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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 *Nde*I 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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Keywords: Loop-mediated isothermal amplification; *Trypanozoon*; Sleeping sickness; Diagnosis

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

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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), periodontis (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 Thekisoe 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 either 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 1.0×10^6trypanosomes/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 was processed 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 and buffy 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 20,800*g* 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′GTTCCCACCCCGTTGGCG and RIME 2, 5′CGTGGGCGCCCAGCCGTG 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 six LAMP primers targeting 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 *Nde*I 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 M and $MgSO_4$ from 0 to 4 mM. The reactions were optimised at 2.0 μM for each FIP and BIP primer, 0.8 μM for each loop primer (LF and LB), 0.2 μM for each of the F3 and B3 outer primers, 200 μM for each deoxynucleoside triphosphate, 0.8 M betaine (Sigma, St. Louis, Mo, USA), 20 mM Tris–HCl (pH8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 and 8 U of *Bst*DNA 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 *Nde*I (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[a]	Identification code	Origin	Year of isolation	Original host
Trypanosoma brucei rhodesiense	LVH 56[b]	Lambwe valley, Kenya	1978	Human
T. b. rhodesiense	LVH 108[b]	Lambwe valley, Kenya	1980	Human
T. b. rhodesiense	KETRI 1883[c]	Lambwe valley, Kenya	1970	Reedbuck
T. b. rhodesiense	KETRI 1900[c]	Lambwe valley, Kenya	1971	Hyena
T. b. rhodesiense	KETRI 2544	Lambwe valley, Kenya	1981	Human
T. b. rhodesiense	KETRI 2492[c]	Lambwe valley, Kenya	1980	Tsetse fly
T. b. rhodesiense	KETRI 2532[c]	Lambwe valley, Kenya	1980	Cow
T. b. rhodesiense	KETRI 3537[c]	Bugoma, Kenya	1998	Human
T. b. rhodesiense	KETRI 3624[c]	Busia, Kenya	1998	Human
T. b. rhodesiense	KETRI 3639[c]	Busia, Kenya	1999	Human
T. b. rhodesiense	KETRI 3739[c]	Busia, Kenya	2001	Dog
T. b. rhodesiense	KETRI 3007[c]	Busia, Kenya	1987	Pig
T. b. rhodesiense	EATRO 149[c]	Nyanza, Kenya	1961	Human
T. b. rhodesiense	KETRI 2473[c]	Nyanza, Kenya	1970	Human
T. b. rhodesiense	UTRO 2509[b]	Uganda	–	Human
T. b. rhodesiense	WB56[b]	Uganda	–	Human
T. b. rhodesiense	UTRO 2504[c]	Busoga, Uganda	1979	Dog
T. b. rhodesiense	KETRI 1911[c]	Busoga, Uganda	1971	Cow
T. b. rhodesiense	KETRI 2355[b]	Busoga, Uganda	1977	Human
T. b. rhodesiense	JE1	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 JM	Kasulu, Tanzania	2001	Human
T. b. rhodesiense	TMRS 58	Mpanda, Tanzania	2006	Human
T. b. rhodesiense	TMRS 4M	Urambo, Tanzania	2006	Human
T. b. rhodesiense	TMRS010[b]	Kasulu, Tanzania	1991	Human
T. b. rhodesiense	TMRS127[b]	Mpanda, Tanzania	1994	Human
T. b. rhodesiense	ATCC 30027	Tanganyika	1934	Human
T. b. rhodesiense	Gambella II[b]	Ethiopia	1968	Human
T. b. rhodesiense	058[b]	Luangwa valley, Zambia	1974	Human
T. b. rhodesiense	TRPZ320[b]	Zambia	1983	Human
T. b. rhodesiense	EATRO 2636[c]	Mozambique	1983	Human
Trypanosoma brucei gambiense	MOS[b]	(Mbam) Cameroon	1974	Human
T. b. gambiense	Boula[b]	Bouenza, Congo	1989	Human
T. b. gambiense	NW2[b]	Uganda	1992	Human
T. b. gambiense	Dal 972[b]	Daloa, Ivory Coast	1978	Human
T. b. gambiense	Mba[b]	Daloa, Ivory Coast	1978	Human
T. b. gambiense	PT41[b]	Ivory Coast	1992	Human
T. b. gambiense	PT16[b]	Ivory Coast	1992	Human
T. b. gambiense	B014[b]	Fontem, Cameroon	1988	Human
T. b. gambiense	Font 1[b]	Fontem, Cameroon	1993	Human
T. b. gambiense	NW5[b]	Uganda	1992	Human
T. b. gambiense	JE16	Adjuman, Uganda	1992	Human
T. b. gambiense	JE17	Adjuman, Uganda	1992	Human
T. b. gambiense	KETRI 2565[c]	Sudan	1982	Human
Trypanosoma brucei brucei	LUMP 266[b]	Kiboko, Kenya	1969	Fly, *Glossina pallidipes*
T. b. brucei	KETRI 1814	Kenya	1970	Rhino
T. b. brucei	KP2N[b]	(Kouassi-Perita) Ivory coast	1982	Fly, *G. palpalis*
T. b. brucei	B8/18[b]	(Nsukka) Nigeria	1962	Pig

