Development of Loop-mediated Isothermal Amplification (LAMP) Technique for Diagnosis of Trypanosomosis and Bovine Theileriosis and its Application to Epidemiological Studies

（**LAMP** 法によるトリパノソーマ症および牛タイレリア症診断法の開発と疫学調査への応用に関する研究）

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Abbreviations

B *B. bigemina*: *Babesia bigemina*

B. bovis: *Babesia bovis*

B. caballi: *Babesia caballi*

B. equi: *Babesia equi*

B. gibsoni: *Babesia gibsoni*

BIP: backward inner primer

B3: backward outer primer

BLASTn: basic local alignment search tool for nucleotide sequences

B. caudatus: *Bodo caudatus*

B. saltans: *Bodo saltans*

BSF: bloodstream form

Bst: *Bacillus stearothermophilus*

C CATT: card agglutination test for trypanosomosis

CD: corridor disease

C. oncopelti: *Crithidia oncopelti*

D DDW: double distilled water

DNA: deoxyribonucleic acid

dNTP: deoxynucleotide triphosphate

E ECF: east coast fever

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

F FBS: fetal bovine serum

FD: fluorescent detection reagent

FIP: forward inner primer

F3: forward outer primer

G gDNA: genomic DNA

H HAT: human African trypanosomosis

HEK 293T: human embryonic kidney 293T cell

H. a. anatolicum: *Hyalomma anatolicum anatolicum*

H. hystricis: *Haemaphysalis hystricis*

I IFAT: indirect fluorescent antibody test

Ig: immunoglobulin

ILRI: International Livestock Research Institute

ITS: internal transcribed spacer

K kDNA: kinetoplast DNA

L LAMP: loop-mediated isothermal amplification

LB: loop B primer

LF: loop F primer

L. major : *Leishmania major*

L. donovani: *Leishmania donovani*

L. tarentolae: *Leishmania tarentolae*

M MCF: metacyclic form

MEM: Eagle's minimum essential medium

M. meles: *Meles meles*

MHCT: microhemtocrit centrifuge test

MI: mouse inoculation test

N *N. caninum*: *Neospora caninum*

O OIE: Office International Des Epizooties

O. moubata: *Ornithodoros moubata*

P PBS: phosphate buffered saline

PCF: procyclic form

PCI: phenol-chloroform-isoamyl alcohol

PCR: polymerase chain reaction

PFR A: paraflagellar rod protein A

P0: ribosomal P0 protein

P. serpens: *Phytomonas serpens*

R *R. appendiculatus*: *Rhipicephalus appendiculatus*

RH: relative humidity

RNA: ribonucleic acid

rRNA: ribosomal RNA

RT: room temperature

S SFG: spotted fever group

S. caffer: *Syncerus caffer*

SRA: serum resistance associated gene

S. scrofa: *Sus scrofa*

T TAE: Tris-acectic acid-EDTA

T. annulata: *Theileria annulata*

Taq: *Thermus aquaticus*

TBS: thin blood smear test

T. avium: *Trypanosoma avium*

T. b. brucei: *Trypanosoma brucei brucei*

T. b. gambiense: *Trypanosoma brucei gambiense*

T. b. rhodesiense: *Trypanosoma brucei rhodesiense*

T. buffeli: *Theileria buffeli*

T. congolense: *Trypanosoma congolense*

T. cruzi: *Trypanosoma cruzi*

T. cyclops: *Trypanosoma cyclops*

T. dionisii: *Trypanosoma dionisii*

TE: Tris-EDTA

T. equiperdum: *Trypanosoma equiperdum*

T. evansi: *Trypanosoma evansi*

T. gondii: *Toxoplasma gondii*

T. lewisi: *Trypanosoma lewisi*

T. mega: *Trypanosoma mega*

T. microti: *Trypanosoma microti*

T. mutans: *Theileria mutans*

T. orientalis: *Theileria orientalis*

T. parva: *Theileria parva*

T. pestanai: *Trypanosoma pestanai*

T. rangeli: *Trypanosoma rangeli*

T. rotatorium: *Trypanosoma rotatorium*

T. sergenti: *Theileria sergenti*

T. sp. KG1: *Trypanosoma* species KG1

T. sp. Wombat H26: *Trypanosoma* species Wombat H26

T. taurotragi: *Theileria taurotragi*

T. theileri: *Trypanosoma theileri*

T. varani: *Trypanosoma varani*

T. velifera: *Theileria velifera*

T. vivax: *Trypanosoma vivax*

U UV light: ultraviolet light

USDA: United States department of agriculture

V VSG: variant surface glycoprotein

W WHO: World Health Organization

Unit abbreviations

D ^{o}C: degree celcius

F fg: femtogram

H h: hour

K kDa: kilo Dalton

M µg: microgram

µl: microliter

µm: micrometer

mg: milligram

min: minute

ml: milliliter

mm: millimeter

mM: milliM

N ng: nanogram

P pg: picogram

pmol: picomol

V v/v: volume/volume

General Introduction

1. Trypanosomosis

Trypanosomosis is a disease caused by protozoan parasites of the genus *Trypanosoma* infecting both human and domestic animals. In Africa trypanosomosis is a serious constraint to both animal agriculture and human health. Estimates of the total losses due to trypanosomosis range from 1.3 to 5 billion US$ including meat and milk production losses (98). In the year 2000, the United Nations (www.unfoundation.org/unwire/) estimated that there maybe as many as 300,000 new cases of human trypanosomosis annually. Over 11 million people are believed to be infected with American trypanosomosis (34) and 100 million people are considered at risk of infection (146). The genus *Trypanosoma* is divided into two sections, salivarian trypanosomes which are transmitted by biting flies in sub-Saharan Africa (1, 36, 125) and stercorarian trypanosomes which are transmitted by bugs and fleas through contaminated faeces in Central and South America (138).

1.1 Salivarian trypanosomes

The salivarian trypanosomes include the *Trypanosoma brucei rhodesiense* and *T. b. gambiense* which cause Human African Trypanosomosis (HAT) or sleeping sickness in East and West Africa (125) respectively. Other salivarian trypanosomes include *T. b. brucei, T. congolense* and *T. vivax* which cause a disease called Nagana in cattle in Western, Central, Eastern and Southern Africa (10, 52, 103). *T. evansi* is the most widely distributed salivarian trypanosome causing a disease called Surra and is transmitted mechanically by tabanid biting flies (63, 88). It affects domesticated livestock in Africa, Asia and South America (88, 147) and it infects buffaloes, llamas, cattle, horses, donkeys, goats, sheep and pigs (111, 119). *T. equiperdum* is distributed in African and Asian countries and causes a disease called

dourine in horses and donkeys and is transmitted by copulation from animal to animal (20, 22, 23). In contrast to other salivarian trypanosomes with complex life cycles, *T. evansi* and *T. equiperdum* are monomorphic.

When tsetse fly takes a blood meal, it injects metacyclic form (MCF) trypomastigotes into the mammalian host that transforms into bloodstream form (BSF) trypomastigotes which then multiplies by binary fission in various body fluids e.g. blood, lymph and spinal fluid. When a tsetse fly takes another blood meal from infected mammalian host the BSF is ingested and transforms into procyclic form (PCF) in the tsetse midgut (61). The PCF leaves the midgut and transforms into the epimastigote form which multiplies in the salivary gland/mouthparts and transforms into the MCF which will be injected into mammalian host when the fly takes another bloodmeal (51).

1.2 Stercorarian trypanosomes

The stercorarian trypanosomes infect all forms of vertebrate classes and are generally non-pathogenic (138). *T. theileri* is a non-pathogenic stercorarian trypanosome with a cosmopolitan distribution among domestic cattle (138) and is transmitted by tabanid flies (54). The stercorarian trypanosomes of medical importance are *T. cruzi, T. rangeli* and *T. lewisi* and are transmitted by bugs and fleas through contaminated faeces (33,138). *T. rangeli* and *T. lewisi* are non-pathogenic rodent trypanosomes which share the same geographical location and same insect vectors with *T. cruzi* (141), hence, necessitating accurate diagnosis.

T. cruzi infects human beings and causes Chagas disease in Central and South America. When the *T. cruzi* trypomastigotes are ingested with the blood meal by vector insect, they differentiate into epimastigotes which multiply in the midgut and ultimately differentiate into infective MCFs. Transmission of *T. cruzi* to humans happens when bug infected extcreta contaminates the mucous membranes or

breaks in the skin. Once beyond the barrier of the skin, host cells are invaded and MCFs differentiate into amastigotes in the cytoplasm which at some point will differentiate into BSFs (41).

1.3 Diagnosis of trypanosomosis

Clinical signs associated with salivarian trypanosome infections may include intermittent fever, oedema, abortion, anaemia, wasting, reduced productivity and often mortality (18,119). Additionally, in later stages of HAT parasites invade the central nervous system causing meningoencephalitis and excessive daytime sleepiness whereby without treatment the disease progresses to coma and death (18, 91). In the acute Chagas disease caused by *T. cruzi*, local inflammatory lesions appear at the site where the MCFs enter. After dissemination symptoms of cardiac insufficiency develop in small number of patients reflecting underlying severe myocarditis, meningoencephalitis may also occur especially in immunosuppressed patients (41).

Microscopic methods such as Giemsa-stained smears, buffy coat, and examination of the wet smears by hematocytometer are commonly used for diagnosis of trypanosome infections (39, 93, 136). Serological assays such as card agglutination test for trypanosomosis (CATT) and enzyme linked immunosorbent assay (ELISA) are also widely used in detection of trypanosomes (7, 111). Polymerase chain reaction (PCR) which amplifies pathogen deoxyribonucleic acid (DNA), is used as a tool allowing precise identification of the infecting trypanosome taxa from blood or tissue samples (30, 31, 80, 93), and to detect trypanosomes in the vector (141), or for stage determination of sleeping sickness by amplifying trypanosome DNA from the cerebrospinal fluid (140).

2. Theileriosis

Theileriosis is a tcik-borne disease caused by piroplasmic parasites of the genus *Theileria* infecting bovids (57, 107), ovids (133) and equids (99). Bovine theileriosis syndromes, include East Coast fever (ECF) or Corridor disease (CD) caused by *Theileria parva* (35, 135); tropical theileriosis caused by *T. annulata* (58), cerebral theileriosis 'Ormilo' caused by *T. taurotragi* (89) and benign theileriosis caused by *T. buffeli/orientalis/sergenti* group (82), *T. mutans* and *T. velifera* (58).

2.1 *Theileria parva*

T. parva which causes ECF and CD commonly infects cattle and buffaloes in the Eastern and Southern African countries respectively (35, 57, 107), and these diseases are of economic importance in the respective countries. With 25 million cattle at risk in Eastern, Central and Southern Africa, economic impact of ECF on the livestock sector in these regions has been estimated at 169 million US$ per annum (127). The main vector of the *T. parva* parasites is a three – host tick *Rhipicephalus appendiculatus* (83, 94). Life cycle stages of *T. parva* include sporozoites that develop in the salivary glands of the vector and are introduced into the mammalian host during tick feeding (109).

The sporozoites then invade lymphocytes where they further develop into intracytoplasmic multinucleate schizonts (12). Some parasites form merozoites and are released into the bloodstream by rupture of the host cell, where they invade erythrocytes and develop into intra-erythrocytic forms called piroplasms (48). Ticks ingest the piroplasms from a bloodmeal and following a sexual cycle in the gut, kinetes migrate to the salivary glands of the tick. Sporogony is initiated when the tick attaches to a host animal, resulting in the release of sporozoites into the salivary glands, ready for transmission to the host (12, 109).

2.2 Diagnosis of theileriosis

Animals infected with *T. parva* show clinical signs such as enlarged lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea, occasional diarrhoea, anaemia and jaundice (119), and if untreated *T. parva* infections results into death of infected cattle (83). During the acute phase of *T. parva* infection, microscopical detection of the parasite in the lymph node and blood smears by Giemsa-stain is possible because of the relatively high numbers of parasites present (85, 135).

Furthermore, the most commonly used method to identify the infection in *R. appendiculatus* ticks involves detection of the infective sporozoite stage of *T. parva* in stained tick salivary glands (117). The most widely used diagnostic test for *T. parva* infections is the indirect fluorescent antibody test (IFAT) (85, 86), additionally, ELISA has also been utilized for detection of *T. parva* infections (81). Recently, PCR diagnostic techniques have been widely used for detection of *T. parva* infections, both in cattle and tick vectors using a variety of gene targets (48, 117, 135) and has been reported as highly specific and sensitive than microscopy and serology.

3. Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method characterised by rapidity, simplicity, and high sensitivity whereby a few copies of DNA can be amplified to 10^9 copies in less than an hour under isothermal conditions with greater specificity (115).

3.1 LAMP primer design

The LAMP primers are designed using primer explorer software available in the EIKEN Chemical Co. Ltd., website (http://primerexplorer.jp/e/). As shown in Figure 1, the Forward inner primer (FIP) consists of the F2 region that is

complimentary to the F2c region on the target gene at the 3' end, and the same sequence as the F1c region on the target gene at the 5' end. The forward outer primer (F3) consists of the F3 region that is complimentary to the F3c region on the target gene. The backward inner primer (BIP) consists of the B2 region that is complimentary to the B2c region on the target gene at the 3' end, and the same sequence as the B1c region on the target gene at the 5' end. The backward outer primer (B3) consists of the B3 region that is complimentary to the B3c region on the target gene.

3.2 Mechanism of LAMP reaction

Reagents for DNA amplification by LAMP method include four primers (FIP, F3, BIP and B3), *Bst* DNA polymerase with strand displacement activity, a reaction buffer with deoxynucleotide triphosphates (dNTPs) and the target DNA (115). As shown in Figure 2, target DNA amplification by LAMP technique includes a series of stages as described by Notomi *et al.* (115) and Mori *et al.* (101). Briefly, the FIP penetrates the double stranded DNA of the target gene and anneals to the region containing a sequence complementary to its own, whereby it hybridizes to F2c in the target DNA and initiates complimentary strand synthesis. The outer primer F3, which is a few bases shorter and lower in concentration than FIP, slowly hybridizes to F3c on the target DNA and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which can form a looped out structure at one end. This single stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis leading to the production of a dumb-bell form DNA, which is quickly converted to a stem-loop DNA by self-primed DNA synthesis. This stem-loop DNA serves as a starting material for the second stage of LAMP reaction called LAMP cycling.

To initiate the LAMP cycling, FIP hybridizes to the loop in the stem-loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem-loop DNA with an additional inverted copy of the target sequence in the stem and a loop formed at the opposite end via a BIP sequence. Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA and one gap repaired stem-loop DNA with a stem elongated to twice as long (double copies of the target sequence) and a loop at the opposite end. Both these products then serve as template for a BIP-primed strand displacement reaction in the subsequent cycles. The final products are a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops (101, 115). Additionally two more primers termed loop primers (LoopF and LoopB) can be added to make a total of six primers. Loop primers hybridize to the stem loops (Figure 3) and accelerate the LAMP reaction thereby significantly reducing the reaction time (110).

3.3 Detection of LAMP products

The LAMP products are detected by agarose gel electrophoresis stained with ethidium bromide and visualised under UV light. A smear of multiple bands is seen in a lane of positive LAMP reaction whereas none will be visible in negative LAMP reaction (115). Further, in a positive LAMP reaction magnesium pyrophosphate is formed as a by-product which forms white turbidity in the reaction tube (100, 102, 123). This enables detection by naked-eye further saving time of detection. Additionally, fluorescent detection reagent can be added to the LAMP reaction mixture which reacts with the magnesium pyrophosphate further simplifying detection by naked eye or under UV light (75, 137). Recently, real-time turbidimetry

of LAMP can also be conducted using a device which can measure turbidity released during the reaction which further simplifies detection of LAMP results (101).

4. Objectives of this study

The first line of disease control and management involves diagnosis, followed by treatment, and once again diagnosis to monitor the success of treatment. The same applies to prevention strategies, diagnosis is conducted on a large scale through epidemiological studies, then prevention measures are applied and the success of prevention is further monitored using diagnostic techniques. In addition to clinical manifestations of trypanosome and *Theileria* infections which are generally unspecific, various diagnostic techniques have been developed ranging from simple microscopic tests, serological tests and state-of-the-art molecular assays. Although widely used, microscopic tests are limited by lack of sensitivity during chronic infections which are characterised by low parasitaemia in the host (27, 147). Furthermore, it is difficult to distinguish different species of the same genus or subspecies by microscopy during mixed infections (105). The specificity and sensitivity of serological tests has been greatly improved, however these serological assays cannot distinguish between past and current infections due to persistence of antibodies even after successful treatment (17, 27).

The advent of molecular biological techniques for diagnosis of trypanosomosis and theileriosis has brought in highly sensitive and specific diagnostic techniques that can even identify subspecies (30, 31, 32, 149). PCR has evolved as one of the most specific and sensitive molecular method for diagnosis of infectious diseases (28, 48, 80, 135). However, PCR requires expensive equipment such as a thermocycler, hence, in spite of the excellent specificity and sensitivity it is

not commonly used in the diagnosis of trypanosomosis and theileriosis in countries where these diseases are endemic due to lack of resources (67, 84, 123).

LAMP is a simple, rapid, highly sensitive and cost effective technique which can be applied under isothermal conditions (115). The main advantage of LAMP over PCR is that it does not require complicated thermocyclers and that amplification can be conducted within 1 hour in a simple heating device. Based on the above mentioned advantages this novel technique has better chances of overcoming the shortcomings of conventional diagnostic methods for application in confirmatory diagnosis in the field, clinics, hospitals and large scale epidemiological studies.

Therefore, this study was designed with the general objective of development of LAMP for diagnosis of animal and human trypanosomosis, and bovine theileriosis caused by hemoprotozoan parasites of the genera *Trypanosoma* and *Theileria*.

Specific objectives included:

(i) comparative evaluation of LAMP, PCR and microscopic tests for detection of *T. evansi* infections in experimentally infected pigs (Chapter 1),

(ii) development of species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of *T. b. gambiense, T. b. rhodesiense, T. congolense, T. cruzi* and *T. evansi* (Chapter 2),

(iii) evaluation of the stability of LAMP reagents when stored at different temperatures, assessment of its amplification efficiency on different trypanosome DNA templates and determination of its tolerance to inhibitory substances (Chapter 3),

(iv) identification, determination of phylogenetic position and development of LAMP and PCR for detection of a trypanosome species isolated from naturally infected *Haemaphysalis hystricis* ticks in Kagoshima Prefecture, Japan (Chapter 4),

(v) development of universal LAMP primers for diagnosis of bovine theileriosis caused by *T. parva, T. annulata, T. mutans* and *T. taurotragi* and specific LAMP primers for detection of *Theileria parva* infections in the host and the vector (Chapter 5).

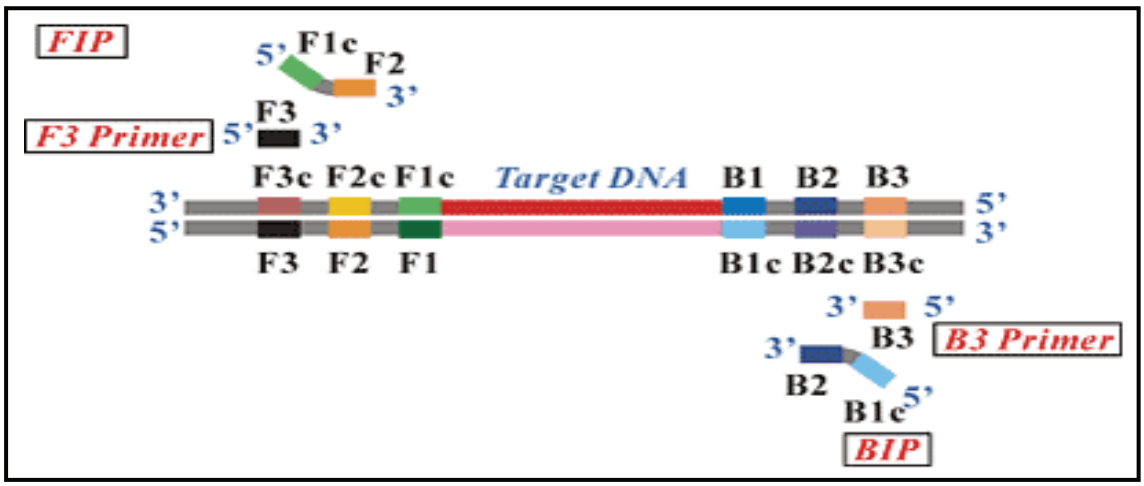

FIG 1. Schematic illustration of LAMP primer design showing four LAMP primers and their positions on the target DNA. Adopted from Eiken Chemical Co. Ltd., website: http://loopamp.eiken.co.jp/e/lamp/primer.html.

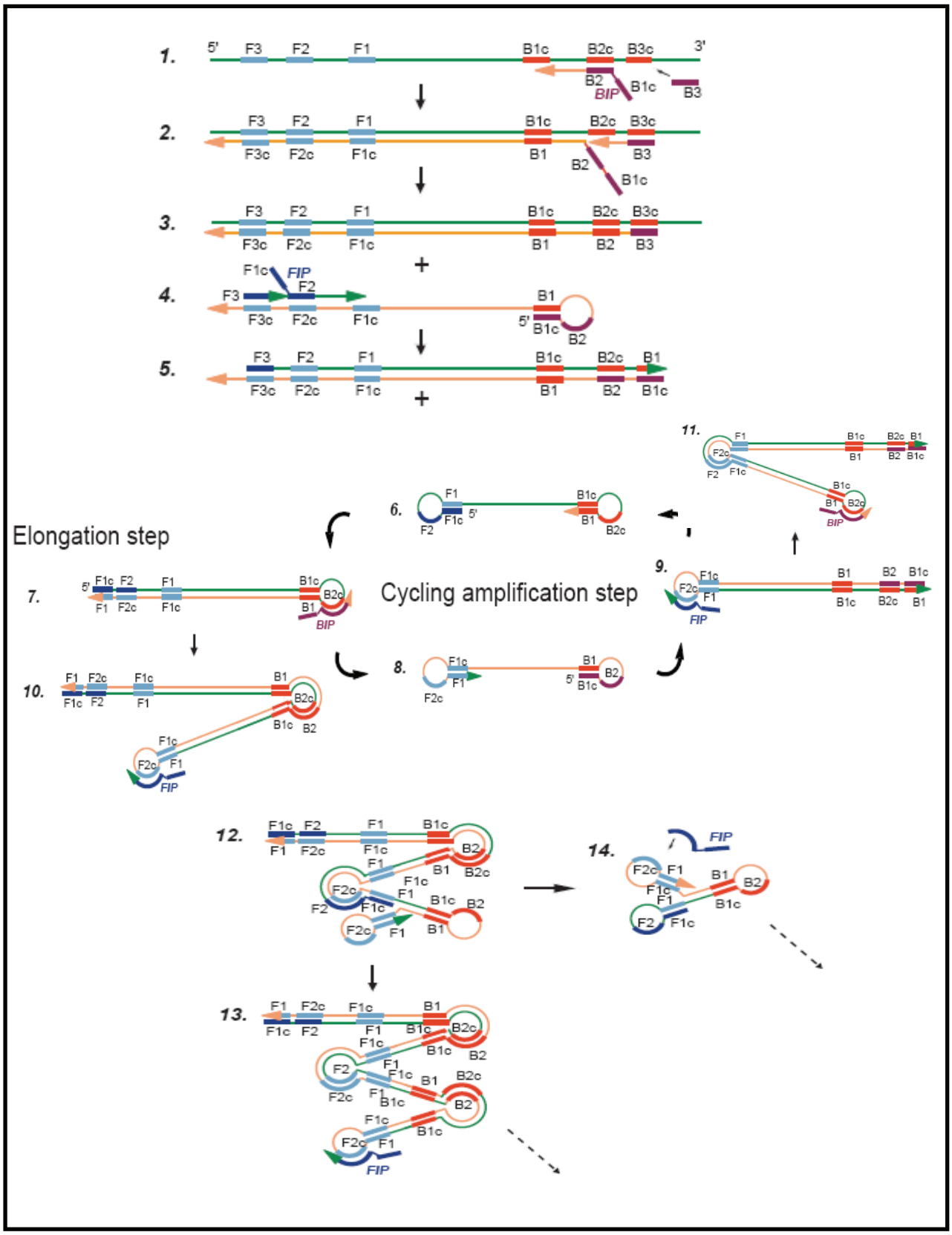

FIG 2. Schematic illustration of LAMP reaction mechanism. Adopted from Nagamine *et al*. (110).

