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A Comparison of Parasitological Techniques (Microscopy) and Loop-mediated Isothermal Amplification (LAMP) of DNA in Diagnosis and Monitoring Treatment of *Trypanosoma brucei rhodesiense* Infection in Vervet Monkeys (*Chlorocebus aethiops*)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LMC, WFM and JBM designed the study and wrote the protocol. Authors PSO, DOO, SE, FOO, MKW, GOM and JMN wrote the first draft of the manuscript, managed the literature searches, analyses of the study, performed the structural equation modelling and discuss the conclusion. All authors read and approved the final manuscript.

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ABSTRACT

Microscopy and LAMP were compared in diagnosis and treatment follow-up of HAT with *T. b. rhodesiense* infected vervet monkeys (*Chlorocebus aethiops*) treated with diminazene aceturate (Berenil®) and Melarsoprol (Mel-B®). Saponin method and heat treatment were used to extract pure and crude DNA, amplified on thermocycler and water bath. SYBR-green and UV-illumination in agarose gel were used to assess results. Parasitaemia, CSF-parasitosis, PCV, WBC and CSF-protein concentration were determined. DNA was detected 7dpi in blood and serum and 21dpi in CSF. It cleared 56dpi in blood and serum of both monkeys and re-appeared 77dpi and 129dpi in blood and serum of monkey A and B respectively after Berenil® treatment. DNA cleared 40 and 90 days and 90 and 150 days in blood and serum and CSF of A and B respectively after Mel-B® treatment. Using blood and CSF, detection of microscopy was 28.21% and 21.18% while LAMP was 60.26%, 55.13% and 79.49% using blood, serum and CSF respectively. X^2 was 16.734 (p=0.000) and 38.023 (p=0.000) between LAMP and microscopy in blood and CSF, pure DNA using thermocycler it was 60.27%, 55.13% and 78.12%, and 46.15%, 48.72% and 75.64% on water bath in blood, serum and CSF respectively. Crude DNA had %detection of 56.41%, 56.41% and 76.92% using thermocycler and 48.72%, 44.87% and 64.10% on water bath using blood, serum and CSF respectively. Crude DNA using thermocycler, LAMP and microscopy had Kappa (k) of 0.397 and 0.602 and X^2 13.141 (p=0.000) and 35.247 (p=0000) using blood and CSF respectively. In late stages, k and X^2 of 0.600 and 15.000 (p=0.000) were obtained using CSF. For treatment follow-up, k and X^2 were 0.472 and 19.429 (p=0.000) and 0.527 and 21.346 (p=0.000) in blood and CSF respectively. Heat treatment and amplification using water bath may be used for sample preparation and amplification respectively.

Keywords: Loop-mediated isothermal amplification (LAMP); diagnosis; post-treatment follow-up; Human African Trypanosomiasis (HAT); Trypanosoma b. rhodesiense.

1. INTRODUCTION

Rhodesian form of human African trypanosomiasis (HAT) or sleeping sickness was an emerging public health problem in most parts of the world [1]. Although it was under control last two decade, [2] it has been neglected in terms of drug development and research in understanding the pathogenesis, immunological and pathological changes. The disease is caused by infection with *T. b. rhodesiense* transmitted by a bite of tsetse flies of *Glossina spp* and it's fatal if left untreated [3]. It occurs in eastern, central and southern parts of Africa [4]. Other *Trypanozoon* are *T. b. gambiense*, *T. b. brucei* and *T. evansi*. *Trypanosoma b. gambiense* cause a devastating chronic form of the disease in western and central Africa. *Trypanosoma b. brucei* is lysed by human serum thus not infective but cause disease in livestock along with *T. evansi*. However, research has indicated a human case of *T. evansi* infection reported in India [5,6], highlighting the need to develop definitive diagnostic tests for trypanosomes in human infection.

Devastating epidemics of *T. b. rhodesiense* in the past occurred in Uganda and western Kenya [7]. However, diagnosis has heavily relied on direct microscopic visualization of the trypanosomes in clinical samples [8]. This is less sensitive as result of low parasitaemia. Despite improved laboratory diagnostic methods such as m-AECT [9], PCR [10] and dipstick test [11], diagnosis still remain unsatisfactory. Undiagnosed patient remains untreated and are bound to die soon or later. At the same time, the patient becomes a burden to the family and acts as a reservoir of the disease. Diagnosis of HAT involves a combination of parameters such as origin of the patient, symptoms and detection of trypanosomes by microscopy. Clinical signs are non-specific, variable and inconstant. The paucity of these definitive diagnostic test means that some patients go undetected and become potential source of infections to other people.

Drugs available for therapy and prophylaxis are limited due to toxicity and resistance [12]. Treatment of early stage HAT is easier and safer though side effects have been reported upon the use of suramin [13]. For late-stage, long treatment schedule requires hospitalization and considerable care in the use of toxic drugs [14]. Melarsoprol, the only drug effective for the late stage is toxic [15]. Modification of treatment regime has not been able to reduce mortality and failure has been reported in the field [16].

Definitive diagnostic tests are thus crucial for the early detection cases to minimize false positive and exposure of patients to toxic drugs with unguaranteed efficacy [17]. The molecular technique; Loop-mediated isothermal amplification of DNA [18] has been used diagnosis of HAT [19,20,21,22]. The present study evaluates of LAMP in diagnosis, staging and post-treatment follow-up of HAT in vervet monkeys infected with *T. b. rhodesiense*.

