

Microscopy and loop-mediated isothermal (LAMP) of DNA were compared in diagnosis and treatment follow-up of HAT in two vervet monkeys (*Chlorocebus aethiops*) infected with *T. b. rhodesiense* and treated with diminazene aceturate (Berenil®), 35dpi and later with Melarsoprol (Mel B®), 113dpi and 171dpi respectively. Saponin method and heat treatment were used in pure and crude DNA extraction respectively and amplified on thermocycler and water bath. SYBR green I and UV illumination of DNA in agarose gel were used to assess results. Parasitaemia, CSF-parasitosis, FCS, WBC-counts and CSF-protein concentration were determined. DNA was detected 7dpi in blood and serum and 21dpi in CSF. After Berenil® treatment, DNA 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 Mel B® treatment, DNA cleared after 40 and 90 days and 90 and 150 days in blood and serum and CSF of vervet monkey A and B respectively. The efficiency of microscopy was microscopy 28.21% and 21.18% in blood and CSF respectively. In blood, serum and CSF, the efficiency of LAMP was 60.26%, 55.13% and 79.49% respectively. The X^2 was 16.734 ($p=0.001$) and 38.023 ($p=0.000$) between LAMP and microscopy in blood and CSF respectively. The efficiency of pure DNA on a thermocycler was 60.27%, 55.13% and 78.12%, and 46.15%, 48.72% and 75.64% on a water bath in blood, serum and CSF respectively. Crude DNA had an efficiency of 56.41%, 56.41% and 76.92% on a thermocycler and 48.72%, 44.87% and 64.10% on a water bath in blood, serum and CSF respectively. In crude DNA on thermocycler, LAMP and microscopy had k of 0.397 and 0.602 and X^2 13.141 ($p=0.000$) and 35.247 ($p=0.000$) in blood and CSF respectively. In late stages, k and X^2 of 0.600 and 15.000

($p=0.000$) were obtained in 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 on a water bath may be used for sample preparation and amplification respectively.

Key Words: Loop-mediated isothermal amplification (LAMP); Diagnosis; post-treatment follow-up; **HAT**; *Trypanosoma b. rhodesiense*.

INTRODUCTION

Rhodesian form of human African trypanosomiasis (HAT) or sleeping sickness was an emerging public health problem [9]. Although it's currently under control, it has been neglected in terms of drug development and research in understanding the pathogenesis, immunological and pathological changes [23]. 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 [1]. It occurs in eastern, central and southern parts of Africa [15]. 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, recent research indicated a human case of *T. evansi* infection reported in India [30, 28], 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 [2]. However, diagnosis has heavily relied on direct microscopic visualization of the trypanosomes in clinical samples [29]. This is less sensitivity as result of low parasitaemia. Despite improved laboratory diagnostic methods such as m-AECT [12], PCR [8] and dipstick test [3], diagnosis still remain unsatisfactory. Undiagnosed patient will remain untreated and will die soon or later. In the mean 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

58 test means that some patients go undetected and become potential source of
59 infections to other people.

60 Drugs available for therapy and prophylaxis are limited due to toxicity and
61 resistance [34]. Treatment of early stage HAT is easier and safer though side effects
62 have been reported upon the use of suramin [36]. For late-stage, long treatment
63 schedule requiring hospitalization and considerable care in the use of toxic drugs is
64 needed [32]. Melarsoprol, the only drug effective for the late stage is toxic [22].
65 Modification of treatment regime has not been able to reduce mortality and failure
66 has been reported in the field [14]. Definitive diagnostic tests are thus crucial for the
67 early detection cases to minimize false positive and exposure of patients to toxic
68 drugs with unguaranteed efficacy [6] The molecular technique; Loop-mediated
69 isothermal amplification of DNA [21] has been used diagnosis of HAT [11, 19, 20,
70 24]. The present study evaluates of LAMP in diagnosis, staging and post-treatment
71 follow-up of HAT in vervet monkeys infected with *T. b. rhodesiense*.