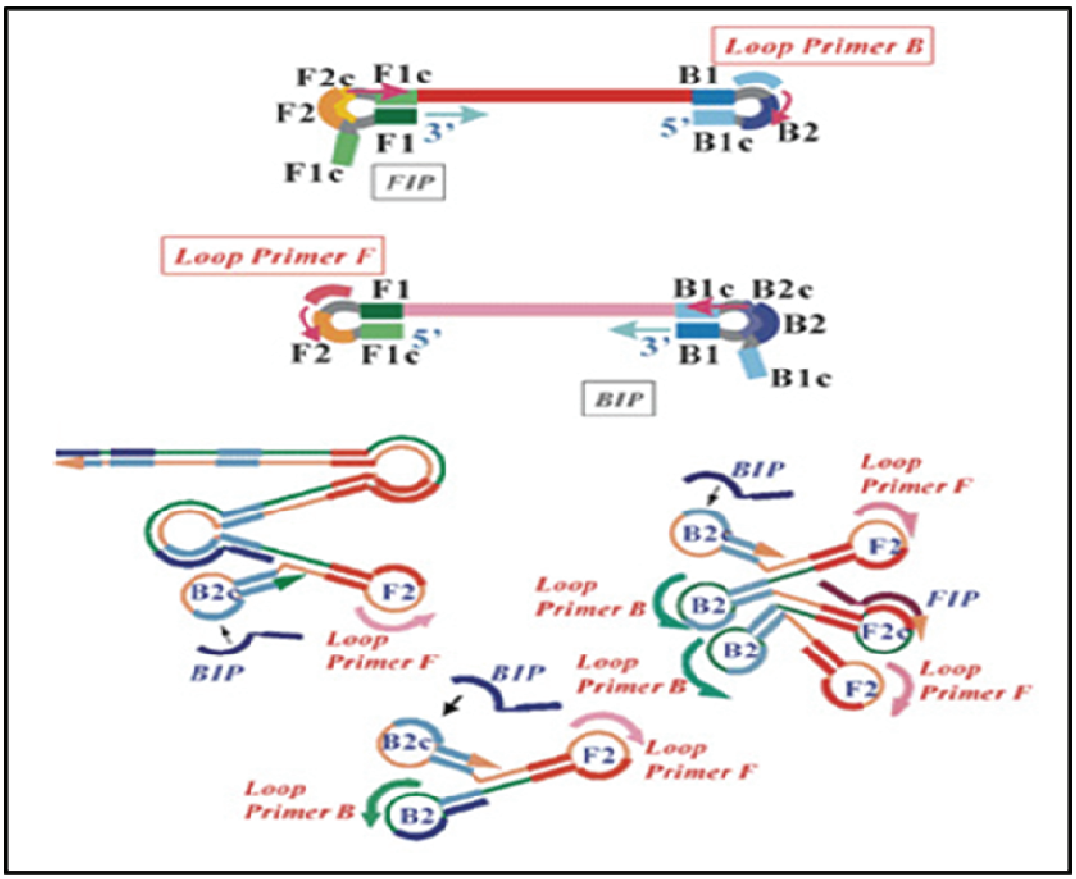

FIG 3. Schematic illustration of LAMP mechanism using loop primers which accelerates the reaction. Adopted from the Eiken Chemical Co. Ltd., website: http://loopamp.eiken.co.jp/e/lamp/loop.html

CHAPTER 1

Evaluation of Loop-mediated Isothermal Amplification (LAMP), PCR and Parasitological Tests for Detection of *Trypanosoma evansi* in Experimentally Infected Pigs

1-1. Introduction

Trypanosoma evansi is the most widely distributed pathogenic trypanosome. It causes epidemics commonly called surra and is transmitted mechanically by biting flies such as tabanids. It affects domesticated livestock in Asia, Africa and South America (88, 147). *T. evansi* infects and produces a mild or subclinical form of the disease in buffaloes, llamas, cattle, donkeys, goats, sheep and pigs (120). Diagnosis of surra is usually based on the demonstration of parasites in the blood, but hematological, biochemical and serological tests are also commonly applied. On the other hand, the PCR test using DNA probes specific for *T. evansi* genome is still under evaluation (118). LAMP is a novel method that can amplify a few copies of DNA to 10^9 copies in less than an hour under isothermal conditions. The test is simple and easy to perform, as it requires only 4 specific primers, *Bst* DNA polymerase, and a regular laboratory heat block or a water-bath for the reaction (115).

Recently, Kuboki *et al.* (84) reported the successful use of LAMP for detecting African trypanosome DNA from *in vitro* cultures and *in vivo* (mice) with high sensitivity. Furthermore, they reported that LAMP primers targeting the paraflagellar rod protein A (PFR A) amplify both *T. brucei* subspecies DNA as well as that of *T. evansi*. This study was therefore designed to evaluate the performance of different diagnostic methods for *T. evansi* infection including LAMP, PCR, microhematocrit centrifuge test (MHCT), thin blood smear test (TBS) and the mouse inoculation test

(MI) in experimentally infected pigs. The MI is generally regarded as the most sensitive method for detecting animal trypanosomosis (126), hence, it has been used as a gold standard in the current study.

1-2. Materials and Methods

Experimental animals and sampling

Six of 8 piglets (6 weeks old) were infected intravenously with 0.6 x 10^6 *T. evansi* parasites isolated from naturally infected horse in Khonkaen – Thailand, whilst 2 were kept as negative controls. This work was carried out in year 2003 at the National Institute of Animal Health, Bangkok – Thailand. Blood samples were then collected weekly from each animal over a period of 12 weeks and microscopic examination was conducted on each blood sample collected, additionally DNA was extracted and molecular diagnostic tests were conducted.

Microscopic tests

The MHCT, TBS and MI were conducted in accordance with OIE manual of standards for diagnostic tests and vaccines (118). For MHCT ~70 µl of blood was collected into heparinised capillary tubes (75 x 1.5 mm) which were then sealed at the dry end and centrifuged with sealed end down at 3000 x *g* for 10 minutes. Two pieces of glass (25 x 10 x 1.2 mm) were glued to the slide and the capillary tube placed between them. A cover-slip was placed on top at the level of the buffy coat junction where the trypanosomes are presumed to be concentrated. The space around this part was flooded with immersion oil, and the buffy coat area examined under the microscope (x 100 – 200).

For TBS, a drop of blood was placed on a clean microscope slide and a thin film was drawn out in the usual way. The film was then briefly air-dried, fixed in methyl alcohol for 2 minutes and allowed to dry. Thereafter the smears were stained

by Giemsa solution (one drop Giemsa + 1 ml PBS, pH 7.2) for 25 minutes. The preparation was then poured off, the slide was washed in tap water and left to dry. Slides were then examined at high magnification (x 400 -1,000).

For MI, 0.5 ml of blood from the infected piglets treated with anticoagulant sodium heparin was inoculated intraperitoneally into mice (6 weeks old). The mice were then bled from the tail and the parasites were examined from wet smears on a hemocytometer slide.

DNA extraction

DNA was extracted with QIAamp® DNA blood kit according to manufacterer's instructions (Qiagen, Inc., Valencia, CA, USA). Briefly, twenty microlitres of QIAGEN protease (or proteinase K) was pipetted into the bottom of a 1.5 ml microcentrifuge tube and 200 µl of blood sample as well as 200 µl of buffer AL were added, mixed by pulse-vortexing for 15 seconds, and then incubated at 56 ^{o}C for 10 min. Thereafter 200 µl of ethanol (99.5%) was added, mixed by pulse-vortexing and the mixture was then applied on a QIAamp spin column with a 2 ml collection tube and centrifuged at 4,000 x *g* for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube, 500 µl of buffer AW1 was added and the mixture was centrifuged once again at 4,000 x *g* for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube, 500 µl of buffer AW2 was added and the mixture was centrifuged once again at 10,000 x *g* for 3 min. The QIAamp spin column was finally placed in a clean 1.5 ml collection tube, 200 µl of buffer AE was added and the mixture was incubated at room temperature (RT) for 5 min and then centrifuged at 4,000 x *g* for 1 min. The resultant DNA was stored at -20 ^{o}C until used for LAMP and PCR assays.

LAMP

The LAMP primer set designed from the PFR A (GenBank accession number X14819), was used in this study as described by Kuboki *et al.,* (84) with the following nucleic acid sequences:

FIP: 5'-TCAGAAGCGTCGAGCTGGGATTTTATCGACAATGCCATCGCC-3';

F3: 5'-TCACAACAAGACTCGCACG-3';

BIP: 5'-CGCAAGTTCCTGTGGCTGCATTTTTTCCCAAGAAGAGCCGTCT-3';

B3: 5'-GGGCTTTGATCTGCTCCTC-3'.

Each reaction mixture with a total volume of 25 µl contained: a LAMP reaction buffer (40 mM Tris-HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM of each dNTP), 1 µl (8 units) of *Bst* DNA polymerase (both provided in the Loopamp DNA Amplification Kit - Eiken Chemical Co. Ltd., Tokyo, Japan), primer mix (FIP and BIP at 40 pmol each and F3 and B3 at 5 pmol each) and 2 µl of template DNA. The reaction mixture was incubated in a heat block (Dry Thermounit DTU 1B, TAITEC Co., Saitama, Japan) at 63^{o}C for 1 hour and then at 80^{o}C for 2 min. to terminate the reaction. LAMP products were electrophoresed in 1.5% Tris-acetic acid-EDTA (TAE) agarose gels and bands were visualized by staining with ethidium bromide solution.

PCR

Primers known as MP1 and MP2 (32) which give a product size of 362 bp were used in a PCR reaction. The primers had the following nucleic acid sequences: MP1: 5'-CAACGACAAAGAGTCAGT-3' and MP2: 5'-ACGTGTTTTGTGTATGGT-3'.

The PCR reaction mixture with a total volume of 50 µl contained: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM of each dNTP, 5 pmol of each primer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosytems, CA, U.S.A.). Reactions were conducted according to the method described by Artama *et al.* (6)

except that the number of cycles was increased to 35. Briefly, reactions were incubated at 94^{o}C for 1 min (denaturaion step), 55^{o}C for 2 min (annealing step) and 72^{o}C for 2 min (extension). PCR products were electrophoresed in 1.5% TAE agarose gels and bands were visualized by staining with ethidium bromide solution.

Data analysis

Sensitivities and specificities of the different diagnostic methods were calculated by two by two analysis and significant differences were calculated using the chi-square test.

1-3. Results

Specificity of the diagnostic methods

All piglets used in this study were free from any infection before the start of experiments. Accordingly, all methods showed the absence of *T. evansi* infection in the negative controls for the entire twelve weeks of the study from the raw data (Table 1). A two by two analysis without a gold standard gave 100% specificity for all diagnostic methods, whilst specificity was 61%, 87%, 91% and 96% by LAMP, PCR, MHCT and TBS respectively, when a two by two analysis with MI as gold a gold standard was used (Table 2).

Sensitivity of the diagnostic methods

There was a 65% (43/66) positive detection of *T. evansi* infection by the MI method, whereas LAMP, PCR, MHCT and TBS detected 45% (30/66), 33% (22/66), 38% (25/66) and 24% (16/66), respectively. In a two by two analysis without a gold standard the highest sensitivity was observed for MI, followed by LAMP, PCR, MHCT and TBS respectively (Table 2). There were significant differences ($p<0.05$) in the detection sensitivity between MI and all other methods and between LAMP and

TBS (Table 3). In a two by two analysis with MI as gold standard, the MHCT protocol gave the highest sensitivity followed by LAMP, PCR and TBS (Table 2).

1-4. Discussion

All methods showed high specificity for detection of *T. evansi* in experimentally infected pigs which is mainly due to the fact that these pigs were known to be free from any other infection, in particular from other trypanosome parasites. This high specificity will be different in detection of Surra in the field especially in African countries whereby mixed infections of trypanosomes are common. *T. evansi* infection produces a mild or subclinical form of the disease in pigs (120), hence the high rate of negative detection by all methods used in the current study due of low parasitaemia and resistance of pigs to the infection.

The MI showed highest detection sensitivity as compared to other diagnostic methods used in the current study which was an expected result as it is commonly known that MI is one of the most sensitive methods for detection of *T. evansi* infections (126, 145). However, MI is not an ideal method for detection of infection where treatment is urgently required as it is time consuming as parasitaemia can be seen only after several days of mouse infection (126, 145) and is expensive as mice will always have to be purchased to conduct this test. With exclusion of the gold standard method, higher level of positive detection using the LAMP method compared to PCR and parasitological tests (MHCT and TBS) was observed, which agrees with the results reported by Kuboki *et al.* (84).

Molecular diagnostic methods especially PCR have higher detection sensitivity than microscopic tests (134, 142). In the current study MHCT protocol appeared to be more sensitive than LAMP and PCR when MI was used as a gold standard method, however this is due to the statistical analysis used in this study

whereby in cases where the standard method (MI) had negative detection, the positive detection by other methods was regarded as false positive. Hence in the raw data without statistical analysis in Table 1, LAMP has clearly higher detection sensitivity than MHCT.

Diagnosis of Surra is problematic (111), because commonly used tests have important limitations. The clinical signs are variable and not necessarily specific to *T. evansi* infections. Parasitological examination frequently fails to detect patent infection especially when parasitaemia is scanty in peripheral blood, such as in the chronic form of the disease. Requirements for infrastructure, equipment and technical skill are major obstacles to the utilization of PCR technique, especially for many laboratories in underdeveloped countries where *T. evansi* is endemic (67).

LAMP offers a better alternative diagnostic protocol because it detects surra infections at a much higher level and possesses advantages such as simplicity and the ability to produce extremely large amounts of amplified products that can be easily detected by visual inspection of the turbidity or fluorescence of the reaction mixture (100, 115). The LAMP primers used in this study are limited for use on samples collected in areas exclusively endemic to surra infections only as it has being previously reported (6, 142) that *T. evansi* and the *T. brucei* group are evolutionarily closely related, thereby resulting in indistinguishable genomic DNA, hence, the PFR A primers amplifies DNA of both trypanosome species. With proper primer design, the LAMP method can be exploited as a useful and alternative molecular diagnostic tool for *T. evansi* infections.

1-5. Summary

Six surra negative piglets (6 week-old) were infected with *T. evansi* and two uninfected piglets were used as negative controls. Detection performances of

various diagnostic tests (LAMP, PCR, and parasitological tests) were compared by analysing blood samples collected weekly over a period of 11 weeks. In the raw data without statistical analysis, MI has highest detection rate of 43 positive out of 66, with LAMP been second highest with 30 positive out of 66, which shows superior detection sensitivity of LAMP over PCR and other microscopic methods. With a two by two analysis without a gold standard, all methods were 100% specific. MI had the highest sensitivity of 65%, while LAMP, PCR, MHCT and TBS had sensitivities of 45%, 33%, 38% and 24%, respectively. However, when the analysis was done using MI as a gold standard, the sensitivity of MHCT was the highest at 53% followed by LAMP, PCR and TBS at 49%, 44% and 35%, respectively. All methods gave high specificity above 60%. This study validates LAMP as an alternative method for the diagnosis of surra.

TABLE 1. Detection performance of each diagnostic method

Pig no.	Methods	Weeks postinfection											
		1	2	3	4	5	6	7	8	9	10	11	12
	LAMP	−	−	+	+	−	−	+	+	+	−	+	−
	PCR	−	−	+	+	−	−	+	+	−	−	−	−
A	MHCT**	−	−	+	+	−	−	+	+	−	+	−	−
	TBS***	−	−	+	−	−	−	+	−	−	−	−	−
	MI****	−	−	+	+	−	+	+	+	+	+	−	−
	LAMP	−	−	+	−	+	+	+	+	+	+	−	+
	PCR	−	−	+	+	−	+	+	+	−	+	+	−
B	MHCT	−	−	−	+	−	+	+	+	−	+	+	+
	TBS	−	−	−	+	−	+	+	+	−	+	+	+
	MI	−	−	−	+	−	+	+	+	−	−	+	+
	LAMP	−	−	−	−	−	−	−	+	+	−	−	−
	PCR	−	−	+	−	−	+	−	−	−	−	−	−
C	MHCT	−	−	+	+	−	−	−	−	−	−	−	−
	TBS	−	−	−	−	−	−	−	−	−	−	−	−
	MI	−	+	+	+	−	+	+	+	+	+	−	−
	LAMP	−	+	−	+	−	+	−	+	−	−	−	−
	PCR	−	−	+	+	−	−	−	−	−	−	−	−
D	MHCT	−	−	+	+	−	−	−	−	−	−	−	−
	TBS	−	−	+	+	−	−	−	−	−	−	−	−
	MI	−	−	+	+	+	−	+	−	−	+	+	−
	LAMP	−	−	+	+	+	+	−	+	−	−	+	−
	PCR	−	−	+	+	+	−	−	−	−	−	+	−
E	MHCT	−	−	+	+	+	+	−	−	−	+	+	−
	TBS	−	−	+	−	+	−	−	−	−	+	−	−
	MI	−	−	+	+	+	+	+	+	+	+	+	−
	LAMP	−	+	+	−	−	+	+	−	−	−	−	−
	PCR	−	−	+	−	+	−	−	−	−	−	+	−
F	MHCT	−	−	+	−	+	−	−	−	−	−	+	−
	TBS	−	−	−	+	+	−	−	−	−	−	−	−
	MI	−	−	+	+	+	+	+	+	−	+	−	−
	LAMP	−	−	−	−	−	−	−	−	−	−	−	−
	PCR	−	−	−	−	−	−	−	−	−	−	−	−
G*	MHCT	−	−	−	−	−	−	−	−	−	−	−	−
	TBS	−	−	−	−	−	−	−	−	−	−	−	−
	MI	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	LAMP	−	−	−	−	−	−	−	−	−	−	−	−
	PCR	−	−	−	−	−	−	−	−	−	−	−	−
H*	MHCT	−	−	−	−	−	−	−	−	−	−	−	−
	TBS	−	−	−	−	−	−	−	−	−	−	−	−
	MI	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

*nd- not done

**MHCT-microhematocrit centrifuge test

***TBS-thin blood smear test

****MI-mouse inoculation test

TABLE 2. Sensitivities and specificities of the diagnostic methods

Methods	No gold standard		MI as gold standard	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
MI**	65	100	nd*	nd
LAMP	45	100	49	61
PCR	33	100	44	87
MHCT***	38	100	53	91
TBS****	24	100	35	96

*- not done

**- mouse inoculation

***- microhematocrit centrifuge test

****- thin blood smear

TABLE 3. Chi-square test for the calculation of significant differences

	No gold standard		MI as gold standard	
	Chi-square	P-value	Chi-square	P-value
MI** vs LAMP	5.2	0.0229	nd*	nd
MI vs PCR	13.3	0.0003	nd	nd
MI vs MHCT***	9.8	0.0017	nd	nd
MI vs TBS****	22.3	0.0001	nd	nd
LAMP vs PCR	2.0	0.1541	0.9	0.3309
LAMP vs MHCT	0.8	0.3774	0.5	0.4922
LAMP vs TBS	6.5	0.0105	2.7	0.0988
PCR vs MHCT	0.3	0.5855	0.1	0.7465
PCR vs TBS	1.3	0.2487	0.5	0.4736
MHCT vs TBS	2.9	0.0905	1.1	0.2919

*- not done

**- mouse inoculation

***- microhematocrit centrifuge test

****- thin blood smear

CHAPTER 2

Species-specific Loop-mediated Isothermal Amplification (LAMP) for Diagnosis of Trypanosomosis and its Application to Epidemiological Studies

2-1. Introduction

Trypanosomosis is an economically important disease affecting animals as well as humans. It is caused by protozoan parasites of the genus *Trypanosoma*; the species that infect mammals are divided into two categories, namely, the salivaria and stercoraria. The salivarian trypanosomes include *T. b. brucei, T. b. gambiense* (subtype I and II), *T. b. rhodesiense*, *T. congolense* (subtype Kilifi, Savana and Forest), and *T. vivax* which are transmitted by tsetse flies and cause sleeping sickness in humans and nagana in domestic animals respectively (74, 138), and *T. evansi* (subtypes A and B), which is mechanically transmitted by biting flies such as tabanids cause surra in various animals (6, 21, 142). *Trypanosoma cruzi* (subtypes I, II and ZIII), a stercorarian trypanosome, is transmitted by bugs belonging to Triatominae (41) and causes Chagas disease in humans (150), other stercorarian trypanosomes include *T. rangeli*, *T. lewisi* and *T. theileri* (138).

Parasitological diagnosis of trypanosomes in humans or animals is usually hampered by the low levels of parasitemia (39). Although antibody detection tests are useful for screening purposes, they do not distinguish between past and present infections, and the current reliability of antigen detection tests is limited (27). Recently, the development of molecular biology techniques such as restriction enzyme polymorphisms, DNA hybridization, and PCR, have provided considerable details with regard to trypanosome species and/or sub-species identification, characterization, and diagnosis at various taxonomic levels (32). PCR has evolved

as one of the most specific and sensitive molecular methods for diagnosis of infectious diseases and has been widely applied for detection of pathogenic microorganisms (47, 53, 80). However, in spite of the excellent specificity and sensitivity, these molecular biology techniques are not commonly used in the diagnosis of trypanosomosis in countries lacking resources where the disease is endemic. This is because skilled personnel and expensive automated thermal cyclers for PCR are not easily available in these countries (67).

LAMP is a new DNA amplification method that is highly specific and sensitive under isothermal conditions. This method relies on auto-cycling strand displacement DNA synthesis that is performed by a *Bst* DNA polymerase with high strand displacement activity (115). Unlike *Taq* DNA polymerase, *Bst* DNA polymerase is hardly inhibited by impurities, such as hemoglobin and/or myoglobin contaminated blood and tissue derived DNA samples (3, 4, 9, 77, 84). LAMP has been successfully developed and applied in detection of various pathogens including African trypanosomes (84), *Babesia gibsoni* (72), *Mycobacterium* species (75), edwardsiellosis in fish (131), and herpes simplex virus (43). Recently, a *T. brucei* group specific LAMP was reported by Kuboki *et al.* (84). To further enhance specific trypanosome detection by LAMP, the current study was aimed at developing LAMP for specifically detecting trypanosome species and sub-species including *T. b. gambiense, T. b. rhodesiense, T. congolense, T. cruzi*, and *T. evansi*. With the use of field samples collected from wild and domestic animals from various countries where animal trypanosomosis is endemic, this study has further demonstrated that LAMP can be applied in the epidemiological studies.