2. MATERIALS AND METHODS

2.1 Animals

Two adult vervet monkeys (*Chlorocebus aethiops*) A and B were acquired from Institute of Primate Research (IPR), Kenya. The animals were housed in quarantine for 90 days, screened for evidence of any diseases including zoonotics. They were dewormed and treated for any ectoparasite infection and became accustomed to staying in individual squeeze-back stainless steel cages and human handling. They were fed on green maize, fresh vegetables (bananas, tomatoes and carrots) and commercial monkey cubes (Monkey pellets®, Unga Feeds Ltd, Kenya) twice daily [23,24]. Drinking water was provided *ad libitum.* Before infection, the animals were transferred to experimental wards and allowed to settle for another three weeks. The institutional animal care and use committee (IACUC) and Institution's scientific ethical review committee (ISERC) of trypanosomiasis Research Centre (TRC) approved the protocol of this study.

2.2 Trypanosomes

The stabilate *T. b. rhodesiense* KETRI 2537 was used. This was a derivative of EATRO 1989 isolated from a human patient in Uganda in 1972 by direct inoculation of blood and lymph node aspirate into a monkey, and later cryopreserved [25].

2.3 Trypanocidal Compounds

Melarsoprol (Mel B; Arsobal®, Specia) and diminazene aceturate (Berenil®, Hoechst) were used. Mel B was presented as 3.6 mg/mL solution. The total volume of administration was made up to 1 mL using water for injection. Berenil was weighed and reconstituted in distilled water to give a volume of 1 mL for injection.

2.4 Experimental Design

Two vervet monkeys were infected by intravenous injection with 10^4 *T. b. rhodesiense* using femoral vein in 1 mL of phosphate saline glucose (PSG). Before and during the course of the disease, a daily clinical evaluation was carried out. Parasitaemia was estimated using the rapid matching method [26] and Haematocrit centrifugation technique (HCT) [27]. At weekly intervals, the animals were anaesthetized with diazepam (May and Baker, U.K) at a dosage of 1mg/Kg bwt and ketamine hydrochloride (Rotexmedica, Trittau, German) at a dosage of 10-15 mg/Kg bwt weighed, and detailed clinical examination undertaken. 5 mL of Ethylene-diamine-tetra-acetic acid (EDTA) blood was collected by femoral vein puncture. 5 mL non-EDTA collected blood was processed into serum. Packed cell volume (PCV) was determined using the standard micro-haematocrit method [28]. 2 mL of CSF was collected by lumbar puncture. When the animals were sick, they were treated with berenil at 5 mg/Kg btw intra muscularly (IM) at 35 dpi for three days. The animals were not cured but developed clinical meningoencephalitis and were treated with Mel B (3.6 mg/Kg bwt) from 113dpi and 171 dpi respectively for three days. The animals were then monitored during a follow-up for a period of 462 days. Within the experimental period there were 78 sampling points where a total of 78 samples (blood, serum and CSF) were collected from each animal which were used to compare the performance of LAMP and parasitological methods. Samples were collected on weekly basis starting at 7 dpi due to ethical considerations when dealing with non-human primate experiments.

2.4.1 Cerebrospinal fluid sampling and analysis

Free fresh CSF collected in a capillary tube was immediately transferred to a microscope for trypanosomes detection. If trypanosomes were not seen, the tube was centrifuged and examined using a microscopy as described by Gould and Sayer [28]. Fortress protein assay kit (Serolab, UK) was used to estimate the total proteins in the CSF. Cerebrospinal fluid white blood cell counting was done using a drop of fresh CSF in a haemocytometer.

2.4.2 Preparation of the DNA template for LAMP test

Crude total DNA was extracted through heat treatment of the samples [19]. 15 µL of the

sample was mixed with 40 µL of ultra pure water (PCR grade) (Fisher Biotec), boiled for 3-5 minutes in a water bath, centrifuged at 20,800 revolutions per minute for 10 minutes. The CSF was boiled 3-5 minutes and centrifuged at 20,800 revolutions per minute without dilution for 10 minutes. 10-15 µL of supernatant was collected for LAMP test. Pure total DNA was extracted using Saponin method [29]. Briefly, 500 µL of the sample was mixed with 500µL of Saponin lysis buffer (0.15% w/v Saponin, 0.2% w/v NaCl and 1mM EDTA) and vortexed. The mixture was then centrifuged at 11,000 revolutions per minute for 10 minutes in a microcentrifuge followed by four washes in the same buffer. The resulting pallet was then re-suspended in 100µl of PCR buffer (50 mM KCl, 1.5 mM MgCl2, 10 mM of Tris-HCl, pH 8.3) and incubated at 95℃ for 20 min, cooled and stored at -20℃.