Table 1 (*continued*)

Species/sub-species[a]	Identification code	Origin	Year of isolation	Original host
T. b. brucei	J10[b]	Luangwa valley, Zambia	1973	Hyena
T. b. brucei	STIB 215[b]	Serengeti, Tanzania	1971	Lion
T. b. brucei	Katerema[b]	Uganda	1990	Cow
T. b. brucei	TSW187/78E[b]	Ivory coast	1978	Pig
T. b. brucei	LVBG 3N[b]	Lambwe valley, Kenya	1980	Cow
T. b. brucei	H3[b]	Luangwa valley, Zambia	1974	Lion
Trypanosoma evansi	SA17[c]	Isiolo, Kenya	2003	Camel
T. evansi	KETRI 2426[c]	Ukunda, Kenya	1978	Camel
T. evansi	KETRI 3093[c]	Colombia, South America	1979	Horse
T. evansi	SA263[c]	Samburu, Kenya	2003	Camel
T. evansi	KETRI 2439[c]	Kulal, Kenya	1979	Camel
T. evansi	KETRI 3565[c]	Athi River, Kenya	1994	Camel
Trypanosoma congolense forest	Cam 22[b]	Mbetta, Cameroon	1984	Goat
T. congolense kilifi	WG5[b]	Kenya	1980	Sheep
*T. congolense*savannah	KETRI 1869[c]	Kenya	–	–
Trypanosoma simiae	Ken 4[b]	Keneba, The Gambia	1988	Fly
T. simiae tsavo	KETRI 1864[c]	Kenya	–	Fly
Trypanosoma godfreyi	Ken 7[b]	Kenya	1988	Fly, *Glossina morsitans*
Trypanosoma vivax	Y58[b]	Nigeria	–	–

The JE samples were processed using a Sigma Genomic DNA extraction kit, USA.
TMRS samples were processed using a Qiagen DNA extraction kit, Australia.
[a] Ten picograms were used for each sample and the reactions were performed in triplicate and repeated after 2 weeks.
[b] Wendy Gibson, University of Bristol, UK. DNA processed through the method of Sambrook and Russell (2001).
[c] Trypanosomiasis Research Centre, Kenya. The DNA was prepared using Qiagen 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 *Nde*I restriction enzyme gave the predicted sizes of 89 and 134 bp. The melting curves for RIME LAMP amplification of *T. b. rhodesiense* human samples JE8, 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 1.0×10^6trypanosomes/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^{-9} 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 $\geqslant$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[a]	Source	Template	Origin	Year of isolation	Original host	Mouse inoculation[b]	Specific PCR tests			RIME LAMP	Species/sub-species[c]
							TBR	SRA gene	TgsGP		
JE2	Blood	DNA	Tororo, Uganda	1991	Human	+	–	–	–	+	*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[d]	Tanzania	2007	Human	nd	nd	nd	nd	+	*T. b. rhodesiense*
TMRS11S	Serum	Direct[d]	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.