2-2. Materials and Methods

Parasites

Trypanosome parasites used in this study for specificity and sensitivity experiments are listed in table 4. Non-trypanosome protozoan parasite species used as controls include *Babesia bovis*, *B. bigemina, B. caballi*, *B. equi* (all USDA strains), *Theileria orientalis* (isolated from infected cattle in Japan), *T. parva* (Muguga strain), *Toxoplasma gondii* (RH strain), and *Neospora caninum* (NC-1 strain).

DNA extraction

DNA of all parasites from *in vitro* cultures and domestic animal blood (cattle and horses) was extracted using the already published method (129). Briefly, extraction buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% sodium dodecyl sulphate) and 100 µg/ml proteinase K were added to the samples and incubated overnight at 55^{o}C. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol, and the pellet was dissolved in 250 µl of double distilled water (DDW). For epidemiological studies of *T. congolense* infection, blood samples were collected from wild animals (buffaloes, impalas, wildebeests, and zebras) and domestic cattle, and were blotted on filter papers (FTA® card, Whatman, United Kingdom) and then left to dry at room temperature until use. DNA template from filter papers was prepared according to the manufacturer's instructions. Briefly, the blood was blotted on the filter paper, then, the paper with blood spot was cut out with a 2 mm hole puncher (2.0 mm Harris Micro Punch; Whatman, United Kingdom). The cut out piece of filter paper was washed 3 times with 200 µl of FTA purification reagent (Whatman, United Kingdom) and twice with 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and thereafter allowed to dry at 50^{o}C for 10 min. This purified cut out piece of filter paper was used as a DNA template. Further,

genomic DNA from 64 blood samples dried on filter papers collected from cattle in Tanzania was extracted using QIAamp DNA blood mini kit according to manufactures instructions (Qiagen Science, Maryland, USA).

LAMP

The LAMP primer sets were designed from 18S rRNA genes for both *T. congolense* and *T. cruzi*, the 5.8S rRNA-internal transcribed spacer 2 (5.8S rRNA-ITS2) gene for *T. b. gambiense*, the SRA gene of *T. b. rhodesiense* and the variant surface glycoprotein (VSG) RoTat1.2 gene for *T. evansi* (Table 5). All the primer sequences were designed using the software program Primer Explorer V2 (Fujitsu, Japan). LAMP reaction was conducted such that each reaction mixture (25 µl total volume) contained 12.5 µl of the reaction buffer (40 mM Tris-HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM of each dNTP), 1 µl (8 units) of *Bst* DNA polymerase, 0.9 µl primer mix with FIP and BIP at 40 pmol each and F3 and B3 at 5 pmol each), 2 µl of template DNA and 8.6 µl of DDW. The reaction mixture was incubated in a heat block (Dry Thermounit DTU 1B, TAITEC Co, Saitama, Japan) at 63^{o}C for 1 h and then at 80^{o}C for 2 min to terminate the reaction. LAMP products were electrophoresed in 1.5% TAE agarose gel and stained with ethidium bromide solution for visualization.

PCR

The F3 and B3 LAMP primers (Table 5) for each trypanosome species with the corresponding target genes were used in the PCR reaction. Briefly, the PCR mixture (total volume 25 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM each of the 4 dNTPs, 5 pmol of each primer, and 5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Ltd., Tokyo, Japan). The reaction mixtures were incubated in a thermocycler (GeneAmp, PCR system 9700, Applied

Biosystem, USA) at 94^{o}C for 10 min as an initial denaturation step, and then subjected to 35 cycles consisting of 45 s at 94^{o}C, 1 min at 57^{o}C, and 1 min at 72^{o}C, followed by a terminal elongation for 7 min at 72^{o}C. PCR products were electrophoresed in 1.5% TAE agarose gel and stained with ethidium bromide solution for visualization.

2-3. Results

The LAMP primer sets for *T. b. gambiense*, *T. congolense*, *T. cruzi*, and *T. evansi* were tested for their species specificity, and they showed high specificity whereby only the target trypanosome DNA was amplified (Figure 5). However there were no LAMP reactions achieved for amplification of *T. b. rhodesiense* using primers designed from the SRA gene (Data not shown). These experiments were repeated several times to ensure reliability and consistency of the results. PCR with the F3 and B3 LAMP primers was also specific for *T. congolense* and *T. evansi* (Figures 5B and D); however, for *T. b. gambiense* and *T. cruzi*, the assay was not specific (Figures 5A and C). As shown in Figure 4, the target region of BIP, F3, and B3 primers among *T. cruzi*, *T. b. brucei* and *T. evansi* are identical. However, nucleic acid sequence of FIP primer regions (FIP-F2 and FIP-F1c) varied among the trypanosome species. In case of *T. b. gambiense* detection, PCR amplification using F3 and B3 primer pair for 5.8S rRNA-ITS2 region has shown cross reactivity among *T. b. gambiense*, *T. b. brucei*, and *T. evansi*. However, LAMP targeting the same gene was highly specific to *T. b. gambiense* because of the sequence diversity within the FIP-F1c and BIP-B2 regions (Figure 4B). Species specificity of LAMP primers for *T. b. gambiense* and *T. evansi* has been further confirmed by using total DNA isolated from various species and strains of trypanosomes as shown in figure 6.

The genomic DNA of *T. b. gambiense, T. congolense, T. cruzi*, and *T. evansi* was quantified from 100 ng up to 1 fg by serial dilution and used to assess the sensitivity of the LAMP primers, and these experiments were also repeated several times to ensure reliability and consistency. The primers showed high sensitivity while detecting trypanosome DNA up to 1 fg for *T. b. gambiense, T. cruzi*, and *T. evansi* (Figures 7A, C and D), whereas *T. congolense* DNA was detected up to 10 fg (Figure 7B). PCR with F3 and B3 primers was as sensitive as LAMP for purified genomic DNA extracted from *in vitro* cultures.

Epidemiological studies were conducted using LAMP for detecting nagana and surra infections in field samples collected from wild and domestic animals in Zambia, Tanzania, Mongolia, and Brazil. As shown in Table 6, all the buffalo, impala, and zebra blood samples collected from Zambia tested negative for *T. congolense* infections, whilst 2 out of 20 and 5 out of 40 wildebeest and cattle samples tested positive, respectively. Sixteen and 1 out 64 cattle samples from Tanzania tested positive for *T. congolense* infections by LAMP and PCR respectively. Further, 1 out of 240 cattle samples collected from Mongolia tested positive for *T. evansi*. In the case of blood samples collected from cattle of both sexes and varying ages (ranging between 8 months to 7 years) in a suburban area of Campo Grande in Brazil, 5 out of 200 samples tested positive for *T. evansi* by LAMP. In addition, all the 22 samples collected from horses (aged between 10 months and 7 years) tested negative for surra (Table 6). Although all these samples had been tested by PCR using F3 and B3 primer pair that was used in LAMP, no positive case was detected by PCR.

2-4. Discussion

Experiments in the current study have demonstrated that a highly specific detection method for different species of trypanosomes could be established by

LAMP primers designed from conserved genes among the trypanosomes, such as 5.8S rRNA-ITS2 (114) and 18S rRNA (69). However, the PCR reaction with F3 and B3 primers was not specific for *T. b. gambiense* because of the high degree of similarity among the nucleic acid sequences of the target region. As shown in Figure 4, LAMP reaction shows a considerable degree of sensitivity to small sequence differences in the regions recognized by the LAMP primers FIP and BIP. Thus, LAMP method has higher species specificity than the PCR even if the target gene used is highly conserved. In fact, it has been reported that sequence specificity of LAMP is higher than that of PCR, and LAMP can be used for the detection of single nucleotide polymorphisms (76).

Trypanosoma brucei subspecies have been reported to be genetically indistinguishable from *T. evansi* species (6, 142), additionally, LAMP primers designed from the PFR A that amplify both these trypanosome species have previously been reported (84). In the current study, a LAMP assay that is specific for detecting *T. evansi* using primers targeting the VSG RoTat 1.2 gene has been developed. The VSG RoTat 1.2 gene is expressed in the *T. evansi* species, and most importantly, not expressed in the *T. brucei* subspecies (20, 21).

Trypanosoma brucei rhodesiense IL 2343 (Table 4) was isolated from a chronic sleeping sickness patient in Ivory Coast and was designated as *T. brucei gambiense*, but was later classified as *T. b. rhodesiense* based on the repetitive DNA data by Hide *et al.* (64) who suggested that this species is indistinguishable from East African *T. b. rhodesiense* and *T. b. brucei* stocks, but Bromidge *et al.* (14), Agbo *et al.* (2), and Gibson (50), referred to it as *T. b. gambiense* type 2. Both LAMP and PCR targeting the *T. brucei gambiense* 5.8S rRNA-ITS2 gene amplifies this trypanosome DNA, suggesting that this trypanosome might be type 2 *T. b.*

gambiense. This interesting finding demonstrates the significant role which LAMP can play in trypanosome identification and taxonomical studies.

Recently, serum resistance associated gene (SRA) was reported to be a *T. b. rhodesiense* sub-species specific gene (29, 49). The *T. b. rhodesiense* DNA can be specifically detected by SRA specific PCR (49, 113, 124). The current study attempted to develop *T. brucei rhodesiense* specific LAMP reaction by targeting the SRA gene with 4 sets of LAMP primers. However, these LAMP primer sets could not amplify the SRA gene (data not shown). One possible reason might be that the annealing step with primer sets designed from this gene is too slow hence no reaction could be produced within 60 minutes.

LAMP is highly sensitive and can detect DNA when it is present in as few as 6 copies in a reaction mixture (115). In this study, LAMP was highly sensitive in detecting *T. b. gambiense, T. cruzi*, and *T. evansi* up to 1 fg and 10 fg for *T. congolense*. A volume of 10 pg of DNA represents approximately 100 trypanosomes (113), implying that 10 fg and 1 fg are equivalent to 0.1 and 0.01 trypanosomes respectively. The sensitivity threshold of trypanosome detection by PCR generally ranges from 1 to 20 parasites/ml of blood (0.001–0.02 parasites/µl) (32). In the current study, the results obtained by PCR using F3 and B3 primers prepared for LAMP with same target genes for the respective trypanosomes was equally sensitive when compared to LAMP, which therefore means LAMP can also detect as little as 1 or less trypanosome parasite.

This study has further demonstrated that LAMP can be applied in epidemiological studies of trypanosomes (*T. congolense* and *T. evansi*) for both domestic and wild animals. These results confirm the existence of nagana in cattle in Zambia and Tanzania as reported previously (80, 95, 108) and earlier reports that wild animals might be harboring trypanosomes (56). However, wild animals are

resistant to trypanosome infections and can eliminate the parasite from their blood system (57, 128), hence the low prevalence in the wildebeests and negative detection in buffaloes, impalas, and zebras.

The current study has also used LAMP to confirm the existence of surra in Brazil and Mongolia as previously reported (22, 23, 63). It is important to highlight the fact that epidemiological results in this study do not reflect the overall status of animal trypanosomosis in the respective countries since only a few samples were collected from small regions of these countries. Except for cattle samples from Tanzania, all samples used in the current epidemiological study were PCR negative. This confirms previous report that PCR has poor sensitivity for trypanosome DNA templates from filter papers (84). Specific LAMP for *T. b. gambiense* and *T. cruzi* will hopefully be applied in future epidemiological studies since the current study was limited by the lack of field samples. LAMP is a rapid and simple technique since it can be carried out in 1 h and requires only a simple heating device for incubation; therefore, it has great potential of being used for diagnosis of trypanosomosis in laboratory research and for epidemiological studies, especially, in countries that lack sufficient resources.

2-5. Summary

In this study, a LAMP technique was developed for the specific detection of both animal and human trypanosomosis using primer sets that are designed from 5.8S rRNA- ITS2 gene for *T. b. gambiense*, 18S rRNA for both *T. congolense* and *T. cruzi,* and VSG RoTat 1.2 for *T. evansi*. These LAMP primer sets are highly sensitive and are capable of detecting up to 1 fg trypanosomal DNA, which is equivalent to ~ 0.01 trypanosomes. Furthermore, the current study has demonstrated that LAMP can be applied in epidemiological studies for detecting nagana and surra by using

DNA extracted from blood. In the epidemiological studies of the domestic and wild animals using samples collected from Zambia, *T. congolense* infections were detected by LAMP in 12.5%, and 10% of the cattle and wildebeest samples, respectively, and from 25% of the cattle samples from Tanzania. Of the blood samples collected from the cattle in Brazil and Mongolia, 2.5% and 0.4% respectively, tested positive for *T. evansi* by LAMP. Above results further demonstrates that LAMP can be a useful diagnostic tool for application in large scale epidemiological studies.

TABLE 4. Trypanosome strains used in this study

Species	Strains	Types	Location	Year
T. brucei brucei	GUTat3.1	ND*	Uganda	1966
T. b. gambiense	IL1922	II	Ivory coast	1952
T. b. gambiense	IL3248	I	Nigeria	1969
T. b. gambiense	IL3250	I	Nigeria	1969
T. b. gambiense	IL3253	I	South Sudan	1982
T. b. gambiense	IL3254	I	South Sudan	1982
T. b. gambiense	IL3301	I	Nigeria	1969
T. b. gambiense	IL3707	II	Nigeria	1968
T. b. gambiense	Welcome	II	ND	ND
T. b. gambiense	IL2343	II	Ivory coast	1978
T. b. rhodesiense	IL1501	ND	Kenya	1980
T. congolense	IL3000	Savana	Kenya/Tanzania border	1966
T. congolense	IL1180	Savana	Serengeti/Tanzania	1971
T. cruzi	Tulahuen	II	Chile	ND
T. evansi	IL1934	Non A or B**	South America	1971
T. evansi	IL1695	A1	Kenya	1978
T. evansi	IL3354	A2	Mali	1988
T. evansi	IL3382	A1	Mali	1988
T. evansi	IL3960	A1	Kenya	1980
T. evansi	IL3962	A1	Sudan	1976
T. evansi	Tansui	Non A or B	Taiwan	ND
T. evansi	Batong tani	ND	Thailand	1996
T. evansi	Khonkaen	ND	Thailand	2000

*No data

**Akinetoplast *T. evansi* species that is neither type A nor B (13, 73).

TABLE 5. Trypanosome target genes and specific LAMP primer sets

Primers	Specificity	Target genes	(Accession #)	LAMP primer sequences
TBG1	*T. b. gambiense*	5.8S-ITS2	(AF306777)	FIP:5'-GCGTTGAACAACACAAAATAGGTGATGCCACATTTCTCAGTGT-3' BIP:5'-CCACCTCTTCTCCTCGTGTGGAAGAAAGAGATGAAAGATATCGTA-3' F3 :5'-AAGCTCTCTCGAGCCATC-3' B3 :5'-TGACATACACAATATGTGCGA-3'
TBR1	*T.b. rhodesiense*	SRA	(AJ345057)	FIP:5'-GGACTGCGTTGAGTACGCATCTGCACAGACCACAGCAACAT-3' BIP:5'-ACGCTCTTACAAGTCTTGCGCCACTTCTGAGATGTGCCCACT-3' F3 :5'-GCGGAAGCAAGAATGACCTT-3' B3 :5'-GCCTGCTGCAGAATGTCCT-3'
CON2	*T. congolense*	18S rRNA	(U22315)	FIP:5'-GCGCATGCGTCGGTGTTATTTTCGCGTGTGTGTTCATGTCA-3' BIP:5'-ACTCTCCCCCCAAAATGGTTGTCCAAGCACGCAAATTCACAT-3' F3 :5'-TGTGTGTTTGTCGTGGAAGC-3' B3 :5'-ATTCGTGACCGCGTCAAA-3'
CRU3	*T. cruzi*	18S rRNA	(AF301912)	FIP:5'-GGTAAAAAACCCGGCTTTCGCAACCGGCAGTAACACTCAGA-3' BIP:5'-CGATGGCCGTGTAGTGGACTGTTTCTCAGGCTCCCTCTCC-3' F3 :5'-GGACGTCCAGCGAATGAATG-3' B3 :5'-CCTCCGTAGAAGTGGTAGCT-3'
TEV1	*T. evansi*	RoTat 1.2	(AF317914)	FIP:5'-TTCGATCGCTGCGAAGTGCGTCTGGAAGCCATTGTGCG-3' BIP:5'-AAGCTCTTGATTTACGCGGCGGGCTGCTAACCCTCTTGCTG-3' F3 :5'-GCCGCCAATGTAGCTCTT-3' B3 :5'-CCGCTGCTCGTATGTGTC-3'

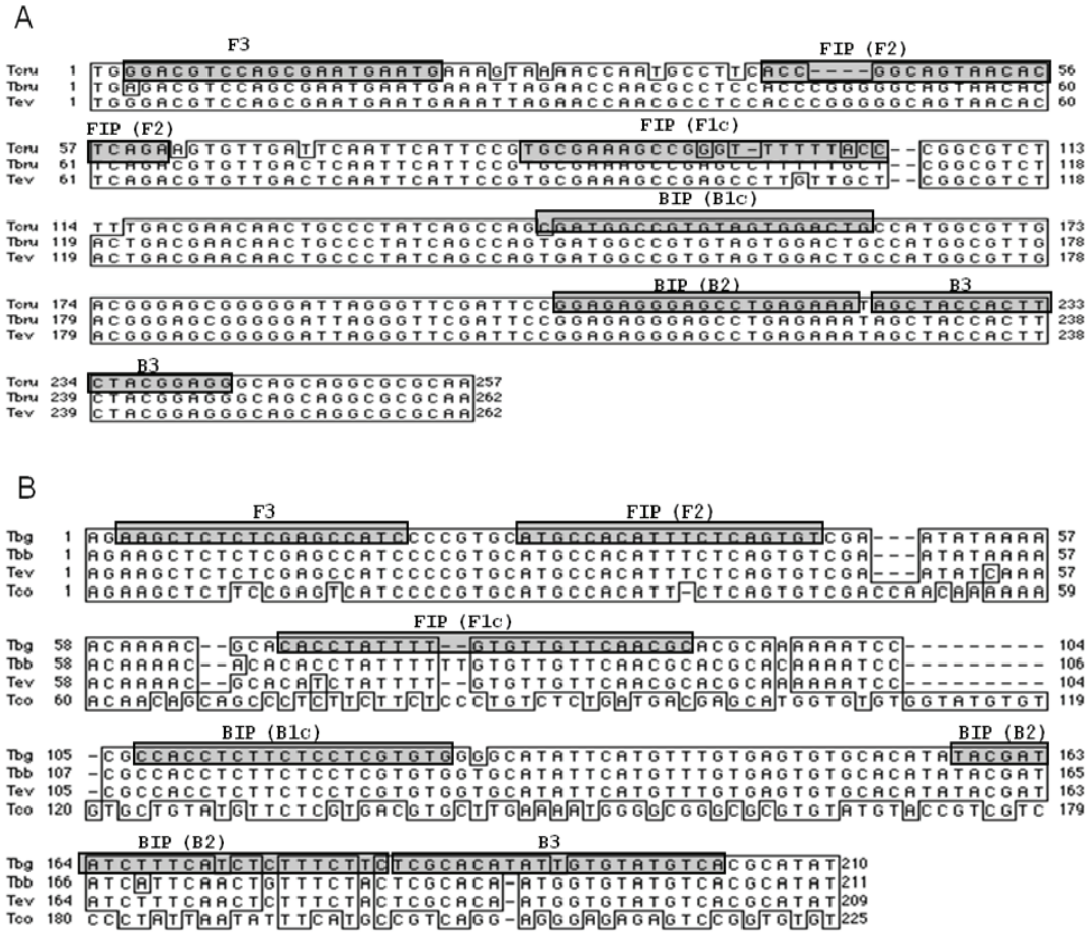

FIG. 4. The nucleotide sequence alignment of target regions of the 18S rRNA (A) and (B) 5.8S-ITS2 genes. The grey boxes indicates target regions of the LAMP primers, F3 (forward outer) and B3 (backward outer); the two target regions for the forward inner primer, FIP (F2 and F1c) and the backward inner, BIP (B2 and B1c). Accession numbers of genes shown in **(A)** and **(B)** are as follows: *T. brucei brucei* 18S rRNA (M12676), *T. brucei brucei* 5.8S-ITS2 (AF306771), *T. brucei gambiense* 5.8S-ITS2 (AF306777), *T. congolense* 5.8S-ITS2 rRNA (U22315), *T. cruzi* 18S rRNA (AF301912), *T. evansi* 18S-5.8S-ITS2 rRNA (D89527).

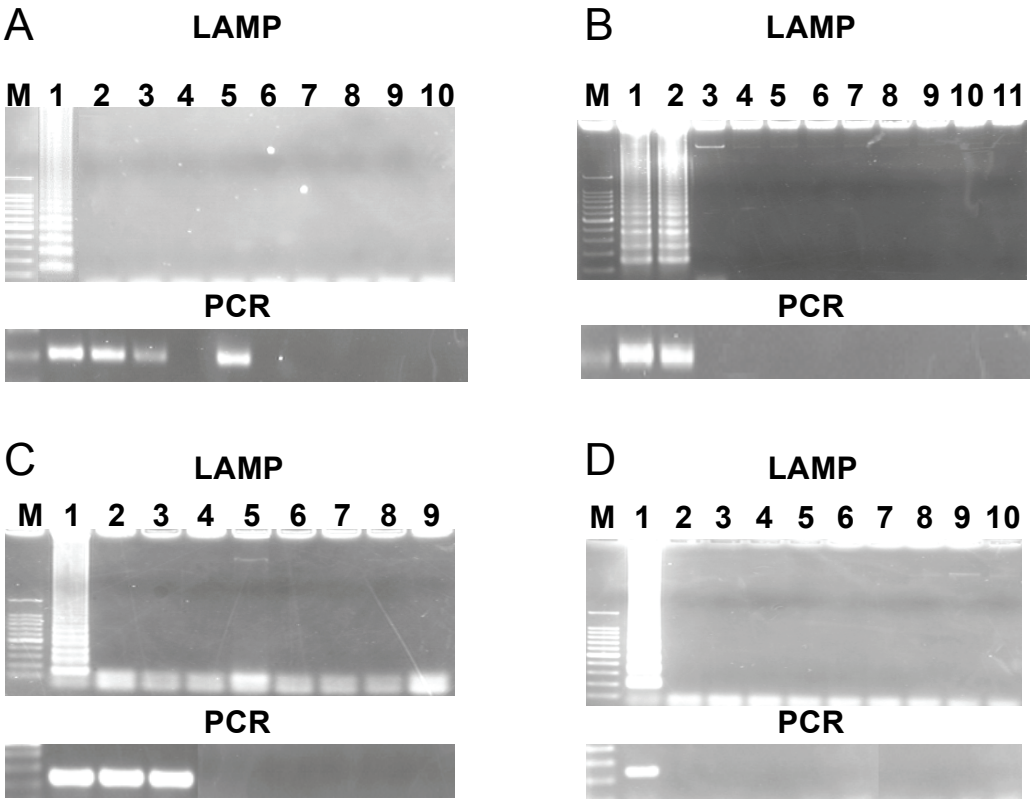

FIG. 5. Comparisons of LAMP and PCR specificity for trypanosome detection.