2.4.3 Oligonucleotide primers

Primer RIME 1, 5'-GTTCCCACCCCGTT GGCGG-3' and RIME 2, 5'-CGTGGGCGCCCAGCCGTG-3' designed from the genetic data bank sequence (Genbank Accession No. K01801) were used to design six LAMP primers [20] for the study. These primers included; two inner primers, forward inner primer (FIP), 5'-GGAATACAGCAGATGGGGCGAGGC CAATTGGCATCTTTGGGA-3' and backward inner primer (BIP), 5'–AAGGGAGACTCTGCC ACAGTCGTCAGCCATCACCGTAGAGC-3' two outer primers; forward outer primer (F3) 5'-CTGTCCGGTGATGTGGAAC-3' and backward outer primer (B3), 5'-CGTGCCTTCGTGAGAGTTTC-3', two loop primers; loop forward primer (LF) 5'–GCCTCCCACCCTGGACTC-3' and loop backward primer (LB) 5'-AGACCGATAGCATCTCAG-3'.

2.4.4 LAMP reaction

LAMP reaction was carried using Loop amp DNA amplification kit (Eiken Chemical Co. Ltd., Japan). The LAMP reaction mixture (25 µL) contained template, 40 pMol each of FIP and BIP, 5 pMol each of F3 and B3, 5pMol of each LF and LB, 8 U of *Bst* DNA polymerase large fragment (New England Biolabs, MA, USA), 1.4 mM dNTPs, 0.8M betaine, 20mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8mM $MgSO_4$, and 0.1% Tween 20. 1 µL of pure total extracted DNA and 2-4 µL of crude total extracted DNA was used as the template for the LAMP reaction. The test was carried out for 30 minutes to 1 hour at 65℃ in a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia) and in water bath. In both cases, termination of the reaction was done by increasing the temperature to 80℃ for 4 minutes.

2.4.5 Detection of LAMP product

Amplification of DNA in the LAMP reaction was monitored through direct visual inspection after addition of 1 µL/mL of 1/10 dilution of SYBR Green I (Invitrogen, Australia) and electrophoresis in 1% ethidium bromide stained agarose gel and the bands visualized after illumination with UV light. To confirm that LAMP amplified the correct target, the product was digested with specific restriction enzyme *Ndei* I (New England Biolabs, Ma, USA) at 37℃ for 3 h, followed by electrophoresis in 1% agarose stained with ethidium bromide solution (1µg/ml). The bands were visualized after illumination using UV light.

3. RESULTS

3.1 Pre-treatment Clinical Findings

The animals developed clinical signs of the disease 5-7 dpi characterized by mild fever, splenomegarly and lymphadenopathy, dullness, reduced appetite and raised hair coat, progressive anaemia, rapid wasting and weight loss. The predictive clinical sign of the diseases such as lethargy, somnolence, ataxia, abnormal posture and muscle tremors were not observed. Parasiteamia developed 5 dpi and remained high characterized by minor fluctuations until treatment. In the CSF trypanosomes were first detected between 21-28 dpi. Before infection, the PCV ranged from 50-61% decrease after infection until treatment.

3.2 Post-treatment Clinical Findings

After berenil treatment at 35 dpi, the clinical signs disappeared for 10 days and re-appeared. The clinical signs disappeared after Mel B treatment at 113 and 171 dpi in two monkeys A and B respectively. Parasiteamia cleared off within 3 days after berenil treatment. In one of the monkey no parasiteamia relapse occurred while the other monkey had parasiteamia relapse between 154 and 168 dpi which cleared off after Mel B treatment at 171 dpi. An increase in PCV after berenil treatment was observed which returned to normal after Mel B treatment.

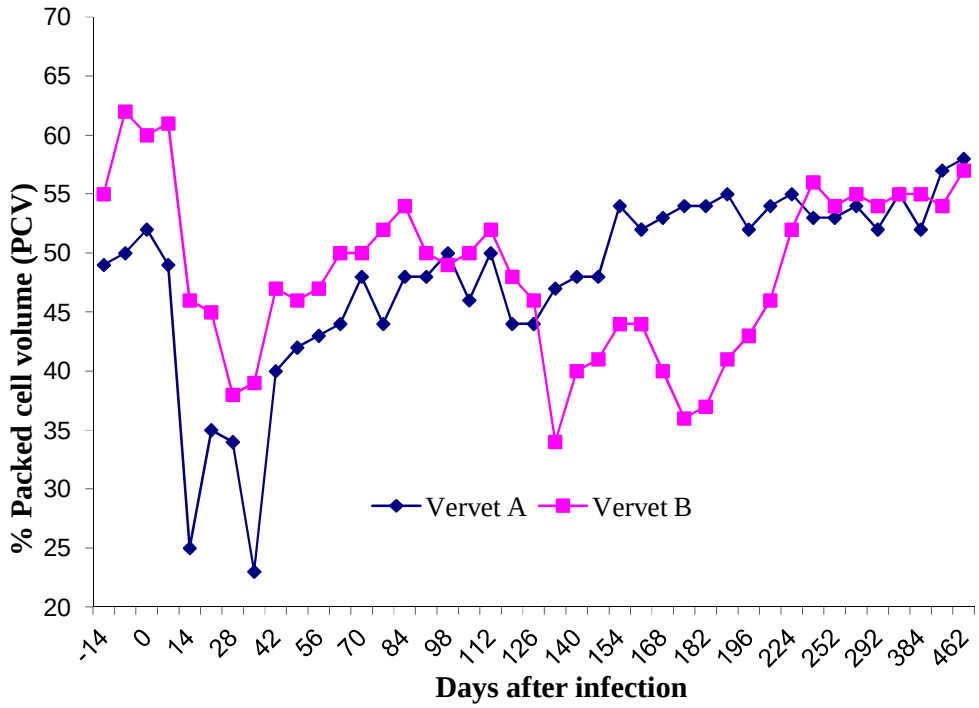

Fig. 1. Packed cell volume (PCV) changes in *T. b. rhodesiense* infected vervet monkeys infected treated with berenil and Mel B