Table 3
Nucleotide sequences for the repetitive insertion mobile element (RIME) loop-mediated isothermal amplification (LAMP) primers

Primer	Type	Sequence (5′–3′)	Length	Amplicon size[a]	Target
RIME-F3	F3	CTGTCCGGTGATGTGGAAC	19	179	RIME
RIME-B3	B3	CGTGCCTTCGTGAGAGTTTC	20		
RIME-FIP	FIP (F1c + F2)	GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA	42		
RIME-BIP	BIP (B1c + B2)	AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC	41		
RIME-LF	LF	GCCTCCCACCCTGGACTC	18		
RIME-LB	LB	AGACCGATAGCATCTCAG	18		

[a] F3 and B3 primers are only used for the initial strand displacement and are not involved in subsequent LAMP reaction (Notomi et al., 2000). Therefore the length between F2 and B2 is 179 bp. However, after amplification, the uppermost amplified amplicon size is 223 bp as the FIP and BIP primers consist of F1c = 22 bp and B1c = 22 bp sequences, respectively.

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. faciparum.*

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 $\geqslant 200$ ng ($\sim 2.0 \times 10^6$ 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 ($\sim$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 $\geqslant 10$ pg ($\sim$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

Table 4
Analytical sensitivity of the repetitive insertion mobile element loop-mediated isothermal amplification (RIME LAMP) assay compared with other *Trypanozoon* sub-genus based tests using pre-heated templates from 10-fold serial dilution of *Trypanosoma brucei rhodesiense* LVH 56, ATCC 30027 and *Trypanosoma brucei gambiense* isolate B014 DNA

Type of Test	Target sequence	Expected specificity	Ten-fold dilutions[a]											Reference
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}[b]	10^{-9}	10^{-10}	10^{-11}	
RIME LAMP (WL)	RIME	*Trypanozoon*	+	+	+	+	+	+	+	+	+	–	–	This study
RIME LAMP (NL)	RIME	*Trypanozoon*	+	+	+	+	–	–	–	–	–	–	–	This study
PFRA LAMP	PFRA gene	*Trypanozoon*	+	+	+	+	–	–	–	–	–	–	–	Kuboki et al. (2003)
TBR1 & 2	Repeatative region	*Trypanozoon*	+	+	+	+	+	+	+	–	–	–	–	Masiga et al. (1992)
pMUTEC	Retrotransposon	*Trypanozoon*	+	+	+	+	–	–	–	–	–	–	–	Wuyts et al. (1994)
RIME A & B	RIME	*Trypanozoon*	+	+	+	–	–	–	–	–	–	–	–	Tilley et al., 2003

WL, with loop primers; NL, without loop primers.
[a] 10^{-1} (~1.0×10^5 tryps/ml), 10^{-2} (~1.0×10^4 tryps/ml) and 10^{-9} dilution (~0.001 tryp/ml).
[b] Detection limit for ATCC 30027 DNA and cold templates.

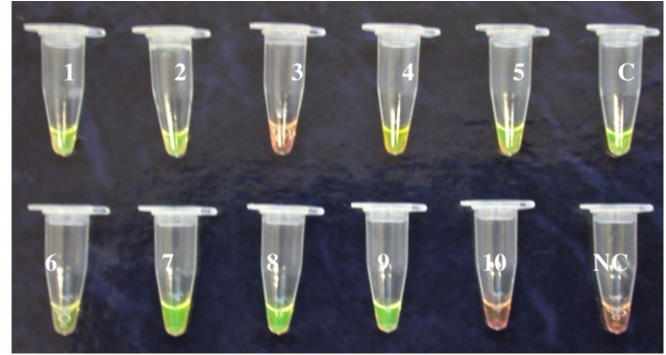

Fig. 1. The visual appearance of repetitive insertion mobile element loop-mediated isothermal amplification products from *Trypanozoon* isolates and human African trypanosomiasis clinical samples (Table 2) using SYBR Green I. The reactions were incubated in a water bath for 35 min at 62 °C. Positive 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).

