMP reaction with DNA concentrations of ≥ 200 ng. The RIME LAMP results were identical when either a Rotorgene 3000 thermocycler or a water bath was
Table 2
Analysis results for 20 human clinical samples from Uganda and Tanzania

| ID  | Source   | Template | Origin     | Year of isolation | Original host | Mouse inoculation | Specific PCR tests | RIME LAMP | Species/sub-species |
|-----|----------|----------|------------|-------------------|---------------|------------------|--------------------|-----------|---------------------|
| JE2 | Blood    | DNA      | Tororo, Uganda | 1991             | Human         | +                | TBR, SRA gene, TgsGP | +         | Trypanosoma brucei rhodesiense |
| JE3 | Blood    | DNA/supernatant | Tororo, Uganda | 2005           | Human         | +                |                     | +         | T. b. rhodesiense    |
| JE4 | Blood    | DNA      | Tororo, Uganda | 2002           | Human         | +                |                     | +         | T. b. rhodesiense    |
| TMRS10B | Blood | Supernatant/buffy coat | Tanzania         | 2007          | Human         | +                |                     | +         | T. b. rhodesiense    |
| TMRS11B | Blood | Supernatant/buffy coat | Tanzania         | 2007          | Human         | +                |                     | +         | T. b. rhodesiense    |
| JE8 | CSF      | DNA      | Tororo, Uganda | 2001           | Human         | +                |                     | +         | T. b. rhodesiense    |
| JE9 | CSF      | DNA/supernatant | Tororo, Uganda | 2001          | Human         | +                |                     | +         | T. b. rhodesiense    |
| JE10 | CSF     | DNA      | Tororo, Uganda | 2001          | Human         | +                |                     | +         | T. b. rhodesiense    |
| TMRS10C | CSF    | supernatant | Tanzania          | 2007          | Human         | +                | nd, nd, nd | +         | T. b. rhodesiense    |
| TMRS11C | CSF    | Supernatant | Tanzania          | 2007          | Human         | –                | nd, nd, nd | +         | T. b. rhodesiense    |
| TMRS10S | Serum  | Direct | Tanzania          | 2007          | Human         | nd               | nd, nd, nd | +         | T. b. rhodesiense    |
| TMRS11S | Serum  | Direct | Tanzania          | 2007          | Human         | nd               | nd, nd, nd | +         | T. b. rhodesiense    |
| OM55 | Blood    | DNA      | N.W Uganda       | 2004           | Human         | +                | –, –, –   | –         | Trypanosoma brucei gambiense |
| OM56 | Blood    | DNA      | N.W Uganda       | 2004           | Human         | +                | –, –, –   | –         | T. b. gambiense      |
| OM66 | Blood    | DNA      | N.W Uganda       | 2004           | Human         | +                | –, –, –   | –         | T. b. gambiense      |
| OM62 | Blood    | DNA      | N.W Uganda       | 2004           | Human         | +                | –, –, –   | –         | T. b. gambiense      |
| OM54 | CSF      | DNA      | N.W Uganda       | 2004           | Human         | +                | –, –, –   | +         | T. b. gambiense      |
| OM64 | CSF      | DNA/supernatant | N.W Uganda       | 2004          | Human         | +                | –, –, –   | +         | T. b. gambiense      |
| OM51 | Blood    | DNA      | N.W Uganda       | 2004           | Human         | +                | –, –, –   | +         | T. b. gambiense      |
| OM52 | Blood    | DNA      | N.W Uganda       | 2004           | Human         | +                | –, –, –   | +         | T. b. gambiense      |

TMRS11B was confirmed through a serum resistance-associated gene LAMP test (data not shown).
B, blood; C, CSF; and S, serum; thus TMR10B,C,S samples are from the same patient.
nd, not done; +, positive; and –, negative results.

a Ethical clearance obtained from the Ugandan Council of Science and Technology (UNCST).
b The first portion was inoculated into mice and the second portion was processed for DNA.
c Identification was confirmed through specific PCR using the samples amplified in mice (first portion).
d Three microliters of native serum was used for loop-mediated isothermal amplification (LAMP) test.
used to heat the reaction mixture. In all cases RIME LAMP showed a lower detection limit than PCR (Table 4). The RIME LAMP assay was specific and no reactivity was recorded with non-target DNA from other trypanosomes, hosts, vectors or P. falciparum.