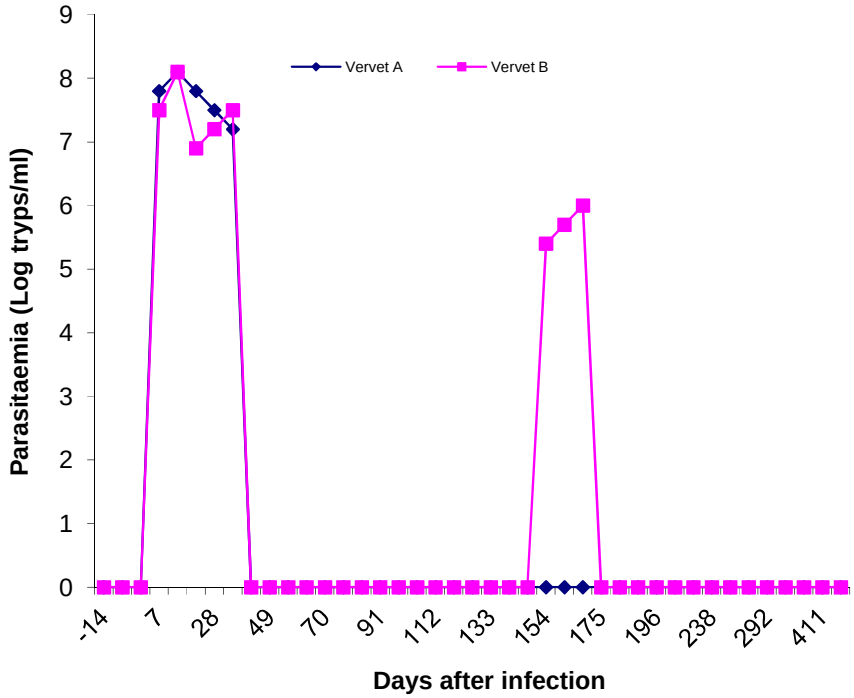

Fig. 2. Parasiteamic changes in *T. b. rhodesiense* infected vervet monkeys infected treated with berenil and Mel B

3.3 Pre-treatment CSF Changes

The CSF white blood cells during pre-infection ranged between 1 and 3 cell/mm^3. After two weeks of infection, the number of white cells increased gradually, reaching between 11-18 cell/mm^3. Before infection, total CSF protein concentration ranged between 23-25 mg/dl. There was an increase in total protein concentration above 25 mg/dl after two weeks of infection.

3.4 Post-treatment CSF Changes

3.4.1 Berenil treatment

Treatment with berenil had no effect on trypanosomes, WBC count and total protein concentration in the CSF. The WBC count and total protein concentration in the CSF fluctuated. The number of white cells some times decreasing <5 cell/mm^3 while the total protein concentration remained above 25 mg/dl.

3.4.2 Melarsoprol treatment

Trypanosomes were cleared off from the CSF by day 3 days after Mel B treatment. It was only after Mel B treatment that the number of WBC decreased <.5 cell/mm^3 dropping to levels < 5 cell/mm^3, by 62 and 53 dpt with Mel B in monkey A respectively. After Mel B treatment, major decreased in the total protein concentration was observed at 78 and 136 dpt with berenil. Trypanosomes were cleared off in the CSF of both monkeys 3 days after Mel B treatment. During the post-treatment follow-up period up to 462, the animals were aparasitaemic with no parasites detected in the CSF.

3.4.3 Detection of trypanosome DNA in the LAMP amplicon

LAMP detected DNA at 7 dpi in the blood and serum disappearing after berenil treatment at 35 dpi and reappeared after 35 dpt with berenil. The DNA disappeared completely in the blood and serum after Mel B treatment. In the CSF, DNA was detected at 21 dpi and disappeared only after Mel B treatment at 113 and 171 dpi in monkey A and B respectively. The percentage detection of positive samples for LAMP to show that it has high capacity of detecting trypanosome DNA was calculated by taking the positive detections divided by the total multiplied by 100 and is presented in Table 1.