72 **MATERIALS AND METHODS**

73 **Animals**

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

86 **Trypanosomes**

87 The stabilate *T. b. rhodesiense* KETRI 2537 was used. This was a derivative of EATRO
88 1989 isolated from a human patient in Uganda in 1972 by direct inoculation of blood
89 and lymph node aspirate into a monkey, and later cryopreserved [4].

90 **Trypanocidal compounds**

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

95 **Experimental design**

96 Two vervet monkeys were infected by intravenous injection with 10^4 *T. b. rhodesiense*
97 using femoral vein in 1 mL of phosphate saline glucose (PSG). Before and during the
98 course of the disease, a daily clinical evaluation was carried out. Parasitaemia was
99 estimated using the rapid matching method [5] and Haematocrit centrifugation
100 technique (HCT) [31] for low parasitaemia. At weekly intervals, the animals were
101 anaesthetized with diazepam (May and Baker, U.K) at a dosage of 1mg/Kg bwt and
102 ketamine hydrochloride (Rotexmedica, Trittau, German) at a dosage of 10-15mg/Kg
103 bwt weighed, and detailed clinical examination undertaken. 5 mL of Ethylene-
104 diamine-tetra-acetic acid (EDTA) blood was collected by femoral vein puncture. 5
105 mL non-EDTA collected blood was processed into serum. Packed cell volume (PCV)
106 was determined using the standard micro-haematocrit method [23]. 2 mL of CSF was
107 collected by lumbar puncture. When the animals were sick, they were treated with
108 berenil at 5 mg/Kg btw intra muscularly (IM) at 35 dpi for three days. The animals
109 were not cured but developed clinical meningoencephalitis and were treated with
110 Mel B (3.6 mg/Kg bwt) from 113dpi and 171 dpi respectively for three days. The
111 animals were then monitored during a follow-up for a period of 250-300 days.

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 [37]. 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.

Preparation of the DNA template for LAMP test

Crude total DNA was extracted through heat treatment of the samples [20]. 15µL 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 20,800g for 10 minutes. The CSF was boiled 3-5 minutes and centrifuged at 20,800g without dilution. 10-15 µL of supernatant was collected for LAMP test. Pure total DNA was extracted using Saponin method [35]. 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 11,000g for 10 minutes in a microcentrifuge followed by four washes in the same buffer. The resulting pellet 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°C for 20 min, cooled and stored at -20°C.

Oligonucleotide primers

Primer RIME 1, 5'-GTTCCCACCCGTTGGCGG-3' and RIME 2, 5'-CGTGGGCGCCAGCCGTG-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 include; two inner primers, forward inner primer (FIP), 5'-GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA-3' and backward inner primer (BIP), 5'-AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC-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'-GCCTCCCACCTGGACTC-3' and loop backward primer (LB) 5'-AGACCGATAGCATCTCAG-3'.

LAMP reaction

LAMP reaction was carried using Loop amp DNA amplification kit (Eiken Chemical Co. Ltd., Japan). The LAMP reaction mixture (25µL) 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), 1.4mM dNTPs, 0.8M betaine, 20mM Tris-HCl (pH 8.8), 10 mM KCl, 10mM (NH₄)₂SO₄, 8mM MgSO₄, and 0.1%Tween 20. 1µL 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°C 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°C for 4 minutes.

Detection of LAMP product

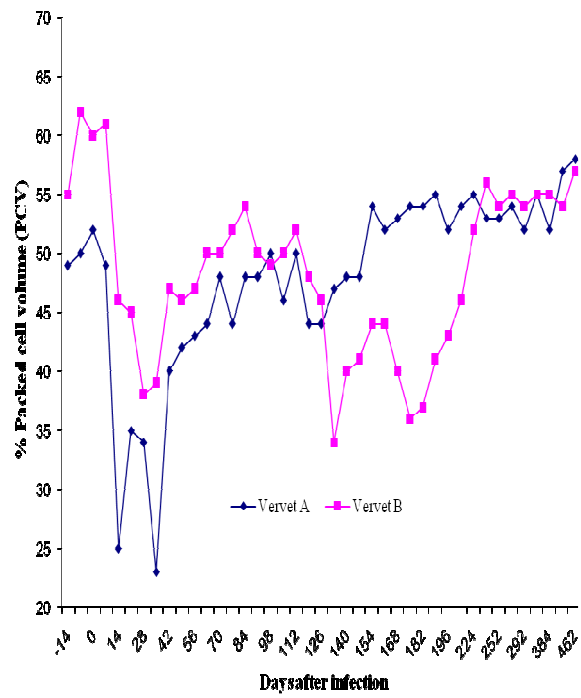
Amplification of DNA in the LAMP reaction was monitored through direct visual inspection after addition of 1 µL/mL of 1/10 dilution of SYBR Green I (Invitrogen, Australia) and electrophoresis in 1% ethidium bromide stained agarose gel and the bands visualized after illumination with UV light. To confirm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