(A) *T. b. gambiense* detection with TBG1 primers targeting 5.8S-ITS2 gene. Lanes M: 100 bp DNA maker; 1: *T. b. gambiense* (IL3253); 2: *T. b. brucei* (GUTat3.1); 3: *T. b. rhodesiense* (IL1501); 4: *T. congolense* (IL1180); 5: *T. evansi* (Tansui); 6: *T. cruzi* (Tulahuen); 7: *Theileria parva* (Muguga stock); 8: *Babesia bovis* (USDA); 9: *Toxoplasma gondii* (RH); and 10: *Neospora caninum* (NC-1). **(B)** *T. congolense* detection with CON2 primers targeting 18S rRNA gene. Lanes: M: 100 bp DNA maker; 1: *T. congolense* (IL1180); 2: *T. congolense* (IL3000); 3: *T. b. brucei* (GUTat3.1); 4: *T. b. gambiense* (IL3253); 5: *T. evansi* (Tansui); 6: *T. cruzi* (Tulahuen); 7: *T. b. rhodesiense* (IL1501); 8: *T. parva* (Muguga stock); 9: *B. bovis* (USDA); 10: *B. bigemina* (USDA); and 11: *N. caninum* (NC-1). **(C)** *T. cruzi* detection with CRU3 primers targeting 18S rRNA gene. Lanes: M: 100 bp DNA maker; 1: *T. cruzi* (Tulahuen); 2: *T. b. brucei* (GUTat3.1); 3: *T. evansi* (Tansui); 4: *T. b. rhodesiense* (IL1501); 5: *T. congolense* (IL1180); 6: *T. parva* (Muguga stock); 7: *B. bovis* (USDA); 8: *B. equi* (USDA); 9: *T. gondii* (RH). **(D)** *T. evansi* detection with TEV1 primers targeting RoTat1.2 gene. Lanes: M: 100 bp DNA maker; 1: *T. evansi* (Tansui); 2: *T. b. brucei* (GUTat3.1); 3: *T. congolense* (IL1180); 4: *T. rhodesiense* (IL1501); 5: *T. parva* (Muguga stock); 6: *T. orientalis*; 7: *B. bovis* (USDA); 8: *B. bigemina* (USDA); 9: *B. equi* (USDA); and 10: *B. caballi* (USDA).

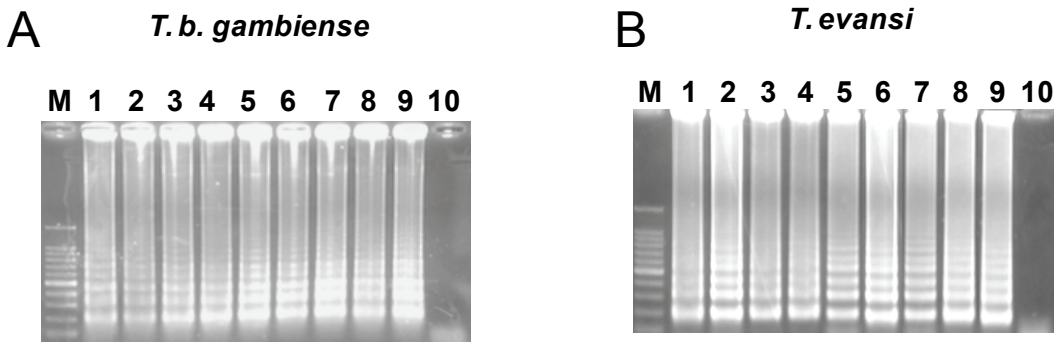

FIG. 6. Specificity of LAMP primers for amplification of DNA from different isolates. (A) *T. b. gambiense* - Lanes M: 100bp DNA maker; 1: IL1922; 2: IL3248; 3: IL3250; 4: IL3253; 5: IL3254; 6: IL3301; 7: IL3707; 8: Welcome; 9: IL2343; 10: Negative control, and (B) *T. evansi* - Lanes M: 100bp DNA maker; 1: IL1695; 2: IL1934; 3: IL3354; 4: IL3382; 5: IL3960; 6: IL3962; 7: Tansui; 8: Batong tani; 9: Khonkaen, and 10: Negative control.

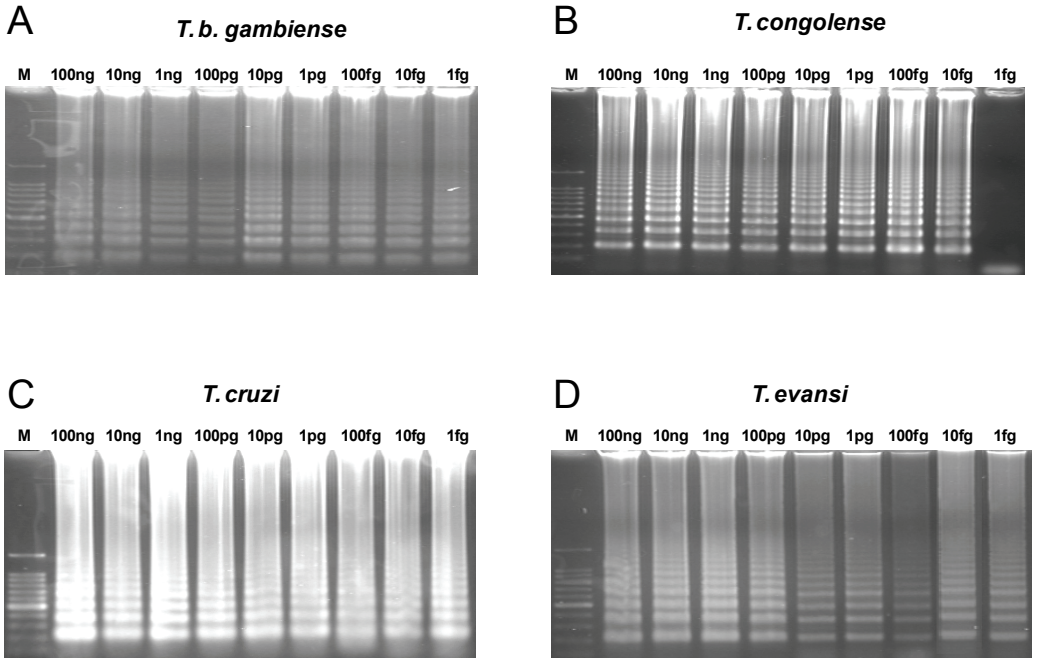

FIG. 7. Sensitivity of LAMP for detection of *T. b. gambiense* (A), *T. congolense* (B), *T. cruzi* (C) and *T. evansi* (D), respectively. Total DNA of the respective trypanosomes was serially diluted from 100 ng to 1 fg.

TABLE 6. Application of LAMP in epidemiological studies of animal trypanosomosis

Trypanosomes	Countries	Animal sp.	Total #	# positive	% positive
T. congolense	Tanzania*	Cattle	64	16	25
T. congolense	Zambia	Cattle[a]	40	5	12.5
T. congolense	Zambia	Wildebeest[a]	20	2	10.0
T. congolense	Zambia	Zebra[a]	8	0	0
T. congolense	Zambia	Impala[a]	8	0	0
T. congolense	Zambia	Buffalo[a]	8	0	0
T. evansi	Brazil	Cattle[b]	200	5	2.5
T. evansi	Brazil	Horse[b]	22	0	0
T. evansi	Mongolia	Cattle[b]	240	1	0.4

[a]Filter paper DNA template.

[b]DNA extracted from total blood by standard method.

*One out of 64 (1.5%) from Tanzania samples was PCR positive.

CHAPTER 3

Stability of Loop-mediated Isothermal Amplification (LAMP) Reagents Stored at Different Temperatures, its Amplification Efficiency on Different Trypanosome DNA Templates and its Tolerance to Inhibitory Substances

3-1. Introduction

LAMP is a method that amplifies DNA with high specificity, sensitivity and rapidity under isothermal conditions. LAMP employs a set of two specially designed inner (FIP and BIP) and two outer (F3 and B3) primers (115). The main advantage of LAMP over PCR is that it does not require complicated thermocyclers and that amplification can be conducted within 1 hour in a laboratory heat block or water bath. LAMP results can be visualized by agarose gel electrophoresis, addition of fluorescent dyes that can be observed under UV light (37, 75, 137) and by turbidity that can be seen by the naked eye (19, 100, 132). *Bst* DNA polymerase large fragment with optimal temperatures ranging from 60 – 65^{o}C is utilized in LAMP reaction, and is inactivated at 80^{o}C (115). With the above-mentioned advantages of LAMP, most of the literature on development of this method for diagnosis of various infections concludes that this method is a strong candidate for application of molecular diagnosis particularly in the field where cost and environmental constraints prohibit the use of PCR (71, 72, 84, 115). Moreover, *Bst* DNA polymerase appears not to be affected by known inhibitors of PCR found in blood components (4, 55, 84).

There is therefore need to conduct research that can determine the practicability of LAMP method being applied for diagnosis in the field, such as conducting LAMP experiments at field ambient temperatures, and determining simple DNA template preparations which could also be easy to apply in the field. This study, therefore evaluates the stability of LAMP reagents when stored at 25^{o}C, 37^{o}C and − 20^{o}C. Furthermore, the current study also assesses detection performance of LAMP on different DNA template preparations including, crude and hemolysed blood, filter papers and genomic DNA extracted by standard phenol-chloroform method, and lastly this study seeked to determine the possible *Bst* DNA polymerase inhibitors.

3-2. Materials and Methods

Experimental set-up

The first set of experimental reactions were conducted over a period of 15 days on genomic DNA (gDNA) kept at − 30^{o}C (general storage temperature for DNA) of *T. b. brucei* (GUTat 3.1) and *T. congolense* (IL3000) extracted from *in vitro* cultures whereby LAMP reagents (LAMP buffer with dNTPs, 4 LAMP primers, DDW and *Bst* DNA polymerase) were stored in 25^{o}C and 37^{o}C dry incubators. The *T. b. brucei* gDNA was used as negative control for reactions with *T. congolense* LAMP primer set, alternatively, *T. congolense* gDNA was used as negative control for reactions with *T. b. brucei* LAMP primer set.

The second set of experimental reactions was conducted over a period of 30 days, whereby, 5 mice (6-week old female BALB/c) were infected with *T. congolense* (IL1180) and another 5 were kept as controls. Blood was

collected at 3-day intervals and LAMP was conducted with reagents stored at above-mentioned temperatures for a period of 30 days on different DNA template preparations described below. Furthermore, LAMP was also conducted on same DNA preparations from mice with reagents stored at −20°C as control reactions. Parasitaemia was also monitored during the entire experiment by wet smears.

In the third set of experiments, hemoglobin from bovine blood (Sigma-aldrich, Germany), heparin Na (Wako Pure Chemical Industries, Japan) and EDTA (Dojindo, Japan) all available in powder form were prepared at concentrations of mg/ml serially diluted from 1 mg/ml down to 1 µg/ml. The human IgG and IgM (Oriental Yeast Co., LTD, Japan), readily available at 10.1 and 10.2 mg/ml respectively, were also serially diluted down to 1 µg/ml. Then, 1 µl of each dilution of the above mentioned substances was added to LAMP and PCR reactions for amplification of *T. b. brucei* DNA using the PFR A primers (Table 7), in order to determine their possible inhibitory effects for *Bst* DNA polymerase used in LAMP.

Genomic DNA (gDNA)

The gDNA was extracted with a standard phenol-chloroform-isoamyl alcohol (PCI) method (129) from *in vitro* cultured *T. b. brucei* (GUTat 3.1) and *T. congolense* (IL3000), and from blood of *T. congolense* (IL1180) infected mice. Briefly, extraction buffer containing 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% sodium dodecyl sulphate and 100 µg/ml proteinase K was added to the samples and incubated overnight at 55°C. DNA was extracted with PCI (25:24:1) and precipitated with isopropanol and the pellet was dissolved in 250 µl of DDW.

Crude and hemolysed blood as DNA templates

One microliter of crude blood collected from each *T. congolense* (IL1180) infected mouse was added into the reaction tube-containing LAMP reagents ready prepared for amplification. For hemolysed blood, 10 µl of blood collected from a *T. congolense* (IL1180) infected mouse was mixed with 1 ml DDW and spun at 500 x *g* for 10 minutes, thereafter supernatant was discarded and the precipitate was suspended in 10 µl Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). Two microlitres was added as DNA template for LAMP reactions.

Filter paper DNA

Blood was collected from *T. congolense* (IL1180) infected mice, blotted on filter papers (FTA card®; Whatman, U.K.) and left to dry at room temperature for 1 hour. Filter papers were purified according to manufacturer's instructions. Briefly, 2 mm disc was cut out with a hole puncher (2.0 mm Harris Micro Punch; Whatman, U. K.) from blood blotted filter paper and washed 3 times with 200 µl of FTA purification reagent (Whatman, U. K.), and twice with 200 µl of TE buffer. Thereafter, they were left to dry at 50^{o}C for 10 min in a heat block (Dry Thermounit DTU 1B, TAITEC Co, Saitama, Japan) and then used as DNA templates.

LAMP

Table 7 shows LAMP primer sets used in this study for *T. b. brucei* and *T. congolense* as designed by Kuboki *et al.* (84). LAMP reactions were conducted as described by Notomi *et al.* (115). Briefly, the reaction mixture (total volume 25 µl) contained 12.5 µl of the reaction buffer (40 mM Tris-HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6

M Betaine, 2.8 mM of each dNTP), 1 µl (8 units) of *Bst* DNA polymerase, 0.9 µl primer mix (FIP and BIP at 40 pmol each and F3 and B3 at 5 pmol each), 2 µl of template DNA and 8.6 µl DDW. However, in reactions where 1 µl for crude blood or 2 µl of hemolysed blood was used as template DNA, the DDW volume was increased in order to adjust the total reaction volume. Reaction mixture was incubated in a heat block at 63°C for 1 hour and then at 80°C for 2 min to terminate the reaction. LAMP products were electrophoresed in 1.5% TAE agarose gel and stained with ethidium bromide solution for visualization.

PCR

The F3 and B3 LAMP primers were used in PCR for amplification of the PFR A gene of *T. brucei* and ribosomal P0 of *T. congolense* (Table 7). Briefly, the PCR mixture (total volume 25 µl) contained a reaction buffer with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM each of the 4 dNTPs, 5 pmol of each primer, and 5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Ltd., Tokyo, Japan). The reaction mixtures were incubated in a thermocycler (GeneAmp, PCR system 9700, Applied Biosystem, USA) at 94^{o}C for 10 min as an initial denaturation step, and then subjected to 35 cycles consisting of 45 s at 94^{o}C, 1 min at 57^{o}C, and 1 min at 72^{o}C, followed by a terminal elongation for 7 min at 72^{o}C. PCR products were electrophoresed and visualized as described above for LAMP products.

3-3. Results

LAMP reactions with reagents stored at different temperatures

The *T. b. brucei* (GUTat 3.1) (Figure 8A and B) and *T. congolense* (IL 3000) gDNA (Data not shown) extracted from *in vitro* cultures and stored at −

30°C were amplified by LAMP from day 1 to day 15 (time when experiment was terminated) with reagents stored at 25°C and 37°C for a period of 15 days. The typical multiple band pattern of positive LAMP reactions was seen in agarose gel electrophoresis (Figure 8A and B), whilst there were no multiple band pattern formation in negative control reactions. Same results were obtained for amplification of *T. congolense* (IL1180) gDNA (Data not shown). As shown in Figure 8B, irregular band patterns (Day 1and 9) of positive LAMP reactions were occasionally observed when using the reagents stored at 37°C.

LAMP detection performance on different DNA template preparations

Parasitaemia in *T. congolense* (IL1180) experimentally infected mice was monitored during the entire experiment and was low during the first 8 days of infection and highest between day 12 and 24 (Figure 9), however, LAMP detection sensitivity was not affected by fluctuations in parasitaemia. The average LAMP detection performance with reagents stored at 37°C on different types of DNA templates was 70%, 47.5%, 77.5% and 100% for crude blood, hemolysed blood, filter paper and gDNA extracted by standard PCI method respectively (Figure 10). For reagents stored at 25°C, the average LAMP detection performance on different types of DNA templates was 72.5%, 57.5%, 75.0% and 100% for crude blood, hemolysed blood, filter paper and gDNA extracted by standard method respectively (Figure 10). Furthermore, the average LAMP detection performance with reagents kept at −20°C for DNA templates from crude blood, hemolysed blood, filter paper and gDNA was 73%, 47.5%, 80%, and 100% respectively (Figure 10).

DNA polymerase inhibitors

PCR is one the most widely applied molecular method for diagnosis of various pathogens, however it is well known that detection sensitivity of PCR is significantly influenced by a variety of inhibitory substances contained in DNA templates. Hence, this study assessed the inhibitory effect of some of these substances on LAMP method. As a result, PCR reactions in this study were inhibited by hemoglobin, IgG and IgM at concentrations shown in table 8, whilst none of these substances used showed inhibitory effects for LAMP at concentrations used in this study (Table 8).

3-4. Discussion

In the current study LAMP was performed with reagents stored at 25^{o}C and 37^{o}C, which are possible ambient temperatures in tropical and sub-tropical countries where protozoan diseases such as trypanosomosis are endemic. There were no significant differences in detection performance of LAMP with reagents stored at the above mentioned temperatures as compared to those stored at −20^{o}C which was used as standard in this study. With DNA polymerase being the critical reagent in gene amplification reaction, it seems that *Bst* DNA polymerase can amplify DNA even when reagents are not kept at freezing temperatures. However occasional irregular band patterns observed in Figure 8B suggests that LAMP reagents and/or reaction pattern might be affected by higher storage temperature. Since the LAMP method amplifies its target genes with very complicated amplification pattern, LAMP amplification pattern is not always identical even in the target specific amplification (84, 115).

In order to simplify procedures for DNA template preparation in LAMP, this study has further used DNA templates prepared by different methods. As a result, LAMP method detection efficiency was best (100%) when using gDNA template extracted by standard PCI method despite storage of reagents at different temperatures. This is probably due to the fact that gDNA is highly purified. LAMP detection performance was slightly lower ranging from 75 to 80% with DNA templates on filter papers. This is in contrast to previous report in which LAMP detected DNA of *T. b. gambiense* infected mice on all days post infection (84). It has been observed that occasionaly filter paper DNA templates would be not completely purified, as some would still have red pigment even after purification. In addition, the chance of positive detection from filter paper preparations significantly relies on the parasite distribution on the paper, because only 2 mm diameter area on the blood spot is utilized as a template DNA source. Therefore the reaction has to be repeated several times to confirm true negative samples when the filter paper preparation is utilized as a template DNA source. The lowest detection performance of LAMP was from crude and hemolysed blood. LAMP in all the three conditions whereby reagents were kept at 37^{o}C, 25^{o}C and –20^{o}C detected gDNA from infected mice for the whole 30 days of the study despite fluctuations in parasitaemia.

The critical factor for accurate and reliable amplification seems to be the DNA template. Table 9 shows a summary of advantages and disadvantages of various DNA template preparations used in the current study. Crude and hemolysed blood DNA templates were easy to prepare, but they offer unspecified DNA which affects the specificity of gene amplification

method. However, Poon *et al.* (123) has reported that LAMP detected *Plasmodium* DNA from heat-treated blood, which is a promising simple DNA template preparation method.

Highly purified DNA template is desirable for precise gene amplification. However, the current standard procedures for gDNA extraction and purification are tedious and labour-intensive (5). Filter paper DNA template preparation is relatively easy and rapid. However, it offers partially purified DNA template. In reactions with gDNA, positive LAMP results can be seen by turbidity in the reaction tube (100, 101, 115). However it is not possible when using crude and hemolysed blood as DNA templates, whilst it is possible but difficult to observe turbidity when using filter paper DNA templates.

Hemoglobin, heparin, EDTA, and IgG have been previously reported as inhibitors for *Taq* DNA polymerase used in PCR (4, 5, 112), whilst *Bst* DNA polymerase used in LAMP has been presumed to be unaffected by these inhibitors based on results obtained on blood blotted filter papers (84). The current study has confirmed that LAMP has superior tolerance to these inhibitors as compared to PCR, whereby PCR was inhibited by minimal concentrations of hemoglobin, IgG and IgM, whilst none inhibited LAMP. Additionally, Kaneko *et al.* (78) have reported that LAMP has superior tolerance than PCR to substances used in *in vitro* cultures including saline, phosphate buffered saline (PBS), Eagle's minimum essential medium (MEM), aqueous and vitreous humor, and that it is only inhibited by more than 1% of each serum, plasma and urine.

In conclusion, this study revealed that LAMP reagents are relatively stable when stored at 25^{o}C and 37^{o}C that are warmer temperatures than

recommended storage temperatures by the manufactures. This further highlights the possibility of application of LAMP in field conditions as previously reported (55, 71, 72, 78, 115). However, further improvements on rapid and simpler DNA extraction and purification methods are required to enhance the practicability of applying LAMP method for diagnosis in the field.

3-5. Summary

This study evaluated the stability of LAMP reagents when stored at 25^{o}C and 37^{o}C, assessed its detection efficiency on different DNA template preparations and determined its tolerance to inhibitory substances. Accordingly, LAMP with reagents stored at 25^{o}C and 37^{o}C amplified DNA of *in vitro* cultured *T. b. brucei* (GuTat. 3.1) and *T. congolense* (IL3000) from day 1 to day 15 of reagent storage. Further, LAMP with reagents stored at above-mentioned temperatures amplified DNA from *T. congolense* (IL1180) infected mice from day 1 to day 30 post-infection which was also a period of reagent storage. Detection sensitivity of LAMP was not affected by fluctuations in parasitaemia. There were no significant differences in detection sensitivity of LAMP between reagents stored at 25^{o}C, 37^{o}C and －20^{o}C (Recommended storage temperature). The average detection efficiency for LAMP was best achieved with gDNA template extracted with standard PCI method, followed by filter paper, crude and hemolysed blood. The 25^{o}C and 37^{o}C temperatures are possible ambient temperatures in the field, clinics and hospitals, and this study has shown that LAMP reagents stored at those temperature conditions does not affect detection efficiency of the method. LAMP was also conducted in the presence of known PCR inhibitors including EDTA, hemoglobin, heparin,

IgG and IgM and none inhibited LAMP reactions, whilst PCR was inhibited by hemoglobin, IgG and IgM with variable concentrations ranging from 1 mg/ml to 0.01 mg/ml. These results demonstrated the superiority of LAMP as compared to PCR on tolerance to above-mentioned inhibitors.

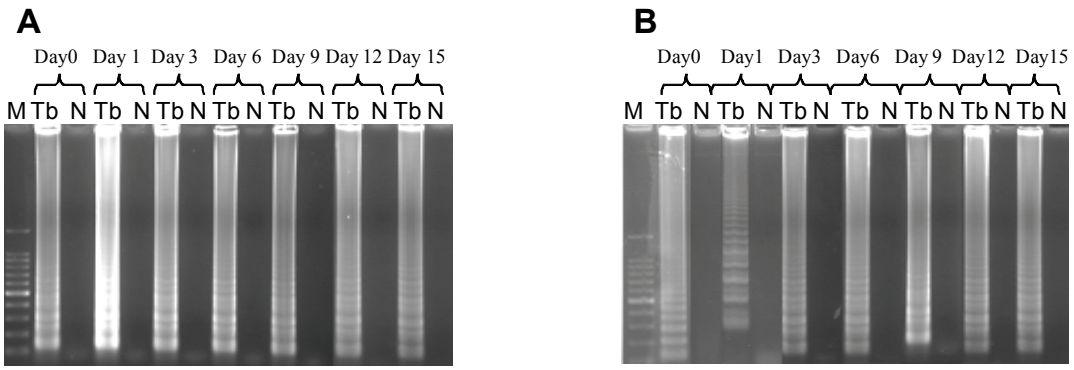

FIG. 8. Amplification of *T. b. brucei* DNA from *in vitro* culture by LAMP method with reagents stored at (A) 25°C and (B) 37°C over a period of 15 days. Day0 reactions were conducted with LAMP reagents from –20°C (recommended storage temperature), thereafter reagents were then stored at 25°C and 37°C. M: 100 bp marker, Tb: *T. b. brucei* DNA, and N: negative control.