3.3. Detection of sub-genus Trypanozoon isolates

RIME LAMP detected all 75 Trypanozoon isolates analysed (Table 5). The TBR primers detected 52/59 (88.1%) of T. b. rhodesiense and T. b. gambiense isolates while the SRA gene-specific PCR test (Gibson et al., 2002) detected 21/46 T. b. rhodesiense isolates. The TgsGP PCR (Radwanska et al., 2002) failed to detect two T. b. gambiense isolates (Table 5).

3.4. Results for clinical samples

The results of the analysis of 20 archived human samples are shown in Table 2. The RIME LAMP assay detected all T. b. rhodesiense in blood, CSF supernatant and unprocessed serum, while it failed to detect two samples of T. b. gambiense DNA. There was 100% agreement in LAMP test replicates. The TBR PCR only detected 5/18 samples while specific PCR tests gave negative results. The RIME LAMP assay was specific and no amplification was recorded with DNA or supernatant prepared from CSF and blood of non-infected humans.

4. Discussion

Human African Trypanosomiasis (HAT) often presents non-specific clinical symptoms. Diagnosis is even more complicated as the disease progresses, since the clinical symptoms can mimic those of other diseases that are common in the endemic areas (Atouguia and Kennedy, 2000). Patients normally consult a health professional when the disease is advanced and irreversible brain damage has probably occurred (Robays et al., 2004). Development of a sensitive and reliable test for HAT is therefore a priority for early treatment and implementation of appropriate control measures. Furthermore, a test that is rapid and can give results at the point of care, would be ideal in the expansive and remote endemic areas of Africa. In the present study, we have demonstrated the use of LAMP technology in diagnosis of HAT using laboratory propagated isolates, as well as clinical samples. The RIME LAMP test that we have developed is rapid, and results are obtained within 35 min using a normal water bath to maintain the temperature at 62 °C. The analytical sensitivity was the equivalent of 0.001 trypanosomes/ml, indicating that it would be possible to detect very low parasitaemias in patients.

LAMP has inherent characteristics that make it advantageous as a diagnostic test for the rural endemic regions in Africa: (i) the Bst enzyme is active at relatively high temperatures (60–65 °C), reducing the prospect for non-specific priming, (ii) using six primers that recognise eight targets increases sensitivity and specificity (Nagamine et al., 2002), and (iii) the ability to read results visually eliminates the need for gel electrophoresis. In our hands, the RIME LAMP test could amplify DNA from clinical samples within 20–30 min. However, we optimised the tests for 35 min to ensure detection of DNA at very low concentrations. Purified DNA at ≥200 ng (~2.0 × 10^6 trypanosomes/ml) had an inhibitory effect on LAMP reaction as monitored in real time, and showed very weak bands on the agarose gel. It is, however, unlikely that such a high concentration of DNA would be found in a human host. The DNA concentrations in the 10^-7 to 10^-5 dilutions (~0.1 to 10 trypanosomes/ml) gave the best results (efficient reaction in real time). Using the same dilutions, we observed an optimum concentration of ≥10 pg (~100 trypanosomes/ml) for LAMP tests based on maxicircle COX-1 and 18S genes, with detection limits below 10^-7 (results not shown), a factor that is probably associated with the number of copies of each gene. In their studies to detect Trypanozoon sub-genus, Kuboki et al. (2003) used a low-copy gene (PFRA) and four primers. Their detection limit was 1 pg (10 trypanosomes/ml), observations that were also confirmed in this study (Table 4). In the RIME LAMP test, we have used a target that is a multicopy gene and six primers, which may explain the higher sensitivity.

The robustness of RIME LAMP was further demonstrated when various templates were used. Heat-treated blood, serum and CSF supernatants were sufficient for amplification. Further, LAMP amplified DNA using 1–4 μl of largely native sera and Buffy coat with no inhibitory effects in a 25 μl reaction. The ability to use heat-treated
samples without compromising sensitivity eliminates the need for DNA extraction, and further shortens the LAMP reaction. Other studies have shown superior tolerance of LAMP for biological substances (Enomoto et al., 2005; Kaneko et al., 2007; Yamada et al., 2006), and heat-treated blood has been used successfully in the detection of malaria (Poon et al., 2006). During LAMP reactions, DNA separation is achieved through destabilisation of the DNA helix by betaine (Notomi et al., 2000). The increase in sensitivity associated with pre-heating of the template could therefore be due to faster DNA strand separation, translating into a more efficient LAMP test. Since HAT LAMP tests appear to be amiable to several templates, further work is needed in selecting and streamlining the best protocols for template preparation.