RESULTS

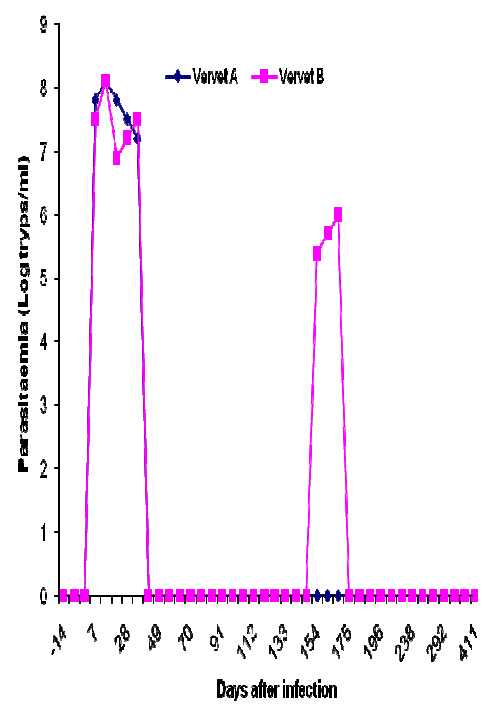
Pre-treatment clinical findings

The animals developed clinical signs of the disease 5-7 dpi characterized by mild fever, splenomegaly 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. Parasitaemia developed 5dpi and remained high characterized by minor fluctuations until treatment. In the CSF trypanosomes

170 were first detected between 21-28dpi. Before infection, the PCV ranged from 50-61%
171 decrease after infection until treatment.



172



173

174 Figure 1: PCV and parasitaemia changes in *T. b. rhodesiense* infected vervet monkeys
175 infected treated with berenil and Mel B

176 *Post-treatment clinical findings*

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

184 *Pre-treatment CSF changes*

185 The CSF white blood cells during pre-infection ranged between 1 and 3 cell/mm³.
186 After two weeks of infection, the number of white cells increased gradually, reaching
187 between 11-18 cell/mm³. Before infection, total CSF protein concentration ranged
188 between 23-25 mg/dl. There was an increase in total protein concentration above 25
189 mg/dl after two weeks of infection.

190

191 *Post-treatment CSF changes*

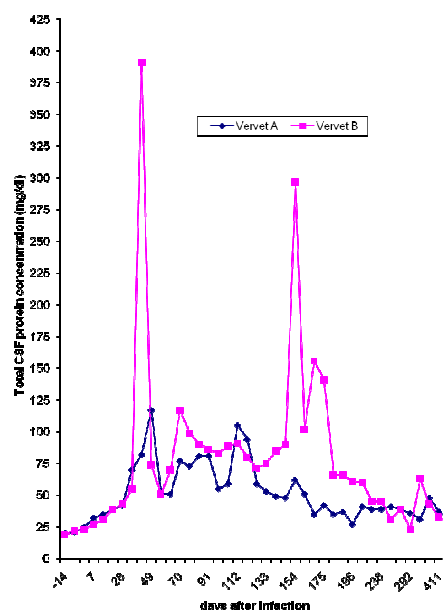
192 *Berenil treatment*

193 Treatment with berenil had no effect on trypanosomes, WBC count and total protein
194 concentration in the CSF. The WBC count and total protein concentration in the CSF
195 fluctuated. The number of white cells some times decreasing <5 cell/mm³ while the
196 total protein concentration remained above 25 mg/dl.