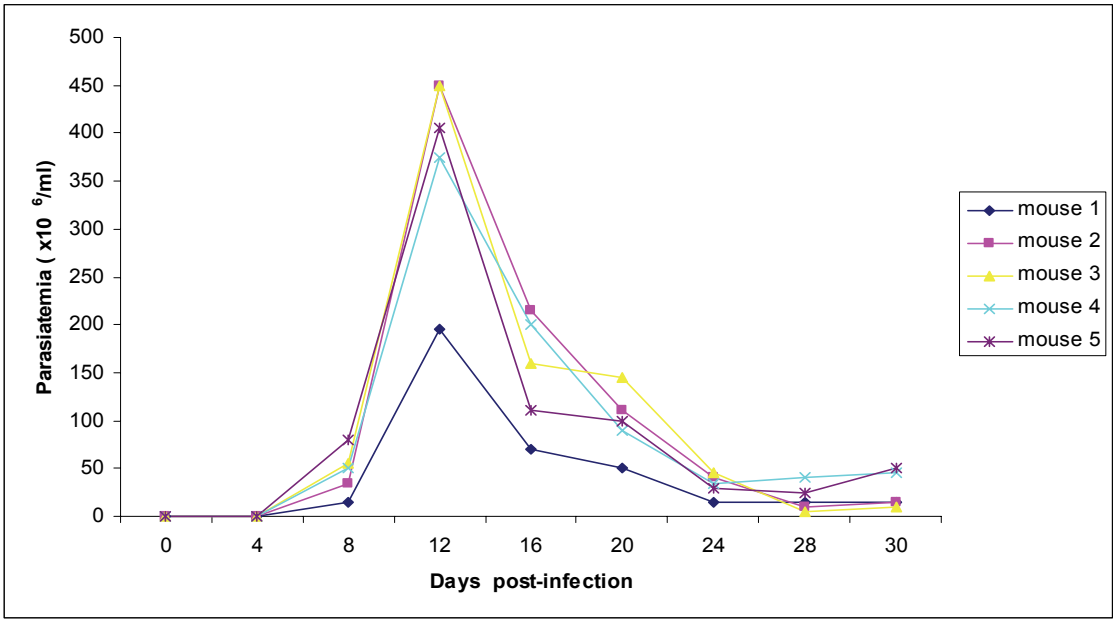

FIG. 9. Parasitaemia in five mice infected with *T. congolense* (IL1180).

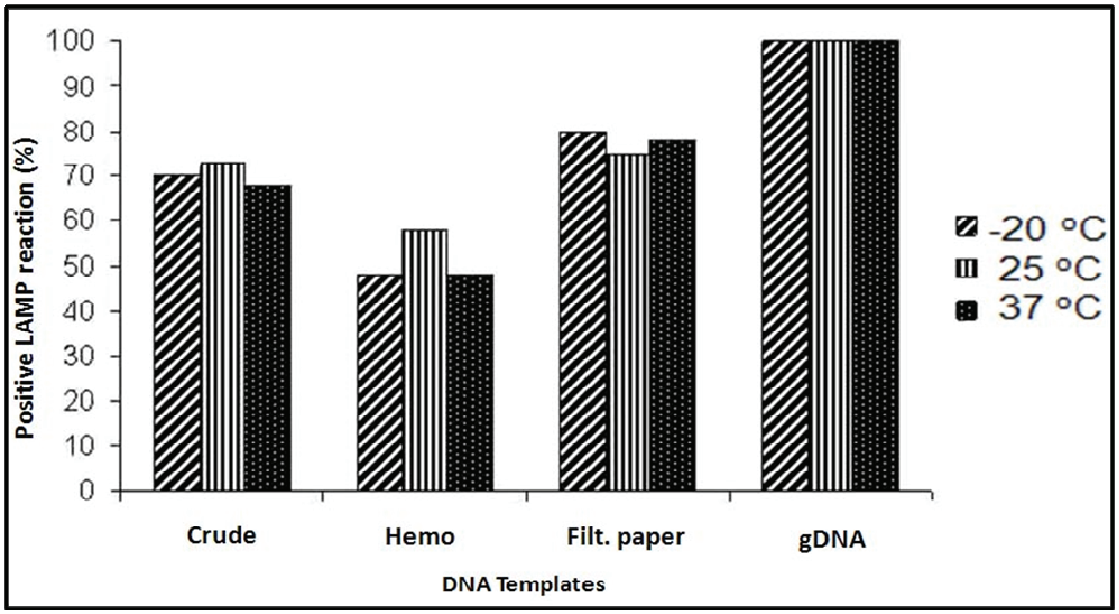

FIG. 10. Detection performance of LAMP method with reagents stored at -20°C, 25°C and 37°C on different DNA template preparations. Percentages for each DNA preparation were calculated from averages of LAMP positive reactions for 5 infected mice from day 4 to 30. Crude: crude blood; Hemo: hemolysed blood; Filt. paper: filter paper and gDNA: genomic DNA.

TABLE 7. LAMP primers used in this study

Parasites	Target genes	Accession #	Nucleotide sequences
T. b. brucei	PFR A	X14819	FIP: 5'TCAGAAGCGTCGAGCTGGGATTTTATCGACAATGCCATCGCC3'
			BIP: 5'CGCAAGTTCCTGTGGCTGCATTTTTTCCCAAGAAGAGCCGTCT3'
			F3 : 5'TCACAACAAGACTCGCACG3'
			B3 : 5'GGGCTTTGATCTGCTCCTC3'
T. congolense	P0	AB056702	FIP: 5'ATCCGTCGCCTTGCTGTCCTTTTTATGGGGAAGAAGACGCTTCA-3'
			BIP: 5'CAAGCAGCTGCTGTGCGGTATTTTTGATCTCCGTAACGTCCTCG-3'
			F3 : 5'CGTGGTAAGGGTGAATTGGT3'
			B3 : 5'GTGTCCGTCCAACACCTTC3'

TABLE 8. Effects of DNA polymerase inhibitors on LAMP and PCR

	LAMP					PCR				
	mg/ml					mg/ml				
Inhibitors	0	1.0	0.1	0.01	0.001	0	1.0	0.1	0.01	0.001
Heparin	+	+	+	+	+	+	+	+	+	+
Hemoglobin	+	+	+	+	+	+	-	-	-	+
EDTA	+	+	+	+	+	+	+	+	+	+
IgG	+	+	+	+	+	+	-	-	+	+
IgM	+	+	+	+	+	+	-	+	+	+

+: No inhibition, positive reaction

-: Reaction inhibited, negative reaction

TABLE 9. Summary of advantages and disadvantages of different trypanosome DNA templates preparations for LAMP

Types of DNA template	Simplicity	Preparation time	Reagents required	Purity of DNA	Turbidity
Crude blood	Easy to prepare	< 5 min	None	Crude	Not visible
Hemolysed blood	Easy to prepare	< 0.5 hours	DDW, TE buffer	Crude	Not visible
Filter paper blood	Easy to prepare	~1.5 hours	FTA purification reagent and TE buffer	Partially purified	Unclear
Genomic DNA	Complicated	~12 to 24 hours	Proteinase K, PCI, chloroform, NaOAc, Isopropanol, 70% EtOH, DDW	Purified	Visible

CHAPTER 4

A Trypanosome Species Isolated from Naturally Infected *Haemaphysalis hystricis* Ticks in Kagoshima Prefecture, Japan

4-1. Introduction

The genus *Trypanosoma* comprises unicellular flagellates that are parasites of all vertebrate classes. The vectors can be haematophagous arthropods for mammalian, avian, reptilian as well as amphibian trypanosomes, whereas fish, certain amphibian and reptilian trypanosomes are transmitted by leeches (60). Salivarian trypanosomes are known to be transmitted by biting flies of the genus *Glossina* commonly called tsetse flies (36, 125, 138) with the exception of *T. evansi* and *T. equiperdum* which are transmitted mechanically by the *Stomoxys* and *Tabanid* flies and by copulation, respectively (15, 50). Furthermore, the stercorarian trypanosomes such as *T. cruzi*, *T. lewisi* and *T. rangeli* are transmitted by bugs and fleas through contaminated faeces (138).

The current study reports on the unknown trypanosome species that has been isolated from naturally infected *Haemaphysalis hystricis* ticks isolated in Kagoshima Prefecture in Japan. The *H. hystricis* tick is a vector of *Ehrlichia* and *Rickettsia* sp. that causes spotted fever group (SFG) ehrlichiosis and rickettsiosis respectively (92, 121). This unknown trypanosome is referred to as *Trypanosoma* KG1 isolate. With the aim of identifying the taxonomic status of this unknown trypanosome species, a series of experiments were conducted including the establishment of an *in vitro* culture system for *Trypanosoma* KG1 isolate (KG1), an attempt to determine the laboratory mammalian

host, experimental infection of other tick species and the development of PCR and LAMP for specific detection of this trypanosome. Furthermore, this study cloned and sequenced the ITS1, 18S rRNA, large subunit 28S rRNA and kinetoplast DNA (kDNA) genes of *Trypanosoma* KG1 isolate.

4-2. Materials and Methods

Isolation of parasites from ticks

Three male and 3 female adult *H. hystricis* ticks were collected in year 2003 from vegetation at Uchinoura-cho of Osumi Peninsula in Kagoshima prefecture, situated on the Southernmost part of Kyusyu island, Japan. Average temperature, relative humidity and rainfall in Kagoshima prefecture is 15-23^{o}C, 60-75% relative humidity (RH), and 2,200-2,900 mm respectively. Vegetation is a typical sub-tropical forest, in which *Cinnamomum camphora* (Camphor tree) and *Cycas revoluta* (Sago palm) plantations are dominant. Midguts of the ticks were removed aseptically and suspended as pooled samples in the sucrose-potassium-glutamate medium. Suspension of the pooled midgut contents was then inoculated into the monolayer of L929 cells cultivated with MEM (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 2% fetal bovine serum (FBS) and incubated at 33^{o}C (68). However in the current study the parasites were propagated at 37^{o}C with HEK 293T cell line as feeder cells. The KG1 cultures were maintained in Medium 199 (Sigma® Aldrich, U.K.) supplemented with 2% FBS and Penicillin-Streptomycin (100U-100 µg/ml) by replacing the culture medium every second day.

Tick infection

Twelve micro litres of KG1 culture supernatant (containing approximately 10^3 parasites) was experimentally injected into the haemocoel of 12 *Ornithodoros moubata* ticks. Another set of ticks was made to feed on the culture supernatant containing 1 x 10^6 parasites/ml through an artificial membrane (Figure 11) as described previously (143). However, in this study a laboratory film (Parafilm® M, Pechiney Plastic Packaging, Chigago, IL) was used as an artificial feeding membrane. The presence of the parasites was examined by Giemsa-stained smears made from haemolymph, salivary glands, and midgut every 5 days for a period of 30 days. All ticks were kept at 25^{o}C and 50 – 60% relative humidity in continuous darkness throughout the experiment.

Infection of laboratory animals and parasite detection

Five female ICR mice, BALB/C mice, and SD rats aged 6 weeks were inoculated intraperitoneally with 1 ml culture supernatant containing approximately 1 x 10^6 *Trypanosoma* KG1 parasites, and corresponding number of each animal was kept as uninfected control group. One Japanese white rabbit was also inoculated with 1 x 10^6 parasites, and another was kept as control. Furthermore, one splenectomized rat and sheep were also inoculated with 1 x 10^6 and 2 x 10^6 *Trypanosoma* KG1 respectively. While a corresponding number of each animal species were kept as uninfected controls. All the controls of the above-mentioned mammalian species were inoculated with 1 ml of the new culture medium (Medium 199) (Sigma® Aldrich, U.K.). The inoculated animals were bled every 3 days for a period of 30 days and parasitological examinations (microscopy of wet smears, Giemsa-stained thin blood smears, and buffy coat preparations), PCR and LAMP were performed in order to detect the parasite from

blood samples. All animals were euthanized thirty days post infection. In order to clarify infectivity of the KG1 parasite in the animal species tested, total DNA of spleen, heart, liver, kidneys, and lymph nodes were examined by PCR and LAMP.

DNA extraction

Total DNA of KG1 isolate from blood, and visceral organs was extracted as previously described (129). Briefly, the extraction buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% sodium dodecyl sulphate) and 100 µg/ml proteinase K were added to the samples and incubated overnight at 55^{o}C. DNA was extracted with PCI (25:24:1) and precipitated with isopropanol. DNA was dissolved in 250 µl of TE (10 mM Tris-HCl, 1mM EDTA, pH8.0) buffer.

PCR

In the initial experiments, several primers of commonly known trypanosomes, including the *Trypanosoma* (*Trypanozoon*) group, *T. evansi*, *T. rangeli*, Kinetoplastida (32), and the *T. lewisi* primers (33), were used in attempts to amplify DNA of *Trypanosoma* KG1 isolate. Primers for eukaryotic 18S rRNA (24) and primers designed from the 18S rRNA of *Trypanosoma pestanai* (Accession no: AJ009159) were also used for amplification of KG1 DNA and 28S rRNA LSU (Accession no: X14553) of *T. brucei* group (Table 10 and Figure 12). The PCR reaction mixture contained 10x PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), 2 mM of each dNTP, 5 pmol of each primer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Japan) in a final volume of 50 µl. The reaction mixture was heated at 94^{o}C for 10 min and subjected to 35 cycles at 94^{o}C for 45 sec, 1 min at 55^{o}C, and 1 min at 72^{o}C with a final extension at 72^{o}C for 7 min.

LAMP

The following LAMP primer set targeting ITS-1 gene of *Trypanosoma* KG1 isolate was designed using the Primer Explorer V2 software program (Fujitsu, Japan):

FIP: 5'-GAT TCC AGC TGC AGG TTC ACC AAT AGT AGG GAA GCA AAG TC-3', BIP: 5'-GCA TGT ATG TAT GTG TAG TAT GCG TTA GAA GCT GTT GCT TCA TAC C-3', F3: 5'-ACC GAA AGT TCA CCG TAT T-3', and B3: 5'-TTG TGT GCG AAG AGA ACA-3'. The reaction was carried out as previously reported (115), briefly, in a final volume of 25 µl reaction mixture containing 12.5 µl of x2 LAMP reaction buffer (40 mM Tris-HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM of each dNTPs), 2 µl template DNA and 1 µl (8 units) *Bst* DNA polymerase (New England BioLabs Inc., U.S.A.), 0.9 µl of primer mix (FIP and BIP at 40 pmol each, and F3 and B3 at 5 pmol each), and 8.6 µl of distilled water. The reaction mixture was incubated at 63^{o}C for 1 hour followed by 80^{o}C for 2 min for termination of the reaction. The F3 and B3 LAMP primers were also used for PCR with the same PCR conditions as mentioned above except for annealing temperature that was modified to 57^{o}C. Both the PCR and LAMP products were electrophoresed in 1.5% TAE agarose gel and stained with ethidium bromide for visualization.

Gene cloning, sequencing, and analysis

The PCR products were purified using the QIAquick gel extraction Kit (Qiagen, U.S.A.) and cloned to pT7Blue-T vector (Novagen Inc., Germany) with DNA ligation kit Ver 2.1 (Takara Bio Inc., Japan). The plasmid was cut with *Bam* HI and *Xba* I restriction enzymes, and the nucleic acid sequence was determined with the BigDye terminator

cycle sequencing kit (Applied Biosystems, Japan). In order to analyze similarities between the nucleic acid sequences from KG1 and the known sequences of other trypanosomes, the sequences of KG1 were subjected to NCBI BLASTn search (www.ncbi.nlm.nih.gov/BLAST). Furthermore, the KG1 18S rRNA sequence was aligned with other selected stercorarian trypanosome genes using ClustalW program, and phylogenetic trees were constructed by the bootstrapped maximum likelihood and maximum parsimony methods using the PHYLIP 3.6 package program downloaded from University of Washington website (http://evolution.gs.washington.edu/phylip.html). Maximum likelihood is a method for predicting and evolutionary tree that uses an expected pattern of mutational changes from one DNA base to another and probability calculations to find the most likely arrangement of branches that generates the set of sequences (106). The maximum parsimony is a method for predicting an evolutionary tree that best fits the sequence variation in each column of a multiple sequence alignment. This tree will provide the minimal number of evolutionary steps to produce the sequences (106). The Seqboot and Consense programs were used to statistically assess the strength of the trees using bootstrap resampling. The model used for the maximum likelihood and maximum parsimony classification is one that allows for inequalities of equilibrium base composition and for inequalities of the rate of transitions and transversions. It is related to the model reported by Felsenstein (45) but generalizes it to allow for unequal rates of transitions and transversions (46).

4-3. RESULTS

Identification of the taxonomic status of the *Trypanosoma* KG1 isolate

Giemsa-stained smears of KG1 from *in vitro* cultures clearly show common morphological features of trypanosomes such as nucleus, kinetoplast, undulating membrane, flagella pocket and the flagellum (Figure 13A). The trypomastigote-like form of KG1 is 15-30 μm in length and has a 2-5 μm width, and free flagellum length varied amongst cells with minimum of 5 μm and maximum of 20 μm. Out of the several primers of commonly known trypanosomes that were used in an attempt to amplify the unknown trypanosome DNA, only the *T. lewisi* primers targeting the ITS1-5.8S, and the universal primers for the 18S rRNA of eukarytic cells, 28S rRNA and the kDNA genes, with product size of approximately 600 bp, 2,000 bp, 200 bp and 500 bp, respectively, amplified *Trypanosoma* KG1 isolate DNA (Figure 14). These PCR products were successfully cloned and sequenced. However, for the 18S rRNA gene only a nucleotide sequence which was about 1000 bp in length was obtained due to the quality of the sequencing reaction. Two sets of primers designed from the 18S rRNA of *T. pestanai* (Table 10 and Figure 12) with PCR products of 513 bp and 681 bp in order to obtain additional sequences for more reliable phylogenetic analysis based on the 18S rRNA gene sequence which is at least 2,000 bp. Locations of EUK-A and B, TPE F1 and B1, and TPE F2 and B2 are schematically indicated in Figure 12. As a result, a 2,154 bp sequence of 18S rRNA from *Trypanosoma* KG1 was determined. The partial gene sequences were submitted in the GenBank as ITS1 (AB259643); 18S rRNA (AB281091); kDNA (AB259645) and 28S rRNA (AB259646).

Accordingly, the nucleic acid sequences of the above-mentioned genes mainly showed close homology to a group of non-pathogenic trypanosomes as well as some *Bodo*, *Leishmania* and the *Crithidia* species by BLASTn search (Data not shown). Similarly, in the phylogenetic trees constructed with the partial 18S rRNA gene of *Trypanosoma* KG1 which is a conserved region with a length of 2,154bp, *Trypanosoma* KG1 is clustered together with *Trypanosoma* sp. wombat H26 and *T. pestanai* in the bootstrap majority-rule consensus unrooted tree obtained from 100 maximum likelihood and maximum parsimony replicates (Figures 15 and 16). *Bodo caudatus* was used as an outgroup parasite in both phylogenetic analyses.

***In vitro* and *in vivo* propagation of *Trypanosoma* KG1 isolate**

Ticks were collected in the field with the aim of determining *Ehrlichia* infections hence, initially tick extracts were cultured with L929. However, actively motile trypanosome-like flagellates were observed in the cultures. In the current study, *Trypanosoma* KG1 isolate actively propagated in the presence of HEK 293T cell as a feeder cell layer. There were no HEK 293T cells found to be infected with the parasite throughout the culture period. Giemsa-stained smears of the haemolymph showed the presence of the *Trypanosoma* KG1 isolate in the *O. moubata* ticks for up to 30 days post-injection (time at which the experiment was terminated) into the haemocoel (Figure 13B). In another set of *O. moubata* ticks that were made to feed on a medium containing KG1 parasites through an artificial membrane, the trypanosomes were detected in the tick midgut and salivary glands for up to 30 days post-infection. Two different life cycle forms (trypomastigote-like and epimastigote-like) of this trypanosome were observed in both midgut and salivary glands of the tick, whereas in the

haemolymph only the epimastigote-like form was visible (Figures 13C-F). No parasites were observed from haemolymph of ticks fed through artificial membrane. Following attempts to infect mice, rats, rabbits and sheep with *Trypanosoma* KG1 isolate, the parasite could not be detected by microscopic examinations as well as specific gene amplification tests (data not shown).

Development of LAMP and PCR for specific detection of *Trypanosoma* KG1

LAMP detection method targeting ITS1 gene of *Trypanosoma* KG1 isolate has proved to be specific (Figure 17A) and sensitive whereby this method detected the genomic DNA up to 10 fg (Figure 17B). The LAMP primers F3 and B3 have been used for PCR and were also specific (Figure 17C), with same sensitivity as LAMP (Figure 17D).

4-4. DISCUSSION

This study reports on the unknown trypanosome species that has been isolated in naturally infected *H. hystricis* ticks. The clear visibility of common morphological features such as the kinetoplast, nucleus, the flagellar pocket, undulating membrane and the flagellum in the Giemsa-stained smears strongly suggests that the organism belongs to the genus *Trypanosoma.* The nucleic acid sequences of the cloned PCR products of the ITS1, 18S rRNA, 28S rRNA and kDNA genes of this unknown trypanosome isolate indicated close homology to groups of non-pathogenic trypanosomes. According to phylogenetic trees constructed in this study from nucleic acid sequences of the 18S rRNA, *Trypanosoma* KG1 is clustered together with *T. (Megatrypanum) pestanai* which has been reported to infect badgers (*Meles meles*),

however the vector is unknown (66) and *T.* sp. wombat H26 isolated from wombat (*Vombatus ursinus*), whereby the wombat flea (*Lycopsylla nova*) has been suggested as a possible vector (116), as are ticks, based on report where trypanosomes were found in tick nymphs (*Ixodes holocyclus*) in Australia (90).

In the phylogenetic analysis of the 18S rRNA and GAPDH (62, 69, 70) *T. pestanai* clustered with *T. (Megatrypanum) theileri* and *T. (Megatrypanum) cyclops*. Whilst, in the phylogenetic analysis of the SSU rRNA *T. pestanai* clustered only with *T.* sp. wombat H26 (62). However, the clustering in the phylogenetic analyses of the current study is different probably because of the differences in the bootstrap support whereby they are generally low or absent for the above-mentioned analyses. Another possible reason could be due to different lengths of sequences used in alignments which are longer or shorter than those used in the current study or the fact that the model of evolution in the maximum likelihood analysis of the current study is different to evolution models used in above-mentioned analyses.

In particular, *T.* (*Megatrypanum*) *theileri* has also been reported to be naturally infecting the *Hyalomma anatolicum anatolicum* ticks (87). However *Trypanosoma* KG1 is morphologically different from *T.* (*Megatrypanum*) *theileri*. Accordingly, the trypomastigote-like form of KG1 has a curved pointed shape with 15-30 µm length (68). In contrast, *T.* (*Megatrypanum*) *theileri* is one of the largest mammalian trypanosomes with mean length of 60-70 µm (138). There is great variability within trypanosome species, and a lot of incomplete knowledge about these parasites. New trypanosome species as well as new localities of known trypanosomes are constantly being described

(79). Hence, this unknown trypanosome is referred to as *Trypanosoma* KG1 isolate, with reference to the locality of isolation, Kagoshima Prefecture of Southern Japan.