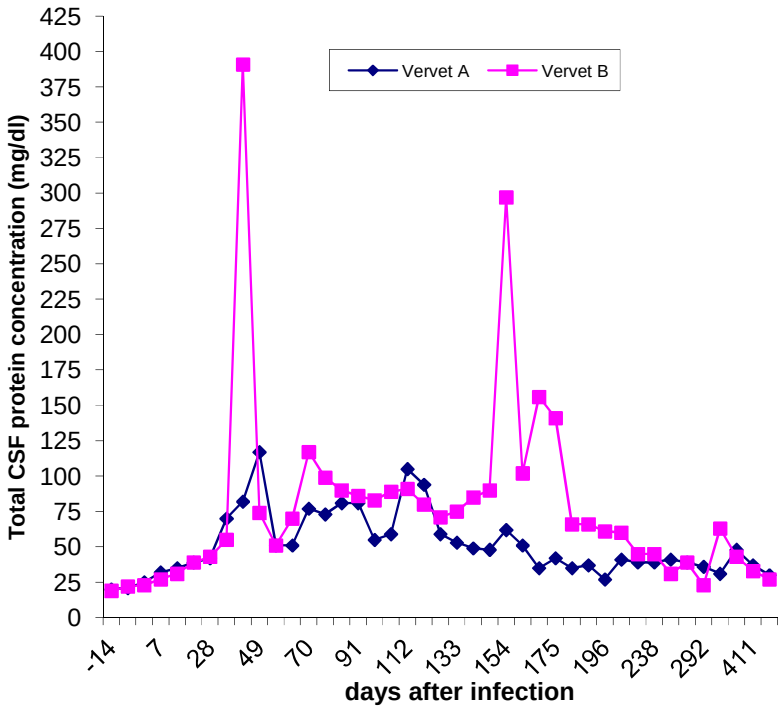

Fig. 3. Total CSF protein concentration in *T. b. rhodesiense* infected vervet monkeys with berenil and Mel B

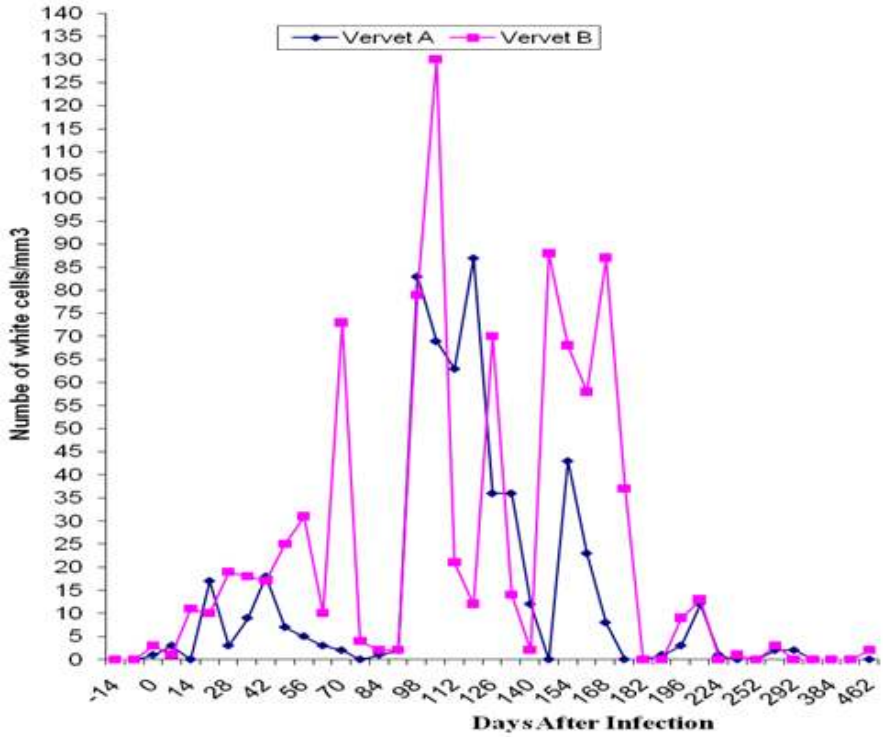

Fig. 4. Total number of white blood cells (No/mm^3) in *T. b. rhodesiense* infected vervet monkeys with berenil and Mel B

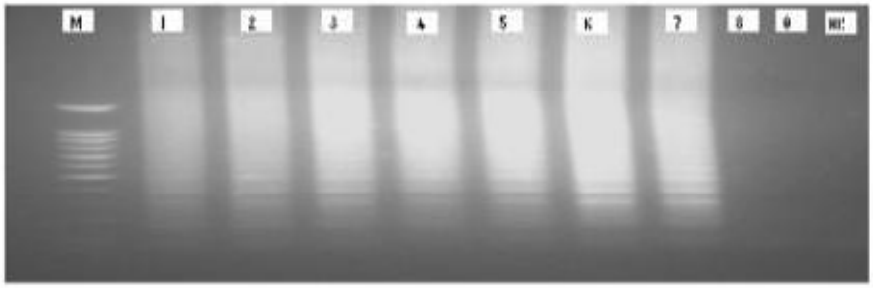

Fig. 5. Electrophoresis analysis of LAMP amplified products after crude DNA extraction. M, 100 bp marker, lane 1-7 positive samples, lane 8 and 9 negative samples, NC, negative control

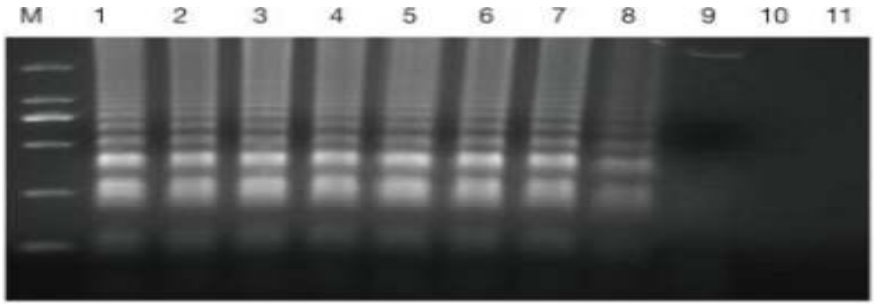

Fig. 6. Electrophoresis analysis of LAMP amplified products after crude DNA extraction. M, 100 bp marker, lane 1-8 positive samples, lane 9 and 10 negative samples and lane 11 negative control