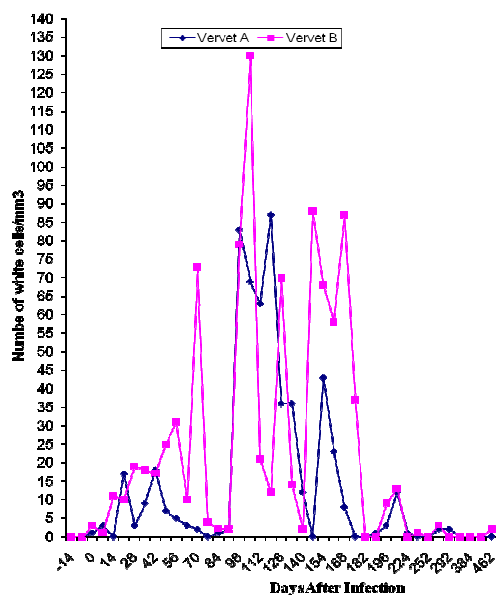
197 *Melarsoprol treatment*

198 Trypanosomes were cleared off from the CSF by day 3 days after Mel B treatment. It
199 was only after Mel B treatment that the number of WBC decreased <.5 cell/mm³
200 dropping to levels < 5cell/mm³, by 62 and 53 dpt with Mel B in monkey A
201 respectively. After Mel B treatment, major decreased in the total protein
202 concentration was observed at 78 and 136 dpt with berenil. Trypanosomes were
203 cleared off in the CSF of both monkeys 3 days after Mel B treatment. During the

204 post-treatment follow-up period up to 462, the animals were aparasitaemic with no
205 parasites detected in the CSF.



206



207

208 **Figure 2** Total CSF protein concentration and number of white blood cells
209 (No/mm³) in *T. b. rhodesiense* infected vervet monkeys with berenil and Mel B.

210

211 *Detection of trypanosome DNA in the LAMP amplicon*
 212 LAMP detected DNA at 7 dpi in the blood and serum disappearing after berenil
 213 treatment at 35 dpi and reappeared after 35 dpt with berenil. The DNA disappeared
 214 completely in the blood and serum after Mel B treatment. In the CSF, DNA was
 215 detected at 21 dpi and disappeared only after Mel B treatment at 113 and 171 dpi in
 216 monkey A and B respectively.

Table 1: The efficiency of LAMP in different DNA extraction and amplification methods

Sample	Method of DNA extraction and amplification and percentage (%) detection			
	Pure DNA; thermocycler (%)	Pure DNA; water bath (%)	Crude DNA; thermocycler (%)	Crude DNA; water bath (%)
Blood	60.27	46.15	56.41	48.72
Serum	55.13	48.72	56.41	44.87
CSF	78.21	75.64	76.92	64.10

217

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

DNA extraction and amplification method	Chi-Square value (X ²); p-values		
	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)

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	Percent agreement (k)
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LAMP	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 serum and CSF versus blood

Sample	Difference or agreement		
	Chi-Square values (X^2); p-values	Percent agreement (k)	Remarks
Serum	0.000 (p=1.000)	0.017	Marginal agreement
CSF	9.510 (p=0.002)	0.205	Marginal agreement

220

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

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)

221 *treatment by Mel B

222 No significant differences in different DNA extraction and amplification methods on
 223 LAMP were observed in the blood, serum and CSF. The agreements of different
 224 DNA extraction and amplification methods on LAMP were marginal in the blood,
 225 serum and CSF. However, for crude DNA on a thermocycler and a water bath, the
 226 agreement was good in the CSF. Thus the performance of LAMP on the different
 227 DNA extraction and amplification methods was similar in the blood, serum and CSF
 228 (Table 3). In different samples LAMP test showed no significant difference and
 229 marginal agreements between different DNA extraction and amplification methods
 230 (Table 3 and Table 4).