Hard ticks of the genera *Hyalomma* and *Rhipicephalus* have been reported to harbour trypanosome (40, 87) and *Leishmania* (25) parasites respectively. *Trypanosoma* KG1 isolate was also isolated in the hard tick *H. hystricis.* Additionally, in the current study this trypanosome was successfully propagated *in vivo* by infecting the soft tick *O. moubata* and the parasite could be isolated in the midgut and the salivary glands. This observation suggests that this trypanosome has the ability to infect both the hard and soft tick species. Some of the stercorarian trypanosomes of which *Trypanosoma* KG1 isolate is closely related to, have a narrow host range for their vertebrate hosts where they live extracellularly, primarily in the bloodstream (130). This could explain unsuccessful attempts in this stduy to propagate *Trypanosoma* KG1 isolate in the experimental animals (mice, rats, rabbits and sheep).

According to Yamaguti *et al*. (148), *H. hystricis* distribution is restricted to a subtropical and temperate belt of Eastern Asia, and the adult ticks have a fairly wide host range including humans, domestic dogs, buffalo, pigs, wild boars, tigers, hog-badgers, goat-like deer (*Muntiacus reevesi*), short-eared rabbit (*Lepus sinensis*) and the Ryuku black rabbit (16, 92, 121, 148). This wide host range of *H. hystricis* suggests that one or more of the above-mentioned mammals could be harbouring *Trypanosoma* KG1 isolate, particularly the wild pig (*Sus scrofa*) and as it has been reported to be hosts of *H. hystricis* in Kagoshima (148), badgers as well which are hosts for *T. pestanai* which is clustered together with KG1 are possible hosts. In Sudan, Morzaria *et al*. (104) demonstrated the first biological transmission of *T.* (*Megatrypanum*) *theileri* to cattle by

the tick *H. a. anatolicum*. It is therefore highly possible that *Trypanosoma* KG1 might be transmitted by the ticks during feeding since this parasite was detected in the salivary glands of experimentally infected *O. moubata* tick.

LAMP reaction amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions using only simple incubators (115), and has recently been developed for diagnosis of African trypanosomes (84), whilst PCR is already an established and widely used diagnostic technique for trypanosomes (26, 31, 32, 149). Thus, this study has also developed sensitive and specific LAMP and PCR methods that could later be used in attempts to identify vertebrate host(s) and epidemiological studies of *Trypanosoma* KG1 isolate.

In conclusion, this study has revealed some of the characteristics of *Trypanosoma* KG1, including morphology, its ability to propagate in soft ticks, its disability to infect commonly used laboratory animals, and its phylogenetic relationship with non-pathogenic stercorarian trypanosomes which are also suspected to be harboured by ticks. However, a challenge of identifying the possible vertebrate host(s) still remains, and if uncovered may lead to better understanding of *Trypanosma* KG1 transmission and parasite host-relationship. There is also need for further studies of other trypanosome species and ticks as this might give information on non-survival of other trypanosomes in ticks, eventually identifying responsible molecules that could be used as anti-trypanosome vaccine candidates.

4-5. Summary

Common arthropod vectors for trypanosomes are flies, fleas and bugs. This study reports on an unknown trypanosome species isolated from naturally infected *H. hystricis* ticks, hereby, referred to as *Trypanosoma* KG1 isolate. The parasite has been successfully cultured *in vitro* with L929 or HEK 293T cell line as feeder cells. This trypanosome cannot survive *in vitro* without feeder cells. Following experimental infections of ticks, the trypomastigote-like and the epimastigote-like forms of this trypanosome could be detected by Giemsa-stained smears in the midgut and salivary glands of *O. moubata* ticks which were made to feed on a culturing medium containing *Trypanosoma* KG1 isolate through an artificial membrane. *Trypanosoma* KG1 isolate could also be detected from Giemsa-stained smears of the haemolymph up to 30 days post inoculation into the *O. moubata* haemocoel. *Trypanosoma* KG1 isolate cannot be propagated in laboratory animals including mice, rats, rabbits and sheep. Phylogenetic trees constructed with the 18S rRNA gene using the maximum likelihood and maximum parsimony methods indicate that *Trypanosoma* KG1 is a member of the stercorarian trypanosomes.

TABLE 10. PCR primers used for amplification of *Trypanosoma* KG1 genomic DNA

Primer name	Sequence	Target gene	Specificity	Primer position*	
				5’	3’
EUK-A and B	A: 5’-AACCTGGTTGATCCTGCCAGT-3’	18S rRNA	Eukaryote cell	ND**	ND
	B: 5’-GATCCTTCTGCAGGTTCACCTAC-3’			ND	ND
TPEF1 and B1	F1: 5’-CCATGCATGCCTCAGAATCACTGC-3’	18S rRNA	*T. pestanai*	ND	ND
	B1: 5’-GGCACTGCCGGCTCTATTTC-3’			345	364
TPEF2 and B2	F2: 5’-GCAGCGAAAAGAAATAGAGCCGG-3’	18S rRNA	*T. pestanai*	335	357
	B2: 5’-GTTCGTCCTGGTGCGGTCTAAG-3’			1,073	1,094
LSU1 and 2	LSU1: 5’-TGGAAATGCGAAACACTTGC-3’	28S rRNA	*T. brucei* group	ND	ND
	LSU2: 5’-ACACCCCAGGTTTTTGCTT-3’			ND	ND
KIN1 and 2	KIN1: 5’-GCGTTCAAAGATTGGGCAAT-3’	kDNA	Kinteplastida	ND	ND
	KIN2: 5’-CGCCCGAAAGTTCACC-3’			ND	ND
TRYP IS and IR	TRYP-IS: 5’-CGTCCCTGCCATTTGTACACA-3’	ITS1-5.8S	*T. lewisi*	ND	ND
	TRYP-IR: 5’-CGATGGATGACTTGGCTTCC-3’			ND	ND

* Primer position on the nucleotide sequence of *Trypanosoma* KG1 18S rRNA (Accession no: AB281091)

** Not Determined

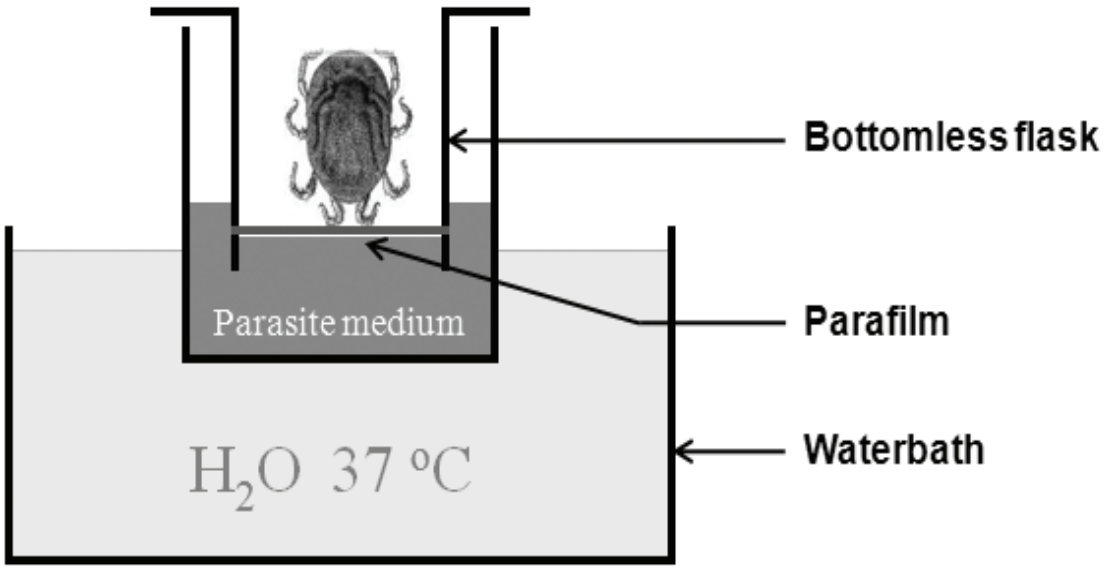

FIG. 11. An illustration of an artificial tick feeding membrane setup.

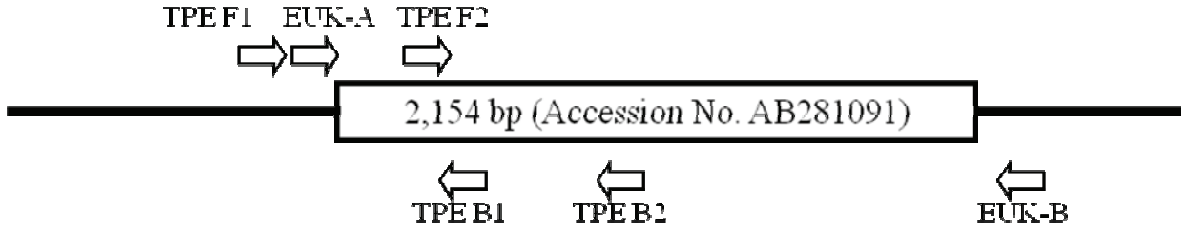

FIG. 12. Schematic diagram of primer positions for 18S rRNA. Entire sequence of 18S rRNA of *Trypanosoma* KG1 is indicated by solid line. The sequence used for phylogenetic tree construction is shown by open box with its length and accession number. Each primer location is indicated by open arrow and primer name.

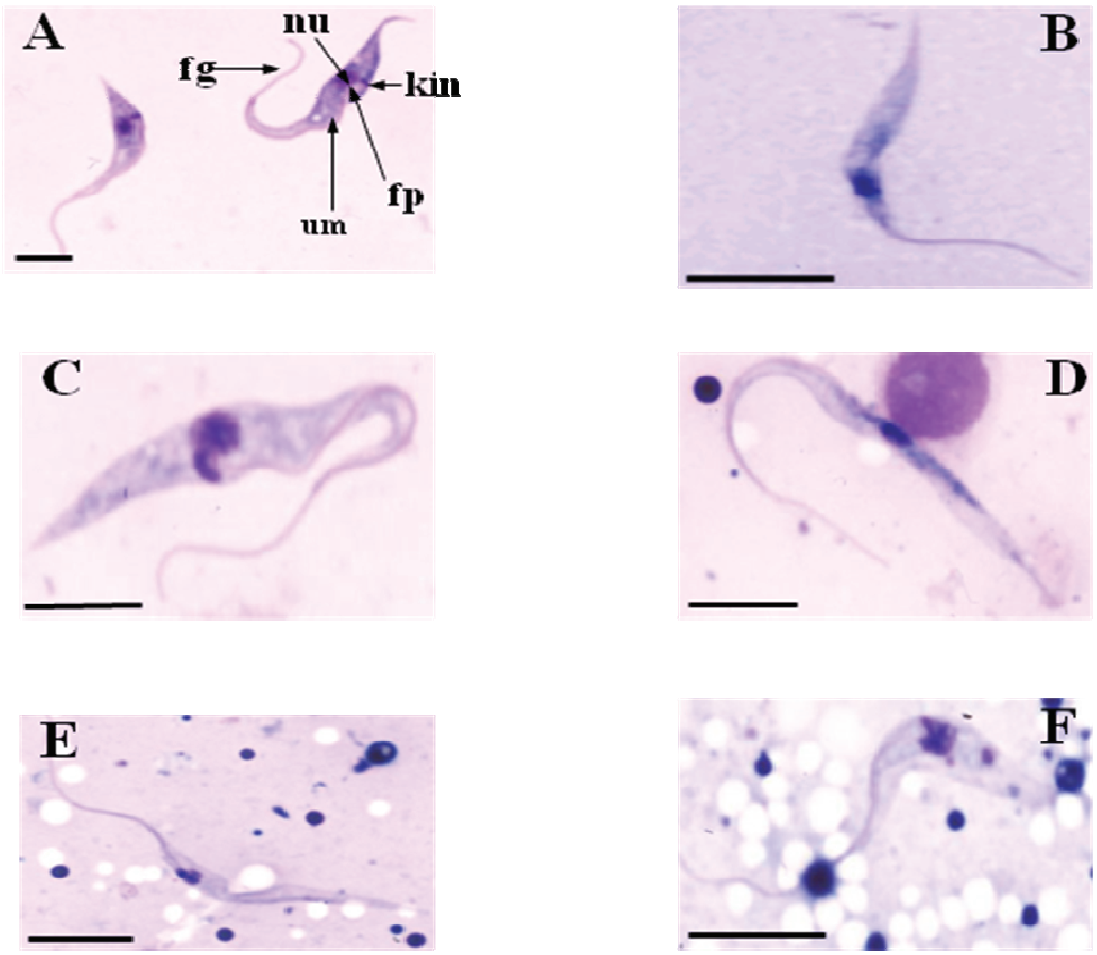

FIG. 13. Giemsa stained slides of different forms of *Trypanosoma* KG1 from in *vitro* culture and within different parts of *O. moubata* tick. Bar = 10 µm. (A) Trypomastigote-like form in the *in vitro* culture with nucleus (nu), kinetoplast (kin), flagellar pocket (fp), undulating membrane (um), and the flagellum (fg). (B) Trypomastigote-like form in the haemocoel. (C) Trypomastigote-like form in the midgut. (D) Epimastigote-like form in the midgut. (E) Trypomastigote-like form in the salivary glands. (F) Epimastigote-like form in the salivary glands.

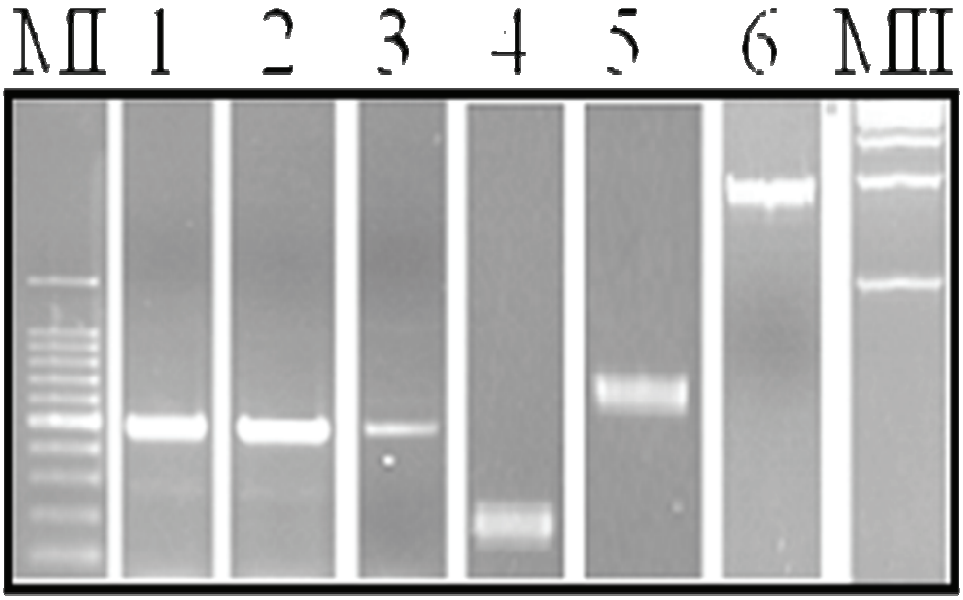

FIG. 14. Amplification of *Trypanosoma* KG1 genomic DNA by PCR with different primers targeting different genes. Lane MI, 100 bp Marker; Lane 1, kDNA; Lane 2, TPEF1B1 for 18S rRNA; Lane 3, TPEF2B2 for 18S rRNA; Lane 4, LSU 28S rRNA; Lane 5, ITS1-5.8S; Lane 6, EUK-AB for 18S rRNA; and Lane MII, 1 kb Marker.

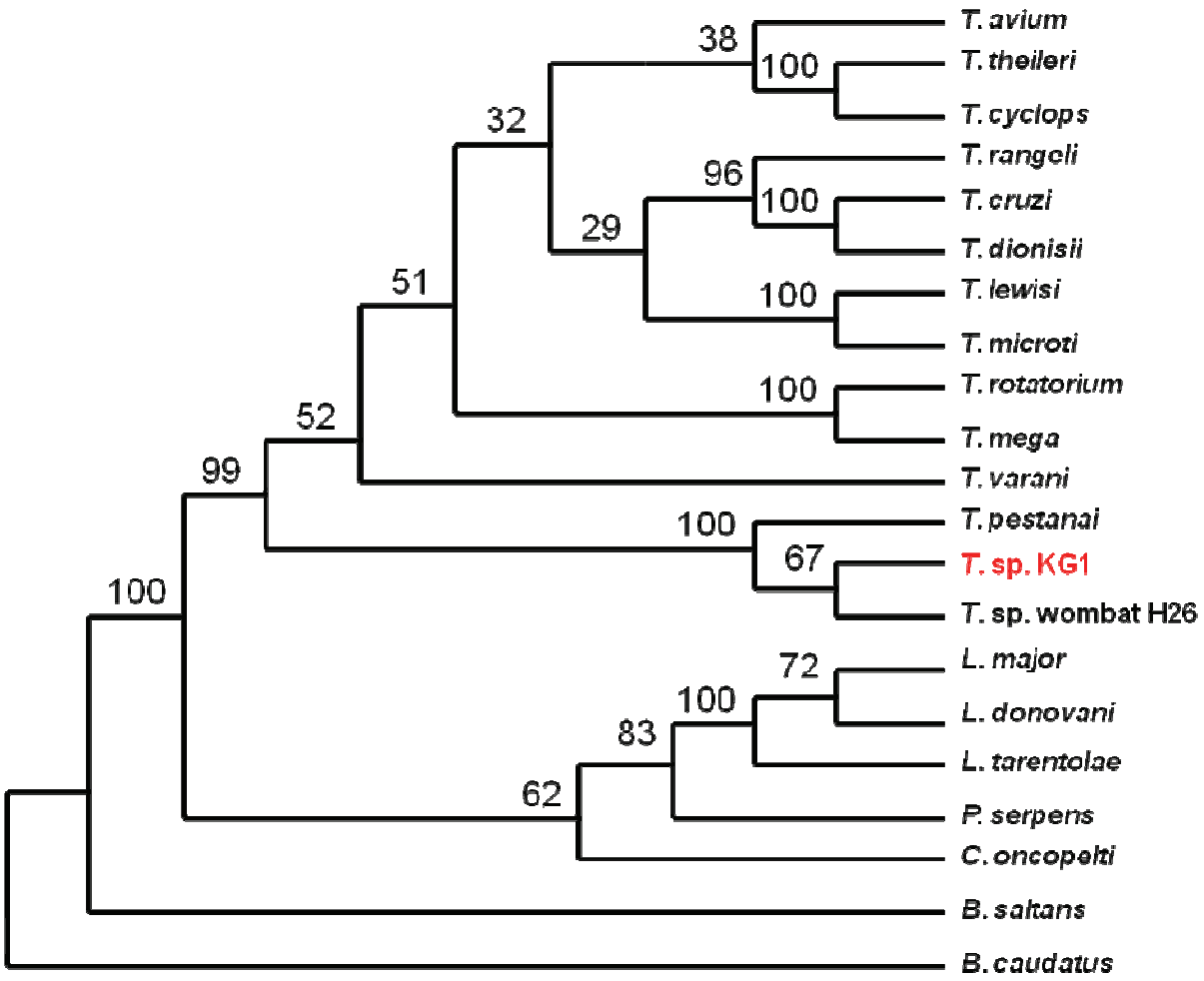

FIG. 15. Phylogenetic tree based on bootstrapped maximum likelihood analysis of the 18S rRNA gene sequences performed using PHYLIP 3.6 program. Tree includes 21 taxa, values at the nodes are bootstrap values (%:100 replicates), and the length of the 18S rRNA gene sequence of KG1 for alignment is 2,154 bp. The accession no. of *T*. sp. wombat H26 sequence is AJ009169. The sequences of other trypanosomes, *Leishmania* parasites, bodonids, *Phytomonas serpens* and *Crithidia oncopelti* were obtained from the GenBank database according to accession numbers published by Hughes and Piontkivska (69).

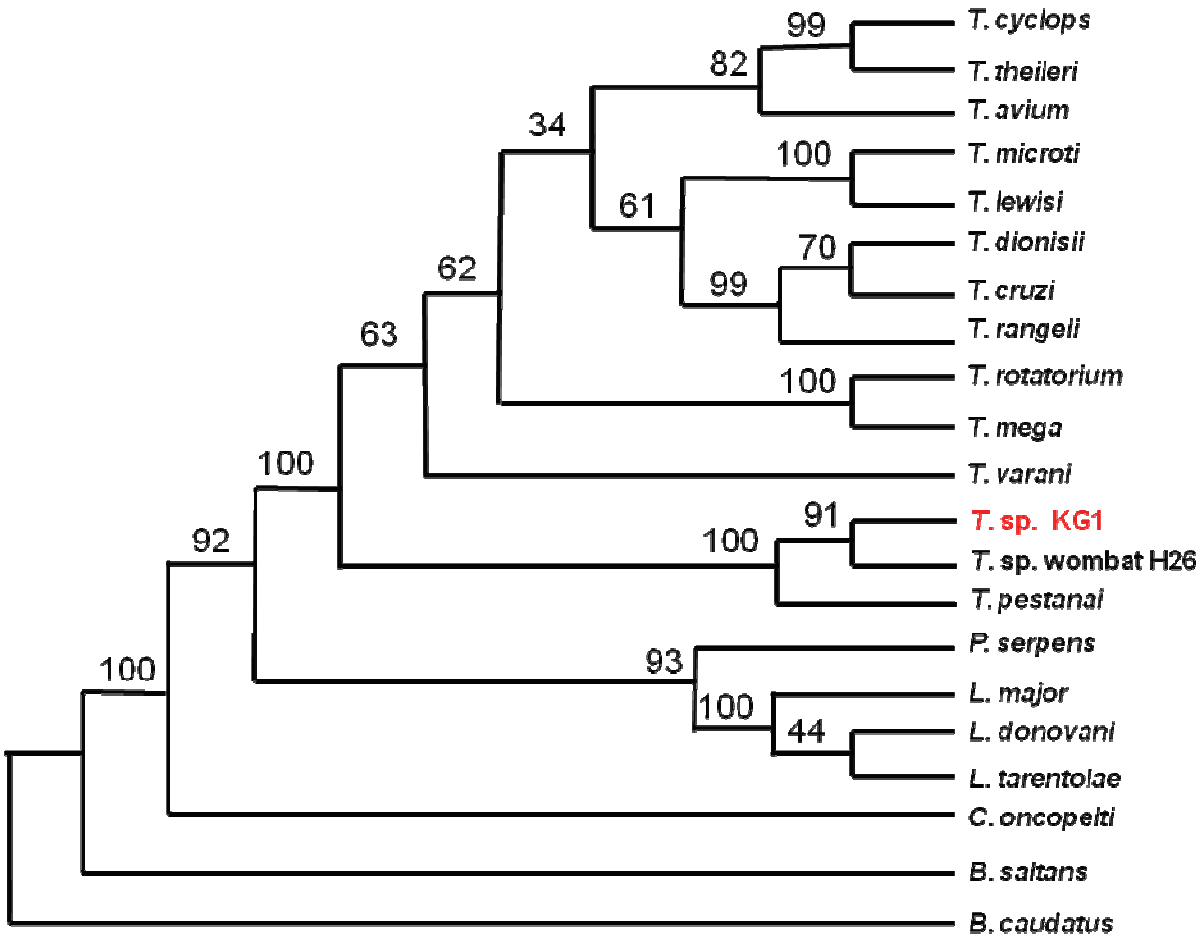

FIG. 16. Phylogenetic tree based on bootstrapped maximum parsimony analysis of the 18S rRNA gene sequences performed using PHYLIP 3.6 program. Tree includes 21 taxa, values at the nodes are bootstrap values (%:100 replicates), and the length of the 18S rRNA gene sequence of KG1 for alignment is 2,154 bp. The accession no. of *T.* sp. wombat H26 sequence is AJ009169. The sequences of other trypanosomes, *Leishmania* parasites, bodonids, *Phytomonas serpens* and *Crithidia oncopelti* were obtained from the GenBank database according to accession numbers published by Hughes and Piontkivska (69).