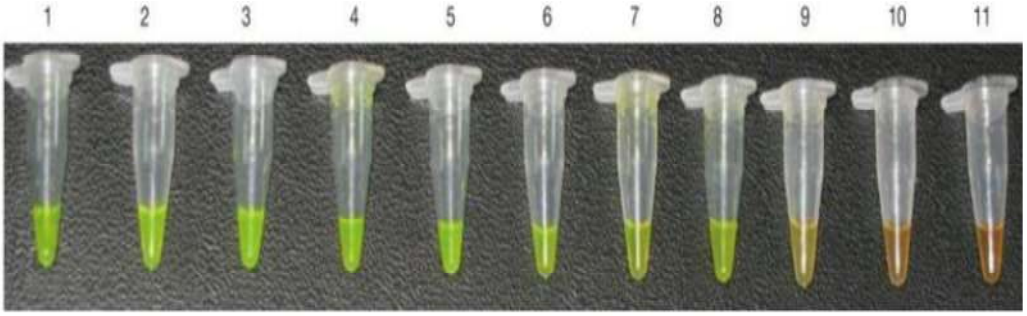

Fig. 7. Visual appearance of LAMP reactions from samples after the addition of SYBR green I. The positive samples produce a green colour (tubes 1, 2, 3, 4, 5, 6, 7 and 8) while negative control and negative samples remain orange (tube 9, 10 and 11)

No significant differences in different DNA extraction and amplification methods on LAMP were observed in the blood, serum and CSF. The agreements of different DNA extraction and amplification methods on LAMP were marginal in the blood, serum and CSF. However, for crude DNA on a thermocycler and a water bath, the agreement was good in the CSF. Thus the performance of LAMP on the different DNA extraction and amplification methods was similar in the blood, serum and CSF (Table 3). In different samples LAMP test showed no significant difference and marginal agreements between different DNA extraction and amplification methods (Table 3 and Table 4). Serum has higher detection percentage than blood (Table 1), however the percentage detection agreement between serum and blood was marginal (Table 4).

The performance of LAMP on the different DNA extraction and amplification methods was evaluated and found to be similar in thermocycler but water bath on pure and crude DNA varied significantly (Table 4). Crude DNA on a thermocycler was used in evaluating the performance of LAMP different samples. Blood and serum as samples in detection of trypanosome using LAMP for crude DNA on a thermocycler (Table 4) had no significant. A significant difference was observed between blood and CSF. The agreements between blood and serum and between blood and CSF were marginal. Microscopy and LAMP had marginal and good agreements in blood and CSF respectively. The X^2-Statistics (Table 6) between LAMP and microscopy in detection of trypanosomes in the blood and CSF at different stages of the disease indicated no significant difference in blood. A significant difference was observed in the CSF during early stage. In the late stage, there was no significant difference in the blood while a significant difference was observed in the CSF. After treatment, significant differences were observed in the blood and CSF.

The percentage agreement (k) (Table 6) between LAMP and microscopy in the detection of trypanosomes in both blood and CSF was marginal in the early and late stages. In the CSF, the agreement was good. After treatment, LAMP and microscopy had good agreements both in the blood and CSF.

4. DISCUSSION

In this study, fever, wasting, spleenomegaly and lymphadenopathy were observed. However, the development of these clinical signs in different vervet monkeys infected with the same stabilate [24] were different from those encountered in this study. This suggests that clinical parameters such as fever, wasting, spleenomegaly and lymphadenopathy among others are not sufficient in diagnosis of the disease. The different progression of parasitaemia in the two vervet monkeys and failure to detect trypanosomes in the CSF confirmed the insufficient nature of clinical signs and microscopy for diagnosis of HAT. Therefore, for proper diagnosis, clinical signs and microscopy should be supplemented with other diagnostic methods like the one described in this study.

Staging and post-treatment follow-up of HAT is done by the examination of CSF for trypanosomes, elevated total protein concentration and WBC counts. Increased WBC counts >5 cell/mm^3 and total protein concentration >25 mg/dl starting from 14 dpi indicated the initiation of late stage HAT with trypanosomes detected 21 dpi. One limitation of

Table 1. The percentage detection of LAMP in different DNA extraction and amplification methods

Extraction method	**Pure DNA**		**Crude DNA**		**Control**	
Amplification method	**Thermocycler**	**Water bath**	**Thermocycler**	**Water bath**	**Negative**	**Positive**
Serum	60.27	46.15`	56.41	48.72	0	100
Blood	55.13	48.72	56.41	44.87	0	100
CSF	78.21	75.64	76.92	64.10	0	100

Table 2. The differences between DNA extraction and amplification method in detection of trypanosome DNA using LAMP

DNA extraction and amplification method	**Chi-Square value (X^2); p-values**		
Sample type	**Blood**	**Serum**	**CSF**
Pure DNA Vs crude DNA; thermocycler (N=78)	0.026 (p=0.873)	0.646 (0.421)	1.53 (p=0.216)
Pure DNA Vs Crude DNA; water bath (N=78)	0.273 (p=0.626)	0.026 (p=0.872)	0.037 (p=0.848)
Pure DNA; thermocycler Vs Pure DNA water bath	3.153 (p=0.078)	0.642 (p=0.423)	0.001 (p=0.975)
Crude DNA; Thermocycler Vs Crude DNA; Water bath	1.258 (p=0.262)	3.108 (p=0.078)	2.026 (p=0.155)

the study was that CSF sampling was done on weekly basis because of ethical issues as daily collection of CSF would have caused suffering to the animals. However, the study shows that the vervet monkey model was demonstrated to be applicable in staging of the disease.