231 Therefore, crude DNA on a thermocycler was used in evaluating the performance of
 232 LAMP different samples. The X²-Statistics between blood and serum in detection of
 233 trypanosome using LAMP for crude DNA on a thermocycler (Table 4) had no
 234 significant. A significant difference was observed between blood and CSF. There
 235 agreements between blood and serum and between blood and CSF were marginal.
 236 Microscopy and LAMP had marginal and good agreements in blood and CSF
 237 respectively. The X²-Statistics (Table 6) between LAMP and microscopy in detection
 238 of trypanosomes in the blood and CSF at different stages of the disease indicated no
 239 significant difference in blood. A significant difference was observed in the CSF
 240 during early stage. In the late stage, there was no significant difference in the blood
 241 while a significant difference was observed in the CSF. After treatment, significant
 242 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.

DISCUSSION

Diagnosis of HAT conventionally has relied on clinical signs and microscopy which are not always accurate. In this study, fever, wasting, splenomegaly and lymphadenopathy were observed. However, the development of these clinical signs in different vervet monkeys infected with the same stabilate [33] were different from those encountered in this study. This was replicated in the study when the two vervet monkeys infected with the same stabilate *T. b. rhodesiense* KETRI 2537. This suggests that clinical parameters such as fever, wasting, splenomegaly 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/mm³ 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 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 experiment, parasites were undetectable in blood and CSF 3 days after Berenil® and Mel B® treatment but the DNA was detected. 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 [10]. 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 are not specific [18] 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 and a 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 [20].

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 [20]. Visualization of colour changes after addition of fluorescent dye, SYBR Green I dye give results immediately [16]. 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 parasite waves. The significant difference between the two methods in detection of trypanosomes suggests the sensitivity of LAMP compared to microscopy. The

301 results from this study are in agreement with the finding other studies [13, 20]. The
 302 high efficiency of LAMP in the CSF regardless of DNA extraction and amplification
 303 method is attributed to its purity. Blood and serum may contain PCR inhibitors
 304 which may also inhibit LAMP by acting as impurities [11]. Although marginal
 305 agreement and significant difference were noted between blood and serum in
 306 LAMP, use of blood is preferred owing to tiresome and time consuming procedure
 307 of processing blood for serum.

308 Trypanosome DNA was detected at 7 dpi in the blood and serum although it should
 309 have been detected much earlier. However, samples were collected on weekly basis
 310 starting at 7 dpi due to ethical considerations when dealing with non-human
 311 primate experiments. The marginal agreements and insignificant difference noted
 312 between LAMP and microscopy during early stages of the infections needs to be
 313 investigated. For proper evaluation of LAMP during early stages in such studies, it
 314 is recommended that the samples be collected on daily basis. Trypanosome DNA
 315 was detected earlier than trypanosomes confirming the sensitivity of LAMP. As the
 316 disease progressed to the late stage, good agreement in terms of diagnosis and
 317 significant differences were noted between LAMP and microscopy in the CSF. In the
 318 blood sample a marginal agreement and insignificant difference was note between
 319 LAMP and microscopy. Due to the low sensitivity of CSF parasitosis, findings from
 320 this study suggest that LAMP may be a good test for staging the disease. Another
 321 advantage of LAMP is that it is a specific test as compared to WBC count and protein
 322 concentration.

323 The good agreements and significant differences noted between LAMP and
 324 microscopy after curative Mel B® treatment suggest the superiority of LAMP.
 325 Microscopy could not detect trypanosomes both in the blood and CSF 3 days after
 326 treatment but trypanosome DNA was still present and could be detected by LAMP
 327 in the CSF 120 to 150 dpt. This further suggests the limitations of microscopy. There
 328 are different PCR assays that have been developed in detection of trypanosome
 329 DNA although none of them was evaluated and validated [8, 28, 29]. However, the

use of LAMP technique has been evaluated in diagnosis of HAT [13, 24, 25]. 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 [18] 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 [11, 19, 20, 24].