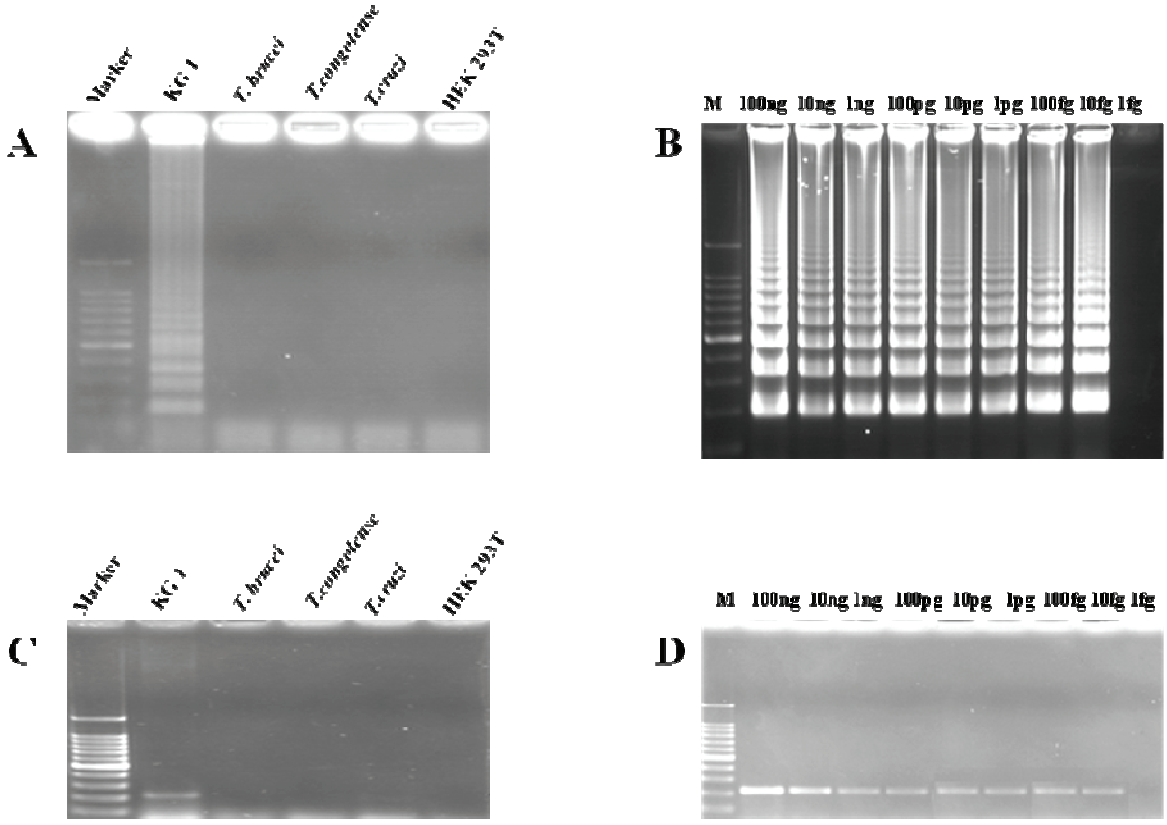

FIG. 17. LAMP and PCR for detection of ITS1 gene of *Trypanosoma* KG1 species.

(A) Specificity of LAMP. (B) Sensitivity of LAMP. (C) Specificity of PCR. (D) Sensitivity of PCR. M and Marker indicate 100 bp ladder DNA size marker up to 1,000 bp and additional band of 1,500 bp.

CHAPTER 5

A Loop-mediated Isothermal Amplification (LAMP) Assay for Detection of Bovine *Theileria* Infections

5-1. Introduction

The genus *Theileria* comprises tick-transmitted sporozoan protozoa that are the causative agents of a variety of disease syndromes in domestic and wild ruminants, and are collectively responsible for economic losses amounting to hundreds of millions of dollars annually in sub-Saharan Africa and Asia (12). Bovine theileriosis syndromes, include ECF or CD caused by *T. parva* (35, 135); tropical theileriosis caused by *T. annulata* (58), cerebral theileriosis 'Ormilo' caused by *T. taurotragi* (89) and benign theileriosis caused by *T. buffeli/orientalis/sergenti* group (59, 82), *T. mutans* and *T. velifera* (58). In particular, ECF caused by *T. parva* continues to pose a serious threat to cattle productivity in Eastern, Central and Southern Africa and is estimated to cost US$ 169 million annually within the region (11). A carrier state of *T. parva* defined as the persistence of a tick transmissible infection, is common among naturally recovered host animals, both in cattle and the major wildlife host, African buffalo (*Syncerus caffer*) (35).

Diagnosis of bovine *Theileria* parasites is commonly by observation of clinical signs on infected animals (119), microscopy from body fluids and tick organs (97, 117), serological assays such as ELISA and IFAT (59, 85, 86). These methods have variable limitations ranging from unspecific clinical signs amongst different infections, lower sensitivity, cross reactivity and failure to distinguish between past and current infections

in case of serology (8). Recently, molecular diagnostic techniques such as PCR have solved issues of specificity and sensitivity (135, 144), however they are negated by the need for expensive equipment and labour intensiveness (67, 84, 123).

In the year 2000, Notomi and colleagues at the EIKEN Chemical Co. Ltd., developed a gene amplification technique termed LAMP method. LAMP is a simple technique which amplifies DNA with high sensitivity, rapidity under one constant temperature (115). This study was aimed at the development of LAMP with universal primers for detection of major bovine *Theileria* species (*T. parva, T. annulata, T. mutans, T. taurotragi* and *T orientalis*) and further, development of specific LAMP for detection of *T. parva* infections and its application in detection of the parasite from bovine blood and vector ticks.

5-2. Materials and Methods

Parasite DNA

The DNA of *T. parva, T. annulata, T. mutans* and *T. taurotragi* was obtained from the International Livestock Research Institute (ILRI) – Kenya. The *T. orientalis* was obtained from blood of an infected cow in Chitose, Hokkaido - Japan. This study further used DNA from other protozoan parasites as controls namely, *Babesia bovis* (Texas); *B. bigemina* (Argentina); *B. equi* (USDA), *T. b. brucei* (GuTat 1.3) and DNA extracted from blood of a cow free of infection at a stable of the National Research Center for Protozoan Diseases of Obihiro University of Agriculture and Veterinary Medicine – Japan.

Field samples

Forty blood samples blotted on filter papers (FTA® card, Whatman, U.K.) and thin blood smears were prepared from blood collected from cattle of unknown age on 10 farms near Dar es Salaam and 24 samples from cattle on seven farms near Arusha (animal age ranged between 4 months – 8 years). Furthermore 99 blood samples were collected from African buffaloes in 3 provinces of the Republic of South Africa, 85 samples from Kwazulu-Natal province, 5 samples from Limpopo province and 9 samples from Mpumalanga province. Thirty two DNA samples prepared from whole tick (*Rhipicephalus appendiculatus*) extracts smeared on filter papers (FTA® card, Whatman, U.K.) collected from infested cattle in Shibuyunji Lusaka West – Zambia were used in the evaluation of the newly developed LAMP assay for detection of *T. parva* infections in the vector.

DNA extraction from filter papers

DNA was extracted from filter papers using a QIAamp DNA blood mini kit (Qiagen Science, Maryland, USA) according to the manufacture's instructions. Briefly, Three punched-out circles (discs) from filter papers (FTA card, Whatman, UK) spotted with whole tick extracts (Zambia tick samples) and whole blood (Tanzania's Arusha and Dar es Salaam cattle samples) were placed into a 1.5 ml microcentrifuge tube and 180 µl of buffer ATL was added, then incubated at 85^{o}C for 10 min. Thereafter 20 µl of proteinase K stock solution was added, mixed by vortexing and incubated at 56^{o}C for 1h and then briefly centrifuged. A 200 µl of buffer AL was added, mixed by vortexing and

then incubated at 70^{o}C for 10 min, and centrifuged briefly. Two hundred microlitres of ethanol (99.5%) was added, mixed by vortexing and briefly centrifuged. The mixture was then carefully applied to the QIAamp Spin Column (In a 2 ml collection tube), centrifuged at 4,000 x *g* for 1 min., thereafter the QIAamp spin column was placed in a clean 2 ml collection tube. A 500 µl buffer AW1 was added to the QIAamp spin column, centrifuged at 4,000 x *g* for 1min. Thereafter the QIAamp Spin Column was placed in a clean 2 ml collection tube. A 500 µl buffer AW2 was added to the QIAamp spin column, centrifuged at full speed 15,000 x *g* for 3 min. A 100 µl of buffer AE, was added to the QIAamp spin column, incubated at RT for 1 min, and then 4,000 x *g* for 1 min. Eluted DNA was stored at -20^{o}C until use.

DNA extraction from whole blood

The gDNA was extracted whole blood collected from buffaloes with a standard PCI method as described by Sambrook and Russell (129). Briefly, extraction buffer containing 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% sodium dodecyl sulphate and 100 µg/ml proteinase K was added to the samples and incubated overnight at 55^{o}C. DNA was extracted with PCI (25:24:1) and precipitated with isopropanol and the pellet was dissolved in 200 µl of DDW.

LAMP

The LAMP primers (Table 11) were designed from the HSP70 (Accession no. U40190), p104 (Accession no. AY034071) and the p67 (Accession no. U40703) using the primer explorer software of the EIKEN Chemical Co.,Ltd (http://primerexplorer.jp/e/). The LF and LB primers for both the p104 and p67 were designed manually. A

schematic diagramn of the distance ranges between LAMP primers is shown in figure 18. LAMP reaction was conducted as described by Notomi *et al.* (115) with minor modifications. The LAMP reaction with HSP70 primers was conducted at 60^{o}C; 63^{o}C; 65^{o}C; and 68^{o}C for 60 to 75 min and terminated at 80^{o}C for 2 min. The reaction mixture with a total volume of 25 µl contained: 12.5 µl of LAMP reaction buffer (40 mM Tris-HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM of each dNTP), 1 µl (8 units) of *Bst* DNA polymerase, 0.9 µl primer mix (FIP and BIP at 40 pmol each and F3 and B3 at 5 pmol each), 2 µl of template DNA and 1 µl of fluorescent detection reagent. In the case of real-time turbidimetry, the fluorescent detection reagent was not added.

The LAMP reactions with p104 and p67 primers (4 primers: FIP, BIP, F3 and B3) were initially conducted under conditions described above, however, following unsuccesfull attempts to get LAMP reactions the following modifications were done. Two additional LAMP primers termed LF and LB were added (20 pmol each). All the LAMP reaction mixtures were incubated in a laboratory heat block (Dry Thermounit DTU 1B, TAITEC Co., Saitama, Japan) and a real-time turbidimetry heatblock (Loopamp, LA 200, Teramics, Japan). LAMP products were visualized using the fluorescent detection reagent (FD) (EIKEN Chemical Co.,Ltd.) and by electrophoresis in a 1.5% TAE agarose gels and bands were visualized by staining with ethidium bromide solution. The standard positive detection threshold for real-time turbidimetry is 0.1 (101).

PCR

The F3 and B3 LAMP primers for the HSP70, p104 and p67 genes of *T. parva* were used in the PCR reactions. Briefly, the PCR mixture (total volume 25 µl) contained

10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM each of the 4 dNTPs, 5 pmol of each primer, and 5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Ltd., Tokyo, Japan). The reaction mixtures were incubated in a thermocycler (GeneAmp, PCR system 9700, Applied Biosystem, USA) at 94^{o}C for 10 min as an initial denaturation step, and then subjected to 35 cycles consisting of 45 s at 94^{o}C, 1 min at 57^{o}C, and 1 min at 72^{o}C, followed by a terminal elongation for 7 min at 72^{o}C. PCR products were electrophoresed in 1.5% TAE agarose gel and stained with ethidium bromide solution for visualization.

Thin blood smears

Thin blood smears were prepared according to procedures of the OIE commission (119) from 40 and 24 samples collected cattle in Tanzania's Dar es Salaam and Arusha regions respectively. Briefly, a drop of blood was placed on a clean microscope slide and a thin film was drawn out in the usual way. The film was then briefly air-dried, fixed in methyl alcohol for 2 minutes and allowed to dry. Thereafter the smears were stained by Giemsa solution (one drop Giemsa + 1 ml PBS, pH 7.2) for 25 minutes. The preparation was then poured off, the slide was washed in tap water and left to dry. Slides were then examined at high magnification (x 400 -1000).

5-3. Results

Specificity and sensitivity of HSP70 LAMP primers

LAMP primer set designed from HSP70 gene of *T. parva* amplifies DNA of major bovine *Theileria* species including *T. parva*, *T. annulata, T. mutans* and *T. taurotragi*

with exception to *T. orientalis* DNA (Figure 19a). Furthermore, the PCR with F3 and B3 LAMP primers similarly amplifies DNA of the above mentioned *Theileria* parasites also with exception to *T. orientalis* DNA (Figure 19a). Serially diluted *T. parva* DNA is amplified by LAMP primer set designed from the HSP70 gene from 100 ng down to 1 fg (Figure 19b). The PCR with F3 and B3 LAMP primers similarly amplified *T. parva* DNA from 100 ng down to 1 fg (Figure 19b).

Detection of *Theileria* infections from field samples using HSP70 primers

There were no *Theileria* infections detected by thin blood smear method from field samples collected from cattle in Tanzania's Arusha and Dar es Salaam. Forty five percent and 22.5% of DNA samples from Dar es Salaam were positive for *Theileria* species infections by LAMP and PCR respectively, whilst there were no *Theileria* infections detected by both LAMP and PCR from Arusha's DNA samples (Table 12).

Optimization of LAMP reactions for p104 and p67 LAMP primers

LAMP reactions with primers designed from p104 and p67 genes specifically amplify *T. parva* DNA (Figures 20A and B; Figure 21A). PCR with p104 F3 and B3 LAMP primers also specifically amplifies *T. parva* DNA (Data not shown), however no PCR reactions were achieved with p67 F3 and B3 LAMP primers. LAMP with p104 primers detected serially diluted *T. parva* DNA up to 100 pg when conducted at 60 min and when reaction time was increased with 5 min to 65 min detection limit became 10 fg (Figure 21B).

LAMP reactions were achieved when conducted at 63^{o}C for 60 min using 6 primers. There was no amplification of *T. parva* DNA with LAMP reactions conducted at

60°C, 65°C and 68°C in a 60 min amplification period. However, amplification of *T. parva* DNA by LAMP reactions with p104 and p67 primers at 60°C, 65°C and 68°C was achieved with prolonged amplification time of 75 min (Table 13).

Detection of *T. parva* infections from buffalo and tick DNA samples

Ninety out of 99 DNA samples from African buffaloes were positive for *T. parva* infection by LAMP using primers designed from the p104 gene. PCR with F3 and B3 for p104 gene also positively detected the same number of *T. parva* infections from the same samples (Table 14). There was no *T. parva* infections detected from 32 DNA samples from *R. appendicultus* ticks from Zambia by both LAMP and PCR targeting the p104 gene (Table 14).

5-4. Discussion

This study has developed a LAMP assay for diagnosis of bovine *Theileria* species infections. LAMP with primers designed from the HSP70 gene of *T. parva* amplifies DNA of major bovine *Theileria* species including *T. parva, T. annulata, T. mutans* and *T. taurotragi*, suggesting that these primers can be used as universal primers. Further, these primers are highly sensitive whereby they amplified serially diluted *T. parva* DNA 100 ng down to 1 fg. This is an indication that LAMP with these primers will be able to detect bovine *Theileria* infections during chronic stage of theileriosis whereby parasitamia is very low in the host fluids, thereby overcoming the detection sensitivity problem usually experienced when using common diagnostic techniques such microscopy and serology (81).

The HSP70 LAMP primers have further been used in detection of bovine *Theileria* species infections from cattle samples collected in Arusha and Dar es Salaam regions of Tanzania. As a result, the current study has showed high prevalence of bovine *Theileria* infections in Dar es Salaam than in Arusha. During the collection of the samples in Arusha, no ticks were observed on the sampled animals which probably explain the absence of *Theileria* species infections by diagnostic methods used in this study. Furthermore, there were no antibodies detected by the indirect fluorescent antibody test (IFAT) against *Theileria* species in seven serum samples collected from animals in Arusha (data not shown). In contrast, the fact that *Theileria* species infections were detected from the Dar es Salaam samples is not surprising as bovine *Theileria* species infections are common in this region (107,139). In particular, it has also been reported that animals which survive *T. parva* infections remain reservoirs of the parasite (12, 35, 139).

LAMP reactions with primers designed from the p104 and p67 genes specifically amplify only the *T. parva* DNA. This is an expected result because the p104 and p67 genes are specifically expressed only in *T. parva* (38, 81). However, LAMP reactions were not successful when conducted according to the basic protocol as published by Notomi *et al*. (115) with incubation period ranging from 30 – 60 min using 4 primers (FIP, BIP, F3 and B3). The reason for this discrepancy appears to be the fact that the primers are slow to initiate amplification within 60 min. The loop primers (LF and LB) accelerate LAMP reaction and also improve the sensitivity of the reaction (101). Hence, following optimization reactions the optimal reaction temperature for primers designed from the p104 gene is 63^{o}C and the optimal incubation period is 60 min with inclusion of LF and

LB primers, thereby making a total of 6 primers used in the reaction. The LAMP reactions with p67 primers were very inconsistent and less satisfactory, hence optimization reactions were discontinued. An increase in primer concentrations did not have effect in sensitivity of the reactions. However an increase in incubation time improves the sensitivity, hence p104 primer detection limit in 60 min 100 pg but when reaction time is increased with 5 min the detection limit is 10 fg. However the desired reaction time is 60 min or less, furthermore increased reaction time above 60 min increases the chances of primer dimer formation which can result in false positive detection.

Field samples collected from African buffaloes in 3 provinces of the Republic of South Africa and from *R. appendiculatus* ticks infesting cattle in Shibuyunji Lusaka West in Zambia were screened for *T. parva* infections using p104. As a result, there was high prevalence 90/99 (90.9%) of *T. parva* infections whilst none of the ticks showed *T. parva* infections. PCR with p104 F3 and B3 primers gave similar results for buffalo and tick samples. *R. appendiculatus* ticks are common vectors of *T. parva* parasites (12, 35). The absence of *T. parva* infections in the sampled ticks can be due to absence of infection from the host (cattle in this case) which can be explained by the fact that the farmer mentioned that he frequently subject his cattle to acaricide treatment. Whilst buffaloes usually harbor *T. parva* parasites (12, 35) and act as reservoirs of infection especially where they graze together with domestic livestock (57).

In conclusion, LAMP showed higher detection sensitivity than PCR and microscopy for diagnosis of bovine *Theileria* species infections in cattle samples from Tanzania. Similar superior detection sensitivity by LAMP as compared to PCR and

microscopy has previously been reported by Kuboki *et al.* (84) for detection of trypanosome infection in mice. The HSP70 LAMP primers can therefore be used as universal primers for gene amplification of bovine *Theileria* species DNA. Furthermore, the p104 LAMP primers can be used for specific detection of *T. parva* infections in both host and the vector.

5-5. Summary

This study has developed LAMP assay for detection of *Theileria* infections. The LAMP primer set designed from the HSP70 gene amplifies DNA of *T. parva, T. annulata, T. mutans* and *T. taurotragi* and further amplifies serially diluted of *T. parva* DNA up 1 fg. LAMP with HSP70 primers has further been applied in detection of bovine *Theileria* species infections in field-derived cattle samples from Tanzania. No parasites were detected by microscopy. There were no bovine *Theileria* species Infections detected by LAMP and PCR from all the 24 samples collected from Arusha. Eighteen and 9 out of 40 samples from Dar es Salaam were positive by LAMP and PCR for *Theileria* species. infection respectively. The LAMP primer sets designed from the p104 and p67 specifically amplify *T. parva* DNA. The p104 primers amplified serially diluted *T. parva* DNA from 100 ng up to 100 pg in 60 min, whilst when reaction time is increase with 5 min to 65 min the detection limit of serially diluted DNA is 10 fg. Optimization reactions for the p67 primers were discontinued due to their inconsistency. This study has further applied p104 LAMP primers in detection of *T. parva* infections in field-derived African buffalo (*S. caffer*) samples from the Republic of South Africa and *R. appendiculatus* tick samples from Zambia. As a result no *T. parva* infections were detected from tick

samples by LAMP and PCR, whilst 90/99 (90.9%) were *T. parva* positive by both LAMP and PCR.