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

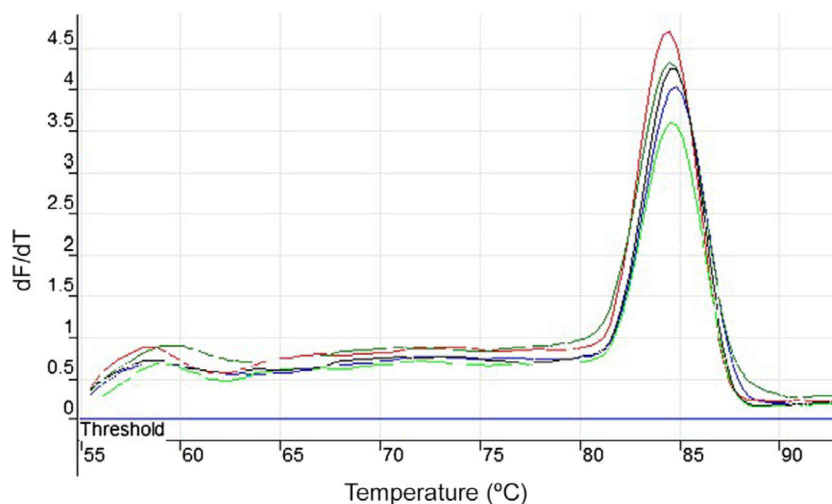

Fig. 2. Melting curves for *Trypanosoma brucei spp* repetitive insertion mobile element loop-mediated isothermal amplification product as monitored in Rotor Gene 3000. The curves from top to bottom are: *T. b. rhodesiense* JE8, TMRS10S, *Trypanosoma brucei gambiense* OM64, OM51 and LVH 56 positive control. 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 (T_m) 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 LAMP[f]
		TBR[a]	SRA[b]	TgsGP[c]	COX-1[d]	Minicircle[e]	
Trypanosoma brucei rhodesiense	46	40 (86.9%)	21 (45.7%)	–	nd	nd	46 (100%)
Trypanosoma brucei gambiense	13	12 (92.3%)	–	11 (84.6%)	nd	nd	13 (100%)
Trypanosoma brucei brucei	10	10 (100%)	–	–	10 (100%)	nd	10 (100%)
Trypanosoma evansi	6	6 (100%)	–	–	–	6 (100%)	6 (100%)
Other trypanosomes[g]	7	–	–	–	–	–	–
Host and vector[h]	4	–	–	–	–	nd	–
Plasmodium falciparum	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.

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

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 *Nde*I, 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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References

Atouguia, J.L.M., Kennedy, P.G.E., 2000. Neurological aspects of human African trypanosomiasis. In: Davis, L.E., Kennedy, P.G.E. (Eds.), Infectious Diseases of the Nervous System. Butterworth–Heinemann, Oxford, pp. 321–372.

Bhattacharya, S., Barke, A., Bhattacharya, A., 2002. Mobile genetic elements in protozoan parasites. J. Genetics 81, 73–86.

Boehme, C.C., Nabeta, P., Henastroza, G., Rubhana, R., Rahim, Z., Gerhardt, M., Sanga, E., Hoelscher, M., Notomi, T., Hase, T., Perkins, M.D., 2007. Operational feasibility of using loop-mediated isothermal amplification (LAMP) for the diagnosis of pulmonary TB in microscopy centres of developing countries. J. Clin. Microbiol. 45, 1936–1940.

Cobb, B., Clarkson, JM., 1994. A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. Nucleic Acids. Res. 22, 3301–3805.

Deborggraeve, S., Claes, F., Laurent, T., Mertens, P., Leclipteux, T., Dujardin, J.C., Herdewijn, P., Büscher, P., 2006. Molecular dipstick test for diagnosis of sleeping sickness. J. Clin. Microbiol. 44, 2884–2889.

Enomoto, Y., Yoshikawa, T., Ihira, M., Akimoto, S., Miyake, F., Usui, C., Suga, S., Suzuki, K., Kawana, T., Nishiyama, Y., Asano, Y., 2005. Rapid diagnosis of herpes simplex virus infection by a loop-mediated isothermal amplification method. J. Clin. Microbiol. 43, 951–955.

Gibson, W., Backhouse, T., Griffiths, A., 2002. The human serum resistance associated gene is ubiquitous and conserved in *Trypanosoma brucei rhodesiense* throughout East Africa. Infect. Genet. Evol. 25, 1–8.

Hasan, G., Turner, M.J., Cordingley, J.S., 1984. Complete nucleotide sequence of an unusual mobile element from *Trypanosoma brucei*. Cell 37, 333–341.

Hong, T.C., Mai, Q.L., Cuong, D.V., Parida, M., Minekawa, H., Notomi, T., Hasebe, F., Morita, K., 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe respiratory syndrome Coronavirus. J. Clin. Microbiol. 42, 1956–1961.