LAMP has been used in diagnosis of HAT using archived clinical samples [11, 19, 20, 24]. In this study, LAMP based on primers derived from *RIME* gene were used. It has been suggested that LAMP may be suitable for both diagnosis and post-treatment follow-up because it utilizes *Bst* DNA polymerase large fragment with optimal temperature ranging from 60 °C to 65 °C inactivated at 80 °C after the reaction [21]. This reduces the prospects of non-specific priming which tends to result into false positive results. A set of two specially designed inner and outer primers; forward inner primer (FIP) and backward inner primer (BIP) and outer primers; forward outer primer (F3) and backward outer primer (B3) are used [21]. Two more primers; loop forward primer (LF) and loop backward primer (LB) may be included. This increases sensitivity and acceleration of the reaction, reducing the time by a half the time for PCR [17]. The use 4-6 primer pairs that recognize 6-8 region of targeted DNA increases specificity and sensitivity of the test. LAMP results can be visualized by addition of fluorescent dyes such as SYBR Green I under UV

light and could be seen by naked eyes [7]. The dye, SYBR Green I was used and similar results noted in the study. This 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 use of crude and pure DNA samples in LAMP was also assessed. The results of crude and pure DNA on LAMP had marginal agreements and no significant difference in detection of trypanosome DNA in whole blood, serum and CSF. This 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 [20]. Blood and serum had similar results in detection of trypanosome DNA using LAMP. Thermocycler and simple incubators such as heat block and water bath were previously noted to allow low concentration of DNA to be amplified within 35 minutes in a laboratory [20]. 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 [20]. 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 [20] eliminate the need for pure DNA extraction, shortening further the time for the LAMP reaction.

Previous studies have also shown that the use of reagents stored at varied temperatures between 25 °C and 37 °C which are above the recommended storage temperature for LAMP reagents had no effects on the detection sensitivity [26]. This further suggests the cost friendliness of LAMP and promisibility for use and storage in tropical climate like most parts of the African continent where high temperatures are common. The use of different DNA template preparation and amplification methods further promises the practicability of LAMP in diagnosis, staging and monitoring treatment of HAT [20, 26]. Therefore, developing LAMP for detection of animal and human trypanosomes is of economic importance [11, 24, 25] owing to the difficulties posed by diagnosis. However, further improvements on rapidity and

388 simplicity of DNA preparation methods are required to enhance the practicability of
389 LAMP for diagnosis, staging and monitoring treatment of HAT.

390 **CONCLUSIONS**

391 This study was aimed at solving the problems associated with diagnosis, staging and
392 monitoring the treatment of HAT. Molecular diagnostic methods for HAT including
393 PCR and dipstick test have major limitations making them unfeasible in field
394 settings due to the need of sophisticated equipments and cost involvement. The
395 present study indicates that LAMP can be used to exclude false negative results
396 caused by the insensitive 'Gold standard' microscopy in diagnosis, staging and
397 monitoring treatment of HAT. Despite the fact that LAMP has been promising as a
398 molecular method for the diagnosis and post-treatment follow-up of animal
399 trypanosomiasis further research has to be done to evaluate its use and potential use
400 in the field. Due to the zoonotic nature of African trypanosomiasis, development of
401 LAMP based on a universal gene for all trypanosomes will be a breakthrough in
402 solving the problem of diagnosis and monitoring treatment. Thus development of
403 LAMP able to detect the DNA as a molecular marker in all groups of trypanosomes
404 should be a research priority in the diagnosis of HAT.

405 **RECOMMENDATIONS**

406 The results from this vervet monkey model suggest that LAMP may be a strong
407 candidate in diagnosis and monitoring treatment of HAT. Blood and serum may be
408 used as samples for LAMP during haemolytic stage. However, blood is the preferred
409 sample owing to the long and tiresome procedure of processing blood for serum.
410 Heat treatment of the samples may be adopted as the sample preparation method
411 with amplification in a water bath. Visualization using SYBR Green I dye which give
412 results immediately after amplification is thus recommended. Due to low sensitivity
413 of microscopy and some of the markers used in staging, LAMP is preferred during
414 stage determination of the disease. Therefore LAMP can be performed for early and
415 late diagnosis of the disease and also during post-treatment follow-up. However,
416 further research on its applicability in the field settings is needed in appropriate

417 experimental animal model to supplement the already finished and ongoing
418 research for proper evaluation.

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