Comparisons of methods for detection of amplicons, including the addition of SYBR Green I, gel electrophoresis (ladder-like appearance) and real time monitoring, gave the same results, confirming the specificity of the three methods. The combination of SYBR Green I with the double-stranded DNA (amplicon) initiates a colour change from orange to green (Iwamoto et al., 2003). This colour change is rapid and eliminates the need to use ethidium bromide, which is potentially mutagenic, to visualise the products. In three laboratories (each in Kenya, Uganda and Tanzania), it was possible to process a template, perform the LAMP test and read results for 10 samples in 1 h, while the procedures for PCR took up to 4 h, excluding the time required for DNA isolation. The efficiency of amplification in a LAMP reaction is higher than that of PCR because there is no loss of time in the thermal change, since the reactions occur at a constant temperature. The test was reproducible in the field and no false positives were observed. Further, the usefulness of this assay was confirmed by its ability to detect both T. b. rhodesiense and

| Type of Test | Target sequence | Expected specificity | Ten-fold dilutions |
|-------------|----------------|----------------------|--------------------|
| RIME LAMP (WL) | RIME | ++ | 10^{-1} |
| RIME LAMP (NL) | RIME | ++ | 10^{-3} |
| RIME LAMP | PFRA gene | ++ | 10^{-6} |
| RIME LAMP (NL) | PFRA gene | ++ | 10^{-9} |
| RIME LAMP (NL) | Repeated region | ++ | 10^{-12} |
| RIME LAMP (NL) | Retrotransposon | ++ | 10^{-15} |
| RIME LAMP (NL) | Rhode L | ++ | 10^{-18} |
| RIME LAMP (NL) | TBR1 & 2 | ++ | 10^{-21} |
| RIME LAMP (NL) | pMUTEC Retrotransposon | ++ | 10^{-24} |
| RIME LAMP (NL) | RIME A & B | ++ | 10^{-27} |

Detection limit for ATCC 30027 DNA and cold templates.

Fig. 1. The visual appearance of repetitive insertion mobile element loop-mediated isothermal amplification products from Trypanozoon isolates and human African trypanosomiasis clinical samples (Table 2) using SYBR Green I. The reactions were incubated in a water bath for 35 min at 62°C. Positive samples turn green within 1 min and negative samples remain orange, enabling direct inspection of the results. 1. Trypanosoma brucei rhodesiense (Gambella II), 2. Trypanosoma brucei gambiense (MOS), 3. Trypanosoma vivax (Y58), 4. JE2, 5. TMRS10B, C – positive control [T. b. rhodesiense (LVH 56)], 6. JE9, 7. OM52, 8. OM56, 9. TMRS11C, 10. OM66 and NC – negative control (PCR water).
T. b. gambiense directly from archived human blood and CSF samples (Table 2). The superior sensitivity demonstrated by detection of infections below the limits of other molecular techniques reported to date and negative results for P. falciparum, a co-endemic parasite in sub-Saharan Africa, favours adaptation of the assay.

There is no current consensus on the diagnostic criteria for CNS involvement in HAT (Kennedy, 2007). Demonstration of trypanosomes in the CSF is the clearest indicator that CNS invasion has occurred. However this is always difficult to determine as the number of parasites in CSF is persistently low, leading to use of indirect and inconsistent markers such as white blood cell counts (WHO, 1998). Demonstration of CNS involvement is critical as it forms the grounds for the therapeutic choice, either early- or late-stage drugs. Some novel molecular tests such as proteomic signature analysis (Papadopoulos et al., 2004) and a PCR test (Jamonneau et al., 2003) have shown sensitivity and specificity of ≥96%, however their adaptability in the endemic region is still a challenge. The high sensitivity and specificity of the RIME LAMP test recorded in this study and its ability to detect parasite DNA in the CSF samples (Table 2) could prove useful in confirming the presence or absence of parasites after treatment.

An equivocal confirmation that the LAMP test amplifies the target sequence is essential when the test is being developed. This is because LAMP yields a range of product sizes that appear as a ladder on agarose gels, unlike in PCR.