After the start of the experiment, parasites were undetectable in blood and CSF 3 days after Berenil® and Mel B® treatment but the DNA was detected. The parasites were still below the detectable levels for microscopy but LAMP is able to amplify small quantity of the parasite DNA and hence its detection. The decrease in WBC counts to normal level between 50-150 days post Mel B® treatment indicated clearance of trypanosomes in the CSF. An increased WBC count is regarded as a reliable test for staging the disease but its effectiveness is doubtful as the accuracy of the haemocytometer is about equal to the upper limit of normal count and limited by cell lysis [23]. The total protein concentration in the CSF decreased after Mel B® treatment but was still >25 mg/dl at end of the monitoring period. Increase in total protein concentration and WBC counts for staging HAT

Table 3. Agreement of LAMP between different DNA extraction and amplification method in detection of trypanosome DNA in different samples

Sample preparation/amplification method for LAMP	Percent agreement (k)		
Sample type	**Blood**	**Serum**	**CSF**
Pure DNA Vs crude DNA; thermocycler	0.039	0.013	0.116
Pure DNA Vs Crude DNA; water bath	0.038	0.052	0.038
thermocycler Vs water bath; Pure DNA	0.141	0.064	0.025
Thermocycler Vs Water bath; Crude DNA	0.088	0.012	0.435

Table 4. Chi-square values (X^2) of LAMP in detection of trypanosome DNA in blood, serum and CSF versus blood

Sample	Difference or agreement		
	Chi-square values (X^2); p-values	**Percent agreement (k)**	**Remarks**
Blood	1.020 (p=0.004)	0.109	Marginal agreement
Serum	0.000 (p=1.000)	0.017	Marginal agreement
CSF	9.510 (p=0.002)	0.205	Marginal agreement

Table 5. Percentage detection, agreement (k) and difference (X^2) between LAMP and microscopy in detection of trypanosome DNA

Sample	Detection method			
	Microscopy (% detection)	**LAMP (% detection)**	**Agreement (k)**	**Difference (X^2; p-values)**
Blood	28.21	60.26	0.397	13.141 (p=0.000)
CSF	21.18	79.49	0.602	35.247 (p=0.000)

Table 6. Percentage agreement (k) and difference (X^2) between LAMP and microscopy in detection of trypanosomes at different stages of the disease

Sample	Percent agreement (k) and Chi-square value (X^2); p-values		
	Percent agreement (k)	**Remarks**	**(X^2); p-values**
Early stage (0-35 dpi)			
Blood	0.143	Marginal agreement	0.000 (p=1.000)
CSF	0.088	Marginal agreement	0.168 (p=0.042)
Late stage (35 dpi - until treatment)			
Blood	0.233	Marginal agreement	3.270 (p=0.071)
CSF	0.600	Good agreement	15.000 (p=0.000)
After treatment (post-treatment follow-up period)			
Blood	0.472	Good agreement	19.249 (=0.000)
CSF	0.527	Good agreement	21.346 (p=0.000)

are not specific [30] while detection of trypanosomes in the CSF was difficult. Therefore, the two methods cannot be separately used for staging of HAT but used in complementarity.

The performance of LAMP on the different DNA extraction and amplification methods was found to be similar in efficiency with that of both thermocycler but water bath on pure and crude DNA varied. These differences were not significant and the agreements were marginal. This means that crude DNA on a water bath may also be adopted for use in LAMP. Thus, the invention of a heat block has resolved the challenges of controlling the temperature. This further extends the application of LAMP in areas where thermocyclers cannot be used besides reducing the operation cost of the test. The results of crude and pure DNA on LAMP showed marginal agreements and no significant difference. This implies that the expensive pure DNA extraction step which requires expertise can be omitted further shortening the time for the test and reducing the cost. Similar findings on diagnosis of trypanosomiasis using *RIME* gene on archived sample were reported [19].

Detection of trypanosome DNA in LAMP amplicon by electrophoresis in ethidium stained agarose gel, a post-amplification processing increases the time for this test to give results and may also introduce risks for contaminations [19]. Visualization of colour changes after addition of fluorescent dye, SYBR Green I dye give results immediately [31]. Omitting a post-amplification step significantly reduced the detection time and also the cost of this test was cut. Microscopy had low efficiency in detection of trypanosomes at different times than LAMP due to undulating parasiteamic waves. The significant difference between the two methods in detection of trypanosomes suggests the sensitivity of LAMP compared to microscopy. The results from this study are in agreement with the finding other studies [32,19]. The high efficiency of LAMP in the CSF regardless of DNA extraction and amplification method is attributed to its purity. Blood and serum may contain PCR inhibitors which may also inhibit LAMP by acting as impurities [19]. Although marginal agreement and significant difference were noted between blood and serum in LAMP, use of blood is preferred owing to tiresome and time consuming procedure of processing blood for serum.