TABLE 11. LAMP primer sequences for amplification of *Theileria* DNA

Parasites	Target genes	Sequences
T. parva	HSP70	FIP: 5'-TGGGTTACGGGCTTCTTGGTTTCCTACGTCGCATTCACTGAC-3'
		BIP: 5'-ATTTTCGACGCCAAGAGGCTCAAATGGCCAGTGCTTCATGTC-3'
		F3 : 5'-GGAAACAGGACAACGCCG-3'
		B3 : 5'-CCGTTTGGTCCGTTGGTAA-3'
T. parva	p104	FIP: 5'- TCTCAGGGAGAGTTTCCTTAAGTATAGGTCTAAGGAAACTAAGACAAC-3'
		BIP: 5'- CTGGAACTCCCTTTACCACTCCTCCTTAGGTGGCTCAAAG -3'
		F3 : 5'- GATTACAGTAAAGCTGCAAGT-3'
		B3 : 5'- ACTAGACGGTGAATCAGGA-3'
		LF : 5'- TTCAAATGATTCATCAAATGC-3'
		LB : 5'- GTGCCACCAAAACGTCCCA-3'
T. parva	p67	FIP: 5'- TCTTGAAGATGAACCCGGACCGGTTCATCTACAGATACAAGAC-3'
		BIP: 5'- CAGTAACAAGGCCGGTACCACCCATATACTCAAAAAAACAAACCT-3'
		F3 : 5'- CAGTACCAACACGACCAC-3'
		B3 : 5'- ATCCATTGCCCTATTACCTAA-3'
		LF : 5'- CGGAATAATTATTAGAG-3'
		LB : 5'- CGGAATAATTATTAGAG-3'

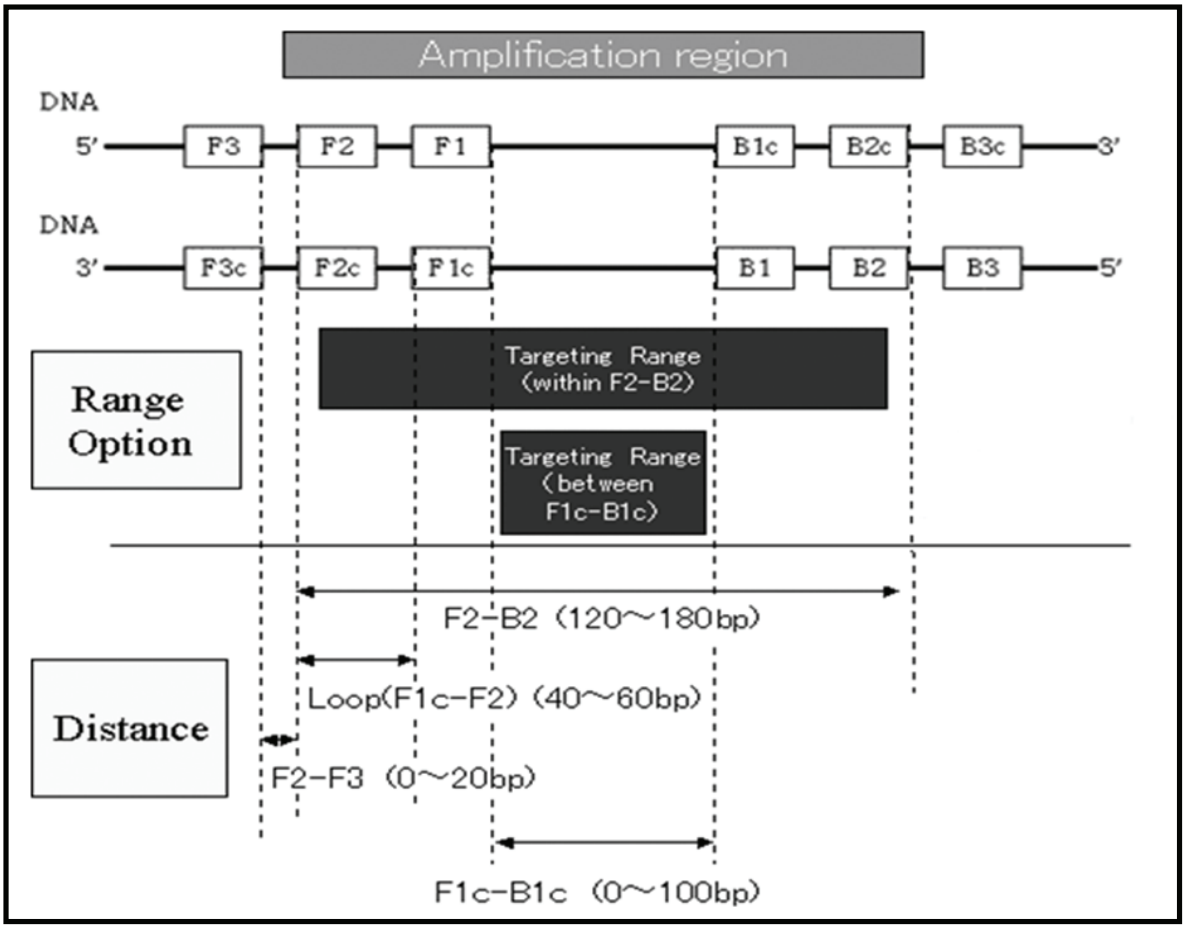

FIG. 18. Schematic illustration of LAMP primers design showing option ranges and distance ranges between the primers of the target DNA. Adopted from Eiken Chemical website: http://primerexplorer.jp/e/v4_manual/03.html#4. F1c and F2 are components of FIP primer whilst the B1c and B2 are components of BIP primer. The LF primer is situated between the F3 and F2 region of the target DNA whilst the LB primer is situated between the B2 and B3 region of the target DNA.

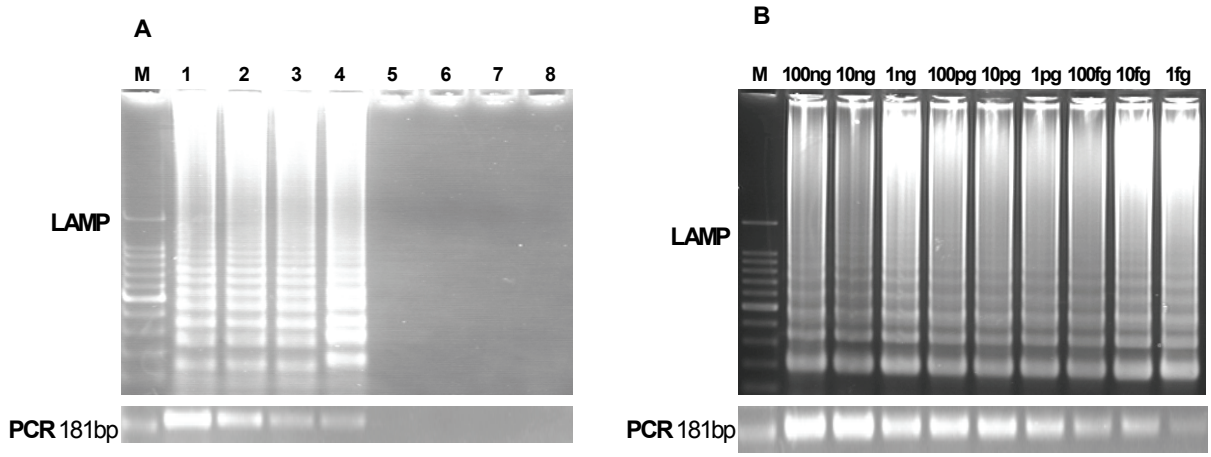

FIG. 19. Specificity and sensitivity of LAMP and PCR with HSP70 primers. (A) Specificity. Lanes M: 100 bp maker; 1: *T. parva*; 2: *T. annulata*; 3: *T. taurotragi*; 4: *T. mutans*; 5: *T. orientalis*; 6: *B. bovis*; 7: *T. brucei*; 8: Bovine blood. (B) Sensitivity of LAMP and PCR (F3 and B3) primers for detection of *T. parva* DNA quantified with a spectrophotometer (Smart Spec 3000, U.S.A.) and serially diluted from 100 ng to 1 fg.

TABLE 12. Detection of *Theileria* sp infections from Tanzania cattle samples

Method	Arusha	Dar es Salaam
LAMP	0/24 (0%)	18/40 (16.6%)
PCR	0/24 (0%)	9/40 (22.5%)
TBS*	0/24 (0%)	0/40 (0%)

* Thin blood smear

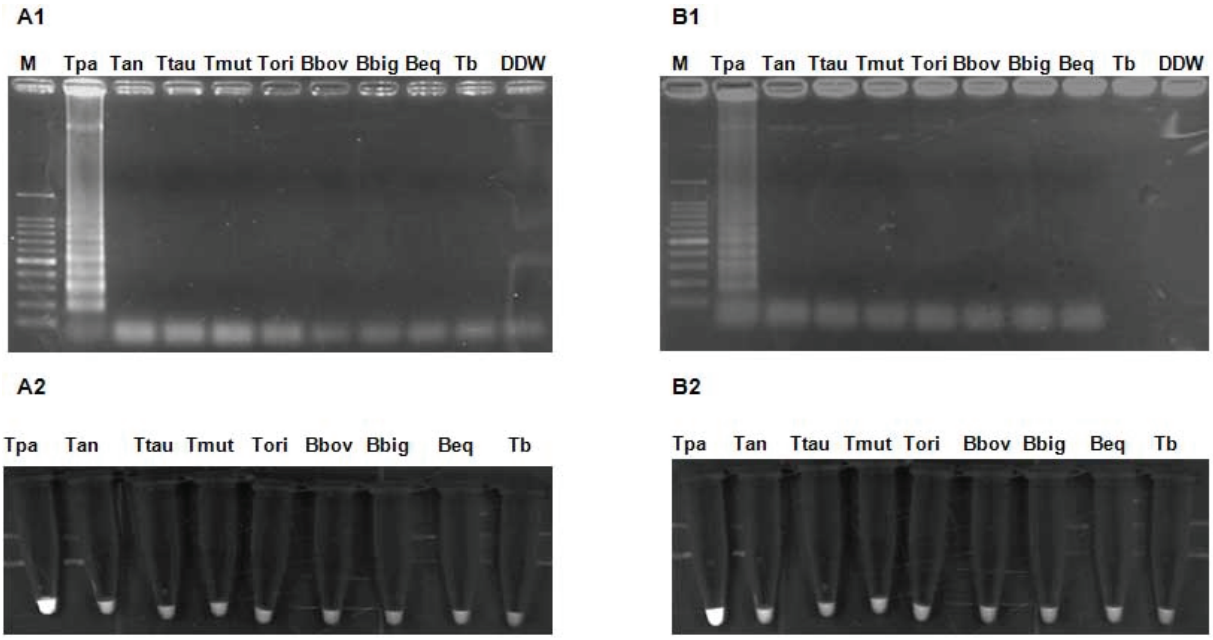

FIG. 20. Specificity LAMP with (A) p104 and (B) p67 primers. (A1 and B1) Agarose gel electrophoresis. (A2 and B2) Fluorescent detection reagent visualised under UV light. Lanes M: Tpa: *T. parva*; Tan: *T. annulata*; Ttau: *T. taurotragi*; Tmut: *T. mutans*; Tori: *T. orientalis*; Bbov: *B. bovis*; Bbig: *B. bigemina*; Beq: *B. equi*; Tb: *T. brucei* and DDW: Double distilled water.

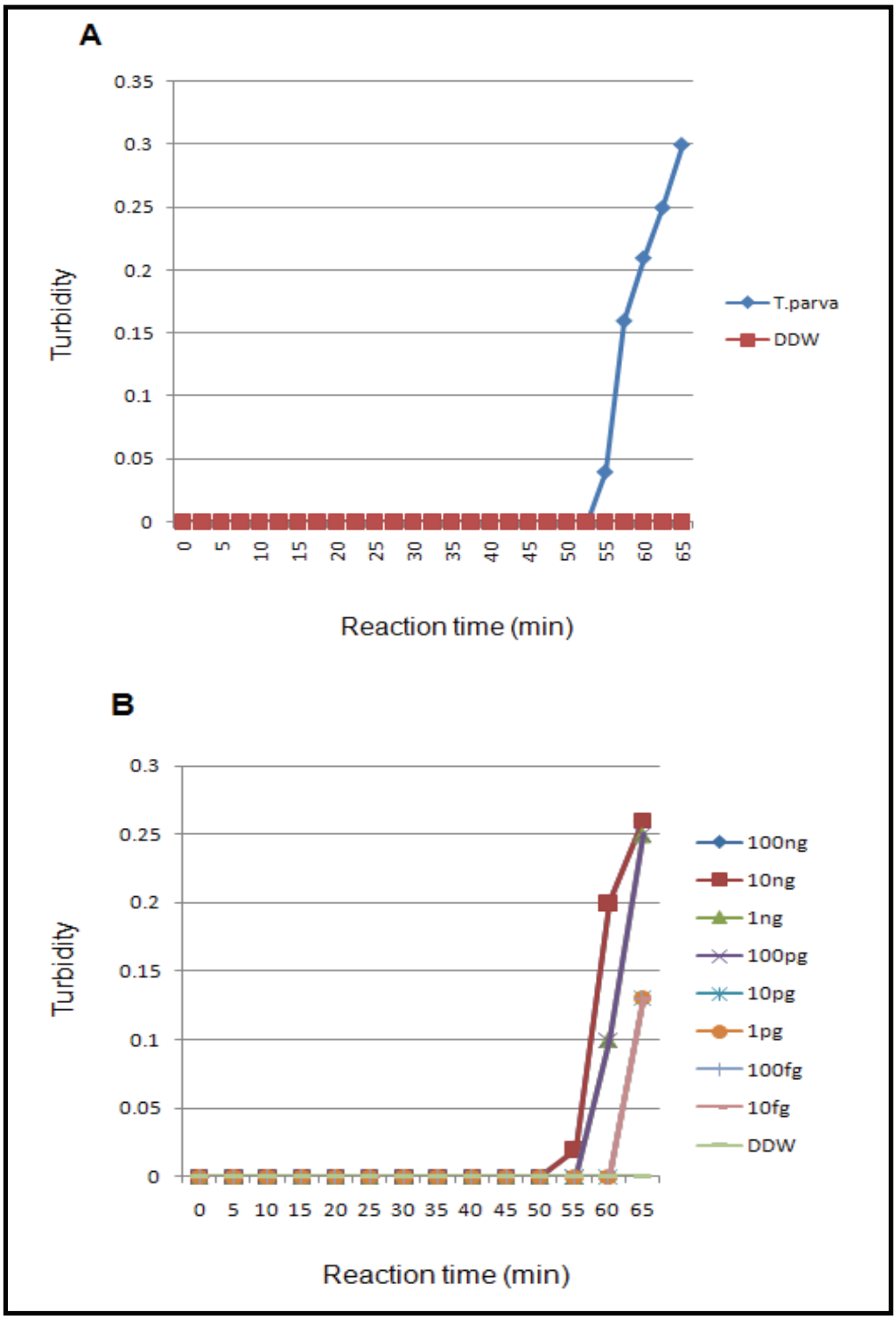

FIG. 21. Real-time turbidimetry LAMP for amplification of *T. parva* DNA with p104 primers. (A) Specificity reaction. (B) Sensitivity reactions with serially diluted *T. parva* DNA from 100ng down to 1fg. DDW – Double distilled water.

TABLE 13. Optimization of LAMP reactions with p104 and p67 primers

	Time (min)			
Temperature (^{o}C)	30	45	60	75
	p104 primers			
60	-	-	-	-
63	-	-	+	+
65	-	-	-	+*
68	-	-	-	+
	p67 primers			
60	-	-	-	-
63	-	-	+*	+*
65	-	-	-	+*
68	-	-	-	+*

- = No reaction

+ = Reaction

+* = Reaction achieved but inconsistent following repetitions.

TABLE 14. Detection of *T. parva* infections from field-derived blood and tick extracts with p104 primers

Countries	Hosts	Methods	Total # of samples	# positive
South Africa	Buffalo	LAMP	99	90 (90.9%)
		PCR	99	90 (90.9%)
Zambia	Ticks*	LAMP	32	0 (0%)
		PCR	32	0 (0%)

**R. appendiculatus*

General discussion

This study was aimed at the development of LAMP assays for specific diagnosis of trypanosome and bovine *Theileria* species of medical, veterinary and economical importance. The trypanosome species targeted by the current study included *T. brucei gambiense*, *T. b. rhodesiense*, *T. congolense*, *T. cruzi* and *T. evansi*. In addition this study developed LAMP for detection *Trypanosoma* KG1 isolate, a trypanosome of scientific importance due to its unusual characteristic of infecting ticks. The *Theileria* species targeted by this study included *T. parva, T. annulata, T. mutans, T. taurotragi* and *T. orientalis*.

Kuboki *et al*. (84) reported that the LAMP primer set designed from the PFRA gene amplifies DNA of *T. brucei* subspecies including *T. b. brucei, T. b. gambiense, T. b. rhodesiense* and *T. evansi*. Hence there was a need to validate the use of these primers for detection of *T. evansi* DNA, especially from infected animal model. In Chapter 1, I compared detection performance of LAMP to that of PCR and microscopical methods from *T. evansi* experimentally infected pigs. LAMP detection sensitivity for *T. evansi* DNA was proved to be higher than that of PCR and microscopy. However PFRA primers can only be used for detection of surra in countries outside the tsetse belt because the PFRA gene is also expressed in other tsetse transmitted *T. brucei* subspecies and because it is difficult to distinguish them from *T. evansi* due to high genetic similarity (6, 13, 73, 142). In order to address this shortcoming, in chapter 2, I have further developed species-specific LAMP for detection of *T. evansi* targeting the VSG RoTat1.2 gene which is specifically expressed in *T. evansi* and not in tsetse

transmitted *T. brucei* subspecies. The LAMP assay with the VSG RoTat1.2 primers is highly sensitive and has proved to be useful in screening of field samples from Brazil and Mongolia.

Furthermore, I have developed species-specific and highly sensitive LAMP assay for detection of *T. b. gambiense* infections targeting the 5.8S-ITS2 gene. *T. b. gambiese* together with *T. b. rhodesiense* cause a devastating HAT sleeping sickness in Western and Eastern Africa respectively (17). However attempts to develop LAMP for detection of *T. b. rhodesiense* with primer sets targeting the SRA gene were unsuccessful. LAMP primers designed from the SRA gene seem to be slow for annealing and initializing DNA synthesis in a required 60 minute incubation period of LAMP reaction. *T. cruzi* is a trypanosome of medical importance which causes Chagas disease in Central and South America (34). I have also developed a specific and highly sensitive LAMP assay for detection of *T. cruzi* targeting the 18S rRNA gene.

In addition to LAMP primers designed from the ribosomal P0 gene which specifically amplify of *T. congolense* DNA as reported by Kuboki *et al.* (84), I have further developed a specific and highly sensitive LAMP assay for detection of *T. congolense* DNA with a primer set targeting the 18S rRNA. This primer set has further being applied in screening of field-derived cattle and wildlife samples from Tanzania and Zambia. The superior detection sensitivity of LAMP against PCR has been demonstrated in this study especially when filter papers are used as DNA templates. Similar poor sensitivity by PCR for amplification of *T. brucei gambiense* DNA from filter papers has previously being reported (84).

Since the advent of LAMP method, many researchers have been engaged in basic research from a variety of perspectives (102). LAMP method has been developed for sexing of bovine preimplantation embryos (65), *in situ* LAMP (96) and largely for diagnosis of various pathogens including protozoan (72, 84,123), bacterial (44, 75), fungal (42) and viral infections (37, 122). As a result, LAMP is constantly recommended as a possible gene amplification method with the potential to be applied in field conditions due to its simplicity and rapidity. In chapter 3, I have further assessed the stability of LAMP reagents when stored at 25^{o}C and 37^{o}C, and its detection efficiency on different DNA templates. LAMP reagents were fairly stable when stored at 25^{o}C and 37^{o}C, and they amplified trypanosome DNA from *in vitro* cultures when reactions were performed on 3 day intervals until 15 days of reagent storage. Moreover *T. congolense* DNA from *in vivo* (infected mice) was also amplified whereby reactions were conducted on 4 day intervals until 30 days of reagents storage at 25^{o}C and 37^{o}C.

LAMP can also amplify trypanosome DNA from crude and hemolysed blood although reactions were inconsistent over a couple of repetitions. However, this observation indicates that with minor modifications in the future, LAMP would be conducted without any need for DNA extraction from body fluids, thereby significantly reducing reaction and detection time. Although filter paper DNA templates are a quicker method of DNA preparation, results of this study have also revealed that detection efficiency when reactions are conducted with filter paper templates ranges from 75 to 80%. A possible solution might be by using two discs instead of one and by also increasing the number of washes before the filter paper is used in a reaction. I have further shown that minimal amounts of hemoglobin, heparin, EDTA, and IgG do not

inhibit LAMP reactions. These substances are well known inhibitors for *Taq* DNA polymerase used in PCR (4, 5, 112).

I have further conducted identification studies of *Trypanosoma* KG1 isolated from naturally infected *Haemaphysalis hystricis* ticks in Kagoshima. As revealed in chapter 4, KG1 can now be described as a stercorarian trypanosome species which according to phylogenetic analysis of the conserved 18S rRNA appears in the same clade with *T.* (*Megatrypanum*) *pestanai* of which its vector is unknown and *T.* sp. wombat H26 which is suspected to be harbored by ticks (90, 116). This trypanosome propagates *in vitro* in the presence of feeder cells and can infect both hard (*H. hystricis*) and soft ticks (*Oornithodoros moubata*) as shown by this study. KG1 cannot infect common laboratory animals including mice, rats rabbits and sheep. Morphologically the trypomastigote-like form is C-shaped, a common characteristic in genus *Trypanosoma* and the epimastigote-like form appears to be elongated. A highly sensitive LAMP has been developed for detection of KG1 targeting the ITS1 gene. This LAMP assay would be useful in future studies aimed at isolation of this trypanosome from suspect mammalian hosts such as wild pigs (*S. scrofa*) and badgers (*M. meles*) which are found in Kagoshima and are hosts to *H. hysricis* tick.

In chapter 5, LAMP has been developed for detection of major bovine *Theileria* species DNA including *T. parva, T. annulata, T. mutans* and *T. taurotragi*, with exception to *T. orientalis* using primer set designed from the HSP70 gene. Furthermore a *T. parva* specific LAMP has been developed with primers designed from the p104 and the p67 genes. This study has also shown that the HSP70 and p104 LAMP assays can be applied in detection of *Theileria* species infections from field-derived cattle samples

from Tanzania, buffalo samples from Republic of South Africa and *R. appendiculatus* tick samples from Zambia. In particular the p104 LAMP assay will be useful in diagnosis of East Coast Fever a devastating disease of cattle caused by *T. parva* in Eastern and Southern Africa (81).

Conclusions

Common diagnostic methods currently being used for diagnosis of trypanosomosis and theileriosis have variable shortcomings ranging from specificity, low sensitivity due to low parasitaemia and cross reactivity, inability to distinguish past and current infections and requirement of expensive equipment for some of the methods. LAMP with its characteristics of rapidity, simplicity, high sensitivity and specificity, and most importantly its non-requirement for complicated equipment for incubation, can easily overcome the shortcomings experienced when using common diagnostic methods. This study has therefore developed LAMP assays for diagnosis of *T. b. gambiense, T. congolense, T. cruzi, T. evansi* and *T*. KG1 isolate. Attempts for development of *T. b. rhodesiense* LAMP with SRA gene were unsuccessful, however further attempts with other target genes remain to be pursued. There is also a need to further develop LAMP assays for other trypanosome species including *T. vivax* and *T. theileri* as they co-exist with other trypanosome species in African countries which then need to be distinguished, this study was limited by lack of DNA for the latter trypanosome species. Similarly, development of specific LAMP for the non-pathogenic stercorarian trypanosomes including *T. rangeli* and *T. lewisi* is a subject that remains to be pursued. Once again this study was limited by lack of DNA of the above mentioned stercorarian trypanosome species.

Further, LAMP assays have also been developed for detection of bovine *Theileria* infections with the first assay targeting the HSP70 gene collectively amplifying DNA of *T. parva, T. annulata, T. mutans* and *T. taurotragi.* The second assay targeting

the p104 and p67 genes, specifically amplifies DNA of *T. parva*. However, the p104 LAMP assay needs further improvement of shortening the reaction incubation period and imporving its sensitivity. Another option will be to develop primers from other target genes which are only expressed in *T. parva* such as p150 and PIM genes. With bovine theileriosis being the number one problematic disease in cattle in the Eastern Africa, these LAMP assays will be useful in diagnosis of *Theileria* infections as the first step for their control programs.

This study has further showed that LAMP reagents are stable when stored at 25^{o}C and 37^{o}C which are possible ambient temperatures in the field. This observation brings LAMP a step closer to actually being conducted in field situations for a number of days without keeping a cold chain for reagents. However, improvements of simpler DNA preparation protocol are required, as DNA template is a critical factor that will assist in application of LAMP outside the laboratory. Previously LAMP proved to have superior tolerance than PCR to substances used in *in vitro* cultures including saline, PBS, MEM, aqueous humor and vitrous, additionally this study has demonstrated superior tolerance by LAMP over PCR to EDTA, hemoglobin, heparin, IgG and IgM which are substances found in blood or used in preparation of blood and DNA.

The rate of adoption of diagnostic DNA technology by laboratories in developing countries appears to be limited not only due to the high cost but also due to a widespread perception that these techniques are complicated. LAMP is a rapid (amplification in 1 h) and simple (requires only a water bath/heatblock) technique, it amplifies DNA at a constant temperature, can produce large amounts of DNA that can be visualized by the naked eye as white turbidity indicating positive amplification or by

using fluorescent dyes or turbudimeter thereby reducing detection time. All these advantages indicate that LAMP has the potential to replace PCR and be used even by moderately trained technicians in countries that lack sufficient resources. Although there is still a need for improvement on some of the LAMP primers, LAMP assays developed in this study will hopefully be useful as alternative diagnostic tools for confirmative diagnosis and in epidemiological studies of trypanosomosis and theileriosis in countries where these diseases are endemic.

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