Fig. 2. Melting curves for Trypanosoma brucei spp repetitive insertion mobile element loop-mediated isothermal amplification product as monitored in Rotor Gene 3000. The curves from top to bottom are: T. b. rhodesiense JE8, TMRS10S, Trypanosoma brucei gambiense OM64, OM51 and LVH 56 positive control. The curves were acquired after loop-mediated isothermal amplification amplification for 1 h at 62 °C and enzyme denaturing at 80 °C on the FAM channel using 1 °C steps and a hold of 30 s at each step from 60 to 96 °C. All isolates had a melting temperature (Tm) of 84.5 °C, indicating similar sequences, and hence similar amplicons. dF/dT = fluorescence.

Table 5
Summary of PCR and repetitive insertion mobile element loop-mediated isothermal amplification (RIME LAMP) results for samples used in the study

| DNA source                  | No. of isolates | Specific PCR tests | RIME LAMPf |
|-----------------------------|-----------------|--------------------|------------|
| T. b. rhodesiense           | 46              | 40 (86.9%)         | 46 (100%)  |
| T. b. gambiense             | 13              | 12 (92.3%)         | 13 (100%)  |
| T. b. brucei brucei         | 10              | 10 (100%)          | 10 (100%)  |
| T. evansi                   | 6               | 6 (100%)           | 6 (100%)   |
| Other trypanosomesg         | 7               | –                  | –          |
| Host and vectorsh           | 4               | –                  | –          |
| Plasmodium falciparam       | 1               | –                  | –          |
| nd, not done; –, negative.  | a TBR 1 and 2 test (specific for subgenus Trypanozoon) (Masiga et al., 1992).
| b T. b. rhodesiense PCR (Gibson et al., 2002).
| c T. b. gambiense PCR (Radwanska et al., 2002).
| d T. b. brucei Maxicircle COX-1 PCR (Njiru et al., 2006).
| e T. evansi PCR (Masiga and Gibson, 1990).
| f The resulting amplicon was detected using SYTO-9 fluorescence dye in a real-time thermocycler; visual observation after the addition of SYBR Green I and by gel electrophoresis.
| g Trypanosoma congolense clade, Trypanosoma simiae clade, Trypanosoma godfreyi, Trypanosoma lewisi and Trypanosoma vivax.
| h Human, bovine, camel and tsetse fly.
where a characteristic single band size is observed. Furthermore, the test kit should be developed with a focus on reading colour change, to limit post-reaction DNA manipulations. In the present study, amplification of the target sequence was confirmed with specific restriction enzyme digestion using NdeI, melting curves (Fig. 2), and unequivocally through sequence analysis. Since the technique uses six primers, higher specificity and sensitivity were achieved. Real time analysis forms an important component in diagnostic test development, since it allows the monitoring of the test instantaneously. It was possible to monitor the LAMP amplification, obtain the melt curves and cut-off point through monitoring fluorescence of the double-stranded DNA intercalating dye – SYTO-9. To our knowledge this is the first time that SYTO-9 has been used in LAMP studies. The data obtained was reproducible, robust and consistent. The SYTO-9 dye has an advantage over other intercalating dyes in that it has a less inhibitory effect, shows a broader working range of dye concentration and does not selectively bind to amplicons (Monis et al., 2005). This wider working flexibility makes SYTO-9 an effective option in LAMP studies.

In summary, this study shows that the RIME LAMP test is robust and has great potential as a test that can be deployed easily in endemic countries. The emerging information suggests: (i) that pre-heating of the template prior to its addition into the reaction mixture increases the test sensitivity by 10-fold, (ii) that amplification can easily be achieved using unprocessed template (buffy coat, supernatant and native serum) without inhibition or compromising RIME LAMP sensitivity and (iii) that a normal water bath is sufficient to reproduce results in endemic countries. This study provides direct evidence that addition of loop primers to the RIME LAMP test increased the test sensitivity and efficiency to a new level. It is apparent that a RIME LAMP test is promising as a diagnostic test and may be used as a back up to other tests in active or passive screening for HAT in endemic areas where diagnostic equipment are minimal. Determination and optimisation of protocols for processing the template to make the assay more user-friendly is a crucial next step. The data presented in this study will not only form an excellent comparator for further LAMP studies but will be useful towards development of a HAT test kit.

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Z.K. Njiru et al. / International Journal for Parasitology 38 (2008) 589–599