Trypanosome DNA was detected at 7 dpi in the blood and serum although it should have been detected much earlier. The marginal agreements and insignificant difference noted between LAMP and microscopy during early stages of the infections needs to be investigated. For proper evaluation of LAMP during early stages in such studies, it is recommended that the samples be collected on daily basis. Trypanosome DNA was detected earlier than trypanosomes confirming the sensitivity of LAMP. As the disease progressed to the late stage, good agreement in terms of diagnosis and significant differences were noted between LAMP and microscopy in the CSF. In the blood sample a marginal agreement and insignificant difference was note between LAMP and microscopy. Due to the low sensitivity of CSF parasitosis, findings from this study suggest that LAMP may be a good test for staging the disease. Another advantage of LAMP is that it is a specific test as compared to WBC count and protein concentration.

The good agreements and significant differences noted between LAMP and microscopy after curative Mel B® treatment suggest the superiority of LAMP. Microscopy could not detect trypanosomes both in the blood and CSF 3 days after treatment but trypanosome DNA was still present and could be detected by LAMP in the CSF 120 to 150 dpt. This further suggests the limitations of microscopy. There are different PCR assays that have been developed in detection of trypanosome DNA although none of them was evaluated and validated [5,8,10]. However, the use of LAMP technique has been evaluated in diagnosis of HAT [22,32,33]. The observations of all the studies suggest that LAMP can be used as an alternative method in monitoring the treatment outcomes of the disease. This study shows that for post-treatment diagnosis follow-up at least 5 months may be adequate for LAMP. The current methods used for post-follow-up are done for a period of 24-36 months. In this context therefore, it's important to note that LAMP could be useful in diagnosis, staging and monitoring treatment outcome.

The trypanosomes-infected vervet monkey model of HAT [30] has been shown to develop both clinical and pathological changes mimicking the human disease. However, no comparable studies have been conducted in vervet monkey model for the purpose of diagnosis and monitoring treatment outcome using LAMP. The present study investigated the use of LAMP in diagnosis, staging and monitoring treatment of *T. b. rhodesiense* form of HAT in vervet monkey model. The findings from this study are in

agreement with those that involved the use of archived samples [19,20,21,22].

LAMP has been used in diagnosis of HAT using archived clinical samples [19,20,21,22]. In this study, LAMP based on primers derived from *RIME* gene were used. The optimal temperature ranging from 60℃ to 65℃ inactivated at 80℃ after the reaction [18] reduces the prospects of non-specific priming which tends to result into false positive results. Use of multiple (4 to 6) primer pairs recognizing 6-8 region of targeted DNA [18] increases specificity and sensitivity and acceleration of the reaction, reducing the time by a half the time for PCR [34,35]. The use of the dye, SYBR Green I [31] eliminates the need for electrophoresis in ethidium bromide stained agarose gel, a post- amplification processing and reduce the exposure to ethidium bromide, a potential mutagen.

The marginal agreements and no significant difference in detection of trypanosome DNA using whole blood, serum and CSF using crude and pure DNA implies that the pure DNA extraction step can be omitted further shortening the time for the test. Similar findings using RIME gene were reported by other studies [19]. Blood and serum had similar results in detection of trypanosome DNA using LAMP. Thermocycler and simple incubators such as heat block and water bath can amplify low concentration of DNA within 35 minutes in a laboratory [19]. This makes LAMP cost friendly as compared to other molecular methods for detection of trypanosome DNA. In previous studies, LAMP showed superior tolerance to biological substances such as proteins found in clinical sample [19]. This was also noted in the study where both pure and crude DNA extraction methods were used to prepare sample for LAMP. The use of various heat-treated templates; blood, serum, CSF, native sera and buffy coat [19] eliminate the need for pure DNA extraction, shortening further the time for the LAMP reaction. Studies on the use of reagents stored at varied temperatures between 25℃ and 37℃ don't affect the sensitivity [36] and different DNA template preparation and amplification methods [19,36] justify the cost friendliness and practicability of LAMP. Developing LAMP for detection of animal and human trypanosomes is of economic importance [19,22,33] owing to the difficulties posed by diagnosis. Further improvements on rapidity and simplicity of DNA preparation methods are required to enhance the practicability of LAMP for diagnosis, staging and monitoring treatment of HAT.

5. CONCLUSIONS

Blood and serum as samples for LAMP during haemolytic stage. Heat treatment of the samples as sample preparation method with amplification in a water bath, visualization using SYBR Green I dye had similar performance thus recommended. LAMP has better performance during stage determination of the disease than microscopy and some of the markers used in staging.

6. RECOMMENDATIONS

Research should be done to validate that LAMP may be a strong candidate in diagnosis, staging and monitoring treatment of HAT on different samples, sample preparation and sample amplification methods. Thus development of LAMP able to detect the DNA as a molecular marker in all groups of trypanosomes should be a research priority in the diagnosis of HAT. Further research on its applicability in the field settings is needed in appropriate experimental animal model to supplement the already finished and ongoing research for proper evaluation. Due to the zoonotic nature of African trypanosomiasis, development of LAMP based on a universal gene for all trypanosomes will be a breakthrough in solving the problem of diagnosis and monitoring treatment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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