Inojosa, W.O., Augusto, I., Bisoffi, Z., Josenado, T., Abel, P.M., Stich, A., Whitty, C.J.M., 2006. Diagnosing human African trypanosomiasis in Angola using a card agglutination test: observational study of active and passive case finding strategies. BMJ 332, 1479.

Iwamoto, T., Sonobe, T., Hayashi, K., 2003. Loop-mediated amplification for direct detection of *Mycobacterium tuberculosis*, *M. avium* and *M. intracellulare* in sputum samples. J. Clin. Microbiol. 41, 2616–2622.

Jamonneau, V., Solano, P., Garcia, A., Lejon, V., Dje, N., Miezan, T.W., N'Guessan, P., Cuny, G., Buscher, P., 2003. Stage determination and therapeutic decision in human African trypanosomiasis: value of polymerase chain reaction and immunoglobulin M quantification on the cerebrospinal fluid of sleeping sickness patients in Cote d'Ivoire. Trop. Med. Int. Health 8, 589–594.

Kaneko, H., Kawana, T., Fukushima, E., Suzutani, T., 2007. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J. Biochem. Biophys. Methods 70, 499–501.

Kennedy, P.G.E., 2004. Human African trypanosomiasis of the CNS: current issues and challenges. J. Clin. Invest. 113, 496–504.

Kennedy, P.G.E., 2007. Diagnostic and neuropathogenesis issues in human African trypanosomiasis. Int. J. Parasitol. 36, 505–512.

Kuboki, N., Inoue, N., Sakurai, T., Di Cello, F., Grab, D.J., Suzuki, H., Sugimoto, C., Igarashi, I., 2003. Loop-mediated isothermal amplification for detection of African trypanosomes. J. Clinic. Microbiol. 41, 5517–5524.

Legros, D., Fournier, C., Gastellu, E.M., Maiso, F., Szumillin, E., 1999. Therapeutic failure of melarsoprol among patients treated for late stage *T. b. gambiense* human African trypanosomiasis in Uganda. Bull. Soc. Pathol. Exot. 92, 171–172.

Lejon, V., Boelart, M., Jannin, J., Moore, A., Buscher, P., 2003. The challenge of *Trypanosoma brucei gambiense* sleeping sickness diagnosis outside Africa. Lancet. Infect. Dis. 3, 804–808.

Lumsden, W.H., Kimber, C.D., Evans, D.A., Doig, S.J., 1979. *Trypanosoma brucei*: miniature anion-exchange centrifugation technique for detection of low parasitaemias: adaptation for field use. Trans. R Soc. Trop. Med. Hyg. 73, 312–317.

Magnus, E., Vervoort, T., Van Miervenne, N., 1978. A card agglutination test with stained trypanosomes (CATT) for the serological diagnosis of *T. b. gambiense* trypanosomiasis. Ann. Soc. Belg. Med. Trop. 58, 169–176.

Masiga, D.K., Gibson, C., 1990. Specific probes for *Trypanosoma* (*Trypanozoon*) *evansi* based on kinetoplast DNA minicircles. Parasitology 40, 279–284.

Masiga, D.K., Smyth, A.J., Hayes, P., Bromidge, T.J., Gibson, W.C., 1992. Sensitive detection of trypanosomes in tsetse flies by DNA amplification. Int. J. Parasitol. 22, 909–918.

Matovu, E., Enyaru, J.C.K., Legros, D., Schmid, C., Seebeck, T., Kaminsky, R., 2001. Melarsoprol refractory *T. b. gambiense* from Omugo, North Western Uganda. Trop. Med. Int. Health 6, 407–411.

Minami, M., Ohta, M., Ohkura, T., Ando, T., Torii, K., Hasegawa, T., Goto, H., 2006. Use of a combination of brushing technique and the loop-mediated isothermal amplification method as a novel, rapid, and safe system for detection of *Helicobacter pylori*. J. Clin. Microbiol. 44, 4032–4037.

Monis, P.T., Giglio, S., Saint, C.P., 2005. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal. Biochem. 30, 24–34.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Comm. 289, 15–154.

Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Prob. 16, 223–229.

Njiru, Z.K., Constantine, C.C., Masiga, D.K., Reid, S.A., Thompson, R.C.A., Gibson, W.C., 2006. Molecular characterization of *Trypanosoma evansi* type B. Infect. Genet. Evol. 6, 20–25.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic. Acid. Res. 28, E63.

Papadopoulos, M.C., Abel, P.M., Agranoff, D., Stitch, A., Tarelli, E., Bell, B.A., Planche, T., Loosemore, A., Saadoun, S., Wilkins, P., Krishna, S., 2004. A novel and accurate diagnostic test for human African trypanosomiasis. Lancet 363, 1358–1363.

Pépin, J., Milord, F., 1994. The treatment of human African trypanosomiasis. Adv. Parasitol. 33, 1–17.

Poon, L.L., Wong, B.W., Ma, E.H., Chan, K.H., Chow, L.M., Abeyewickreme, W., Tangpukdee, N., Yuen, K.Y., Guan, Y., Looareesuwan, S., Peiris, J.S., 2006. Sensitive and inexpensive molecular test for *falciparum* malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clinic. Chem. 52, 303–306.

Radwanska, M., Claes, F., Magez, S., Magnus, E., Perez-Morga, D., Pays, E., Buscher, P., 2002. Novel primer sequences for polymerase chain reaction-based detection of *Trypanosoma brucei gambiense*. Am. J. Trop. Med. Hyg. 67, 289–295.

Robays, J., Bilengue, M.M., Van der Stuyft, P., Boelaert, M., 2004. The effectiveness of active population screening and treatment for sleeping sickness control in the Democratic Republic of Congo. Trop. Med. Int. Health 9, 542–550.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning, third ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, US.

Schmid, C., Nkunku, S., Merolle, A., Vounatso, P., Burri, C., 2004. Efficacy of 10-day melarsoprol schedule 2 years after treatment for late-stage gambiense sleeping sickness. Lancet 364, 789–790.

Thekisoe, OM., Kuboki, N., Nambota, A., Fujisaki, K., Sugimoto, C., Igarashi, I., Yasuda, J., Inoue, N., 2007. Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomosis. Acta Tropica 102, 182–189.

Tilley, A., Welburn, S.C., Fevre, E.M., Feil, E.J., Hide, G., 2003. *Trypanosoma brucei*: trypanosome strain typing using PCR analysis of mobile genetic elements (MGE-PCR). Exp. Parasitol. 104, 26–32.

Truc, P., Gibson, W., Herder, S., 2006. Genetic characterization of *Trypanosoma evansi* isolated from a patient in India. Infect. Genet. Evol. 7, 305–307.

Van Meirvenne, N., 1999. Biological diagnosis of human African trypanosomiasis. In: Dumas, M., Boutelle, B., Buguet, A. (Eds.), Progress in Human African Trypanosomiasis, Sleeping sickness. Springer, Paris.

Welburn, S.C., Picozzi, K., Fevre, E.M., Coleman, P.G., Odiit, M., Carrington, M., Maudlin, I., 2001. Identification of human-infective trypanosomes in animal reservoir of sleeping sickness in Uganda by means of serum-resistance-associated (SRA) gene. Lancet 358, 2017–2019.

WHO, 2005. A new form of human trypanosomiasis in India. Description of the first human case in the world caused by *Trypanosoma evansi*. Weekly Epidemiol. Rec. 80, 62–63.

WHO, 1998. Control and surveillance of African trypanosomiasis. Report of a WHO expert committee. Technical Report Series, vol. 881, World Health Organisation, Geneva, Switzerland.

Wuyts, N., Chokesajjawatee, N., Panyim, S., 1994. A simplified and highly sensitive detection of *Trypanosoma evansi* by DNA amplification. S.E. Asian. J. Trop. Med. Publ. Health. 25, 266–271.

Yamada, Y., Itoh, M., Yoshida, M., 2006. Sensitive and rapid diagnosis of human parvovirus B19 infection by Loop-mediated isothermal amplification. Br. J. Dernatol. 155, 50–55.

Yoshida, A., Nagashima, S., Ansai, T., Tachibana, M., Kato, H., Watari, Notomi, T., Takehara, 2005. Loop-mediated isothermal amplification method for rapid detection of the periondontopathic bacteria *Porphyromona gingivalis*, *Tannerella forsythia* and *Treponema denticola*. J. Clin. Microbiol. 43, 2418-2.